TOWARDS SELECTIVE ADHESION OF MESENCHYMAL PROGENITOR CELLS FROM THE RAT BONE MARROW

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INTRODUCTION

The public has placed great hope in scientists, who "grow new organs and tissues in the labs" in the past decades. Nevertheless, the journey from the *in vitro* experiments to clinical applications of "off-the-shelf" tissues still presents numerous technical challenges; a native tissue is far more complex than just a mass of cells glued together, and the signals that regulate the extracellular matrix development *in vivo* are still poorly understood. Different test systems and animal models have been developed to qualify the intermediate *in vitro* results of tissue engineering. Whereas human individuals differ in age and many genetic determinants, rodent animal models (mouse, rat) can be manipulated to yield minimal intra-individual variation. Much characterization is needed, however, to find parallelisms between animal models and human systems. Whereas numerous biomaterials used as scaffolds show good *in vivo* performance, the most promising cell sources for tissue engineering are adult stem cells; they are present in every adult individual and give rise to differentiated cells of tissues or organs.

Two main branches of stem cell research have been booming since the 1960s, when Friedenstein and his colleagues documented *in vitro* colony forming fibroblasts from the bone marrow, now called mesenchymal stem / progenitor cells (MSC). First, more detailed basic research deepens into "stemness", differentiation potency and their molecular code, whereas, on the other hand, the more applicative other branch uses the advantages of MSC as cells for cellular therapies (gene delivery, immunosuppression) and neogenesis of mesenchymal tissues (tissue engineering). Both fields are strongly interdependent, providing useful information to the "sister" branch. Likewise, this thesis encompasses both approaches to stem cell research. The first phase included the characterization of MSC from the rat bone marrow, characterization of "accompanying" hematopoietic, cells and their influence on osteogenic differentiation. The outcomes of cell characterization lead us further to test for bone tissue engineering applications as a means of finding biomaterial for selective adhesion of pure MSC.

The materials used as bone substitutes often have the disadvantage of causing fibrous tissue development at the interface with the surrounding tissue when implanted. This process follows the natural wound healing process of fibrin clot formation and inflammation by infiltration of macrophages and other cells of the immune system to the site of an implant[1]. Also *in vitro*, cells often fail to adhere or fail to differentiate and produce enough ECM when seeded onto raw biomaterials. To circumvent these issues, modification of the biomaterial surface can enable the adhesion of desired cells to the biomaterial surface and enhance their differentiation. One possibility is to covalently bind adhesion-promoting sequences, i.e.

ligands for integrins, which are cell adhesion receptors, to the biomaterial surface and at the same time prevent nonspecific cell adhesion to the hydrophobic biomaterial surface using a hydrophilic poly-ethylene-glycol (PEG) layer. To this end, the ligands, for which receptors on the target cells are highly expressed, are bound to the biomaterial surface. Therefore, it is a prerequisite to determine which integrin receptors are actually expressed on "desired cells" (in our case rat MSC) and to which level. Then, the decision has to be made, which ligand will to be bound to the biomaterial surface, to ensure the desired adhesion and cell differentiation.

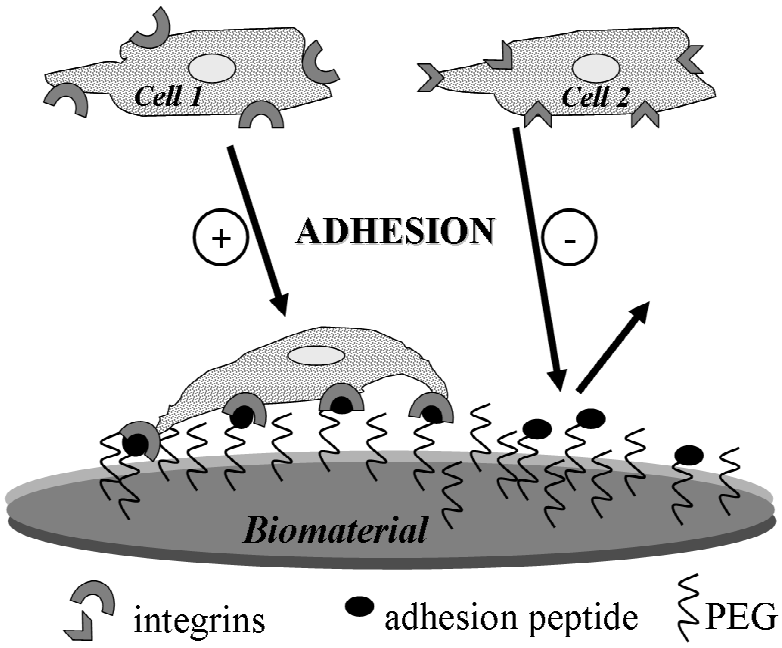

***Figure 1**: The primary goal of the thesis was to discover integrins expressed only on MSC (e.g. Cell 1) to achieve selective surface immobilization and improved differentiation.*

MESENCHYMAL STEM / PROGENITOR CELLS (MSC)

Bone marrow mesenchymal stem / progenitor cells (MSC) were first described in the 60s and 70s by the group lead by Alexander Friedenstein. He and his colleagues showed that bone marrow stroma contains cells that adhere to tissue culture plastic. He determined that these cells (1) belong to a rare population in the bone marrow, (2) did not enter "S" phase until up to 60 hours after initial plating, (3) showed a high replicative capacity *in vitro*, (4) were clonogenic, and (5) formed colonies of irregular shape and density[2]. More importantly, he showed that the cells were capable of forming bone *in vivo*, even after multiple passages *in vitro*.

MSC are isolated mostly from the bone marrow and organs of mesenchymal origin that can be expanded in vitro over several passages (unlimited self renewal) and differentiated into cells of some mesenchymal tissues, such as osteoblasts, chondrocytes, adipocytes, myocytes, tenocytes, and haematopoiesis supporting stromal cells[2-9]. With appropriate culturing conditions (very low seeding density, medium pre-selection, etc.), distinct colonies of spindle-shaped / fibroblastic cells are formed, each of which is derived from a single precursor cell, the colony forming unit – fibroblast (CFU-F)[5]. In addition to bone, cartilage, marrow adipocytes, and formed fibrous tissue could arise from single cell or CFU-F *in vivo*[5]. Due to their high frequency in the bone marrow stroma, they are often named also marrow stromal (mesenchymal stem) cells, although they are also moderately present in the bone marrow (0.001-0.01% of all nucleated cells in the marrow[10]). "Stromal cells" play an important role as a microenvironment (stroma) for the developing hematopoietic stem and progenitor cells in the bone marrow[11]. MSC-like cells have been isolated from periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle, lung and deciduous teeth[4] and human umbilical cord perivascular cells[11]. Scientists still argue, whether the site of isolation is also the site of the MSC origin, or if circulating MSC exist[12].

MSC are generally isolated from an aspirate of bone marrow harvested from the superior iliac crest of the pelvis in humans, though other sites have also been reported, e.g. the tibial and femoral compartments and thoracic and lumbar spine. In larger animals, marrow is often obtained from the same site, and in rodents it is generally harvested from the mid-diaphysis of the tibia or femur[4]. Human bone marrow in particular is often subjected to density gradient centrifugation to enrich the MSC-containing fraction. The cell suspension is placed into tissue culture flasks and cultured in pre-tested medium, supplemented with fetal bovine serum (FBS), and the adherent fraction of cells is expanded and passaged. After

isolation, MSC adhere rapidly and can easily be separated from the non-adherent hematopoietic cells by repeated washing. Non-adherent hematopoietic cells (HC) are removed with media changes, whereas adherent HC disappear from the cultures within several passages or are depleted using immunoselection techniques[4,13]. One of the most important features of MSC culturing is the selection of an appropriate FBS lot, so that it allows for: (1) selective attachment of MSC to the tissue culture flasks, (2) mitotic expansion of MSC, and (3) maintenance of MSC phenotype[14]. Enrichment of CFU-F or increasing their frequency, compared to whole bone marrow seeding, can be achieved by negative immunoselection (CD34+/CD11b+/CD31+ cell depletion) or by positive immunoselection using antibodies against Stro-1, CD49a, D7-FIB and LNGFR (low-affinity nerve growth factor receptor)[5,15].

Multilineage differentiation potential & unlimited self-renewal

According to the definition of a stem cell, MSC should have unlimited proliferative capacity and multilineage differentiation potential. Pittenger at al.[16] showed that only 2 of 6 individual colonies express multilineage differentiation capacity and therefore confirmed the previously observed heterogeneity of CFU-F with regards to size, morphology, and potential for differentiation[5,17,18]. Aubin et al.[3] explain that CFU-F belong to a lineage hierarchy in which only some of the cells are multipotential stem cells or primitive progenitors, whereas others are more restricted. Muraglia et al.[19] demonstrated that only 30% of the in vitro derived MSC clones exhibit a tri-lineage (osteo/chondro/adipo) differentiation potential, while the remainder displayed a bi-lineage (osteo/chondro) or uni-lineage potential (osteo). More importantly, the multi-potential clones progressively loose their adipogenic and chondrogenic potential with repeated passages[2].

When considering the unlimited self-renewal of MSC, it is necessary to stress that the published data are variable, because MSC proliferative capacity depends on a number of culture conditions, including the supplementation of basic media with growth factors (e.g. FGF-2, PDGF, EGF, TGF-β, IGF) and the seeding density (low seeding density increases the fold expansion / population doublings in vitro)[5,11]. Nevertheless, these cell populations, often prematurely named stem cells, are a heterogonous population with regards to their differentiation potential and self-renewal ability. It is believed that multipotent mesenchymal stem cells exist, but that they are lost from the pool of MSC during long-term culturing due to their low frequency in the primary tissue source[3]. Nonetheless, some subclasses of MSC can be enriched and cultured up to 70 population doublings (PD) under select culture conditions,

and they retain the multipotent differentiation potential up to 50 PD[11]. Tri-potent and bi-potent procursor cells are morphologically similar to the multipotent MSC, but differ in their gene transcription repertoire. The progression of MSC to precursor cells (from asymmetric to symmetric division) is considered the first step in stem cell commitment[11].

In vitro osteogenic activation requires the presence of β-glycero-phosphate (phosphate for the mineralization), ascorbic acid (collagen synthesis), dexamethasone (transcriptional activation) and FBS. When cultured in monolayers in the presence of these supplements, the cells acquire an osteoblastic morphology with up-regulation of alkaline phosphatase activity and deposition of a calcium-rich mineralized extracellular matrix[4]. Chondrogenic differentiation occurs when MSC are grown under conditions that include a three-dimensional culture format (e.g. micro mass cultures), a serum-free nutrient medium, and the addition of a member of the TGF-β super-family (especially TGF-β2 and TGF-β3)[4]. When these conditions are met, the cells rapidly loose their fibroblastic morphology and begin to initiate expression a number of cartilage-specific extracellular matrix components, e.g. glycosaminoglycan[4]. MSC cultured in monolayers in the presence of isobutylmethylxanthine, dexamethasone, and indomethacin become adipocytes with the production of lipid-filled vacuoles[15,20]. Adipogenic differentiation is induced by the nuclear receptor and transcription factor peroxisome proliferator-activated receptor-gamma (PPAR-γ) as well as fatty acid synthetase[4]. MSC myogenesis has been induced *in vitro* using 5-azacytidine or amphotericin B, resulting in the formation of multinucleated fibers, resembling myotubes[20]. For stromal differentiation, which enables the maintenance of hematopoietic stem cells, horse serum is added to the culture as well as i-inositol, folic acid, 2-mercaptoethanol, L-glutamine, and hydrocortysone[21]. The differentiation pathways from the MSC to mature mesenchymal lineages are presented in Figure 2.

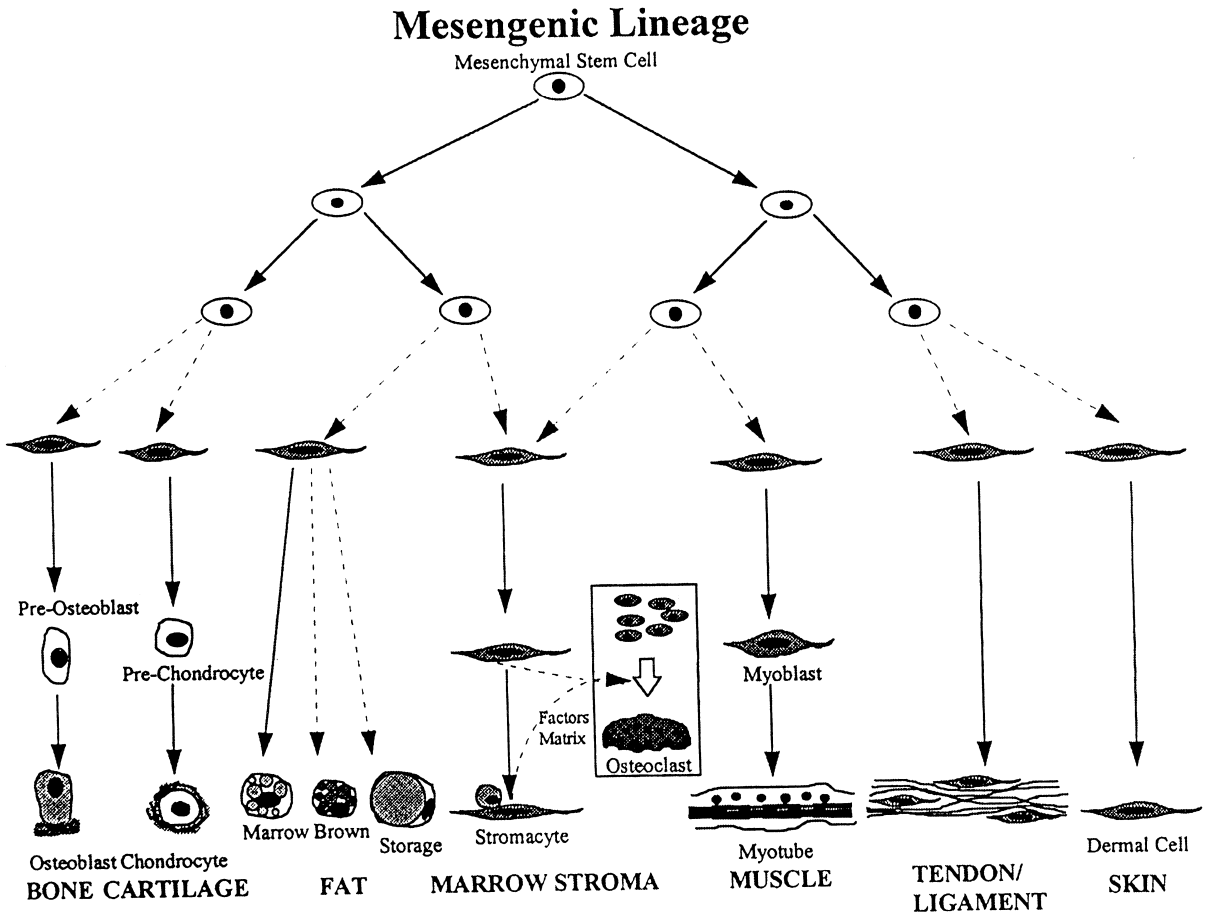

Figure 2: *Mesengenic lineage pathway. The process of mesengenesis involves the generation of multiple mesenchymal end-stage phenotypes from the differentiation of a multipotent mesenchymal stem cell (MSC) through a multistep series of developmental changes in response to microenvironmental stimuli. The lineages are illustrated from left to right in the order of most to least characterized (from Caplan et al.* [14]*).*

Surface marker characterization of MSC

Beside morphological descriptions of MSC, in the past years research has been devoted to finding specific surface markers on MSC that would make them detectable by flow cytometry and would enable the isolation by FACS (fluorescence-activated cell sorting) or MACS (magnetic-associated cell sorting) from freshly isolated tissues. The problem of finding MSC-specific markers is that MSC are described only as cells yielded *in vitro.* Beside that, they express similar features with fibroblastic cells (expression of matrix proteins), endothelial (endoglin and MUC-18), epithelial and muscle cells (α-smooth muscle actin)[5,11]. Several laboratories have developed monoclonal antibodies using MSC as immunogen in order to identify one or more markers suitable for identification and sorting of stromal cell preparations. The nearest approximation has been production of Stro-1, which is highly expressed by stromal cells that are clonogenic[5]. There is a general agreement that MSC lack

typical hematopoietic antigens, namely, CD45, CD34, CD133, CD14 or CD11b and an endothelial marker CD31[4,11]. The most important surface markers expressed on human MSC are listed in Table 1. Detailed reviews of MSC surface marker expression can be found in Deans and Moseley[22], Pittenger et al.[23] and Minguel et al[9]. Data reviewed in Table 1 result from characterization of *in vitro* expanded MSC. There is increasing evidence that MSC-like cells in vivo also express some hematopoietic and other markers and therefore the "true *in vivo* MSC" characterization is still a challenge. Rapidly self-renewing cells (RS cells) observed *in vitro* are an MSC subpopulation of highly mitotic small cells that can be enriched by very low density culturing and can be discriminated by the differential expression of several antigens, including TrkA, Flk-1 and c-kit receptors[2].

However, more primitive adult stem cells than MSC can be isolated from bone marrow and expanded *in vitro*: Multipotent adult progenitor cells (MAPC) can be co-purified with MSC and are actually a pluripotent population, being able to differentiate not only to mesenchymal cells, but also to visceral mesoderm-, neuroectoderm- and endoderm-like cells[24]. Murine MAPC express low levels of Flk-1, Sca-1, and Thy-1 and higher levels of CD13, stage specific antigen I (SSEA-I), and high levels of telomerase, enabling them the expansion over 100 PD. Young et al.[25] isolated so called pluripotent lineage-uncommitted stem cells from several tissues. These cells are small (6-20µm) and lack some typical MSC markers (CD105, CD71, CD117), but express CD10, CD13, CD66e and show telomerase activity[25]. For instance, pluripotent mesodermal stem cells can differentiate to several mesoderm-derived cell types, including hematopoietic cells and intramembranous osteoblasts[25].

***Table 1**: Some common stated MSC typical surface markers.*

CD locus	Marker	MSC	HSC	OB	EC.	Reference
Stem cell markers						
	Stro-1	+	-	+[26]	+/-	2,5,9,12,15,20,20,22,27-29
CD166	SB-10 / ALCAM	+	-	+[26]		2,4,12,20,23,30,31
CD105	SH-2 / endoglin / TGFβRp	+	-	-[26]	+	11,12,14,20,22,23,27,29,31
CD73	SH-3, SH-4	+	-	-		4,11,12,23,29
CD44	H-CAM	+	+	+[32]		9,11,12,23,28-31
CD71	Aka, transferring Rp.	+				11,28
CD90	Thy-1	+	+			11,22,23,27-30,33
CD106	V-CAM	+			+	11,12,22,27,28,30
CD120a	Aka, TNF Rp.	+				11,23
CD124	IL-4 Rp	+				11,23,28
CD271	P75 LNGF Rp	+				11,22
CD133	Prominin / AC133	+/-	+			11,15,23
CD34	Adhesion Rp, HSC marker	-(+)	+			9,11,22,22,29,33
CD10	CALLA, neutr.endpeptidase	+/-		+[32]		22,23
CD13	Aka, aminopeptidase N	+		+[32]		22,23,28,29,31
CD146	MUC-18	+			+	27,30
CD117	c-kit	+ / -	+			23,33
	Sca-1	+	+			29,30,33
Adhesion molecules						
CD29	Integrin β1	+				4,12,22,23,28,28,30
CD49a	Integrin α1	+				4,12,22,23,27,28,30
CD49b	Integrin α2	Low				12,20,22,23,28
CD49c	Integrin α3	Low				12,20,22,23
CD49d	Integrin α4	-	+			20,22,23
CD49e	Integrin α5	+				4,12,22,23,29
CD61	Integrin β3	Low				12,22
CD54	ICAM-1	+		+[32]		12,22,23,31
CD62	selectins	+ / -				12,23,29,30
CD31	PECAM	-			+	23,29
CD38	Adhesion: lymphocytes + EC	-				22,30,33
CD50	ICAM-3, CD54 ligand	-	+			22
Hematopoietic markers						
CD45	LCA	-	+	-[32]		9,11,11,20,22,23,28-30
CD11a	Integrin αL	-	+			20,23
CD11b	Integrin αM	-	+	+[32]		11,20,29,30
CD14	LPS Rp	-		-[32]		9,23
CD18	Integrin β2	-	+			11,20,23
Other	MHC-Class I / HLA-ABC	+				4,20,23,29
	MHC-Class II / HLA-DR	-		+[32]		4,20,22,23,29,30,33
CD40	Cell- and Ab-reg.immunity	-				29
CD80	Aka B7-1, BB1; binds CD28	-		+[32]		23,29
CD86	Aka B7-2, binds CD28	-		+[32]		23,29
CD140	PDGF Rp	+				22,30
	EGF Rp	+				22,30
	IGF-1 Rp	+				22

CD: cluster of differentiation; MSC: mesenchymal progenitor cells; HSC: hematopoietic stem cells; OB: osteoblasts, osteocytes; EC: endothelial cells.

Adhesion molecules

Cells communicate with each other directly via heterotypic or homotypic cell-cell contacts as well as indirectly using their secreted molecules (paracrine, autocrine pathways). The latter can be triggered by a signal from the ECM, which cells (1) adhere to, (2) migrate onto, and (3) produce or degrade. Loss of attachment to the matrix causes apoptosis in many cell types[34]. The bone marrow is an ideal example of MSC communication with surrounding ECM and other cells, including hematopoietic stem and progenitor cells (HSC), endothelial cells, adipocytes, macrophages, reticular cells, fibroblasts, and smooth muscle cells. When isolated from the bone marrow and injected into a host, both MSC and HSC are readily retained in the bone marrow or at the injury site, as observed in cell "homing" studies[4]. Homing depends upon adhesion molecule interactions, which influence the localization, proliferation, and differentiation of the injected cells.

Cell adhesion has been an important part of tissue engineering research, where it was shown that the biomaterial surface dictates how cells adhere and form tissue in contact with the surface. Attachment, adhesion, and spreading belong to the first phase of cell / substrate interactions and the quality of this first phase will influence the cell's capacity to proliferate and differentiate in contact with the implant.

Studies *in vitro* demonstrated the role of adhesion molecules in cell shape, polarization, cytoskeletal organization, cell motility, and survival[35]. Cell adhesion receptors are characterized by their capacity to interact with a specific ligand. These ligands can be situated on the membrane of neighboring cells or can be ECM proteins. Cell adhesion molecules can be divided into 4 major classes: selectins, the immunoglobulin superfamily, cadherins and integrins. Integrins, in contrast to other receptors, primarily mediate adhesion to ECM and to a lesser extent mediate heterotypic cell-cell adhesion to immunoglobulin adhesion receptors (leukocyte integrins).

Integrins

Integrins are transmembrane heterodimers consisting of non-covalently associated alpha and beta subunits[36,37]. Each subunit is made up of a large extracellular domain, a transmembrane domain, and a short cytoplasmic domain. The integrin spanning the cell membrane acts as a linker between the intracellular and extracellular compartments and can translate the attachment of external ligands to internal information, referred to as outside-in signaling. Inside-out signaling can, for example, activate integrins and cause their clustering[36].

Integrins do not bind nonspecifically to ECM proteins, but rather to specific binding sites. The most common binding site is the tripeptide amino acid sequence arginine-glycine-aspartic acid (RGD)[37]. This sequence is present in most of the ECM proteins, like fibronectin, vitronectin, bone sialoprotein, osteopontin, trombospondin, fibrinogen, laminin, collagen, nectipepsin, and others[37,38]. However, binding sites are not always accessible and do not always mediate cell attachment. For instance, an RGD sequence may not always be present at the surface of the protein or may require a second site to perform its function[36].

After the binding of a ligand, the integrins cluster together into focal contacts. This is an area of close contact between a cell and the ECM, consisting of additional cytoskeletal proteins, adapter molecules, and kinases[36], as presented in Figure 3. Integrin activation results in the recruitment of signaling molecules, the reorganization of the cytoskeleton, signal transduction, and the expression of distinct transcription factors. However, integrins can be present on the cell surface in an inactive state, in which they do not bind ligands, and cannot trigger signaling cascades[36]. The activity of an integrin may depend upon a ligand as well as on integrin subtype. Furthermore, some integrins require a second binding site (synergy site) on the ligand for optimal function[36]. Integrins are able to cooperate with growth factor receptors in the regulation of cellular processes in a way that with integrin clustering also growth factor receptors are recruited[36].

Gene deletion studies showed the importance of integrins not only for cell function, but also for the development of an organism. For example, the deletion of β1 can completely block development of blastocyst, and the deletion of α4, α5, αV and β8 can cause major developmental defects or be fatal (deletion of α3, α6, α8, αV, β4, β8). Furthermore, by deleting genes, defects in leukocyte function (αL, αM, αE, β2, β7), inflammation (β6), haemeostasis (αIIb, β3, α2), bone remodeling (β3), and angiogenesis (α1, β3) were observed [39].

Integrins are selective towards their substratum ligand / ligands, although an integrin may target more than a single ECM protein. Integrins α1β1, α2β1, α10β1, α11β1, αIIbβ3, and αVβ8 can all bind collagens, α3β1, α4β1, α5β1, α8β1, αVβ1, αIIbβ3, αVβ3, αVβ6, α4β7, and αVβ8 bind fibronectin, integrins αVβ1, αIIbβ3, αVβ3, and αVβ5 bind vitronectin, and α1β1, α2β1, α6β1, α7β1, α6β4, and αVβ8 bind laminins[36].

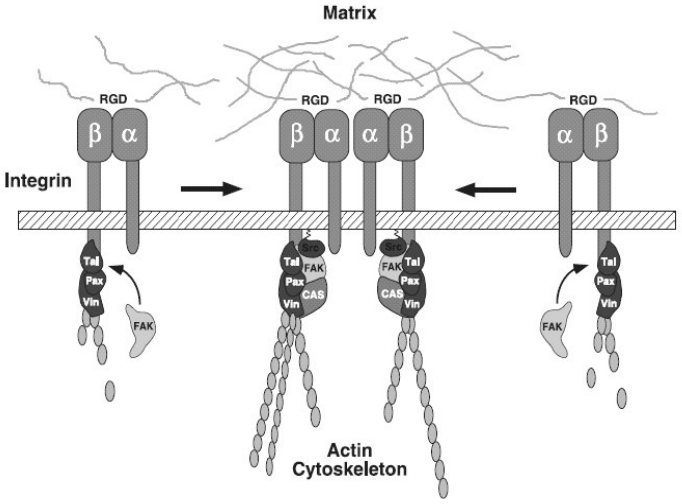

Figure 3: *Matrix binding promotes integrin clustering and association with the cytoskeleton. This in turn promotes further integrin clustering and matrix organization in a positive feedback system.*

RGD, Arg-Gly-Asp integrin-binding motif; Tal, talin; Pax, paxillin; Vin, vinculin; CAS, p130^{CAS}; FAK, focal adhesion kinase; Src, kinase family. (from Giancotti and Ruoslahti [34])

MSC express a large spectrum of cell adhesion molecules of potential importance in cell binding and homing interactions. The characterization of freshly isolated MSC (Stro-1 positive immunoselection) from the human bone marrow showed the expression of α1β1 (VLA-1), α2β1 (VLA-2),. α5β1 (VLA-5), α6β1 (VLA-6), αVβ3 and αVβ5 integrins and substrate / integrin dependent CFU-F colony formation[40]. Cultured human MSC exhibit a high expression of integrins α1, α5 and β1, low expression of α2, α3, α6, αV, β2, and β4, and no expression of α4, αL, and β2[41].

MSC in cell therapies

The fundamental principle of stem cell therapy (SCT) is that undifferentiated cells (autologous or allogenic) can be locally or systemically delivered to the injured host, will migrate to the site of injury, and, under the influence of local signals, will differentiate to the cells of the appropriate phenotype[4,42]. These differentiated cells then contribute to the repair of the injured tissue[4]. It seems clear that MSC, when delivered by intravenous infusion, are capable of specific migration to a site of injury[20]. MSC have already demonstrated efficacy in the treatment of children with osteogenesis imperfecta, for osteoporosis, ostoarthrosis, meniscectomy, hematopoietic recovery as well as cardiovascular repair, lung fibrosis, spinal cord injury, and muscular dystrophy[4,8,9,11,15]. Interestingly, MSC help regenerate not only tissues of mesenchymal origin, but also the tissues of other embryonic layers, including neurons, epithelia, skin, lung, liver, intestine, kidney, and spleen, as shown in animal studies[4,8,9,11,15].

It is also noteworthy that neither autologous nor allogenic MSC induce any immunoreactivity in the host upon local or systemic administration[8,11]. This proposes MSC as an ideal carrier to deliver genes into the tissues of interest for gene therapy applications[8,11]. Moreover, there is accumulating evidence of the hypo-immunogenic nature of MSC. In other words, MSC may actually inhibit inflammation and immunologic responses in the host[15], which leads to the MSC application in reducing so called GVHD (graft versus host disease), a common complication after cell or organ transplantation[4,15]. Although early pre-clinical and clinical data demonstrate the safety and effectiveness of MSC therapy, there are still many questions to be answered surrounding the mechanism of action[4].

BONE

The skeleton consists of highly specialized cells, mineralized and non-mineralized connective tissue matrix, and spaces that include the bone marrow cavity, vascular canals, canaliculi, and lacunae (Figure 4). During development and growth, the shape and the size of the skeleton is achieved by the removal of bone from one site and deposition another (modeling). Once the skeleton has reached maturity, regeneration continues in the form of periodic replacement of the old bone with new at the same location (remodeling)[43]. Removal of the bone is a function of osteoclasts, derived from blood-cell precursors, and new-bone formation is a function of osteoblasts, cells derived from mesenchymal stem / progenitor cells[43].

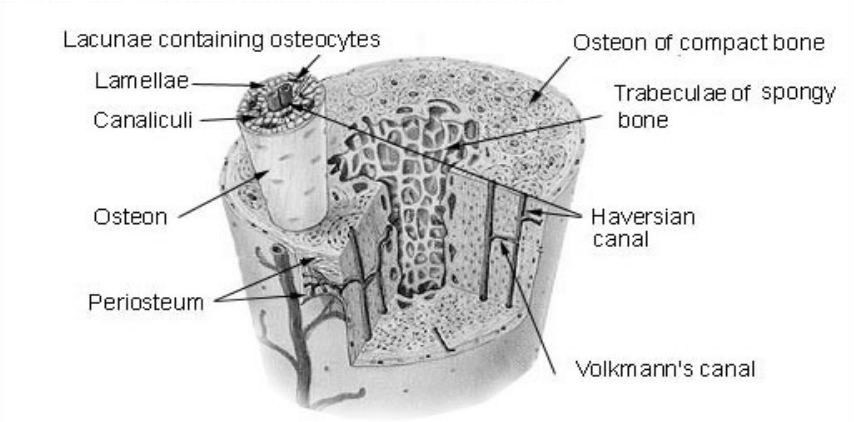

***Figure 4**: Morphological cross-section structure of a long bone with cancellous / spongy bone in the middle and compact / cortical bone surrounding the spongy bone (from [44]).*

Bone is composed of an organic matrix that is strengthened by deposits of calcium salts. Type I collagen constitutes approximately 95% of the organic matrix; the remaining 5% is composed of proteoglycans and numerous non-collagenous proteins (which consist 90% of osteocalcin and osteonectin and the other proteins, e.g. osteopontin, bone sialoprotein)[43,45]. Crystalline salts deposited in the organic matrix of bone under cellular control are primarily calcium and phosphate in the form of hydroxyapatite. Morphologically, there are two forms of bone: cortical (compact) and cancellous (spongy). In cortical bone, densely packed collagen fibrils form concentric lamellae, and the fibrils in adjacent lamellae run in perpendicular planes as in plywood[46]. On the other site, cancellous bone has a loosely organized, porous matrix. Differences in the structural arrangements of the two bone types are related to their primary functions: cortical bone provides mechanical and protective functions and cancellous bone provides metabolic functions[46].

Bone cells

Bone is composed of four different cell types: osteoblasts, osteoclasts and bone lining cells are present on bone surfaces, whereas osteocytes permeate the mineralized interior.

Osteoblasts are fully differentiated cells, originating from MSC. In culture, they are morphologically nearly indistinguishable from fibroblasts, but form cuboidal cells at confluent density, as observed in the middle of the compact colonies - bone nodules[43,47]. They are responsible for the production of bone matrix by secreting collagen I and other non-collagenous proteins[46].

Osteocytes are mature osteoblasts within the bone matrix and are responsible for matrix maintenance. These cells have the ability not only to synthesize the bone matrix, but also to resorb matrix to a limited extent. Each osteocyte occupies a space or lacunae within the matrix with its filopodial extensions in contact with neighboring adjacent cells enabling communications and nutrient / metabolite flow[46].

Bone lining cells are flat, elongated, inactive cells that cover quiescent, non-mineralized collagenous bone surfaces. It has been suggested that these cells are either osteoblast precursors[46] or "resting" osteoblast[43], since no definitive role has been detected.

Osteoclasts are large, multinucleated cells that resorb bone. *In vitro* maturation of hematopoietic stem cells / macrophages into osteoclasts requires the presence of marrow stromal cells or their osteoblast progeny[43]. Osteoclastogenesis is promoted by macrophage colony-stimulating factor (M-CSF) and requires contact between osteoclast precursors, expressing RANK (receptor for activation of nuclear factor kappa B), and stromal cells or osteoblasts, expressing RANK ligand (RANKL). RANKL and RANK are members of the tumor necrosis factor (TNF) and TNF receptor super-families, respectively[43].

Formation of the Skeleton

Formation of the skeleton (ossification) occurs by either direct (intramembranous) or indirect (endochondral) processes. Both require a solid base and a vascular supply for the deposition of the mineralized extracellular matrix.

Intramembranous ossification (Figure 5a) occurs by the direct transformation of mesenchymal cells into osteoblasts during embryonic development. This type of ossification is restricted to those of cranial vault, some facial bones, and parts of mandible and clavicle. The flat bones of the scull grow toward each other from primary ossification centers in each and meet at sutures. The center of a suture contains a proliferating cell population, whose progeny differentiate and move toward adjacent bone surfaces, becoming osteoblasts[46].

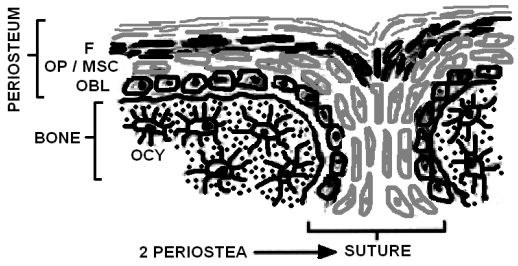

***Figure 5a*: Intramembranous bone formation.** *Cellular relationships in a periosteum and a suture. F, fibroblast; OP, osteoprogenitor cell; OBL, osteoblast; OCY, osteocyte.*
(from Marks and Odgren[46]*)*

Bones that participate in joints and bear weight are formed by endochondral ossification, which is presented in detail in Figure 5b. Endochondral bone formation is also a major factor in bone longitudinal growth, which is a precise balance between chondrocyte proliferation, cartilage matrix production, and mineralization, hypertrophy and vascular invasion[46]. Bone growth in diameter (central portion of the long cylindrical bones) is accomplished most basically by formation at periosteum (externally) and resorption at endosteum (internally, marrow site)[46].

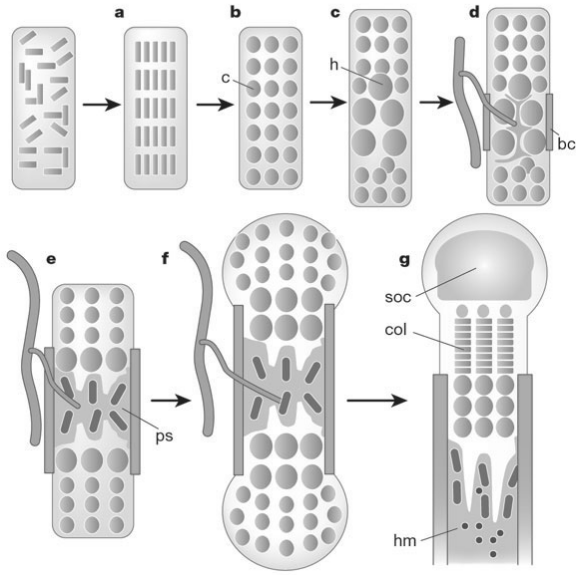

Figure 5b: Endochondral bone formation.
a - b: *Mesenchymal cells condense and become chondrocytes (c, C).* ***c****, C at the centre stop proliferating and become hypertrophic (h, H).* ***d****, Perichondrial cells adjacent to H become osteoblasts . H direct the formation of mineralized matrix, attract blood vessels, and undergo apoptosis.* ***e****, vascular invasion.* ***f****, C continue to proliferate, lengthening the bone. Osteoblasts of primary spongiosa are precursors of eventual trabecular bone; osteoblasts of bone collar become cortical bone.* ***g****, growth plate and secondary ossification center formation.*
bc: bone collar, ps: primary spongiosa, soc: secondary ossification center, col: columns of proliferating chondrocytes, hm: hematopoietic marrow (from Kronenberg H.M.[49]*)*

MSC differentiation to osteoblasts

Two main osteoblast-specific transcripts have been identified and often reported: one encoding Cbfa1 (Runx2), a transcription factor, and the other encoding osteocalcin, a secreted molecule that inhibits osteoblast function[46]. Cbfa1 expression precedes osteoblast differentiation and is restricted to mesenchymal cells destined to become either chondrocytes or osteoblasts, playing a role in endochondral bone formation. Subsequently, Cbfa1 expression becomes limited to the osteoblasts, with a lower level of expression in

hypertrophic chondrocytes. Other transcription factors may control osteoblast differentiation in a Cbfa1-dependent or -independent manner[46]. Cbfa1 binding sites are also present in the regulatory sequences of most genes required for the elaboration of a bone ECM. Interestingly, Cbfa1 gene deletion leads to an absence of osteoblasts and a skeleton made of cartilage, resulting from the incomplete terminal differentiation of chondrocytes (only partial hypertrophy)[48,49]. The existence of an even more specific osteoblast transcription factor has been reported recently: osterix (Osx) is a critical mediator of Cbfa1 action and its deletion leads to inhibition of osteoblastic differentiation without influence on chondrocyte differentiation and maturation[50].

Alkaline phosphatase (ALP) is an osteoblast-membrane bound enzyme expressed in proliferating preosteoblasts and is believed to play a role in mineralization process[43]. ALP activity increases at the beginning of differentiation and decreases when mineralization is well progressed[43]. The expression of the most frequently assayed osteoblast-associated genes, including collagen I (COLLI), alkaline phosphatase (ALP), osteopontin (OPN), osteocalcin (OCN), bone sialoprotein (BSP), and parathyroid hormone receptor (PTH1R), is up-regulated asynchronously, acquired, and/or lost as the progenitor cells differentiate and the matrix matures and mineralizes[3]. All these osteoblast specific markers are upregulated prior to the cessation of proliferation in osteoblast precursors except OCN, which is upregulated only in post-proliferative osteoblasts; in other words, differentiation is well progressed before osteoblast precursors leave the proliferative cycle[3]. Table 2 shows the time-profile of osteogenic marker expression from MSC to osteoblasts and of some differentiation-specific surface markers.

***Table 2**: Osteogenic differentiation from MSC to osteocyte with some differentiation markers.*

	MSC	immature osteoprogenitor	mature osteoprogenitor	preosteoblast	mature osteoblast	osteocyte	Publication
proliferation	++	++	++	+	-	-	
differentiation		+	++	+++	++++	++++	
Cbfa1		++			++		3
ALP (SB-1/RBM211.13/HOB-3)			+	++	+++		3,26
Collagen 1		+	++	++	++	-	3
Osteocalcin	-	-	-	-	+++	-	3
Bone sialoprotein	-	++	-	-/+++	-/+++	-/+++	3
Osteopontin	-/+	-/+	-/+	-/+	-/+++	-/+++	3
Osteonectin / SPARC				+	+		26
PTH/PTHrP-R				+++	+++	++	3
PTHrP				++	++	-	3
CD44				+	++	++	3
Galectin 3 / RCC 455.4				-/+	-/+++	-/+++	26
SH-2	+						26
SB-10	+	+	+				26
Stro-1			+	+	+		26
SB-20 / SB21			+	+	+		26
SB-3				+	+		26
SB-2					+		26
E11					+	+	26
SB-5						+	26

Legend: +: expressed, -: not expressed, + / - : variable data.

Regulation of bone function

Genetic-based studies have revealed how osteoblast differentiation is controlled through growth and transcription factors. In addition to autocrine, paracrine, and endocrine signals, cell-cell and cell-matrix interactions are also required for the development of osteoclasts and osteoblasts. Such interactions are mediated by proteins expressed on the surfaces of these cells and are responsible for the contact between osteoclast precursors with stromal / osteoblastic cells. Adhesion molecules are also involved in the migration of osteoblasts and osteoclast progenitors from the bone marrow to the sites of bone remodeling as well as the cellular polarization of osteoclasts and the initiation and cessation of osteoclastic bone resorption[43].

The only factors capable of initiating osteoblastogenesis from uncommitted progenitors are bone morphogenic proteins (BMP). BMP-2 and BMP-4 in particular stimulate the transcription of Cbfa1, which in turns activates other osteoblast specific genes. Other growth factors, like transforming growth factor beta (TGF-β), platelet derived growth factor

(PDGF), insulin-like growth factors (IGF) and members of fibroblast growth factors (FGF), can all stimulate cell replication and differentiation, but only in committed progenitor cells and not from uncommitted MSC[43,46]. Cytokines are known to play roles in hematopoietic cell development and osteoclastogenesis in the bone marrow. Therefore, they can also influence osteoblast differentiation and bone remodeling. This group includes interleukins (IL-1, IL-3, IL-6, IL-11), leukemia inhibitory factor (LIF), oncostatin (OSM), ciliary neurotropic factor (CNTF), tumor necrosis factor (TNF), granulocyte-macrophage colony stimulating factor (GM-CSF), M-CSF, and c-kit ligand. IL-6 is produced by the cells of stromal / osteoblastic lineage in response to stimulation by a variety of other cytokines and growth factors such as IL-1, TNF, TGF-β, PDGF, and IGF-II[43].

Two systemic hormones of the calcium homeostatic system, namely the parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3, calcitriol), can stimulate osteoclast formation. Calcitonin and thyroid stimulating hormone (TSH), on the other hand, inhibit osteoclast development and activity and promote osteoclast apoptosis. PTH, PTH-related peptide, and 1,25-(OH)2D3 stimulate the production of IL-6 and IL-11 by stromal / osteoblastic cells. Several other hormones, including estrogen, androgen, glucocorticoids, and thyroid hormone thyroxine, exert potent regulatory influences on the development of osteoclasts and osteoblasts by regulating the production and/or action of several cytokines[43]. Bone growth is principally guided already with growth hormone (GH), and also with some other proteins, like osteoprotegrin and leptin[43].

Bone Matrix

Collagens can be defined as "structural proteins of the extracellular matrix, which contain one or more domains harboring the conformation of a collagen triple helix"[51]. The triple helix motif is composed of three polypeptide chains, whose amino acid sequence consists of Gly-X-Y repeats. Due to this particular peptide sequence, each chain is coiled in a left-handed helix, and the three chains assemble in a right-handed triple helix, where Gly residues are in a center of the triple helix and the lateral chains of X and Y residues are on the surface of the helix[51]. In about 1/3 of the cases, X is a proline and Y is a hydroxyproline; the presence of hydroxyproline is essential to stabilize the triple helix and is a unique characteristic of collagen molecules[51]. Type I collegen is a fibrillar collagen and is a most abundant protein in vertebrates. It is present in many tissues, being a major constituent of bone, tendons, ligaments, and skin[51]. Type I collagen is secreted as a soluble propeptide, which after cleavage by specific proteases looses its solubility, and assembles

spontaneously to form fibrils[51]. Finally, cross-linking processes join the collagen molecules covalently. In fibrils, molecules of collagen are parallel to each other. Collagen-forming sheets are composed of Type IV and VIII collagen. Type IV collagen forms a complex three-dimensional network, which is the major component of basement membranes[51], an important component of MSC site of origin.

In bone, hydroxyapatite crystals seem to lie in the gaps between collagen molecules[51]. However, collagen may not be the direct nucleator of hydroxyapatite deposition, but rather serves as scaffolding upon which nucleators - noncollagenous proteins - are oriented[38]. There are a large number of matrix proteins that have been found to bind to collagen, thereby forming fibrils. They belong to the proteoglycans (glycosaminoglycans bound to core proteins) and glycoproteins (noncollagenous proteins). The most prominent nucleator of hydroxyapatite deposition is osteonectin, which has the ability to bind to calcium and collagen[38]. Its constitutive expression in adults is limited to cells associated with mineralized tissues, such as hypertrophic chondrocytes, osteoblasts, odontoblasts, and ion-transporting cells[38].

Fibronectin (FN) exists in a soluble form in micromolar concentrations in blood plasma and in an insoluble form in the ECM[52]. It is synthesized by many connective tissue cells and during early stages of bone formation with upregulation in the osteoblastic cell layer[38]. Circulating FN does not polymerize itself; FN assembly takes place at specialized areas on the cell surface involving integrins $\alpha5\beta1$ and $\alpha V\beta3$[52]. Besides the RGD cell adhesion site, FN has adhesion sites for fibrin, heparin, collagen, and the RGD-independent integrin $\alpha4\beta1$[38,52]. Another serum protein, vitronectin, is also found at low levels in mineralized matrix, though its receptor $\alpha V\beta3$ integrin seem to play an important role in bone biology. Several bone ECM proteins are characterized by the presence of relatively large amounts of carbohydrate sialic acid (SIBLINGs), with osteopontin and bone sialoprotein (BSP) being the best characterized[38]. Both have a high affinity for calcium ions, though only BSP stimulates hydroxyapatite deposition[38]. Osteopontin and BSP both mediate cell adhesion; however, in BSP sequences other than RGD show higher adhesion activity[38]. Osteopontin is produced at late stages of osteoblast maturation just prior to mineralization[38]. Also BSP expression is tightly associated with mineralization phenomena; it is found in low levels in hyperthropic chondrocytes, in osteoblasts at onset of matrix mineralization, and in osteoclasts[38].

TISSUE ENGINEERING

Tissue engineering is an interdisciplinary field which exploits living cells or biological substitutes in a variety of ways to restore, maintain, or enhance tissues and organs[53-56]. In the future, the engineered tissues could potentially not only replace donor tissues and organs, but could also accelerate the development of new drugs, offering complex *in vitro* test systems[53,57,58]. In reconstructive surgery, there is an increasing demand for replacement material to fill defects, especially in bone and cartilage. Usually implants made of synthetic polymers, ceramics, or metals, as well as tissue allografts, are used for tissue repair[57]. However, none of these materials can provide the performance of the original tissue. These artificial materials show different elastic properties, sometimes fracture, induce immunological responses, and are difficult to anchor. In contrast to vital tissues, they lack any process of regeneration. Thus, the challenging goal of tissue repair is the autologous growth or regeneration of the original tissue without remnants of foreign biomaterials[57].

To engineer living tissues *in vitro*, cells are cultured on bioactive and biodegradable scaffolds that provide the physical and chemical cues (e.g. cell adhesion sites, growth factors) to guide cell adhesion, differentiation and assembly into three dimensional (3-D) tissues[53]. The general tissue engineering principle is presented in Figure 6.

There are three principal strategies for treating diseased or injured tissues:

- Implantation of freshly isolated or cultured cells with or without biomaterial scaffold (cell therapy)[53,55]
- Implantation of tissues assembled *in vitro* from cells and scaffolds (3-D tissue is grown *in vitro* and, after reaching "maturity", implanted)[53,55,59]
- *In situ* tissue regeneration (scaffold is implanted directly into the injured tissue and stimulates the body's own cells to promote local tissue repair)[53,54]

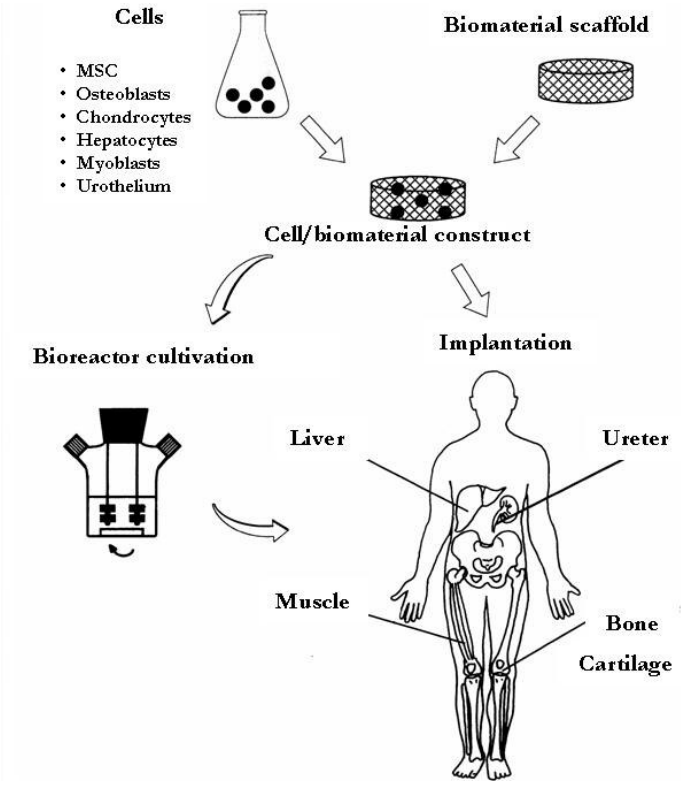

Figure 6: *Schematic representation of the general tissue engineering approach. Specific cell populations are harvested from the appropriate tissue and seeded on a biomaterial scaffold. The cell / biomaterial constructs may undergo a period of dynamic tissue culture in a bioreactor prior to implantation. Organized structural and functional tissues may be produced in this way (from Marter et al.[55])*

Cells for tissue engineering

Sources of cells for implantation include autologous cells from the patient or allogenic cells from a human donor who is not immunological identical to the patient[53,55,59]. Each category may be further delineated in terms of whether the cells are stem cells (adult or embryonic) or a mixture of differentiated cells at different stages of maturation (including stem and progenitor cells); some approaches use cell mixtures, whereas others rely on separation or the enrichment of distinct cell subtype, e.g. stem cells[53]. Cells are most often acquired by biopsy and therefore their initial number has to be increased substantially *in vitro*[57], which can bring additional complications.

Allogenic cells have been used successfully to treat skin ulcers, diabetes and liver disease; patients with diabetes or skin ulcers have been treated with two US Food and Drug Administration (FDA) approved living skin products engineered in the lab[53,59,60]. The FDA has also approved an autologous cell product for the repair of articular cartilage, derived from patients own chondrocytes[53,59,60]. Similar defects have already been healed using human MSC[53,54]. MSC offer another advantage over differentiated cells, as they are less prone to undergo dedifferentiation during culture, as is commonly observed in two-dimensional chondrocyte cultures[57]. Tissue engineers are working to develop several other tissues and organs: besides skin and cartilage, also bone, tendon, liver, bladder, cornea, esophagus, heart valves, etc. [53,59-62].

Scaffolds

Scaffolds are porous, ideally degradable structures fabricated from various materials, which can be divided in four groups: (1) biogenic (collagen, alginate, glycosaminoglycans, fibrin), (2) semisynthetic, (3) synthetic (polyglycolide (PGA), polylactide (PLA), polylactide coglycolide (PLGA)) and (4) inorganic (hydroxyapatite, tricalcium phosphate, titanium alloys)[53,58,59,62,63]. Scaffolds can be sponge-like sheets, gels or highly complex porous structures[53,59]. Materials utilized in tissue engineering have defined prerequisites, such as biocompatibility, sterilizability and the absence of toxicity or carcinogenicity[59]. In addition, their mechanical properties should include permeability, stability, elasticity, flexibility, plasticity, and resorbability at a rate congruent with tissue replacement[55,59,61,63]. However, there is practically no material corresponding to all of these demands. Depending on the tissue or organ to be restored, the optimal scaffold material is chosen.

Biomaterials can be manipulated to direct the growth of cells *in vitro* and *in vivo*[53,57]. The ideal biomaterial for a scaffold would selectively interact with the specific adhesion and growth factor receptors expressed by target cells required for the repair of damaged tissue[45,53]. This "smart" scaffold could guide migration of the target cells and stimulate their growth and differentiation and extracellular matrix production[53,59,64]. Biogenic materials may most closely simulate the native cellular milieu, but their limitations include large batch-to batch variations upon isolation from biological tissues, as well as restricted biomechanical properties, not to mention the risk of viral infection and antigenicity[55,64]. Synthetic matrices can be precisely manipulated with regard to physical and mechanical characteristics; variables such as porosity and degradation rate can be systematically regulated by altering either the material employed or polymer processing methods[55].

Controlling cell-biomaterial interactions

The fate of implants, from chronic inflammation or fibrous encapsulation to osseointegration or tissue regeneration, is determined by the response of cells to the material surface. In both cell culture and implantation, the initial presentation of foreign materials to cells is a layer of spontaneously adsorbed proteins, whose composition and bioactivity provide a biological interpretation of the material's underlying physiological properties[1,36]. Several characteristics of the substrate have been shown to influence protein adsorption. The amount and composition of adsorbed protein and its conformational state subsequently regulate cell adhesion and the expression of ECM binding receptors (integrin)[36,45].

Cell adhesion to the biomaterials in the presence of blood plasma is mostly mediated by vitronectin and fibronectin, although other proteins also adsorb to the surface (e.g. albumin and fibrinogen)[1]. Their adsorption reflects biomaterial surface hydrophilicity / hydrophobicity, surface charge, roughness, and chemistry. It is accompanied by the dehydration of protein and sorbent surfaces, the redistribution of charged groups in the interface and conformational changes in the protein molecule[1]. Independent of serum protein binding, the cells adhere better to hydrophilic surfaces, although proteins adsorb to hydrophilic, as well as to hydrophobic surfaces, resulting in different protein conformations[1]. Cell membranes adhere closely to positively charged surfaces, whereas contact occurs only at distinct points on near-neutral and negatively charged surfaces[1]. Most metals used for orthopedic implants exhibit a net negative charge at physiological pH (passivating oxide layer), as do ceramics such as hydroxyapatite and β-tricalcum phosphate (unsaturated oxygen bonds)[1]. Poly (D,L-lactic acid) also displays a negative zeta potential, although its hydrophobic character seems to dominate biological responses[1]. This numerous information show how nonspecific protein adsorption influences cell behavior and confirms the need to modulate the biomaterial to yield an optimal cell-construct performance *in vivo*.

For enhanced cell adhesion, polymer scaffold surfaces can be modified with collagen, fibronectin, or other adhesion-promoting proteins[36,55,63,64]. The discovery of adhesion domains in fibronectin and other ECM glycoproteins containing the amino acid sequence Arg-Gly-Asp (RGD) has enabled the design of synthetic materials that can modulate cell adhesion[45,53,65]. Covalently bound peptides, however, don't desorb from the surface, as often observed with classical surface coatings involving ECM proteins. Nevertheless, the optimal ligand density should allow for adequate cell adhesion, but not be too dense, in order to enable cell migration[53]. Though the biological activity of short peptides coated on substrates is often lower than that of the complete protein, some RGDs can interact with an inactive integrin, whereas binding to whole ECM usually requires an active integrin[36].

Ligands-antagonists for the cell adhesion receptors have been developed for the inhibition of platelet aggregation in restenosis (ligand for platelet integrin gpIIb-IIIa), neutrophils in sepsis (Mac-1), monocytes in stroke (LFA-1), asthma and multiple sclerosis (α4β1 integrin), endothelial and smooth muscle cells in tumor angiogenesis and restenosis (αVβ3 integrin) and others[37,66]. In tissue engineering the biomaterials are modified with RGD-like peptides and peptidomimetics to control the bioactivity of the materials[37]. The affinity of the RGD peptides for integrin receptors may be affected by the steric conformation of the peptide[37]. In linear peptides, the fourth amino acid alters the binding specificity, whereas cyclization of the peptide shows an increase in activity and a change in integrin specificity from fibronectin to vitronctin[37].

For some integrins, other peptide sequences may be more selective: for example, DGEA and GER are specific for integrin α2β1 and EILDV (or LDV) for α4β1 integrin[52,67]. All of these molecules have the potential to prevent cell adhesion when present in a soluble form, but when immobilized (covalently bound) to the surface of a biomaterial, this is a means to attach receptor-expressing cells. An extensive overview of the RGD peptides and peptidomimetics involved in bone biology has been written by Scaffner and Dard[37].

Bone Tissue Engineering

A loss of bone tissue can occur through infection, loss of blood supply, disease, or as a complication of fracture[55]. Currently, defects can be (1) permanently replaced with a foreign material or (2) restored using transplanted biological material, such as autograft or allograft bone[55]. Nevertheless, the autologous bone grafts, represent the "gold standard" for site specific bone defect repair[68]. However, this technique is associated with complications, such as donor site morbidity, infection, malformation, and subsequent loss of graft function[68].

The need for bone substitutes has led to the formulation of a number of biomaterials geared to functionally replace host bone tissue, as well as to compensate for the limitations imposed by natural bone replacements. These biomaterials include hydroxyapatite [$Ca_5(PO_4)_3OH$] and β-tricalcium phosphate (β-TCP) ceramics, alumina and titanium alloys, synthetic polymers consisting of polyglycolic and polylactic acid, and natural polymers, such as silk fibroin and demineralized bone composites[45,55,59,68]. Material properties including surface roughness, porosity, and cell-binding motifs can be manipulated to further optimize conventional biomaterials to allow for maximal cell attachment, proliferation, osteogenic differentiation, and ultimately mineralized matrix deposition[68]. One major limitation of the creation of a system for bone repair is the development of a scaffold with the requisite mechanical competence as well as porosity and resorbability[59]. On one hand, many biocompatible scaffolds utilized in soft tissue repair often lack the structural integrity required for load bearing, whereas on the other hand, the materials that are appropriately strong are often not resorbable and may involve stress shielding[59]. However, considering improved implant osseointegration, load bearing is often not a primary goal of implantation and therefore FDA-approved suture materials PLA and PLGA are frequently integrated into bone tissue engineering approaches[69]. In addition to suitable implant materials, bone tissue production can be enhanced with the supplementation of growth factors like BMP and other TGF-β family members, which play an important role in endochondral bone formation *in vivo*[56].

GOALS OF THE THESIS

Cell adhesion to the biomaterial surfaces can be directed by modifying the surface with cell adhesion promoting ECM proteins, or smaller peptides, like RGD[37,64]. Modification with synthetic peptides has advantages over the immobilization of natural-derived ECM molecules, because of a reduced risk of infection or immunoreactivity[64]. Cyclic RGD peptide [c(RGDfK)] was shown to be a selective peptide to bind integrins αVβ3 and αVβ5 and therefore its immobilization to the surface of biomaterials should enable adhesion of cells expressing high levels of these integrins, like osteoblasts[37]. Whereas human osteoblast showed RGD-dependent binding, rat bone marrow stromal cells, known to be osteoprogenitors, did not[70]. Therefore, the primary goal was to determine which integrins, and to which extent, are actually expressed on the rat marrow stromal cells. This led us first to characterize the primary cultures (up to 7 days in culture) of rat bone marrow cells adhered to the tissue culture polystyrene (TCPS). These cells are a commonly used source for tissue engineering studies. The characterization of their surface marker expression was performed by flow cytometry (FC, FACSCalibur, BD, USA). FC is a suitable method, as it enables the quantification of up to 6 parameters on a single cell basis in the shortest time. Usually at least 10000 cells are evaluated in each measurement.

The usual rat MSC enrichment protocols employing centrifugal bone marrow isolation from long bones, adherence for 3 days and removal of non-adherent cells following 1-week expansion of the adherent fraction showed that primary cultures are always "contaminated" with varying proportions of CD45-positive hematopoietic cells (HC). For the MSC characterization, a homogenous cell pool is needed. Therefore, in the first study (Chapter 2) we investigated the effects of varying adhesion times (1-4 days) in order to reduce the contamination with HC. In parallel, we assessed the yield of CD45-negative cells (MSC) resulting from the varied adhesion periods.

Knowing that hematopoietic stem cells and MSC share the bone marrow environment and that HC always contaminate the primary MSC cultures, we determined the role of HC in MSC differentiation. It is known that MSC support haematopoiesis, whereas only sparse data is available concerning the opposite interactions. It was shown that megakaryocytes and platelets support MSC proliferation; therefore, we investigated the influence of plastic-adherent HC on MSC osteogenic differentiation (Chapter 3). In cooperation with Nazish Ahmed and Susanne Grässel from the Experimental Orthopedics Department at the University Regensburg-Bad Abbach, the influence of HC on chondrogenic gene expression was also studied (Chapter 2).

In a third step, we characterized surface marker expression on MSC and the contaminating HC. Multi-color FC enabled us to determine integrin-pattern and stem cell marker expression on rat MSC and HC at the same time, without mechanical separation of both populations (Chapter 4). Human and mouse MSC surface marker expression has been already studied by several groups, but for the development of a rat test system (primary goal), integrin expression on rat MSC still needed to be determined. Additionally, MSC-selective integrins and surfaces for selective MSC surface immobilization are discussed in Chapter 4. In Chapter 5, we describe attempts to enhance integrin expression on rat MSC by supplementation with growth factors.

Since it is known that MSC change their phenotype after isolation and with extensive subculturing, we analyzed earlier cultures (day 3) and fresh bone marrow, as well as MSC that were expanded over several passages (Chapter 6). The data obtained could give some cues for further optimization of MSC selection and isolation.

Finally, some data on the effects of two statins (atorvastatin, simvaststin) in our culture systems are reported (Chapter 7). Statins are known as lipid-lowering drugs, but recent data on osteoinductive effects makes them potential candidates for improving bone tissue engineering. Therefore we investigated their dose-dependent effects on MSC differentiation.

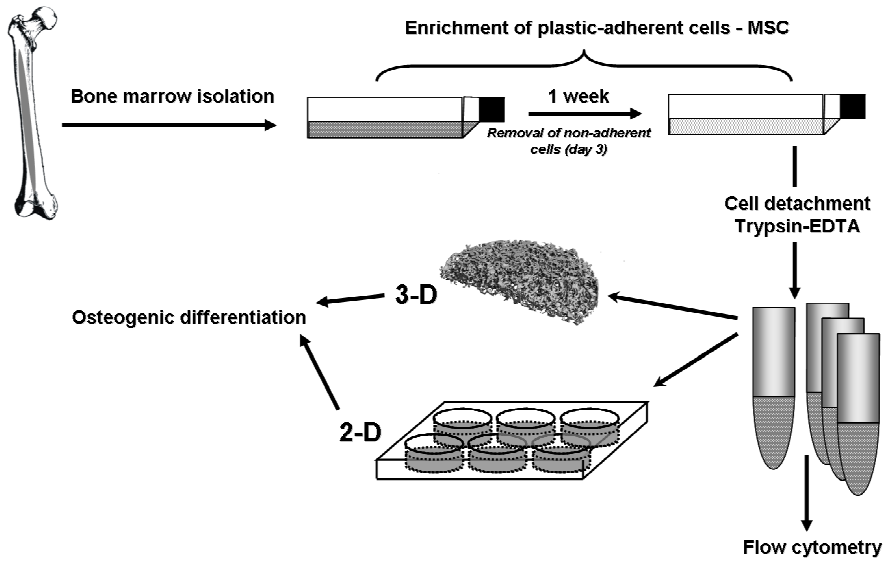

***Figure 7**: Practical approach of MSC enrichment, the characterization of surface marker expression and differentiation, which was used in experiments described in the thesis.*

REFERENCES

(1) *Wilson CJ, Clegg RE, Leavesley DI, Pearcy MJ. Mediation of biomaterial-cell interactions by adsorbed proteins: a review. Tissue Eng. 2005;11:1-18.*

(2) *Phinney D.G. Building a consensus regarding the nature and origin of mesenchymal stem cells. Journal of Cellular Biochemistry. 2002;38:7-12.*

(3) *Aubin J, Triffit JT. Mesenchymal Stem Cells and Osteoblast Differentiation. In: Bilezikian JB, Raisz LG, Rodan GA, eds. Principles of Bone Biology.1. San Diego;San Francisco;New York;Boston;London;Sydney;Tokyo: Academic Press; 2002:59-81.*

(4) *Barry F.P.and Murphy J.M. Mesenchymal stem cells: clinical applications and biological characterization. The International Journal of Biochemistry & Cell Biology. 2004;36:568-584.*

(5) *Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells. 2001;19:180-192.*

(6) *Caplan AI, Bruder SP. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. Trends Mol Med. 2001;7:259-264.*

(7) *Deans R.J. and Moseley A.B. Mesenchymal stem cells: Biology and potential clinical uses. Experimental Hematology. 2000;28:875-884.*

(8) *Ballas CB, Zielske SP, Gerson SL. Adult bone marrow stem cells for cell and gene therapies: implications for greater use 10. J Cell Biochem Suppl. 2002;38:20-28.*

(9) *Minguell JJ, Erices A, Conget P. Mesenchymal Stem Cells. Experimental Biology and Medicine. 2001;226:507-520.*

(10) *Pittenger MF, Mackay AM, Beck SC et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143-147.*

(11) *Baksh D, Song L, Tuan RS. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. J Cell Mol Med. 2004;8:301-316.*

(12) *Roufosse CA, Direkze NC, Otto WR, Wright NA. Circulating mesenchymal stem cells. The International Journal of Biochemistry & Cell Biology. 2004;36:585-597.*

(13) *Prockop D.J. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276, 71-74. 1997.*

(14) *Caplan AI, Reuben D, Haynesworth SE. Cell-based tissue engineering therapies: the influence of whole body physiology. Adv Drug Deliv Rev. 1998;33:3-14.*

(15) *Gregory CA, Prockop DJ, Spees JL. Non-hematopoietic bone marrow stem cells: Molecular control of expansion and differentiation. Experimental Cell Research. 2005;306:330-335.*

(16) *Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca RD et al. Multilineage Potential of Adult Human Mesenchymal Stem Cells . Science 284, 143-147. 1999.*

(17) *Owen M, Friedenstein AJ. Stromal stem cells: marrow-derived osteogenic precursors. Ciba Found Symp. 1988;136:42-60.*

(18) *Kuznetsov SA, Friedenstein AJ, Robey PG. Factors required for bone marrow stromal fibroblast colony formation in vitro. Br J Haematol. 1997;97:561-570.*

(19) *Muraglia A, Cancedda R, Quarto R. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci. 2000;113 (Pt 7):1161-1166.*

(20) *Barry FP, Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. The International Journal of Biochemistry & Cell Biology. 2004;36:568-584.*

(21) *Majumdar MK, Thiede MA, Mosca JD, Moorman M, Gerson SL. Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. J Cell Physiol. 1998;176:57-66.*

(22) *Deans RJ, Moseley AB. Mesenchymal stem cells: Biology and potential clinical uses. Experimental Hematology. 2000;28:875-884.*

(23) *Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res. 2004;95:9-20.*

(24) *Jiang Y, Jahagirdar BN, Reinhardt RL et al. Pluripotency of mesenchymal stem cells derived from adult marrow Nature. 2002;418:41-49.*

(25) *Young HE, Black AC, Jr. Adult stem cells. Anat Rec. 2004;276A:75-102.*

(26) *Bruder SP, Horowitz MC, Mosca JD, Haynesworth SE. Monoclonal antibodies reactive with human osteogenic cell surface antigens. Bone. 1997;21:225-235.*

(27) *Dennis JE, Charbordb P. Origin and Differentiation of Human and Murine Stroma. Stem Cells. 2002;20:205-214.*

(28) *Herzog EL, Chai L, Krause DS. Plasticity of marrow-derived stem cells. Blood. 2003;102:3483-3493.*

(29) *Javazon EH, Beggs KJ, Flake AW. Mesenchymal stem cells: paradoxes of passaging. Exp Hematol. 2004;32:414-425.*

(30) *Short B, Brouard N, Occhiodoro-Scott T, Ramakrishnan A, Simmons PJ. Mesenchymal stem cells. Arch Med Res. 2003;34:565-571.*

(31) *Kassem M, Kristiansen M, Abdallah BM. Mesenchymal stem cells: cell biology and potential use in therapy. Basic Clin Pharmacol Toxicol. 2004;95:209-214.*

(32) *Reyes-Botella C, Montes MJ, Vallecillo-Capilla MF, Olivares EG, Ruiz C. Antigenic phenotype of cultured human osteoblast-like cells. Cell Physiol Biochem. 2002;12:359-364.*

(33) *Huss R. Isolation of Primary and Immortalized CD34– Hematopoietic and Mesenchymal Stem Cells from Various Sources. Stem Cells. 2000;18:1-9.*

(34) *Giancotti FG, Ruoslahti E. Integrin signaling. Science. 1999;285:1028-1032.*

(35) *Hynes RO. Cell adhesion: old and new questions. Trends Cell Biol. 1999;9:M33-M37.*

(36) *Siebers MC, ter Brugge PJ, Walboomers XF, Jansen JA. Integrins as linker proteins between osteoblasts and bone replacing materials. A critical review. Biomaterials. 2005;26:137-146.*

(37) *Schaffner P, Dard MM. Structure and function of RGD peptides involved in bone biology. Cell Mol Life Sci. 2003;60:119-132.*

(38) Gehron Robey P. Bone Matrix Proteoglycans and Glycoproteins. In: Bilezikian JB, Raisz LG, Rodan GA, eds. Principles of Bone Biology.1. San Diego, San Francisco, New York, Boston, London, Sydney, Tokyo: Academic Press; 2002:225-238.

(39) Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell. 2002;110:673-687.

(40) Gronthos S, Simmons PJ, Graves SE, Robey PG. Integrin-mediated interactions between human bone marrow stromal precursor cells and the extracellular matrix. Bone. 2001;28:174-181.

(41) Majumdar MK, Keane-Moore M, Buyaner D et al. Characterization and functionality of cell surface molecules on human mesenchymal stem cells. J Biomed Sci. 2003;10:228-241.

(42) Asahara T, Kalka C, Isner JM. Stem cell therapy and gene transfer for regeneration. Gene Therapy. 2000;7:451-457.

(43) Manolagas S. Birth and Death of Bone Cells: Basic Regulatory Mechanisms and Implications for the Pathogenesis and Treatment of Osteoporosis. Endocrine Reviews. 1980;21:115-137.

(44) Bone Structure. http://training.seer.cancer.gov/module_anatomy/unit3_2_bone_tissue .html . 2006.

(45) Anselme K. Osteoblast adhesion on biomaterials. Biomaterials 21, 667-681. 2000.

(46) Marks SC, Odgren PR. Structure and Development of the Skeleton. In: Bilezikian JB, Raisz LG, Rodan GA, eds. Principles of Bone Boilogy.1. San Diego, San Francisco, New York, Boston, London, Sydney, Tokyo: Academic Press; 2002:3-15.

(47) Aubin JE. Osteoprogenitor cell frequency in rat bone marrow stromal populations: role for heterotypic cell-cell interactions in osteoblast differentiation. J Cell Biochem. 1999;72:396-410.

(48) Zelzer E, Olsen BR. The genetic basis for skeletal diseases. Nature. 2003;423:343-348.

(49) Kronenberg HM. Developmental regulation of the growth plate. Nature. 2003;423:332-336.

(50) Zelzer E, Olsen BR. The genetic basis for skeletal diseases. Nature. 2003;423:343-348.

(51) Rossert J, de Crombrugghe B. Type I Collagen: Structure, Synthesis, and Regulation. Principles of Bone Biology. 2002:189-210.

(52) Magnusson MK, Mosher DF. Fibronectin: structure, assembly, and cardiovascular implications. Arterioscler Thromb Vasc Biol. 1998;18:1363-1370.

(53) Griffith LG, Naughton G. Tissue engineering-current challanges and expanding opportunities. Science. 2002;295:1009-1016.

(54) Service RF. Tissue Engineers Build New Bone. Science. 2000;289:1498-1500.

(55) Marler JJ, Upton J, Langer R, Vacanti JP. Transplantation of cells in matrices for tissue regeneration. Adv Drug Deliv Rev. 1998;33:165-182.

(56) Ripamonti U, Tasker JR. Advances in Biotechnology for Tissue Engineering of Bone. Curernt Pharmaceutical Biotechnology. 2000;1:47-55.

(57) Sittinger M, Bujila J, Rotter N et al. Tissue engineering and autologous transplant formation: practical approaches with resorbable biomaterials and new cell culture techniques. Biomaterials. 1996;17:237-242.

(58) Tabata Y. The importance of drug delivery systems in tissue engineering. PSTT. 2000;3:80-87.

(59) Knight MA, Evans GR. Tissue engineering: progress and challenges. Plast Reconstr Surg. 2004;114:26E-37E.

(60) Ferber D. TISSUE ENGINEERING:From the Lab to the Clinic. Science. 1999;284:423.

(61) Ferber D. TISSUE ENGINEERING:Lab-Grown Organs Begin to Take Shape. Science. 1999;284:422-425.

(62) Terada S, Sato M, Sevy A, Vacanti JP. Tissue Engineering in the Twenty-First Century. Yonsei Medical Journal. 2000;41:685-691.

(63) Hwal S. Tissue Restoration, Tissue Engineering and Regenerative Medicine. Yonsei Medical Journal. 2000;41:681-684.

(64) Rosso F, Marino G, Giordano A, Barbarisi M, Parmeggiani D, Barbarisi A. Smart Materials as Scaffolds for Tissue Engineering. J.Cell.Physiol. 203, 465-470. 2005.

(65) Puleo DA, Nanci A. Understanding and controlling the bone-implant interface. Biomaterials. 1999;20:2311-2321.

(66) Buckley CD, Simmons DL. Cell adhesion: a new target for therapy. Mol Med Today. 1997;3:449-456.

(67) Horton MA, Nesbitt SA, Bennett JH, Stenbeck G. Integrins and Other Cell Surface Attachment Molecules of Bone Cells. In: Bilezikian JB, Raisz LG, Rodan GA, eds. Principles of Bone Biology. San Diego, San Francisco, New York, Boston, London, Sydney, Tokio: Academic Press; 2002:265-286.

(68) Mauney JR, Volloch V, Kaplan DL. Role of adult mesenchymal stem cells in bone tissue engineering applications: current status and future prospects. Tissue Eng. 2005;11:787-802.

(69) Crane GM, Ishaug SL, Mikos AG. Bone Tissue Engineering. Nature Medicine 1, 1322-1324. 1995.

(70) Lieb E, Hacker M, Tessmar J et al. Mediating specific cell adhesion to low-adhesive diblock copolymers by instant modification with cyclic RGD peptides. Biomaterials. 2005;26:2333-2341.

CHAPTER 2

CD 45-POSITIVE CELLS OF HEMATOPOIETIC ORIGIN ENHANCE CHONDROGENIC MARKER GENE EXPRESSION IN RAT MARROW STROMAL CELLS

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ABSTRACT

Adult mesenchymal stem cells (MSC) can be readily isolated from bone marrow, expanded in culture and subsequently subjected to differentiation into various connective tissue lineages. In general, for animal studies MSC separation from other bone marrow derived cells is achieved by sole adherence to plastic surfaces of tissue culture flasks, however, this procedure produces a heterogeneous cell population including CD45-positive hematopoietic cells (HC) and hematopoietic stem cells (HSC). It is well known, that such mixed cell cultures consisting of cocultur differentiated somatic cells with adult stem cells promote differentiation towards specifi lineages. For determining the effect of the CD45-positive cell population on the differentiation potential of MSC we have sorted out the bone marrow derived adherent cells by immunomagnetic technique (MACS™) to attain a subpopulation of CD45-depleted cells. Here, we show that the presence of adherent CD45-positive HC not only promote expression of the chondrogenic marker genes *Col2a1*, *COMP* and *SOX9,* but also of *Col1a1, Col10a1* and to a certain degree *Cbfa1* in MSC when cultured in an appropriate three-dimensional environment. These observations constitute a step towards unravelling the influence of hematopoietic cells on chondrogenic differentiation of MSC.

INTRODUCTION

Adult bone marrow stromal cells regarded as mesenchymal stem cells are progenitors of connective tissue cells, thus are excellent candidates for chondrogenic differentiation studies. The progeny of adult stem cells includes both daughter stem cells and committed progenitors with a more restricted differentiation potential. These progenitors in turn give rise to differentiated cell types. Mesenchymal stem cells (MSC) can differentiate under specific cultural and physical conditions into multiple mesenchymal lineages namely, osteocytes, chondrocytes, adipocytes, astrocytes and myocytes[1,2]. Bone marrow consists of primarily non-adherent hematopoietic cells and hematopoietic stem cells (99%) along with a minor population of MSC (≤1 %). Both of the stem cell types are known to co-exist and have been suggested to cooperate in one another's differentiation[3]. The role of MSC in hematopoietic microenvironment formation is beginning to unravel and it is assumed that the presence of adherent MSC and their progeny facilitates HSC differentiation into granulocytes and erythrocytes both *in vivo* and *in vitro*[4-6]. Conversely, in depth studies concerning the role of HSC in MSC commitment and differentiation towards a distinct lineage are lacking.

Adult rat MSC are routinely isolated from tibio-femoral bone marrow by relying solely on their adherence to the plastic surface of tissue culture flasks[7,8]. The isolated cells constitute a heterogeneous population which always contains HC therefore; obtaining a pure population of non-hematopoietic cells remains difficult. This hematopoietic cell fraction varies depending on the species; being relatively high in initial cultures of mouse marrow cells[9] and less than 30% in human cell cultures. In rat marrow stromal cells this trend has not been studied thoroughly, although the presence of hematopoietic cells in primary (7 days) and secondary (18 days) bone marrow cultures has been indicated[10]. Human and murine MSC studies show subsequent loss of hematopoietic cell surface markers when cultures are maintained for 2 or 3 weeks. Prior to differentiation experiments the hematopoietic cell population is often separated from MSC by employing a magnetic associated cell sorting (MACS) system. The system immunologically separates different cell types by labelling cell surface antigens with magnetic beads followed by sorting through a magnetic column. As a unique identifying marker for MSC is still lacking a negative selection protocol is carried out using CD34 (My10) or CD45 (leukocyte common antigen) for exclusion of undesired positively labelled hematopoietic cells.

One of the major differentiation pathways of MSC occurs along the chondrogenic lineage which can happen autonomously in three separate mesenchymal cell lines, cranial neural crest, sclerotome cells and lateral plate mesoderm cells[11]. The first stage of

chondrogenic differentiation is conversion of undifferentiated MSC to committed osteochondroprogenitor cells leading to cell condensation and growth arrest. The major proteins involved in condensation initiation are either fibronectin or belong to the transforming growth factor ß family of proteins (TGFß-1, -2 and -3). Cells present at the centre of the condensation nodules first form pre-chondrocytes and then chondrocytes which start to produce cartilage matrix marked by up regulation of structural protein genes as *Col2a1, Col9a2*, *Col11a2, aggrecan* and *COMP*. Expression of chondrocyte marker genes is controlled by members of the Sox-family, in particular Sox9 has been characterized as a master transcription factor with essential direct or indirect regulatory effects exerted along the entire chondrogenic differentiation pathway. It is expressed in all chondroprogenitor cells and also in differentiated chondrocytes[12-15] .

In this study we have evaluated the degree of hematopoietic cell coexistence in primary cultures of rat MSC and their influence on the chondrogenic differentiation potential of MSC. We have determined the difference in chondrogenic marker gene expression of heterogeneous MSC isolated by sole adherence to plastic surfaces compared to that of a more homogeneous subpopulation of MACS sorted CD45-depleted cells.

MATERIALS AND METHODS

MSC isolation and primary cell culture

A modified version of the MSC isolation procedure from rat bone marrow was optimized[16,17]. Briefly, 6 week old male Sprague - Dawley rats were killed under CO_2 and tibiae and femora were aseptically removed. The bones were cut from the middle and centrifuged in a 1.5 ml tube at 2000 rpm for 3 min (mini centrifuge - Eppendorf). The centrifuged bone marrow cells were collected and homogenized with 18 g, 21 g and 23 g needles and seeded at the rate of 2.5 x 10^5 cells / cm^2 in 175 cm² tissue culture flasks in proliferation medium containing 5 % glutamate, 1 % antibiotics/antimycotics (Gibco.) and 10 % FBS (Gibco, Invitrogen, UK; Lot No.: 40F7430K) in α-MEM (Sigma Aldrich, Germany).. All non-adherent cells were removed on the 3rd day and the adherent cells were proliferated.

Magnetic separation of cells (MACS)

When the flasks were ~75 % confluent (9 - 11 days) cells were harvested with trypsin digestion. Separation procedure was carried out according to the manufactures instructions; in short, cells were incubated with 4 µl of CD45 antibody / 10^6cells for 5 min at 37°C followed by washing and a second incubation with goat anti-mouse secondary antibody coupled with magnetic beads (Miltenyi Biotech) for 15 min at 4°C. The suspension was passed through a magnetic column (Miltenyi Biotec´s LS-MACS columns); while labelled cells got coupled to the magnetic field the flow through containing unlabelled CD45-negative fraction was collected.

Immunofluorescence

20,000 cells / chamber were seeded on chamber slides (Biocoat slides, BD, Bioscience) and incubated overnight at 37°C and 5 % CO_2. Cells were blocked for 1 h at 37°C in Complete Mini 1:5 (Roche, Germany) plus 5 % normal goat serum and 1 % BSA in PBS. After washing with PBS cells were stained with monoclonal antibodies directed against CD45 (Cb1 1502, Chemicon), CD68 and D7Fib (Acris, Germany) in 1:50 dilution ratio overnight at 4°C. The appropriate Alexa568 or Alexa488 conjugated secondary antibodies (goat anti mouse, 5 µg / ml; Molecular Probes, US) were added for 1 h at RT. After washing slides were permanently mounted with DAKO fluorescent mounting medium (DAKO, USA) and covered with coverslips. Slides were evaluated with scanning laser microscopy (C1 confocal microscope from Nikon) and photos were taken with a Nikon C4 camera and software.

Flow cytometric analysis

Cells obtained from 1 animal (approx. 8x 10^7 cells, as determined after NH_4Cl lysis of the erythrocytes) were seeded in 4 x 150cm^2 flasks (1.5 x 10^5 cells / cm^2). The cells were left to adhere 1, 2, 3 or 4 days before non adherent cells were removed. Cells were trypsinized on day 7 after the isolation and resuspended in PBS. The cell suspension was first blocked for 15 min at 4°C with sheep serum followed by incubation with antibodies against rat CD45-PE (mouse IgG1 against rat CD45, Acris Antibodies, Germany), CD29-FITC (Hamster IgM against rat CD29, BD Pharmingen, USA) and with corresponding isotype controls for 30min at 4°C. Cells were washed with PBS containing sodium azide and bovine serum albumin and resuspended. Shortly before the FACS – analysis propidium iodide was added to each sample in order to discriminate the dead cells which were excluded from the evaluation. All measurements were performed on a FACSCalibur® instrument (Becton Dickenson, USA) with CellQuest data acquisition software (Becton Dickenson, USA). Data analysis was preformed with WinMDI 2.8 software (free access on http://facs.scripps.edu/software.html). A minimum of 1x10^4 viable cells was acquired per data set.

Chondrogenic differentiation

A batch of adherent cells (undepleted) was subjected directly to chondrogenesis favourable condition, the other batch was depleted of CD45-positive cells by MACS prior to chondrogenic differentiation experiments. For chondrogenic differentiation, high density 3-D cultures were attained by preparing cell-alginate amalgam in 1.2 % alginate at a concentration of 10^7 cells / ml. 2 - 3 mm cell-alginate beads containing approx. 10^5 cells / bead, were formed by dropping the suspension into 10^2 mM $CaCl_2$ solution. The culture was carried out in 12 well tissue culture plates for 4 weeks; medium was changed every 2 - 3 days. 10 alginate beads were removed every 7th day for RNA isolation and gene expression analysis. To release the cells from alginate the beads were incubated for 30 min at 37°C on a shaker in 55 mM sodium citrate and 0.15 M sodium chloride buffer; cells were recovered after 3 min spin at 750 g. The chondrogenic medium contained; ITS+ premix (6.25 µg / ml insulin, 6.25 ng / ml selenium acid, 6.25 µg / ml transferrin, 1.25 mg / ml BSA and 5.35 µg / ml linoleic acid. BD Biosciences, USA.), 110 µg / ml pyruvate, 40 µg / ml proline, 0.1 µM dexamethasone, 50 µg / ml ascorbic acid and 10 ng / ml TGFß-3[18] (R&D Systems) in α MEM high glucose (Gibco). Alginate sodium salts were acquired from Sigma Aldrich, Germany (Cat# A0682-100G).

RNA Isolation and reverse transcription

RNA was isolated by an affinity column chromatography method with Ambion's RNAqueous4-PCR kit according to the manufacturer's protocol. For removal of possible DNA contamination, isolated RNA was incubated for 1 h at 37°C in 2 Units of DNaseI enzyme (DNA-free, Ambion). RNA integrity was determined on 0.8 % Agarose-formaldehyde gels. The RiboGreen RNA quantification kit (Molecular Probes) was used for determination of RNA concentration at 585 nm wavelength. For RNA conversion to cDNA, Invitrogen´s SuperScript II reverse transcriptase kit was used with 0.5-1 µg of RNA in 20 µl of total reaction volume in presence of 40 units / µl recombinant ribonuclease inhibitor (RNase OUT®). Reverse transcription was carried out with 500 µg / ml of Oligo-dT primers, 10 mM dNTPs and 200 units of SuperScriptII enzyme in First-Strand Buffer and 0.1 M DTT for 50 min at 42°C followed by an extension period of 15 min at 70°C.

Relative quantitative PCR

Relative quantitative PCR was carried out with SYBR Green Dye I on ABI 7000 Prism Sequence detection system (Applied Biosystems) according to manufacturer's instructions. Briefly, 1 µl of cDNA was amplified in 50 µl final volume of 0.2 µM of each primer suspended in SYBR green master mix (AB Systems).Amplification parameters were same for all primer pairs and were repeated for 40 cycles, denaturation at 95°C for 0.15 min and annealing at 60°C for 1min. Mean relative quantification (RQ) value evaluated from three independent experiments was plotted on a semi logarithmic graph. RQ values were calculated by the software "RQ study application v1.1" (ABI Prism 7000 SDS software v1.1) according to the ΔΔCt method using ß-Actin as endogenous control and undifferentiated MSC (day 0) as calibrator. Primers were designed with either Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) or "Primer Express" software supplied by AB. All primers were manufactured at MWG – Biotech and are listed in Table 1. Prior to RQ studies, a CT value (cycle threshold) standard curve, with 10 folds diluted cDNA, was plotted to determine PCR efficiency of each primer pair only 90-100 % efficient primers were used. The efficiency was determined with 10(1/-S) –1, where S is the slope of the curve[19].

Statistical Analysis

One way ANOVA and student t test were employed for quantitative PCR experiments performed in triplicate. Results obtained by flow cytometry are the average of 4 independent experiments ± standard error of mean. The level of significance was determined by Tukey test.

Table 1: *Primers used for quantitative PCR*

Gene / RefSeq#	Amplicon (bp)	Primer (5´ - 3´)
ß-Actin	104	forward -GTAGCCATCCAGGCTGTGTT-3´
NM_031144		reverse -CTGTAGGTGAATCGACTGTTGC-3´
Col1a1	59	forward -TCCAGGGCTCCAACGAGA-3´
RGD61817*		reverse -CTGTAGGTGAATCGACTGTTGC-3´
Col2a1	60	forward -CCCCTGCAGTACATGCGG-3´
NM_012929		reverse -CTCGACGTCATGCTGTCTCAAG-3´
Col10a1	247	forward -CCC TAT TGG ACC ACC AGG TA -3´
AJ131848*		reverse -TCT CTG TCC GCT CTT TGT GA -3´
COMP	167	forward -TGACTTCGATGCTGACAAGG-3´
NM_012834		reverse -GAACGATCTCCATTCCCTGA-3´
Sox9	140	forward -CTGAAGGGCTACGACTGGAC-3´
XM_343981		reverse -TACTGGTCTGCCAGCTTCCT-3´
Cbfa1	86	forward -GGCCGGGAATGATGAGAACTA-3´
XM_34016		reverse -AGATCGTTGAACCTGGCCACT-3´

*Locus ID

RESULTS

MSC isolation procedure and CD expression profile

To determine the influence of adherence time of rat bone marrow cells on the presence of CD45-positive hematopoietic cells we left the cells to adhere for 3, 4 and 5 days before non-adherent and weakly attached cells were removed. Upon immunofluorescence analysis the adherent cells showed a D7fib+, CD68+ and CD45+/low expression profile regardless of the time of removal of non-adherent cells. Figure 1 shows the representative immunomicrographs of the cells left to adhere for 3 days and compares the staining of non-sorted and MACS- sorted cells after the first passage. Non-sorted cells, left for 3 days to adhere before medium removal, are contaminated with CD45-positive cells (Fig.1A-C). After magnetic sorting, most of the CD45-positive cells were excluded, leaving a CD45-depleted cell fraction (Fig.1D-F). However, we still observe few rounded cells among the CD68 positively stained fraction (Fig1D) of unknown surface antigen characterization. The CD45-positive cell fraction includes cells which stain for CD68 and D7Fib (Fig.1G-I).

Quantification of CD45-positive cell contamination of primary MSC cultures

In order to quantitate the ratio between hematopoietic CD45-positive cells and MSC we employed three colour flow cytometry (FACS) which allowed for a distinction between the two different cell types according to their surface antigens (CD45, CD29) and dead cells (Fig.2). Figure 2b shows that the fraction of CD45-positive hematopoietic cells was not influenced by the adherence time after cell isolation. We mostly found about 20-30 % of the attached cells in bone marrow cell cultures to be CD45-positive when selected according to sole adherence to plastic surfaces. Flow cytometric results were concurrent with the observations made after immunofluorescent staining. In one out of four experiments we detected variations between 10 and 40 % in individual flasks.

We observed that CD45-depleted cells expressed CD29 stronger than CD45-positive cells (Fig.2a). In cultures where non-adherent cells were removed on days 3 and 4, we obtained a significantly increased number of desired CD45-depleted MSC compared to removal on day 1 and 2 (Fig.2c). Apparently, 3 to 4 days were a more appropriate duration for the adherent fraction to get acclimated to the cell culture conditions, hence exhibit a firmer attachment to the cell culture plastics.

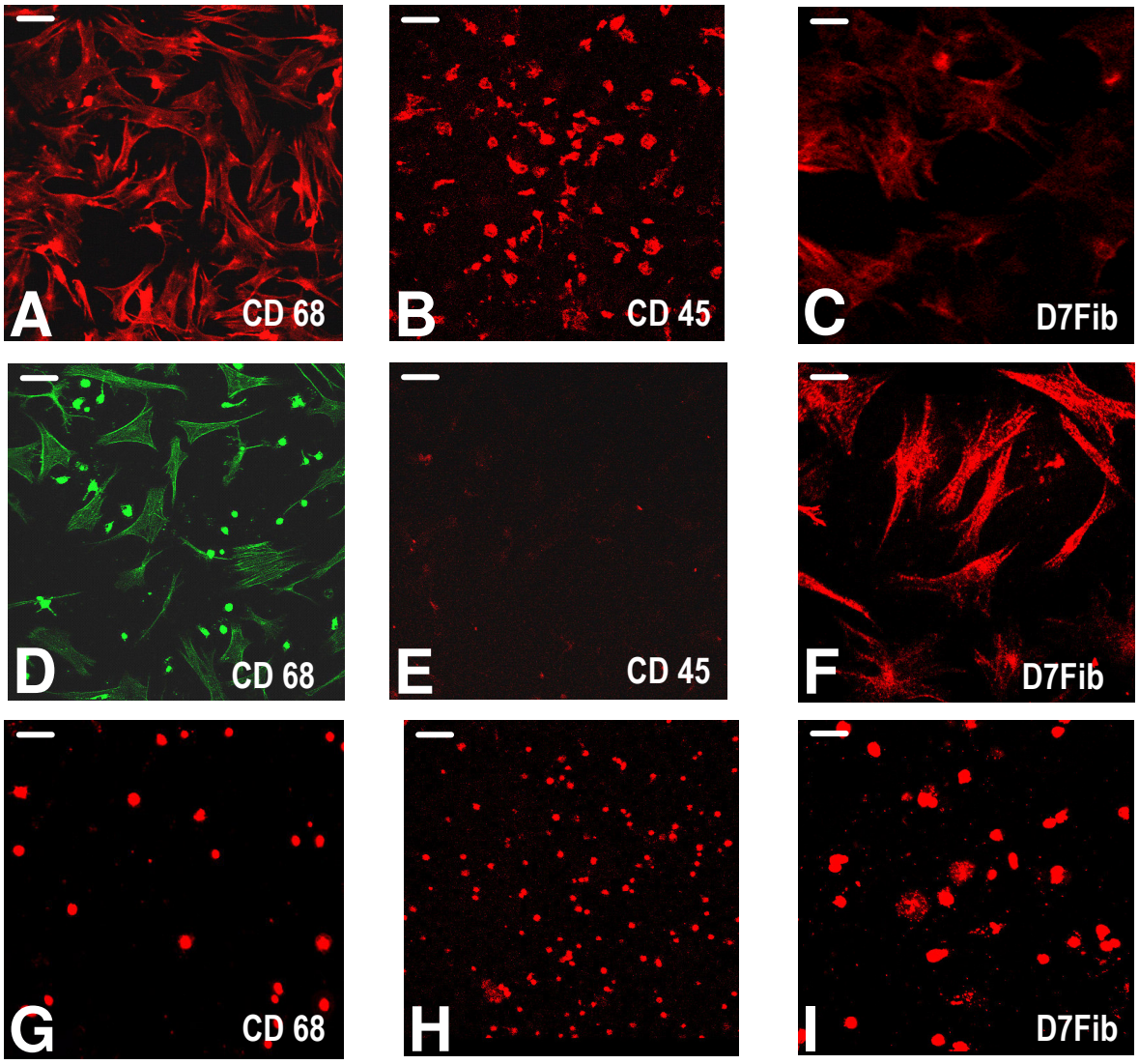

Figure 1: Morphological characterization of MSC
Immunofluorescence imaging of heterogeneous adherent MSC (A, B, C) and CD45-depleted MACS sorted cells (D, E, F), showing typical fibroblast like spindle shaped cells, while CD45-positive cells (G, H, I) are small and rounded in appearance. All 3 cell types are CD68- and D7fib- positive while the CD45-depleted cell population does not exhibit CD45 harbouring cells (E).
Magnification is 20x for all panels, bars are 10 µm.

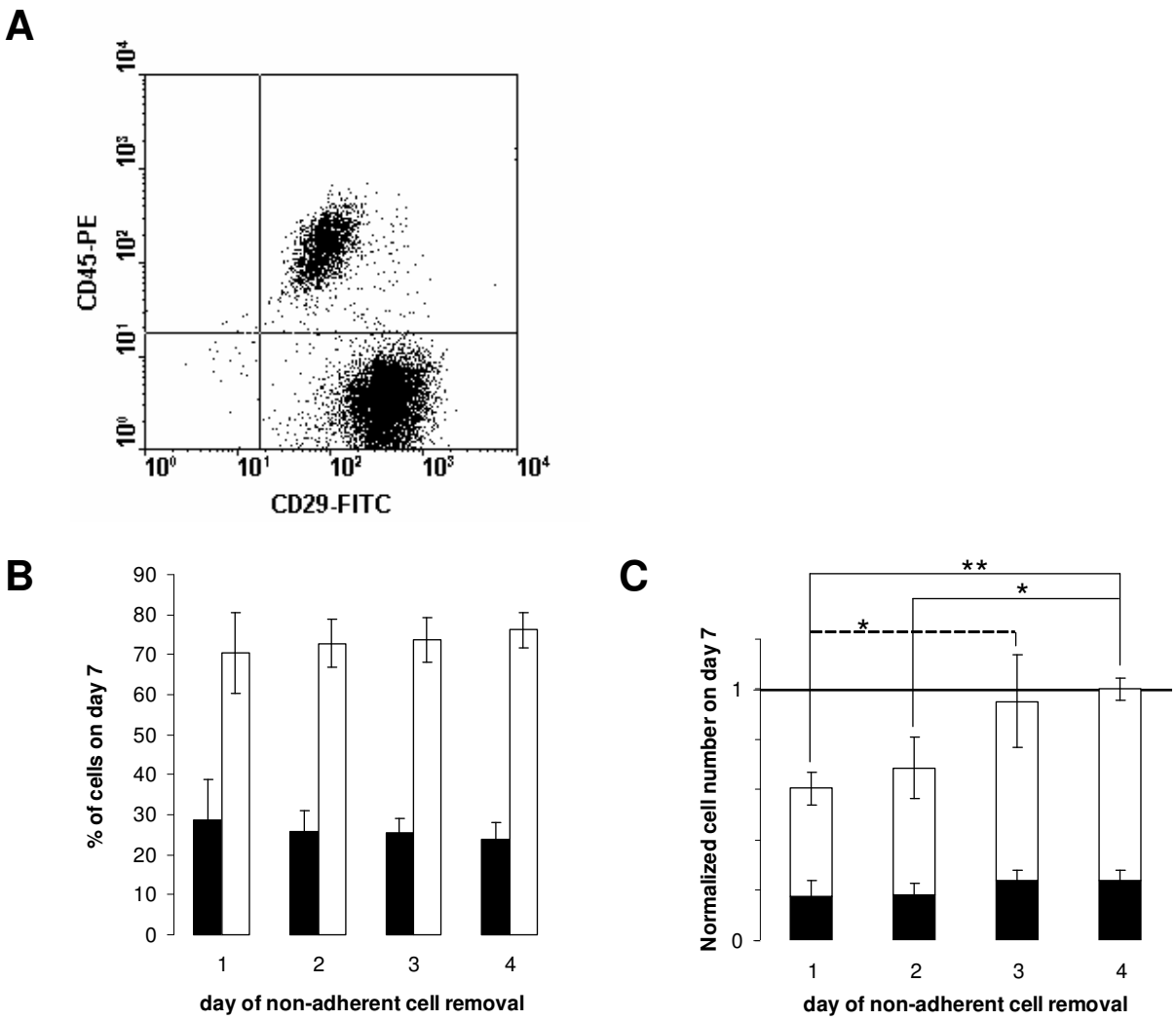

Figure 2: FACS analysis of bone marrow derived stem cells prepared by adherence to plastic

(A) Representative dot-plot (out of 3 experiments) of rat MSC isolated by adherence to plastic after 7 days of cell culture. The lower right quadrant contains CD29 –highly positive and CD45-depleted MSC while the upper right quadrant contains HC with a CD45 – positive and CD29-low expression profile.

(B) Proportion of CD45-positive (black bars) and CD45-depleted (open bars) cells from rat MSC isolated by adherence to plastic in relation to the time of the first medium change and therefore, removal of non-adherent cells.

*(C) Total yield of CD45-depleted (open bars) and CD45-positive (black bars) bone marrow derived cells after 7 days of cell culture (*p< 0,05; **p< 0,01). Yield of cells is normalized to cell number achieved when the non-adherent cells were removed on day 4.*

Effect of CD45-positive cells on gene expression levels of Col1a1, Col2a1, Col10a1, COMP, Sox9 *and* Cbfa1

During the time course of cell culture we observed a strong increase in relative mRNA levels of COMP and collagen II, while Sox9 mRNA levels exhibited a moderate up regulation (Fig.3A, B, D). Collagen X gene expression remained undetectable the first three weeks of cell culture, however, at day 28 expression of this collagen was found to be induced (Fig.3C). The internal control gene, ß-Actin, remained unaltered during the time course of cell culturing (Fig. 3G). Comparing gene expression of CD45-depleted cells and the non-sorted population, we found collagen I gene expression to be increased in the non-sorted cell population during the culture period, while it became decreased in CD45-depleted MSC. However, in CD45-depleted cells collagen I was still expressed at a relative higher level as in freshly isolated hip articular chondrocytes (Fig.3E). Notably, the non-sorted cells displayed a strikingly higher up regulation of collagen II, COMP and Sox9 gene expression compared to the CD45-depleted MSC throughout the culture period. In the non-sorted population collagen II gene expression is up regulated at average about 25 fold, COMP gene expression about 10 fold, collagen X expression about 23 fold and Sox9 mRNA levels about 3 fold if compared to CD45-depleted MSC. For collagen I gene expression we have observed a significant difference only between the two groups at day 14 and 28, where the adherent MSC demonstrated an up regulation resulting in a 3-5 fold higher gene expression if compared to CD45-depleted MSC. Collagen I remained up regulated from day 14 on in adherent cells while CD45-depleted MSC exhibit an increasingly down regulation during the time course of cell culture. We also observed a striking difference in the collagen X expression pattern between non-sorted and CD45-depleted cells. The collagen X mRNA expression profile of CD45-depleted cells experienced only a moderate increase of about 3.3 fold at the end of the culture period while adherent cells up regulated their mRNA level about 76 fold compared to day 0. Notably, Cbfa1 gene expression is differentially regulated with respect to the two cell populations (Fig.3F). On day 1 Cbfa1 mRNA level is significantly higher in adherent cells (4.7 fold) and also in chondrocytes (ca. 2.0 fold) compared to CD45-depleted cells. This pattern became inverted at day 28 with CD45-depleted cells exhibiting an elevated expression (1.7 fold) compared to the adherent MSC. In between these time points, relative gene expression of Cbfa1 is similarly regulated in both cell populations. In remixed control cells (30 % CD45-positive cells with 70 % CD45-depleted cells) collagen II, X, I, Sox9 and Cbfa1 gene expression profile resembles closely that of adherent cells (Fig.3B, C, D, E, F) and exhibits no significant differences. Only COMP gene expression at day 14 of the culture period significantly differs from adherent cells but the level at

day 21 and 28 shows no difference to non-sorted cells (Fig. 3A). ß-actin gene expression profile is similar for all three culture conditions (Fig.3G).

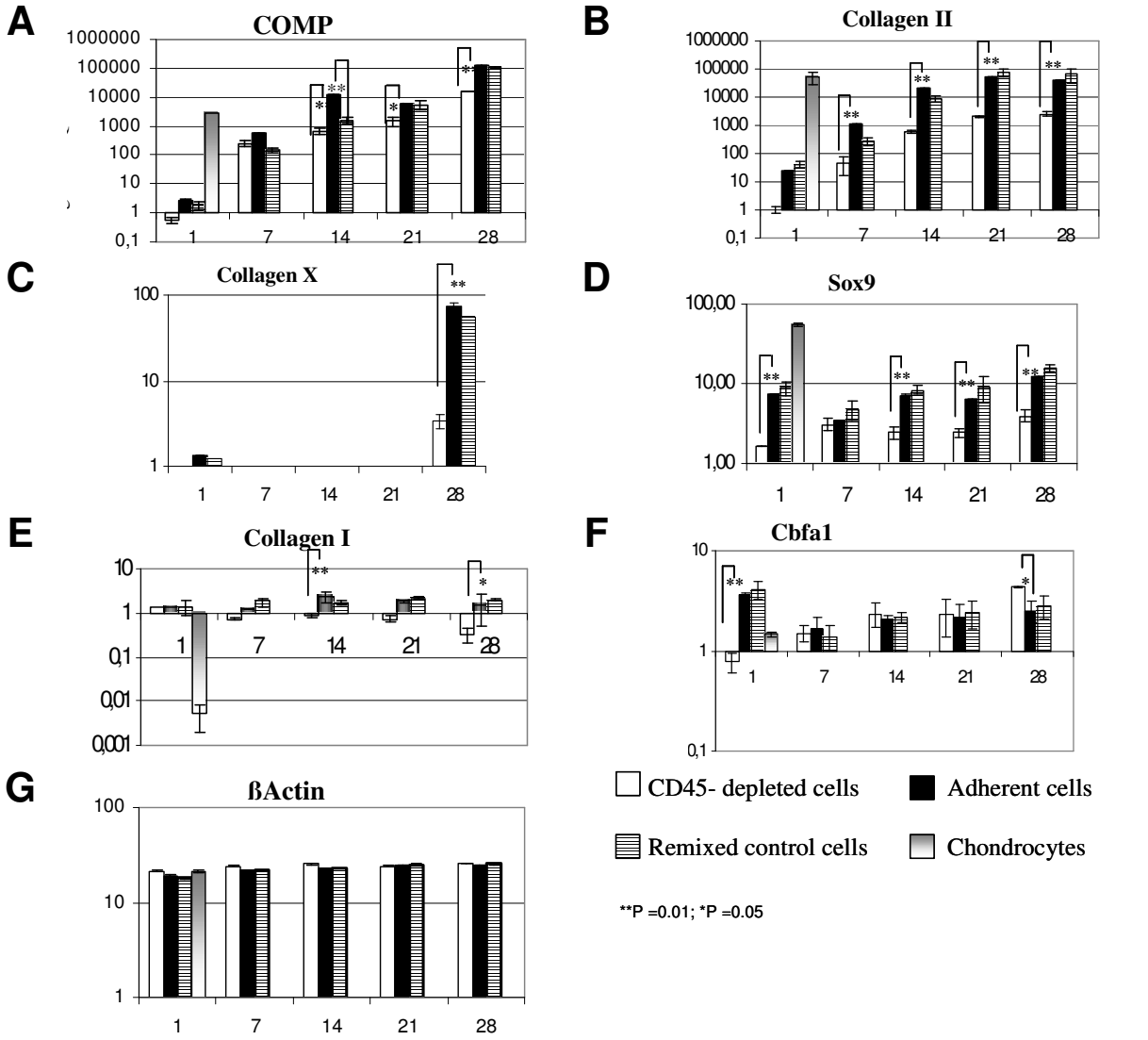

Figure 3: Chondrogenic gene expression profile of adherent MSC, CD45-depleted MSC and remixed control cells.

Relative quantitative mRNA expression level of chondrogenesis specific genes as COMP (A) Col2a1(B) and Col10a1 (C), Sox9 (D), Col1a1(E), and Cbfa1 (F) was determined by employing the ΔΔCT method using SYBR I Green dye. Expression level of housekeeping gene, ß-actin was determined as a controlling indicator of cell viability (G) and is expressed as positive logarithm of CT-values.

Expression level of each gene was compared between adherent (black bars), CD45-depleted (open bars) and remixed control cells (striped bars). Grey shaded bars indicate relative gene expression level of freshly isolated hip articular chondrocytes.

DISCUSSION

Adult bone marrow is the major source of hematopoietic stem cells and mesenchymal stem cells[20]. In animal studies, adherence to tissue culture plastic is accepted as a satisfactory parameter for attaining marrow stromal cell population depleted of hematopoietic cells. However, when employing FACS analysis for surface antigen characterization we observed a contamination of these non-sorted MSC with 20-30% of CD45-positive cells. For additional characterization of sorted and non-sorted cell population we applied immunofluorescence staining for CD68 and D7Fib. The D7Fib antigen constitutes a fibroblast specific molecule of yet unknown function, supposedly a specific marker for MSC as suitable as Stro-1 and CD105[21]. We observed expression of the D7Fib antigen on adherent CD45-positive cells; however, these cells displayed a rounded morphology compared to the fibroblast-like CD45-depleted cells. CD68 and its murine analogue macrosialin, a member of the lamp family, appear to have a rather limited tissue distribution, being found on macrophages, Langerhans cells and dendritic cells[22]. Notably, we have found all adherent cells CD68-positive suggesting that in non-committed, undifferentiated cells, this protein might exhibit a different distribution pattern and possibly exert functions related to cell–cell or cell–ligand interactions[23].

CD45-positive hematopoietic cells were always detected in MSC cultures, regardless of the time of the first change of culture medium. This indicated a persistent presence and attachment of CD45-positive cells in the MSC environment when no further selection based on specific surface antigens is carried out. Adherence to the tissue culture plastic alone is a weak discriminating criterion for a homogeneous MSC population, because neural cells, monocytes and macrophages are also known to be isolated according to their adherence to plastic surfaces[24-26]. The presence of CD45-positive hematopoietic cells and the variation of their proportion in primary cultures of MSC could explain the occurrence of extensive variations usually seen in differentiation experiments with bone marrow derived cells.

The fact, that CD45-depleted cells expressed CD29 stronger than CD45-positive cells offered us an easier separation of two populations and limited the possible existence of more than two major subpopulations in our cultures. The integrin ß1 subunit CD29 exhibits a broad tissue distribution, including lymphocytes, endothelia, smooth muscle, and epithelia[27] and plays an important role in cellular processes, e.g. embryogenesis and HSC development. Although we did not find the hematopoietic cell fraction to vary according to the time of removal of non-adherent cells we observed a correlation between adhesion time and yield of CD45-depleted marrow stromal cells. In parallel, cell adhesion plus proliferation could also be promoted by the prolonged

presence of the non-adherent fraction via cytokine release and / or cell-cell contacts. A comparable effect of hematopoietic cells on MSC proliferation has been observed for megakaryocytes and platelets[28]. According to our results we decided to leave the cells to adhere for 3 days for differentiation experiments.

Differentiation studies of hematopoietic stem cells have demonstrated, that the small number of adherent MSC present in total bone marrow aspirates provide an important microenvironment for growth of HSC and their differentiation into several blood borne cell types. In long-term cultures of HSC, these adherent cells even interact directly with the hematopoietic precursors [29]. Here, we could show that vice versa adherent CD45-positive hematopoietic precursor cells create a microenvironment which may enhance expression of particular marker genes in MSC. For studying the effects of contaminating CD45-positive cells we prepared a relatively homogeneous population of CD45-depleted MSC by immunogenic depletion of CD45-positive cells. Both, the heterogenic adherent cells and the CD45-depleted subpopulation were subjected to chondrogenic favourable 3-D environment by formation of a cell alginate amalgam. For control purposes in order to assure that difference in gene expression profile between sorted and unsorted MSC is due to depletion of CD45-positive cells and not because of general depletion of differentiation competent cells or due to cell damage caused by the sorting process, we prepared a third population of remixed cells (30 % CD45-positive versus 70 % CD45-depleted cells) following MACS preparation which was treated according to the differentiation protocol used for adherent and sorted cells.

We monitored relative gene expression of collagen II30 and COMP[31,32] as examples for structural extracellular matrix proteins highly specific for hyaline cartilage. Additionally, we have investigated Sox9 as a master transcription factor imperative for chondrogenic differentiation[33] and collagen I as differentiation control for a gene normally not active in healthy hyaline cartilage[34] but occurring together with collagen II in fibrocartilage[35]. Gene expression of collagen X, specific for terminal differentiated hypertrophic chondrocytes in the calcifying zone of cartilage and secondary ossification centres within the growth plate[36] indicates maturation of MSC towards an undesired cartilage condition.

Gene expression of Cbfa1, a transcriptional activator for osteoblastic differentiation during the process of endochondral ossification is analyzed for monitoring differentiation towards osteogenic condition. Cbfa1 is also expressed in pre-condensation stage during early development in a cell type with the potential to become either a chondrocyte or an osteoblast. In later developmental stages expression of this transcription factor is restricted to prehypertrophic and hypertrophic chondrocytes where it acts as a hypertrophic differentiation factor[37,38].

The observed gene expression profile indicates not only a beneficial influence of CD45-positive cells on the differentiation process of MSC towards the chondrogenic lineage. It also shows that when culture time is extended beyond 28 days chondrogenically differentiating MSC might enter the prehypertrophic / hypertrophic differentiation cascade and proceed towards terminal differentiation. Interestingly, usage of chondrogenic culture medium does not prevent up regulation of Cbfa1 and collagen X. This indicates either a not yet defined influence of medium components (i.e. TGF-ß or dexamethasone) on intrinsic cell derived factors (i. e. signalling molecules, proteases, transcription factors) which are involved in regulation of the chondrogenic differentiation cascade or a medium independent process occurring by default through paracrine interaction of MSC. A variety of growth factors i.e. members of the TGF-ß superfamily, IGF-1 and bFGF could account for gene modulating effects in our system[39 40 41 42].

In conclusion, we demonstrated that bone marrow derived fibroblast like CD45-depleted rat MSC share a heterogeneous cellular environment with ~20-30% CD45-positive HC, if no other selection procedure than adherence to plastic is employed. Our differentiation results indicate that adherent CD45-positive hematopoietic cells may create a microenvironment promoting differentiation of MSC towards the chondrogenic lineage by up regulating specific genes. However, expression of undesired genes as collagen I and collagen X is likewise promoted by HC. On the contrary, at early condensation stage HC strongly increase gene expression of Cbfa1 thus supporting chondrogenic differentiation of MSC, while at a later differentiation stage when Cbfa1 plays an integral role in hypertrophy the regulation appears to be independent of the presence of HC.

We speculate that the stromal CD45-positive population in toto is responsible for regulation of gene expression. The heterotrophic nature of bone marrow derived cells suggest that in vivo interactions of different cell types is very likely and should be addressed accordingly. Characterization of additional subpopulations of CD45-positive cells according to their surface antigens and determination of their potential influence on gene expression may elaborate this point further more. Studies using conditioned media are needed to clarify whether physical contact between cells or paracrine factors, alone or in concurrence, are necessary for promoting gene expression leading to chondrogenic differentiation and maturation of MSC. Identification and isolation of factors which are capable of modulating genes involved in differentiation and dedifferentiation processes of chondrocytes will be important tools for in vitro engineering of cartilage repair tissue.

REFERENCES

(1) *Pittenger MF, Mackay AM, Beck SC et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143-147.*

(2) *Cancedda R, Dozin B, Giannoni P, Quarto R. Tissue engineering and cell therapy of cartilage and bone. Matrix Biol. 2003;22:81-91.*

(3) *Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells. 2001;19:180-192.*

(4) *Galmiche MC, Koteliansky VE, Briere J, Herve P, Charbord P. Stromal cells from human long-term marrow cultures are mesenchymal cells that differentiate following a vascular smooth muscle differentiation pathway. Blood. 1993;82:66-76.*

(5) *Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science. 1997;276:71-74.*

(6) *Majumdar MK, Thiede MA, Mosca JD, Moorman M, Gerson SL. Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSC) and stromal cells. J Cell Physiol. 1998;176:57-66.*

(7) *Friedenstein AJ, Gorskaja JF, Kulagina NN. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol. 1976;4:267-274.*

(8) *Caplan AI, Bruder SP. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. Trends Mol Med. 2001;7:259-264.*

(9) *Phinney DG, Kopen G, Isaacson RL, Prockop DJ. Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. J Cell Biochem. 1999;72:570-585.*

(10) *Herbertson A, Aubin JE. Cell sorting enriches osteogenic populations in rat bone marrow stromal cell cultures. Bone. 1997;21:491-500.*

(11) *von der MK. Structure and Biosynthesis of Collagens. In: Seibel MJ, Robins SP, Bilezikian JP, eds. Bone and Cartilage Metabolism. Academic Press; 1999:3-29.*

(12) *Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B. Sox9 is required for cartilage formation. Nat Genet. 1999;22:85-89.*

(13) *de Crombrugghe B, Lefebvre V, Behringer RR et al. Transcriptional mechanisms of chondrocyte differentiation. Matrix Biol. 2000;19:389-394.*

(14) *Mundlos S, Olsen BR. Heritable diseases of the skeleton. Part I: Molecular insights into skeletal development-transcription factors and signaling pathways. FASEB J. 1997;11:125-132.*

(15) *Lefebvre V, Behringer RR, de Crombrugghe B. L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. Osteoarthritis Cartilage. 2001;9 Suppl A:S69-S75.*

(16) *Dobson KR, Reading L, Haberey M, Marine X, Scutt A. Centrifugal isolation of bone marrow from bone: an improved method for the recovery and quantitation of bone marrow osteoprogenitor cells from rat tibiae and femurae. Calcif Tissue Int. 1999;65:411-413.*

(17) *Maniatopoulos C, Sodek J, Melcher AH. Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. Cell Tissue Res. 1988;254:317-330.*

(18) *Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res. 1998;238:265-272.*

(19) *Ginzinger DG. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp Hematol. 2002;30:503-512.*

(20) *Pittenger MF, Mackay AM, Beck SC et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143-147.*

(21) *Jones EA, Kinsey SE, English A et al. Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. Arthritis Rheum. 2002;46:3349-3360.*

(22) *Ramprasad MP, Terpstra V, Kondratenko N, Quehenberger O, Steinberg D. Cell surface expression of mouse macrosialin and human CD68 and their role as macrophage receptors for oxidized low density lipoprotein. Proc Natl Acad Sci U S A. 1996;93:14833-14838.*

(23) *Fukuda M. Lysosomal membrane glycoproteins. Structure, biosynthesis, and intracellular trafficking. J Biol Chem. 1991;266:21327-21330.*

(24) *Delles AM, Rittenhouse-Olson K, Morgan J, Oseroff AR. A simple method for the purification of human peripheral blood antigen presenting cells (dendritic cells, monocytes/macrophages, and B lymphocytes). Immunol Invest. 2002;31:233-245.*

(25) *Kiefer F, Wagner EF, Keller G. Fractionation of mouse bone marrow by adherence separates primitive hematopoietic stem cells from in vitro colony-forming cells and spleen colony-forming cells. Blood. 1991;78:2577-2582.*

(26) *Majumdar MK, Thiede MA, Mosca JD, Moorman M, Gerson SL. Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSC) and stromal cells. J Cell Physiol. 1998;176:57-66.*

(27) *Springer TA. Adhesion receptors of the immune system. Nature. 1990;346:425-434.*

(28) *Castro-Malaspina H, Rabellino EM, Yen A, Nachman RL, Moore MA. Human megakaryocyte stimulation of proliferation of bone marrow fibroblasts. Blood. 1981;57:781-787.*

(29) *Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science. 1997;276:71-74.*

(30) *Mendler M, Eich-Bender SG, Vaughan L, Winterhalter KH, Bruckner P. Cartilage contains mixed fibrils of collagen types II, IX, and XI. J Cell Biol. 1989;108:191-197.*

(31) *Hedbom E, Antonsson P, Hjerpe A et al. Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage. J Biol Chem. 1992;267:6132-6136.*

(32) *DiCesare P, Hauser N, Lehman D, Pasumarti S, Paulsson M. Cartilage oligomeric matrix protein (COMP) is an abundant component of tendon. FEBS Lett. 1994;354:237-240.*

(33) *de Crombrugghe B, Lefebvre V, Behringer RR et al. Transcriptional mechanisms of chondrocyte differentiation. Matrix Biol. 2000;19:389-394.*

(34) *Rossert J, Terraz C, Dupont S. Regulation of type I collagen genes expression. Nephrol Dial Transplant. 2000;15 Suppl 6:66-68.*

(35) *Nerlich AG, Boos N, Wiest I, Aebi M. Immunolocalization of major interstitial collagen types in human lumbar intervertebral discs of various ages. Virchows Arch. 1998;432:67-76.*

(36) *Schmid TM, Popp RG, Linsenmayer TF. Hypertrophic cartilage matrix. Type X collagen, supramolecular assembly, and calcification. Ann N Y Acad Sci. 1990;580:64-73.*

(37) *Karsenty G. Minireview: transcriptional control of osteoblast differentiation. Endocrinology. 2001;142:2731-2733.*

(38) *Takeda S, Bonnamy JP, Owen MJ, Ducy P, Karsenty G. Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. Genes Dev. 2001;15:467-481.*

(39) *Bohme K, Winterhalter KH, Bruckner P. Terminal differentiation of chondrocytes in culture is a spontaneous process and is arrested by transforming growth factor-beta 2 and basic fibroblast growth factor in synergy. Exp Cell Res. 1995;216:191-198.*

(40) *Recklies AD, Baillargeon L, White C. Regulation of cartilage oligomeric matrix protein synthesis in human synovial cells and articular chondrocytes. Arthritis Rheum. 1998;41:997-1006.*

(41) *Murakami S, Kan M, McKeehan WL, de Crombrugghe B. Up-regulation of the chondrogenic Sox9 gene by fibroblast growth factors is mediated by the mitogen-activated protein kinase pathway. Proc Natl Acad Sci U S A. 2000;97:1113-1118.*

(42) *Horton WE, Jr., Higginbotham JD, Chandrasekhar S. Transforming growth factor-beta and fibroblast growth factor act synergistically to inhibit collagen II synthesis through a mechanism involving regulatory DNA sequences. J Cell Physiol. 1989;141:8-15.*

CHAPTER 3

MYELOID CELLS SUPPRESS IN VITRO OSTEOGENIC DIFFERENTIATION OF RAT MARROW STROMAL CELLS

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ABSTRACT

Primary cultures of bone marrow stromal cells (MSC), selected by adhesion to tissue culture plastic, contain populations of adherent hematopoetic cells (HC) and endothelial cells. However, little is known about the effect of HC on the differentiation of CD45- MSC to mesenchymal lineages.

Whole rat bone marrow was seeded in tissue culture flasks and selected by adhesion to cell culture plastics. Adherent cells were cultured for seven days in alpha MEM supplemented with 10% fetal bovine serum (FBS). After trypsinization cells were characterized by flow cytometry and separated into CD45+ and CD45- populations using immunomagnetic labeling. Both cell populations were mixed in predetermined proportions and further cultured under osteo-inductive conditions in 2-D and 3-D cultures. Alkaline phosphatase activity (ALP), calcium accumulation, and osteogenic gene expression profiles were examined to assess differentiation.

Flow cytometry showed that CD45- cells from primary MSC cultures expressed CD90, CD29, and CD44, which are markers characteristic for MSC. Additionally, flow cytometry revealed a contamination with CD45+/CD11bc+ HC, referred to as myeloid cells, but not with CD31+ endothelial cells. Purified CD45- cells showed strongly improved differentiation compared to non-separated populations. ALP and calcium accumulation were dose-dependently decreased with increasing percentages of HC. Control experiments excluded an effect of separation procedure or initial cell number. Real time RT-PCR showed increased expression of osteogenic markers in groups without HC in 2-D and 3-D cultures. Finally, trans-well experiments allowed the determination that the observed effect was due to soluble factors.

This study shows that myeloid cells, which contaminate primary rat MSC cultures, modulate osteogenic differentiation negatively, in a fashion independent of heterotypic cell-cell contacts.

INTRODUCTION

Mesenchymal progenitor cells (MPC), or colony forming unit fibroblasts (CFU-F), are a clonogenic subpopulation of bone marrow stromal cells (MSC), that can proliferate *in vitro* as undifferentiated cells up to fifty population doublings[1] and differentiate into cells of connective and supportive tissues like osteoblasts, chondrocytes, adipocytes, myocytes, tenocytes, fibroblast, and haematopoiesis-supporting stromal cells[2-6]. The usual enrichment criterion for bone marrow derived MSC utilizes their ability to adhere to tissue culture plastic, separating them from non-adherent hematopoietic cells, which form the major population isolated from bone marrow. Nevertheless, adherent hematopoietic and endothelial cells contaminate the primary MSC cultures[7,8], although the degree of contamination can be decreased by passaging or medium pre-selection[9]. The MSC phenotype is characterized by the expression of some typical non-specific surface markers, while cultured MSC lack typical hematopoietic markers like CD34, CD45, CD11c and the endothelial marker CD31[2-6].

Bone and bone marrow are anatomically continuous and exhibit marked functional interdependence. Bone marrow contains hematopoietic and non-hematopoietic stem cells, which are progenitors of osteoclasts and osteoblasts, respectively. Non-hematopoietic stromal cells, as well as osteoblasts, create a microenvironment for hematopoietic stem cell proliferation and maturation[10]. Commitment, proliferation, and differentiation of osteogenic or hematopoietic precursor cells are regulated by multiple factors, including cytokines, growth factors, systemic hormones, and transcriptional regulators, which can function in an autocrine or paracrine fashion. In addition, cell-cell and cell-matrix interactions are responsible for the communication between stromal/osteoblastic cells and osteoclast precursors as well as other hematopoietic cells in the bone marrow[11]. MSC have been shown to express interleukins (IL-1α, IL-6, IL-7, IL-8, IL-11, IL-12, IL-14 and IL-15), leukemia inhibitory factor (LIF), stem cell factor (SCF), Flt3 ligand (FL), as well as the granulocyte macrophage- (GM-CSF), granulocyte- (G-CSF), and macrophage-colony stimulating factors (M-CSF). Receptors for IL-1, IL-3, IL-4, IL-6, IL-7, LIF, SCF, G-CSF, interferon γ (IFNγ, tumor necrosis factor (TNFI, TNFII), transforming growth factors (TGFβ1, TGFβ2), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF) and endothelial growth factor (EGF) have also been detected[2,6]. However, most data have been recorded from studies investigating the hematopoietic microenvironment, whereas no comprehensive data on the properties of the microenvironment for mesenchymal progenitors, both in the bone marrow and in other mesenchymal tissues, is available.

Rat MPC have been characterized by Javazon et al.[12] as cells sensitive to plating density, showing rapid expansion at low density culturing. Furthermore, when seeded at densities of 5,000 cells /cm^2 and passaged up to P5, rat MPC express CD90 and CD59, and are negative for CD31, CD4, CD11b, CD43, CD45, and mononuclear phagocyte marker. Assays on colony forming units-fibroblast (CFU-F), CFU-alkaline phosphatase (CFU-ALP), and CFU–osteogenic (CFU-O) have been performed with primary and secondary rat bone marrow cultures. The estimated frequency of CFU-F in secondary cultures was 1/100-1/250 when cultured without dexamethasone, whereas with dexamethasone 1/50-1/100 CFU-F frequencies were found[13]. The frequency of osteoprogenitors CFU-O in the secondary cultures is 1/300-1/500 when stimulated with dexamethasone and less than 1/1000 when no stimulation is applied[13]. Considering all nucleated cells in rat bone marrow, the final frequency of CFU-O is only 1/30,000-1/100,000[13].

This study was performed to investigate the effect of contaminating plastic adherent hematopoietic cells (HC) on the osteogenic differentiation of bone marrow stromal cell cultures *in vitro*. Primary MSC cultures were immunomagnetically separated into CD45+ HC and CD45- MSC. Different percentages of HC were mixed with CD45- cells and cultured under osteo-inductive conditions, using standard osteogenic medium supplements. In the first experiment, we compared the osteogenic differentiation of pure CD45- cells with cultures containing different percentages of CD45+ HC, maintaining a total seeding density of 50,000 cells / cm². We noted a dose-dependant decrease in ALP activity and calcium accumulation with increasing HC percentages. A second experiment was performed as a control, investigating the possible impact of the antibody labeling and separation procedure. Additionally, this second experiment indicated whether the observed effects were caused by the difference in CD45- cell number per well. Neither procedure nor cell number seemed to have an influence on osteogenic differentiation. A third experiment, investigating the gene expression of osteogenic markers in pure CD45- cultures and non-separated cultures containing 20 % HC, confirmed the results of the first two experiments. Finally, to elucidate whether the effect relied upon heterotypic cell-cell contacts or soluble factors, trans-well experiments were performed.

MATERIALS AND METHODS

Suppliers

Gibco, Invitrogen, UK: Foetal calf serum, FCS (Lot No.: 40F7430K); Penicillin-Streptomycin (10,000 units / mL penicillin G sodium and 10,000 µg / mL streptomycin sulphate); Trypsin-EDTA (0.25 % Trypsin and 1 mM EDTA.4Na); TRiZOL

Sigma Aldrich, Germany: L-glutamine (200 mM); Minimum essential medium Eagle, alpha modification (α-MEM; 10.1 g / L); 7-Aminoactinomycin D (7-AAD); Trizma ® base ($C_4H_{11}NO_3$). Corning, Netherlands: tissue culture flasks and wells. Millipore, USA: MilliCell-CM, trans-well inserts.

Polymer scaffolds

For the 3-D experiments, sponge-like scaffolds consisting of poly(DL-lactic-co-glycolic acid) (PLGA, molar ratio 75:25, Resomer® RG756, Boehringer Ingelheim, Ingelheim, Germany) with a pore size of 100 - 300 µm were used. They were fabricated by Solid Lipid Templating published by Hacker et al.[14].

Cell isolation and primary cell culture

Rat bone marrow was isolated from femur and tibia of six week old (150 – 200 g) male Sprague-Dawley rats (Institut für Labortierkunde und -genetik, Medical University Vienna, Austria) by centrifugation[15]. Freshly isolated bone marrow cells were re-suspended in primary medium (10 % FCS, 1 % Penicillin/Streptomycin, 0.5 % L-glutamine, and α-MEM). For each experiment, bone marrow cells of at least three animals were pooled. Cells obtained from one rat (two tibias and two femurs) were seeded in 4 x 150 cm^2 flasks (approx. 1.5x 10^5 nucleated cells / cm^2) and cultured in a humidified atmosphere with 5 % CO_2 at 37°C. After three days, non-adherent cells were removed by rinsing twice with 16 mL / flask PBS. The medium was changed three times a week. On day seven after cell isolation (90 – 100 % confluence), the cells were detached from the flasks using trypsin-EDTA. After trypsin deactivation, cells were passed through a 40 µm cell strainer, centrifuged, re-suspended, and counted using a haemocytometer.

Flow cytometry

The cell suspension was first incubated for fifteen minutes at 4°C with 10 % v/v sheep serum and then for thirty minutes at 4°C with antibodies against rat CD markers or with corresponding isotype controls. Antibodies against the rat markers CD90, CD29, CD11bc, CD3, CD4, CD8b, NKRP1a, CD45RA, and granulocytes were purchased from BD Pharmingen, USA, CD44 and CD45 were obtained from Acris, Germany. Cells were washed and re-suspended in PBS containing sodium azide and bovine serum albumin (BSA) before the measurements, 20 % v/v 7-AAD (20 µg / mL) was added to each sample for three minutes to stain the dead cells, which were then excluded from the evaluation. All measurements were performed on a FACSCalibur® instrument and data acquired using the CellQuest software (both from BD Biosciences, USA). Measurements were evaluated with WinMDI 2.8 Software (free access from http://facs.scripps.edu/software.html). A minimum of 1 x 10^4 viable cells were acquired per data set. The percentage of positive cells was determined as the percentage of cells having a measured fluorescence greater than that of 99.5 % of the cells that had been stained with each associated isotype control. A population was considered positive for a surface marker when the percentage of positive cells for that surface marker was ≥ to 5 %.

Magnetic cell separation

Primary cultures of rat MSC are usually contaminated with CD45+ hematopoetic cells. To separate CD45+ and CD45- cells, cells were re-suspended in separating buffer (PBS pH 7.2 supplemented with 0.5 % BSA and 2 mM EDTA) and incubated with primary phycoerythrin (PE)-labeled antibody against rat CD45 (Acris Antibodies; Hiddenhausen, Germany; Cat. Nr. SM274R) at 4°C in the dark for thirty minutes. After washing, the cell suspension was incubated with secondary anti-PE antibody-coupled magnetic beads (MACS® MicroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 minutes at 4°C in the dark. After washing, cells were re-suspended in separating buffer and applied onto the LS column, which was then placed in the magnet (MidiMACS Separation Unit, Miltenyi Biotech). The negative fraction was first collected (CD45- MSC) and then, after the removal of the column from the magnet, the CD45+ fraction was obtained. Both cell fractions were washed with medium, the viable cells were counted, and the viability and purity of both populations were determined by flow cytometry.

Osteogenic differentiation

For 2-D experiments, cells were plated in well plates at a density of 50,000 cells / cm^2 in primary medium for 24 hours, if not indicated differently. In order to check for the influence of released cytokines, a 3-D experiment was performed. To this end, CD45- cells were dynamically seeded onto 3-D scaffolds in spinner flasks for 24 h[16]. Cell loaded scaffolds (1.8 x 10^6 CD45- cells/scaffold) were placed onto 6-well culture plate inserts (0.4 µm pore size), while CD45+ cells were co-cultured on the bottom of the 6-well plates (1.2 x 10^6 CD45+ cells / well). CD45- cells that were not co-cultured were equally treated, but no CD45+ cells were seeded onto the bottom of the wells.
On day one after seeding, the cells received complete medium (primary medium supplemented with 50 mg / L ascorbic acid, 2.16 g / L β-glycero-phosphate (7mM), and 10^{-8} M dexamethasone). The medium was changed every two to three days and samples were taken at different time points. Wells were washed with PBS and frozen at –20°C.

Cell number

A fluorimetric assay[17] was performed to measure the total amount of DNA and, subsequently, to determine the cell number. The samples were thawed and the cells scraped off the surface and dispersed in PBS. After digestion with papain solution for 18 h at 60°C, the released DNA was quantified using Hoechst 33258 dye. Cell number was calculated using a DNA standard curve (calf thymus DNA (Sigma)) and a predetermined factor of 10 *p*g DNA / cell.

Alkaline Phosphatase (ALP)

Cells were thawed and kept on ice during preparation of the samples. After thawing, cells were scraped and dispersed in TRIS buffer (1 M Trisma base, pH 8). After sonication, the kinetics of p-nitro phenyl phosphate degradation to phosphate and p-nitro phenol was determined using a RANDOX Kit (Randox Laboratories Ltd., Crumlin, UK), measuring the absorbance at 405 nm, and calculating the ALP activity.

Calcium deposition

For the quantification of calcium content, the reaction of the cation with the chromogenic agent o-cresolphtalein complexone to a red complex with an absorbance maximum at 575 nm was employed (Calcium Liquicolor, Rolf Greiner BioChemica, Flacht, Germany). For the von

Kossa stain, cells were fixed with neutral buffered formalin solution (10 %) and incubated with 5 % $AgNO_3$ solution under exposure to day light.

RNA isolation and reverse transcription

Samples were immediately washed with PBS, dispersed, and homogenized in 1 mL TRiZOL. Subsequently, samples were frozen at –70°C. After thawing, samples were transferred into PhaseLock Gel tubes (Eppendorf, Germany) and RNA isolation was performed according to the manufacturer's instructions (TRiZOL, Invitrogen). RNA concentration and purity were determined by absorption measurements at 260 and 280 nm, respectively. Reverse transcription was performed with the High Capacity cDNA Archive Kit (Applied Biosystems, USA) in a thermal cycler (Eppendorf, Germany) for ten minutes at 25°C and 2 h at 37°C. Replicated DNA was stored at –20°C.

Real time PCR

Relative quantitative PCR was performed with *TaqMan* Gene Expression Assays (Table 1) and *TaqMan* 2X Universal Master Mix (both Applied Biosystems) in 96-well optical reaction plates on an Applied Biosystems 7300 Real-Time PCR System according to the manufacturer's instructions. Thermal cycling parameters were an initial incubation at 95°C for ten min, followed by forty PCR amplification cycles of denaturation at 95°C for 15 s, and annealing / extension for one min at 60°C.

Mean relative quantification (RQ) values were calculated according to the ΔΔCt method, using ribosomal 18S RNA as an endogenous control. The results are the average of three (2-D experiment) or four (3-D experiment) samples with double determination.

Table 1: *TaqMan assays for the real time PCR provided by Applied Biosystems (USA).*

Target	RefSeq	TaqMan Assay ID
ALP	NM_037191	Rn00564931_m1
BSP	NM_012587	Rn00561414_m1
Cbfa-1 (Runx2)	AB115746	Rn01512296_m1
Collagen 1	Z78279	Rn00801649_g1
Osteocalcin (Bglap2)	NM_013414	Rn00566386_g1
Osteonectin (Sparc)	NM_012656	Rn00561955_m1
Osteopontin (Spp1)	NM_0128812	Rn00563571_m1
Endogenous control		
Ribosomal 18s mRNA	X03205	4319413E

Experimental Designs

Experiment 1: Aim: Examine the influence of CD45+ HC contamination on osteogenic differentiation at constant total seeding density of 50,000 cells / cm². Methods: ALP activity, Ca^{2+}-content, van Kossa staining. Groups: (1) 100 % CD45- MSC, (2) 85 % CD45- MSC with 15 % CD45+ HC, (3) 71 % CD45- MSC with 29 % CD45+ HC, (4) 57 % CD45- MSC with 43 % CD45+ HC and (5) 42 % CD45- MSC with 58 % CD45+ HC.

Experiment 2: Aim: Control experiment to determine the influences of the antibody labeling and separation procedures on the cell number of CD45- MSC. Methods: ALP activity, van Kossa staining. Groups: (1) separated cells and non-separated in the same proportion as before separation, (2) non-separated cells, which were processed like the separated cells, but without the addition of antibodies, (3) non-separated cells, (4) only CD45- MSC. Groups (1)-(4) were seeded at 50,000 cells / cm².

(5) CD45- MSC seeded at 50,000 cells / cm² with addition of 12,500 CD45+ HC / cm², (6) CD45- MSC only, seeded at 62,500 cells / cm².

Experiment 3: Aim: Examine the influence of CD45+ HC contamination on osteogenic differentiation at constant total seeding density of 50,000 cells / cm². Methods: 2-D culture, real time on day seven. Groups: (1) non-separated cells containing 20 % CD45+ cells, (2) only CD45- MSC.

Experiment 4: Aim: Examine the influence of CD45+ HC contamination in separated co-culture. Methods: trans-well culture, real time on day seven. 3-D: Groups: (1) only CD45- MSC, cultured as monoculture, (2) only CD45- MSC, co-cultured with CD45+ HC, separated by a membrane.

Statistical evaluation

One-way ANOVA and Student t-tests were employed for quantitative PCR experiments and Tukey test was used for multiple group comparisons.

RESULTS

The presented data result from four independent experiments. Each of them showed that purified rat CD45- MSC from primary bone marrow cultures differentiated to the osteogenic lineage significantly better than primary bone marrow cultures containing CD45+ hematopoietic cells (HC).

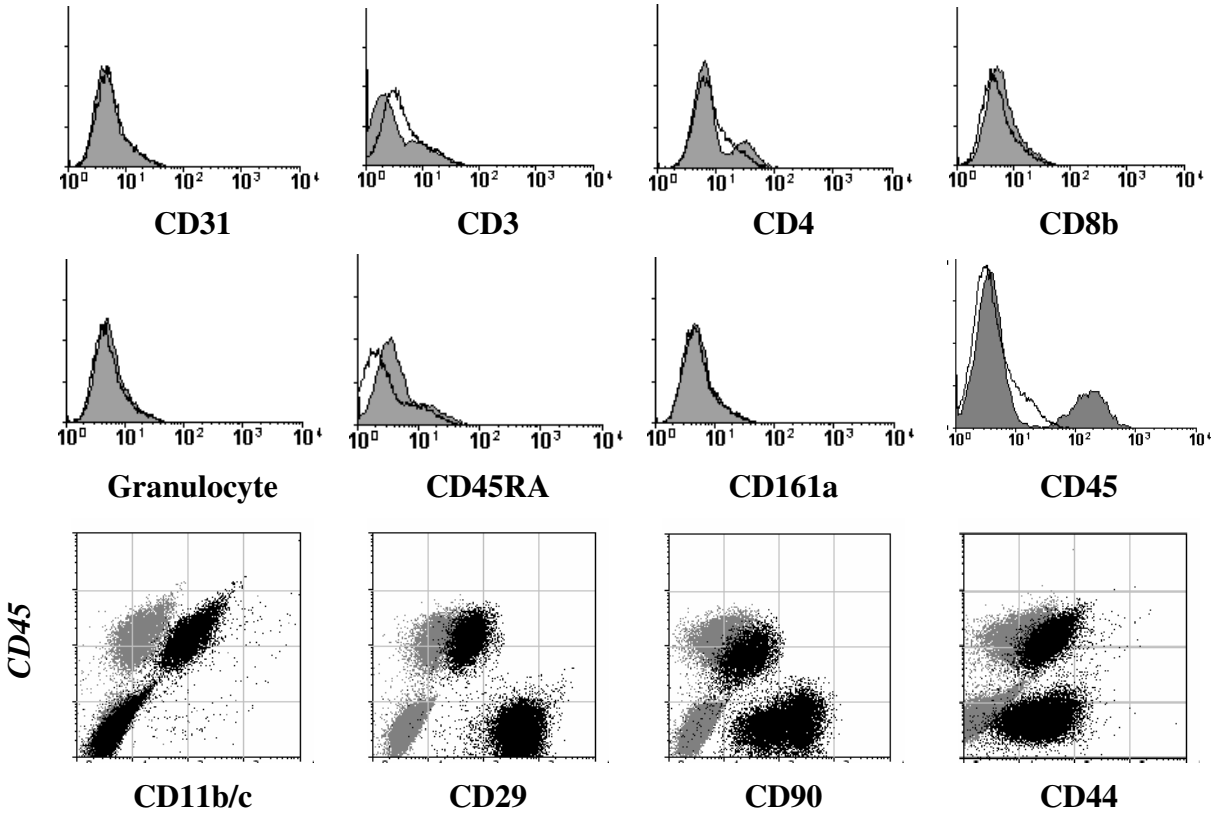

Figure 1: *Flow cytometric adherent bone marrow cell phenotype determination. Representative histograms show the expression of some hematopoietic markers and endothelial marker CD31. The open histograms show the fluorescence intensity of the cells with isotype control and the closed histograms show the fluorescence intensity of the cells with each CD antibody. Dot-plots show the expression of markers on CD45+ and CD45- populations simultaneously. Gray: isotype control, black: CD antibody.*

Flow cytometric characterization of primary cultures

After one week of cultivation, rat bone marrow cultures contained 10-40% CD45+ HC, whereas no CD31+ endothelial cells were detected (Figure 1). We did not detect cells positive for CD3, CD4, CD8b, NKRP1A (CD161a), CD45RA, or granulocyte marker (Figure 1). Most of the primary cells, CD45- as well as CD45+, were positive for both integrin subunit β1 CD29, CD90 and CD44. Most of the cells were identified as CD45-, showing fibroblast-like morphology[18] and expressing MSC markers. The contaminating, generally round-morphology cells were CD45+ HC[18], which co-expressed the myeloid marker CD11b/c.

Effect of HC contamination on osteogenic differentiation of CD45- cells

Since the percentage of HC contamination in primary MSC cultures varied[18], we investigated the influence of different HC proportions on osteogenic differentiation. ALP activity of pure CD45- cultures peaked earlier and was significantly higher than in CD45- cultures containing HC (Figure 2a). Calcium quantification (Figure 2b) and von Kossa staining (Figure 3) supported this delay in differentiation.

Other osteogenic markers were also affected by HC, as seen in figure 4. In that experiment, we compared the gene expression of pure CD45- cultures with that of non-separated cultures (containing 20% CD45+ HC). On day seven, measured mRNA expression of ALP, bone sialoprotein (BSP), and osteocalcin (OC) was four-, five- and six- times greater, respectively, in the pure CD45- group than in the non-separated group (all $p<0.001$). Additionally, the expression of transcription factor Cbfa1, collagen 1 (Col1), osteopontin (OP) (all $p<0.001$) and osteonectin (ON; $p<0.05$) was significantly enhanced in group without CD45+ HC.

Influence of immunomagnetic separation

To confirm that the observed effects were not only a consequence of antibody labeling or the separation procedure, a control experiment was performed (Figure 5, groups one to four). Neither cell labeling with antibodies nor separation procedure affected ALP or cell proliferation significantly. A slightly lowered cell count observed on day one (Figure 5b) in groups one and two (separation procedure), as compared to group three (non-separated group), was probably due to the delay in seeding as a result of the separation procedure.

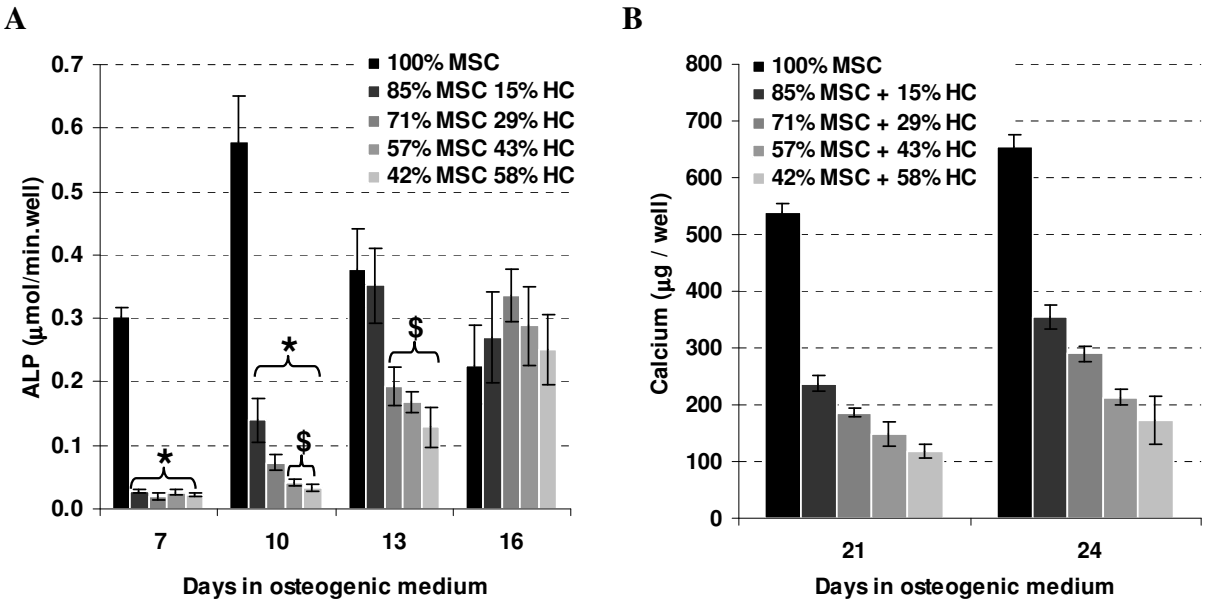

Figure 2: *Influence of CD45+ cell proportion on: A: alkaline phosphatase (ALP) activity and B: calcium accumulation at a total seeding density of 50,000 cells / cm². Results are from the first 2-D experiment, which was preformed in 12-wells. Results are mean (n = 4) ± standard deviation.*
** Significant difference (p<0,01) to 100% MSC group and $: significant difference (p< 0.01) to 85%MSC+15%HC group.*

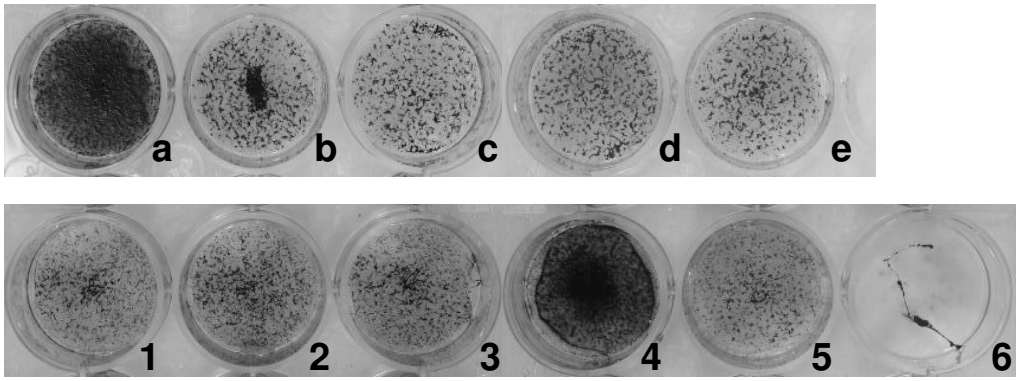

Figure 3: *Von Kossa staining of calcium deposition.*
Upper wells show results of the 1st experiment after 24 days. (a) 100% CD45- MSC, (b) 85% CD45- MSC with 15% CD45+ HC, (c) 71% CD45- MSC with 29% CD45+ HC, (d) 57% CD45- MSC with 43% CD45+ HC and (e) 42% CD45- MSC with 58% CD45+ HC.
Lower wells show results of the 2nd experiment after 21 days in osteogenic medium.
In groups containing CD45+ HC(all 20%), single bone nodules could be seen whereas in groups with CD45- MSC only, the whole cell layer in wells stained positive for calcium. Pure CD45- MSC often tended to detach (4) or rolled together forming coils (6) probably because of higher extracellular matrix production. Groups: (1) separated cells and joined in the same proportion as before separation, (2) non-separated cells, which were processed as the separated, only without antibodies addition, (3) non-separated cells, (4) only CD45- MSC. Groups 1-4 were seeded at 50,000 cells / cm². (5) CD45- MSC seeded at 50,000 cells / cm² with addition of 12,500 CD45+ HC / cm², (6) CD45- MSC only, seeded at 62,500 cells / cm².

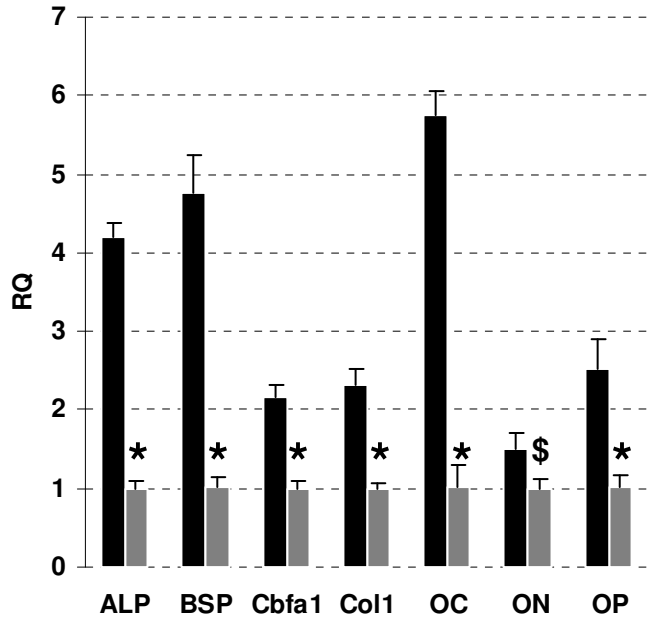

Figure 4: *Relative mRNA gene expression of 2-D cultured cells (6-wells) on day 7. Control (grey bars): non-separated cells with 20% CD45+ cells, CD45 - group (black bars). Results are mean (n = 3) ± standard deviation; significant difference: *: p < 0.001 and $: p < 0.05. ALP: alkaline phosphatase, BSP: bone sialoprotein, Cbfa1: transcription factor, Col1: collagen 1, C: osteocalcin, ON: osteonectin, OP: osteopontin.*

Effect of CD45- cell number

In the first experiment, the total number of CD45- cells in the different groups had been varied to maintain a total cell density of 50,000 cells / cm². Therefore, we evaluated the influence of CD45- cell seeding number, with or without CD45+ HC (Figure 5), on osteogenic differentiation in the control experiment. Groups four and five contained the same number of CD45- cells at the beginning of differentiation, with group five additionally containing 20 % CD45+ cells. We found that pure CD45- cells (group four) showed significantly higher ALP than group five, which contained HC (Figure 5). No difference in ALP and cell number was observed when the initial CD45- cell number was increased from 100 to 125 parts (group four vs. group six). Comparing group one and five – which have the same percentage of CD45+ cells, but different numbers of CD45- cells – revealed no difference.

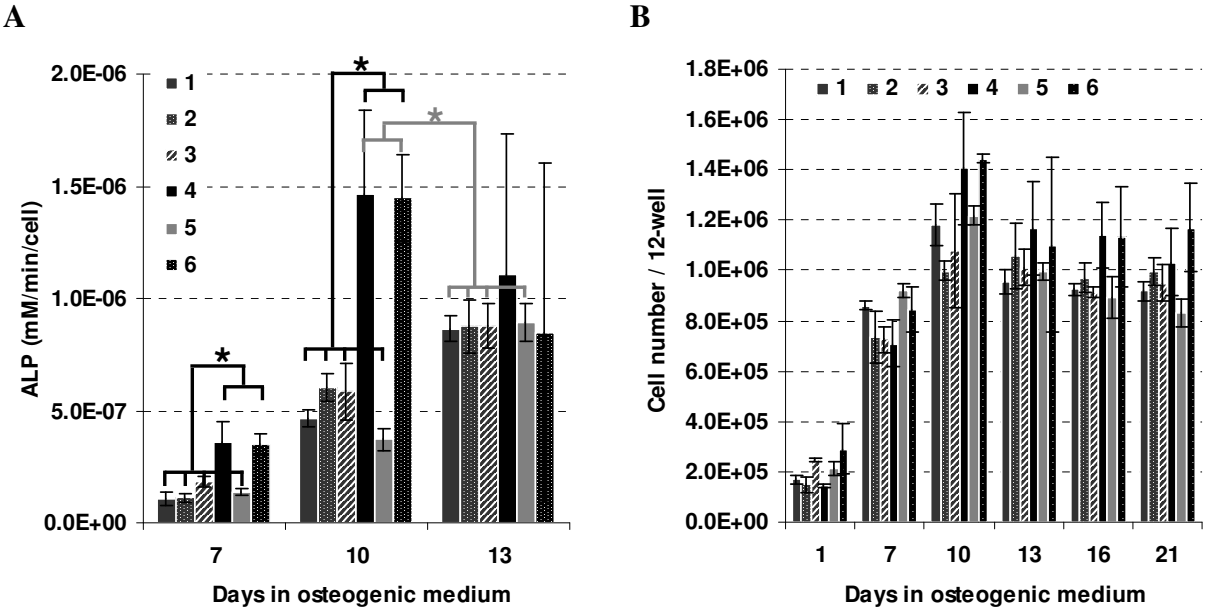

Figure 5: *Second experiment performed 2-D in 12-wells.*
A: Alkaline phosphatase activity during osteogenic induction of CD45- MSC and CD45- MSC co-cultured with CD45+ HC.
B: Proliferation of the cells in osteogenic medium.
Results are mean (n = 4) ± standard deviation.
** Significant difference ($p < 0.01$). Grey bars represent the comparison of maximal ALP activity.*
Groups: (1) separated cells and joined in the same proportion as before separation, (2) non-separated cells, which were processed as the separated, only without antibodies addition, (3) non-separated cells, (4) only CD45- MSC. Groups 1-4 were seeded at 50,000 cells / cm². (5) CD45- MSC seeded at 50,000 cells / cm² with addition of 12,500 CD45+ HC / cm², (6) CD45- MSC only, seeded at 62,500 cells / cm².

Effects in separated co-culture

One week after the passage, we could still observe cells with round morphology "sitting" on the layer of MSC in groups containing HC, as shown in Figure 6. To find out whether CD45+ cells influence CD45- differentiation by heterotypic cell-cell contacts, or if this influence is through released cytokines, we performed another experiment in which we separated CD45- cells from CD45+ cells using 0.4 µm trans-well membranes. CD45- cells were cultured on three-dimensional scaffolds made of PLGA on top of the trans-well membranes, while HC were seeded into the bottom of wells. All cultures received osteoinductive treatment. CD45- cells cultured in absence of CD45+ cells showed increased expression of osteogenic markers as compared to separated, co-cultured cells (Figure 7). In particular, the measured mRNA expression of osteocalcin was forty times, and the expression of ALP and BSP three times, higher than in co-cultured CD45- cells.

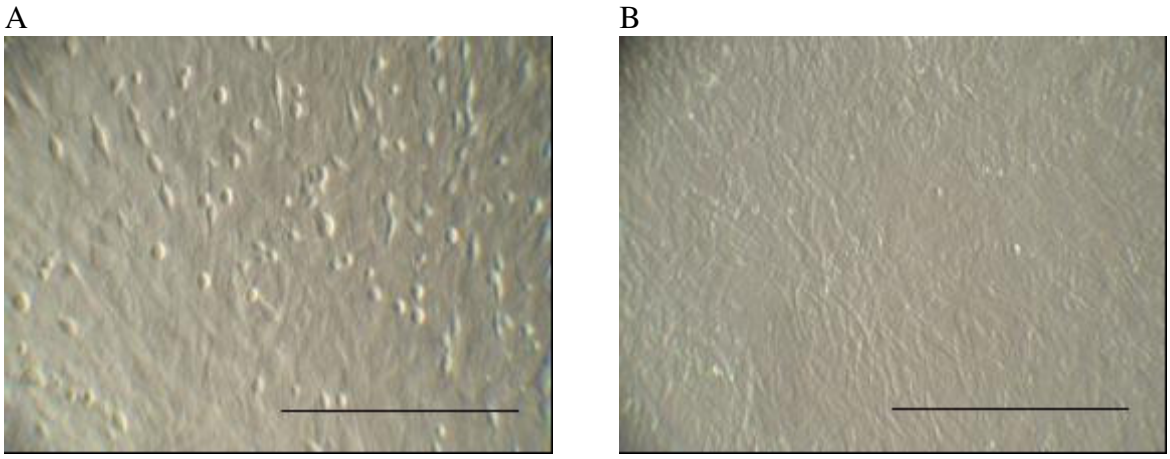

Figure 6: *Interference contrast photographs after 7 days in osteogenic medium. Left: non-separated cells, where round HC-like cells could be observed. Right: CD45- group without round cells.*
Bar = 250 µm.

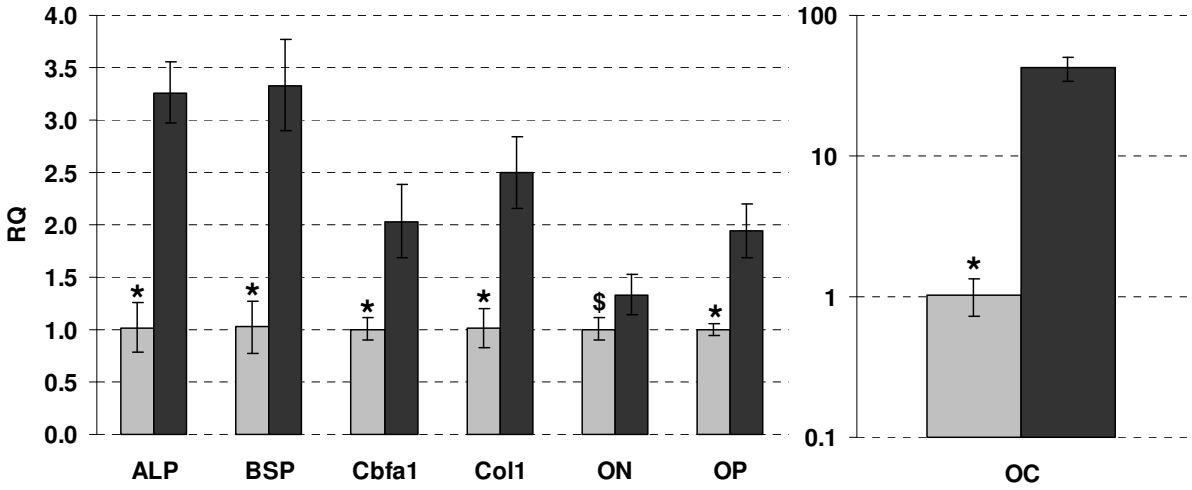

Figure 7: *Effect of soluble factors. Relative mRNA gene expression of 3-D cultured cells. The expression profile of osteogenic markers on day 7 in CD45- group (black bars) and CD45- group which was co-cultured with hematopoietic cells (grey bars), separated with a trans-well membrane. Results are mean (n = 4) ± standard deviation; significant difference: *: $p < 0.001$ and $: $p < 0.05$. ALP: alkaline phosphatase, BSP: bone sialoprotein, Cbfa1: transcription factor, Col1: collagen 1, C: osteocalcin, ON: osteonectin, OP: osteopontin.*

DISCUSSION

It is known that early cultures of bone marrow stromal (MSC) cells also contain hematopoietic cells (HC)[7]. With repeated sub-culturing, the frequency of hematopoietic cells decreases, with the rate of decrease depending on the species and culture conditions[7]. One important difference between primary cultures and passaged MSC is, therefore, the presence of hematopoietic cells[19]. The proportion of HC in rat MSC cultures can also be diminished by dexamethasone supplementation[20]. Regarding the influence of hematopoietic cells on osteogenic differentiation, it is known that either non-adherent bone marrow cells or their conditioned media can increase rat bone nodule formation[13]. Specifically, megakaryocytes and platelets are known to support MSC proliferation and differentiation[21,22].

In a first step, we characterized the fraction of adherent CD45+ cells in our cultures. We used antibodies against common hematopoietic markers, such as CD45 and CD11b/c, as well as several lineage markers. CD45 is a leukocyte-common antigen (LCA) and, therefore, a widely used marker for the detection of hematopoietic cells in general. CD45+ cells in our cultures co-expressed another general leukocyte/myeloid marker, the integrin subunit CD11b/c. The antibody employed (anti CD11b/c, clone OX-42) reacts with the CR3 complement (C3bi) receptor, which is found on most monocytes, granulocytes, macrophages, and dendritic cells[23,24]. This antibody recognizes a common epitope shared by CD11b and CD11c (integrin αM and αX chains) [24], which is present in both mature and immature cells. Because we detected no cells positive for granulocyte marker or NKR-P1A+, typical markers for dendritic cells, we assumed that cells of monocytic-macrophage lineage prevailed in our cultures. Additionally, the detection of a low level of co-expression for the rat Thy-1 antigen CD90, a commonly stated marker for progenitor cell types[25], also indicates the presence of hematopoietic progenitors. We detected no expression of CD3, CD4, CD8b, CD45RA, NKRP1A, or granulocyte positive cells, which indicated that mature cells did not adhere and that precursor cells did not differentiate to these subpopulations (B and T lymphocytes, dendritic cells, granulocytes, erythroblasts) during one week of cultivation under the conditions used. Taking this surface marker profile into consideration, we assume that CD45+ cells in our cultures were a mixed cell population consisting of mature (monocyte-macrophage) and immature (progenitor) cells of the myeloid lineage, which arose from the bone marrow. Additionally, many publications describe similar “primitive” myeloid progenitor cells, or so called “cobblestone area” cells, that adhere to tissue culture plastic in

hematopoietic stem cell studies[19,25]. The other major population in our primary cultures was $CD45^-/CD11bc^-/CD31^-$, but expressed CD90, CD44 and CD29, markers typical for MSC.

Tissue engineering publications utilizing the rat animal model often use primary cultures of plastic adherent, enrichedMSC, for further differentiation and matrix production studies. Primary adherent rat bone marrow cells have been employed for bone[26], fat[27] and cartilage[28] tissue engineering studies without the determination of other bone marrow subpopulations. Because of a loss in their differentiation potential with increasing number of passages[29], early passages that commonly contain adherent hematopoietic cells are generally used. Since we detected varying proportions of CD45+ cells in primary cultures (mostly 10-30%), we assume that this could be one reason for variations observed in osteogenic differentiation experiments. Additionally, effects of some growth factors used for improved bone tissue formation (e.g. TGF-β1) are probably due to the sum of effects on both hematopoietic and mesenchymal populations.

The CD45 marker clearly distinguished both cell populations using flow cytometry (Figure 1). Applying secondary magnetic-bead-coupled antibody against fluorochrom phycoerythrin (PE) enabled specific binding to the anti-CD45 antibody. The efficiency of the magnetic separation procedure was always determined after the separation and the exact proportions were calculated or set for further experiments. First, we wanted to determine if there was an observable effect of hematopietic cells on osteogenic differentiation and whether the strength of this effect depended on the percentage of contamination with hematopoietic cells. Cultures without HC showed remarkably higher alkaline phosphatase activity levels (ALP) than cultures with HC. In the groups containing HC, ALP increased at later time points, which correlated to the proportion of HC contamination. An earlier onset of ALP in pure CD45- cultures was accomplished through earlier mineralization and, consequentially, higher calcium accumulation after 21 and 24 days (Fig. 2b and Fig. 3a).

During the cultivation, we observed some dead cells in cultures containing HC, possibly caused by HC labeling or the separation procedure. To determine the cause, a second experiment was performed (Fig. 5). We compared the differentiation of pure CD45- cells (group four) with that of (1) non-separated cells (group three, control), (2) magnetically labeled and separated cells, which were reunited in the same ratio as before separation (group one), and (3) cells that had experienced the separation procedure without magnetic labeling (group two). We saw that neither labeling of HC nor the separation procedure itself influenced ALP activity profile and proliferation. The only observable difference on the first day after passage and separation was a slightly reduced cell number, which could be explained by the

delay caused by the separation procedure. All groups containing HC showed an ALP activity profile similar to that observed in the first experiment. Again, groups containing CD45- MSC only showed significantly higher ($p < 0.01$) ALP activity than groups with HC. In some wells of the groups with pure CD45- MSC, we observed detaching and aggregating cell layers, possibly caused by cross linking of the secreted collagen matrix and extensive proliferation, that resulted in higher deviations and lowering of ALP activity on day thirteen.

To make sure that the observed improved differentiation of pure CD45- cells was not only due to the higher number of CD45- cells compared to the number in mixed cultures, we analyzed the differentiation of cultures containing confluent numbers of CD45- cells and 20 % additional HC (Fig. 5, group 5). We saw that, within the chosen limits, the initial number of CD45- MSC had no influence on ALP and cell count (Fig. 5).

Alkaline phosphatase is a marker for osteogenic differentiation that increases at the beginning of differentiation and decreases when mineralization has significantly progressed[30]. With the exception of osteocalcin and cbfa-1, the expression of frequently assayed osteoblast-associated genes such as collagen 1 (COLLI), alkaline phosphatase (ALP), osteopontin (OPN), and bone sialoprotein (BSP) is up-regulated prior to cessation of proliferation in osteoblast precursors[30]. We determined the mRNA expression for these genes on day seven, when the cultures had nearly reached their maximum cell number. By choosing this time point, we were able to detect differences between the gene expression of early and late differentiation markers. We compared the expression of pure CD45- cells with that of a non-separated control containing 20 % HC. Confirming ALP and calcium accumulation, pure CD45- cells showed significantly increased mRNA expression for all investigated markers (Fig. 4).

Similar effects on human MSC have been observed in co-cultures with dermal fibroblasts[31]. The effects have been described as dilution effects and are explained by reduced cell-cell contacts between the MSC. To elucidate whether the observed effects of our study also depended on cell dilution, we performed a trans-well co-culture experiment that spatially separated the CD45- cells from the HC. We decided to seed CD45- cells onto three-dimensional scaffolds of poly-(lactic-glycolic acid) primarily to avoid detachment of cell layers and secondarily to enhance osteogenic differentiation. Separated CD45+ hematopoietic cells were plated on the bottom of 6-wells and CD45- MSC seeded constructs were cultured in a 0.4 µm-membrane trans-well insert that was subsequently placed over the HC to prevent cell migration but allow cytokine transport. We compared the mRNA expression of CD45- MSC cultured without HC to that of those co-cultured with HC. We saw that all osteogenic

marker mRNAs were significantly higher in the group that was cultured without HC, suggesting an involvement of cytokines in HC inhibition of CD45- MSC osteogenic differentiation.

Osteoblasts and MSC are known to support haematopoiesis by secreting hematopoietic cytokines that work in both a paracrine and an autocrine way. Some molecules that are known to influence bone metabolism are colony stimulating factors (IL-3, GM-CSF, G-CSF) and IL-1, TNF, lymphotoxin, IFNγ, IL-8, IL-10, IL-4, and the LIF/IL-6 family along with others[32]. Their influence on osteogenesis is still not clearly understood because the studies have been either been performed *in vivo*, where systemic hormones play additional roles, or *in vitro* with osteogenic precursors at different degrees of maturation. Taichman et al.[33] co-cultured human bone marrow CD34+ hematopoietic cells and osteoblasts and showed that HC stimulated osteoblasts to produce higher levels of IL-6 and LIF in a cell-cell contact independent way. On the other hand, levels of G-CSF, GM-CSF, and TGF-β1 remained under the detection limit. The maximal accumulation of IL-6 and LIF occurred within the first 24 hours and correlated with the CD34+ cell number. In rat calvaria (RC) osteoprogenitor cultures, LIF showed an inhibiting effect on bone nodule formation and ALP activity both in the presence or absence of dexamethasone[34]. Moreover, in case of IL-6/LIF cytokines, the receptors seem to play crucial roles. With the exception of the oncostatin M receptor (OSMR), all LIF/IL-6 family receptors, which share a gp130 subunit (LIFR, IL-6R, IL-11R, OSMR), seem to suppress differentiation to the osteogenic line[35]. It has been observed, that when a glucocorticoid is added to the MSC cultures, the expression of IL-6, IL-11 and LIF is reduced[9]. It has been further suggested that TGF-β1 and IL-1 inhibit bone nodule formation in RC cell cultures through the stimulation of LIF production (discussed in [34]). There is little and controversial information, however, on the influence of IL-6/LIF on osteogenic differentiation of early MSC cultures[32,36,37]. Since our experiments were performed with early CD45- cells, we propose that the presence of MSC as well as osteoprogenitors in our culture and, therefore, IL-6 / LIF involvement in the observed effects is possible.

There are several other mechanisms involving HC that could inhibit osteogenic differentiation. For instance, granulocyte-colony stimulating factor (G-CSF) is known to play an important role in bone remodeling. When administered in rats, it produces osteoporosis as a consequence of two mechanisms, an increase in osteoclast numbers and reduced bone formation[32]. Additionally, macrophage-colony stimulating factor (M-CSF) enhanced osteoclast formation and inhibited MSC osteoblast formation *in vitro*[38]. Since MSC are

impacted to varying degrees by cytokines, growth factors, glucocorticoids, and many other effector-molecules in the *in vivo* bone marrow microenvironment, determining the influence of these effectors on MSC, either individually or in combination, is essential to enable understanding and manipulation of MSC involvement in bone formation and bone resorption[9]. In our case, screening studies could bring us a step closer to the identification of the factors that caused the observed effects.

It has already been proposed that *in vivo* MSC need some ”protective mechanism” against the autocrine effects of their secreted growth factors and cytokines to maintain them in an undifferentiated state as stem/progenitor cells[9,36]. These mechanisms may very likely involve other bone marrow populations, particularly hematopoietic stem and progenitor cells. We have to keep in mind the heterogeneity of bone marrow adherent and non-adherent populations and therefore the complexity of already made *in vitro* observations. Beside hematopoietic stem cells, the non-adherent bone marrow fraction *in vitro* contains platelets and megakaryocytes, which stimulate the differentiation and proliferation of MSC, potentially through PDGF and some other mitogenic factors[1,9], whereas our results show that the adherent myeloid cells suppressed osteogenic differentiation.

REFERENCES

(1) *Short B, Brouard N, Occhiodoro-Scott T, Ramakrishnan A, Simmons PJ. Mesenchymal stem cells. Arch Med Res. 2003;34:565-571.*

(2) *Minguell JJ, Erices A, Conget P. Mesenchymal Stem Cells. Experimental Biology and Medicine. 2001;226:507-520.*

(3) *Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells. 2001;19:180-192.*

(4) *Barry FP, Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. The International Journal of Biochemistry & Cell Biology. 2004;36:568-584.*

(5) *Caplan AI, Bruder SP. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. Trends Mol Med. 2001;7:259-264.*

(6) *Deans RJ, Moseley AB. Mesenchymal stem cells: Biology and potential clinical uses. Experimental Hematology. 2000;28:875-884.*

(7) *Prockop D.J. Marrow stromal cells as stem cells for nonhematopoetic tissues. Science 276, 71-74. 1997.*

(8) *Wang QR, Wolf NS. Dissecting the hematopoietic microenvironment. VIII. Clonal isolation and identification of cell types in murine CFU-F colonies by limiting dilution. Exp Hematol. 1990;18:355-359.*

(9) *Caplan AI, Reuben D, Haynesworth SE. Cell-based tissue engineering therapies: the influence of whole body physiology. Adv Drug Deliv Rev. 1998;33:3-14.*

(10) *Majumdar MK, Thiede MA, Haynesworth SE, Bruder SP, Gerson SL. Human marrow-derived mesenchymal stem cells (MSCs) express hematopoietic cytokines and support long-term hematopoiesis when differentiated toward stromal and osteogenic lineages. J Hematother Stem Cell Res. 2000;9:841-848.*

(11) *Compston J.E. Bone marrow and bone; a functional unit. Journal of Endocrinology. 2002;173:387-394.*

(12) *Javazon EH, Colter DC, Schwarz EJ, Prockop DJ. Rat marrow stromal cells are more sensitive to plating density and expand more rapidly from single-cell-derived colonies than human marrow stromal cells. Stem Cells. 2001;19:219-225.*

(13) *Aubin J. Osteoprogenitor Cell Frequency in Rat Bone Marrow Stromal Populations: Role of Heterotypic Cell-Cell Interactions in Osteoblast Differentiation. J Cell Biochem. 1999;72:396-410.*

(14) *Hacker M, Tessmar J, Neubauer M et al. Towards biomimetic scaffolds: anhydrous scaffold fabrication from biodegradable amine-reactive diblock copolymers. Biomaterials. 2003;24:4459-4473.*

(15) *Dobson KR, Reading L, Haberey M, Marine X, Scutt A. Centrifugal isolation of bone marrow from bone: an improved method for the recovery and quantitation of bone marrow osteoprogenitor cells from rat tibiae and femurae. Calcif Tissue Int. 1999;65:411-413.*

(16) *Freed LE, Vunjak-Novakovic G. Culture of organized cell communities. Adv Drug Deliv Rev. 1998;33:15-30.*

(17) *Kim YJ, Sah RL, Doong JY, Grodzinsky AJ. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. Anal Biochem. 1988;174:168-176.*

(18) *Ahmed N. VB, Rohde E, Strunk D, Schulz MB, Grifka J, Grässel S. CD45-positive cells of hematopoietic origin enhance chondrogenic marker gene expression in rat marrow stromal cells. 2005. Unpublished Work*

(19) *Majumdar MK, Thiede MA, Mosca JD, Moorman M, Gerson SL. Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. J Cell Physiol. 1998;176:57-66.*

(20) *Herbertson A, Aubin JE. Cell sorting enriches osteogenic populations in rat bone marrow stromal cell cultures. Bone. 1997;21:491-500.*

(21) *Miao D, Murant S, Scutt N, Genever P, Scutt A. Megakaryocyte-bone marrow stromal cell aggregates demonstrate increased colony formation and alkaline phosphatase expression in vitro. Tissue Eng. 2004;10:807-817.*

(22) *Friedenstein AJ, Latzinik NV, Gorskaya YuF, Luria EA, Moskvina IL. Bone marrow stromal colony formation requires stimulation by haemopoietic cells. Bone Miner 18[3], 199-213. 1992.*

(23) *Banuls MP, Alvarez A, Ferrero I, Zapata A, Ardavin C. Cell-surface marker analysis of rat thymic dendritic cells. Immunology. 1993;79:298-304.*

(24) *Tamatani T, Kotani M, Miyasaka M. Characterization of the rat leukocyte integrin, CD11/CD18, by the use of LFA-1 subunit-specific monoclonal antibodies. Eur J Immunol. 1991;21:627-633.*

(25) *Lucas T, Krugluger W, Samorapoompichit P, et al. Self-renewal, maturation, and differentiation of the rat myelomonocytic hematopoietic stem cell. FASEB J. 1999;13:263-272.*

(26) *Lieb E, Milz S, Vogel T et al. Effects of transforming growth factor beta1 on bonelike tissue formation in three-dimensional cell culture. I. Culture conditions and tissue formation. Tissue Eng. 2004;10:1399-1413.*

(27) *Neubauer M, Fischbach C, Bauer-Kreisel P et al. Basic fibroblast growth factor enhances PPARgamma ligand-induced adipogenesis of mesenchymal stem cells. FEBS Lett. 2004;577:277-283.*

(28) *Crevensten G, Walsh AJ, Ananthakrishnan D et al. Intervertebral disc cell therapy for regeneration: mesenchymal stem cell implantation in rat intervertebral discs. Ann Biomed Eng. 2004;32:430-434.*

(29) *ter Brugge PJ, Jansen JA. In vitro osteogenic differentiation of rat bone marrow cells subcultured with and without dexamethasone. Tissue Eng. 2002;8:321-331.*

(30) *Aubin J, Triffit JT. Mesenchymal Stem Cells and Osteoblast Differentiation. In: Bilezikian JB, Raisz LG, Rodan GA, eds. Principles of Bone Biology.1. San Diego;San Francisco;New York;Boston;London;Sydney;Tokyo: Academic Press; 2002:59-81.*

(31) *Lennon DP, Haynesworth SE, Arm DM, Baber MA, Caplan AI. Dilution of human mesenchymal stem cells with dermal fibroblasts and the effects on in vitro and in vivo osteochondrogenesis. Dev Dyn. 2000;219:50-62.*

(32) *Horowitz MZ, Lorenzo JA. Local Regulators of Bone. In: Bilezikian JB, Raisz LG, Rodan GA, eds. Principles of Bone Biology.2. San Diego;San Francisco;New York;Boston;London;Sydney;Tokyo: Academic Press; 2002:961-978.*

(33) *Taichman RS, Reilly MJ, Verma RS, Emerson SG. Augmented Production of Interleukin-6 by Normal Human Osteoblasts in Response to CD34+ Hematopoietic Bone Marrow Cells In Vitro. Blood. 1997;89:1165-1172.*

(34) *Malaval L, Gupta AK, Aubin JE. Leukemia inhibitory factor inhibits osteogenic differentiation in rat calvaria cell cultures. Endocrinology. 1995;136:1411-1418.*

(35) *Malaval L, Liu F, Vernallis AB, Aubin JE. GP130/OSMR is the only LIF/IL-6 family receptor complex to promote osteoblast differentiation of calvaria progenitors. J Cell Physiol. 2005;204:585-593.*

(36) *Erices A, Conget P, Rojas C, Minguell JJ. gp130 Activation by Soluble Interleukin-6 Receptor/Interleukin-6 Enhances Osteoblastic Differentiation of Human Bona Marrow-Derived Mesenchymal Stem Cells. Experimantal cell research. 2002;280:24-32.*

(37) *Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca RD et al. Multilineage Potential of Adult Human Mesenchymal Stem Cells . Science 284, 143-147. 1999.*

(38) *Gyda M, Corisdeo S, Zaidi M, Troen BR. Macrophage Colony-Stimulating Factor Suppresses Osteoblast Formation. Biochemical and Biophysical Research Communications. 2001;285:328-334.*

CHAPTER 4

TOWARDS SELECTIVE ADHESION OF RAT MARROW STROMAL CELLS: PHENOTYPE CHARACTERIZATION AND ADHESIVE PROPERTIES OF PLASTIC ADHERENT RAT BONE MARROW STROMAL CELLS

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ABSTRACT

Plastic-adherent bone marrow cells are often used as a source of marrow stromal cells (MSC) that can differentiate into cells of supportive tissue. We isolated rat bone marrow cells, seeded them in tissue culture flasks and cultured 3-day adherent cells in alpha MEM supplemented with 10 % fetal bovine serum up to a week. We characterized adherent rat bone marrow cells using 6-parameter flow cytometry in order to determine stem cell markers and adhesion molecules on MSC, as well as the presence of other bone marrow subpopulations. We found that after 1-week in culture; plastic–adherent rat bone marrow cells contained CD45- cells with MSC-like surface marker expression, and a minor population of CD45+ hematopoietic cells (10 – 30 %), while no CD31+ endothelial cells was found. CD45- MSC expressed CD29, CD49e, CD54 and CD90 strongly, CD44 and CD49a moderately and weakly CD49b, CD49c, CD61 and CD106. Hematopoietic cells in our cultures belonged to the myeloid lineage and expressed CD29, CD44, CD49a, CD49e, CD54, and CD90 beside CD45 and CD11a, b/c. Adhesion experiments resembled the expression of adhesion molecules. CD45- cells adhered strongly to fibronectin, collagen 4 and collagen 1, whereas CD45+ cells adhered to fibronectin, vitronectin and bovine serum albumin strongly, while adhesion to laminin and collagens 1 and 4 was weak, making collagens selective substrates for MSC adhesion in the presence of myeloid cells.

INTRODUCTION

Since Friedenstein[1] and his colleagues reported about *in vitro* colony forming fibroblasts from the bone marrow, which form bone when implanted, these cells have been investigated for different kinds of cellular therapies and tissue engineering. Tissue engineering approaches involve biomaterials, the surface of which offers means to influence cell adhesion and function. Modification with adhesion promoting ligands, such as Arg-Gly-Asp (RGD) peptide, facilitate tissue ingrowths and to a certain degree support cell type selective adhesion[2]. The RGD sequence is expressed in many extracellular matrix (ECM) molecules like fibronectin, vitronectin, bone sialoprotein, osteopontin, thrombospondin, fibrinogen, laminin, collagen, nectinepsin and in other molecules[3]. Cell receptors for these proteins include integrins, which belong to a widely distributed family of cell surface molecules that mediate cell adhesion. Integrins are heterodimeric glycoproteins consisting of an α (17 known)and a β subunit (9 known), forming 23 distinct heterodimers[4]. Integrins interact with many peptide motivs in ECM molecules and so are through inside-out and outside-in signaling involved in a range of physiological processes, including embryogenesis, morphogenesis, wound repair, inflammation, tumor cell migration and leukocyte trafficking. Integrin-mediated adhesion also involves other binding sites, e.g. heparin binding site[3]. In addition to matrix proteins, integrins can bind to other cell surface receptors, e.g. members of the imunoglobulin (Ig) superfamily ICAM-1/CD54, VCAM-1/CD106, and soluble proteins like fibrinogen and inactivated complement component C3[5].

First step towards selective osteoprogenitor cell immobilization on peptide-modified biomaterials is to determine which integrins are actually expressed on the cells to be attached. Additionally, the level of receptor expression has to be high enough to enable adequate cell adhesion. After that the decision has to be made which integrin-specific ligand needs to be bound on the biomaterial surface.

Marrow stromal cells (MSC) are plastic adherent fibroblastoid cells isolated from bone marrow, which are often used as cell source for tissue engineering applications. These cells are a heterogeneous population of precursor cells including cells showing stem-cell like characteristics. They are therefore often referred to as mesenchymal stem / progenitor cells (MPC). MPC-like cells have been isolated from bone marrow, periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle and deciduous teeth[6], but are reported to occur in highest frequencies in adult bone marrow[7,8]. They can replicate as undifferentiated cells and have the potential to differentiate to lineages of mesenchymal tissues, including bone,

cartilage, fat, tendon, muscle, and marrow stroma[9]. MPC express a large spectrum of cell adhesion molecules of potential importance in cell binding and homing interactions. The characterization of freshly isolated MPC (Stro-1 positive immunoselection) from the human bone marrow showed the expression of α1β1 (CD49a/CD29), α2β1 (CD49b/CD29), α5β1 (CD49e/CD29), α6β1 (CD49f/CD29), αVβ3 (CD51/CD61) and αVβ5 integrins and substrate / integrin dependent colony formation[10]. For *in vitro* expanded human MPC it was shown, that they highly express integrins α1, α5 and β1, low α2, α3 (CD49c), α6, αV, β3 and β4, and don't express α4 (CD49d), αL (CD11a) and β2 (CD18)[11].

Rat MPC have been characterized by Javazon et al.[12] as cells sensitive to plating density showing rapid expansion at low density culture. Furthermore, when seeded at densities of 5,000 cells / cm^2 and expanded up to 5 passages, they express CD90 and CD59 and are negative for CD31, CD4, CD11b, CD43, CD45 and mononuclear phagocyte marker. Data on the integrin expression of primary rat MSC have been reported before, but only after osteogenic treatment[13]. Furthermore, that study did not distinguish between mesenchymal and hematopoietic or endothelial cells known to reside in the early cultures.

For the purpose of tissue engineering using the rat animal model, differentiation of MSC to the osteogenic[14], chondrogenic[15] and adipogenic[16] lineage has been established. Usually, bone marrow is isolated from femurs and tibias of an animal, and then the cells are re-suspended in proliferation medium without any other separation procedures, seeded and cultured until sub-confluence. Plastic non-adherent cells are removed with medium changes, usually on day 3 or even later. The differentiation medium is generally added to primary or secondary cultures and not later, because of decreased differentiation capacity with increasing passage number[17].

This study intended to characterize plastic adherent cells of primary rat bone marrow cultures after 7 days. Since in a recent study we have shown that CD45+ myeloid hematopoietic cells (HC) suppress osteogenic differentiation of MSC, integrin expression of MSC and HC was investigated to find ways for selective adhesion of MSC in the presence of HC. Confirming these results on different integrin expression patterns on MSC and HC cell adhesion to a panel of ECM proteins was performed using immunomagnetically separated HC and MSC.

MATERIALS AND METHODS

Suppliers

Gibco, Invitrogen, UK: Foetal calf serum, FCS (Lot No.: 40F7430K); Penicillin-Streptomycin (10,000 units / mL penicillin G sodium and 10,000 µg / mL streptomycin sulphate); Trypsin-EDTA (0.25 % Trypsin and 1mM EDTA.4Na); phosphate buffered saline (PBS, w/o Ca and Mg);
Sigma Aldrich, Germany: L-glutamine (200mM); Minimum essential medium Eagle, alpha modification (α-MEM; 10.1 g / L); 7-Aminoactinomycin D (7-AAD);
Corning, Netherlands: Tissue Culture Flasks.

Cell isolation and cell culture

Rat bone marrow was isolated from femur and tibia of 6-week old (150-200g) male Sprague-Dawley rats (Institut für Labortierkunde und -genetik Himberg, Medical University Vienna, Vienna, Austria) by centrifugation[18]. Freshly isolated bone marrow cells were re-suspended in primary medium (10 % FCS, 1 % Penicillin/Streptomycin, 0.5 % L-glutamine and α-MEM). For each experiment bone marrow cells of at least 3 animals were pooled. Cells obtained from 1 rat (2 femurs and 2 tibias) were seeded in 4 x 150 cm^2 flasks (approx. 1,5 x 10^5 nucleated cells / cm^2) and were cultured in humidified atmosphere with 5 % CO_2 at 37°C. After 3 days, non-adherent cells were removed by rinsing twice with 16 mL / flask PBS. The medium was changed three times a week. On day 7 after cell isolation (90 - 100% confluence), the cells were detached from the flasks using trypsin-EDTA. After trypsin deactivation cells were passed through 40 µm cell strainer, centrifuged, resuspended, and counted using haemacytometer.

Flow cytometry

The cell suspension was first incubated for 15 minutes at 4°C with 10 % v/v sheep serum and then for 30 minutes at 4°C with antibodies against rat CD markers or with corresponding isotype controls (Table 1). Because of the constant presence of CD45+ hematopoietic cells, all the stains were done simultaneously with anti-CD45 antibody. Cells were washed and resuspended with PBS containing sodium azide and bovine serum albumin (BSA) before the measurements. Short before measurements 20 % v/v 7-AAD (20 µg / mL) was added to each sample for 3 minutes to stain the dead cells, which were then excluded from the evaluation. For the stains with antibodie against CD49c the cells were fixed and permeabilized using

Fix&Perm® kit (An der Grub, Kaumberg, Austria) according to the manufacturer's instructions. When the stains requested incubations with anti mouse secondary antibodies, anti-CD45 was added at the end, following a blocking step with normal mouse serum.
All measurements were preformed on a FACSCalibur® instrument and data acquired using the CellQuest software (both BD Biosciences, USA). Measurements were evaluated with WinMDI 2.8 Software (free access on http://facs.scripps.edu/software.html). A minimum of $1x10^4$ viable cells of each population (CD45- and CD45-) were acquired per data set.

The Percentage of positive cells was determined as the percentage of cells having a measured fluorescence greater than that of 99.5 % of the cells that had been stained with each associated isotype control. A population was considered positive for a surface marker when the percentage of positive cells for that surface marker was ≥ to 5 %. The level of marker expression (LME) was calculated as the ratio between geometric mean fluorescence intensity (MFI) of sample cells and that of the isotype control[19]. Quantitative results represent mean values of 3 independent experiments ± standard deviation.

Table 1: *Antibodies used in the flow cytometric experiments.*

Antigene / CD	Other name	species	Clone	Ig class	Label	Producer	Cat. Nu.
CD11a	αL integrin	Ms	WT.1	IgG2a κ	PE	Pharmingen	550972
CD11b/c	αM/X integrin	Ms	OX-42	IgG2a κ	FITC	Pharmingen	554861
CD29	β1 integrin	Ham	Ha2/5	IgM κ	FITC	Pharmingen	555005
CD31	PECAM-1	Ms	TLD-3A12	IgG1	FITC	Acris	SM272F
CD44	H-CAM	Ms	OX-50	IgG1	PE	Acris	SM273R
CD45	LCA	Ms	MRC OX-1	IgG1	PE	Acris	SM274R
CD49a	α1 integrin	Ham	Ha31/8	IgG2 λ	-	Pharmingen	555001
CD49b	α2 integrin	Ham	Ha1/29	IgG2 λ	FITC	Pharmingen	554999
CD49c	α3 integrin	Ms	42	IgG1	-	Pharmingen	611044
CD49d	α4 integrin	Ms	TA-2	IgG1	FITC	Serotec	MCA1383F
CD49e	α5 integrin	Ham	HMα5-1	IgG1 κ	-	Pharmingen	553350
Hu CD51/β5	αVβ5 integrin	Ms	P1F6	IgG1	FITC	Chemicon	MAB1961F
CD54	ICAM-1	Ms	1A29	IgG1 κ	PE	Pharmingen	554970
CD61	β3 integrin	Ham	2C9.G2	IgG1 κ	PE	Pharmingen	553347
CD90	Thy-1	Ms	OX-7	IgG1 κ	FITC	Pharmingen	554897
CD106	VCAM-1	Ms	MR106	IgG1 κ	PE	Pharmingen	559229
Ham IgG	2nd step	Ms	G70/204, G94-90.5	IgG1 κ	PE	Pharmingen	554056
Ms Ig	2nd step	Goat	*	*	APC	Pharmingen	550826
Mineral oil	isotype	Ms	MOPC-31C	IgG1 κ	-	Pharmingen	557273
Unknown	isotype	Ms	MOPC-21	IgG1 κ	FITC	Pharmingen	555748
Unknown	isotype	Ms	*	IgG1	PE	Cymbus Biotech.	CBL 600P
TNP-KLH	isotype	Ms	G155-178	IgG2a κ	FITC	Pharmingen	553456
TNP	isotype	Ms	G155-178	IgG2a κ	PE	Pharmingen	555574
TNP-KLH	isotype	Ham	A19-3	IgG1 κ	-	Pharmingen	553969
TNP-KLH	isotype	Ham	A19-3	IgG1 κ	PE	Pharmingen	553972
KLH	isotype	Ham	Ha4/8	IgG2 λ	-	Pharmingen	553962
KLH	isotype	Ham	Ha4/8	IgG2 λ	FITC	Pharmingen	553964
TNP-KLH	isotype	Ham	G235-1	IgM λ	FITC	Pharmingen	553960

*Legend: *: not stated; Ms: mouse; Ham: hamster; Hu: human; FITC: fluorescein-isothiocyanat; PE: phycoerythrin; APC: allophycocyanin; TNP: trinitrophenol; KLH: keyhole limpet hemocyanin.*

Magnetic cell separation

To separate CD45+ and CD45- cells, cells were re-suspended in separating buffer (PBS pH 7.2 supplemented with 0.5 % BSA and 2 mM EDTA) and incubated with primary phycoerythrin (PE)- labeled antibody against rat CD45 at 4°C in the dark for 30 minutes. After washing, the cell suspension was incubated with secondary anti-PE antibody-coupled magnetic beads (MACS® MicroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 minutes at 4°C in the dark. After washing, the cells were re-suspended in separating buffer and applied onto the LS column placed in a magnetic field (MidiMACS separator, Miltenyi Biotech). The negative fraction was first collected (CD45- MSC) and then, after the removal of the column from the magnet, the CD45+ fraction was obtained. Both cell fractions were washed with α-MEM, the viable cells were counted and the viability and purity of both

populations was determined by flow cytometry. The purity of the negative fraction was 99-100 % and 90-95 % of the positive fraction.

Adhesion experiments

After magnetic sorting, the cells were tested for adhesion onto different ECM proteins. We used a 96-well CytoMatrix screening kit (Chemicon, USA) and determined the proportion of adherent cells after crystal violet staining and dye solubilization, with absorbance measurement at 544 nm using a micro plate reader. The assay was performed according to the manufacturer's instructions. Cell densities of 32,000 (CD45-) or 36,000 (CD45+) cells / well and a 3- hour adhesion time were used. Adherence to bovine serum albumin (BSA) and to tissue culture polystyrene (Costar, Corning) in the presence of 10% FBS served as controls.

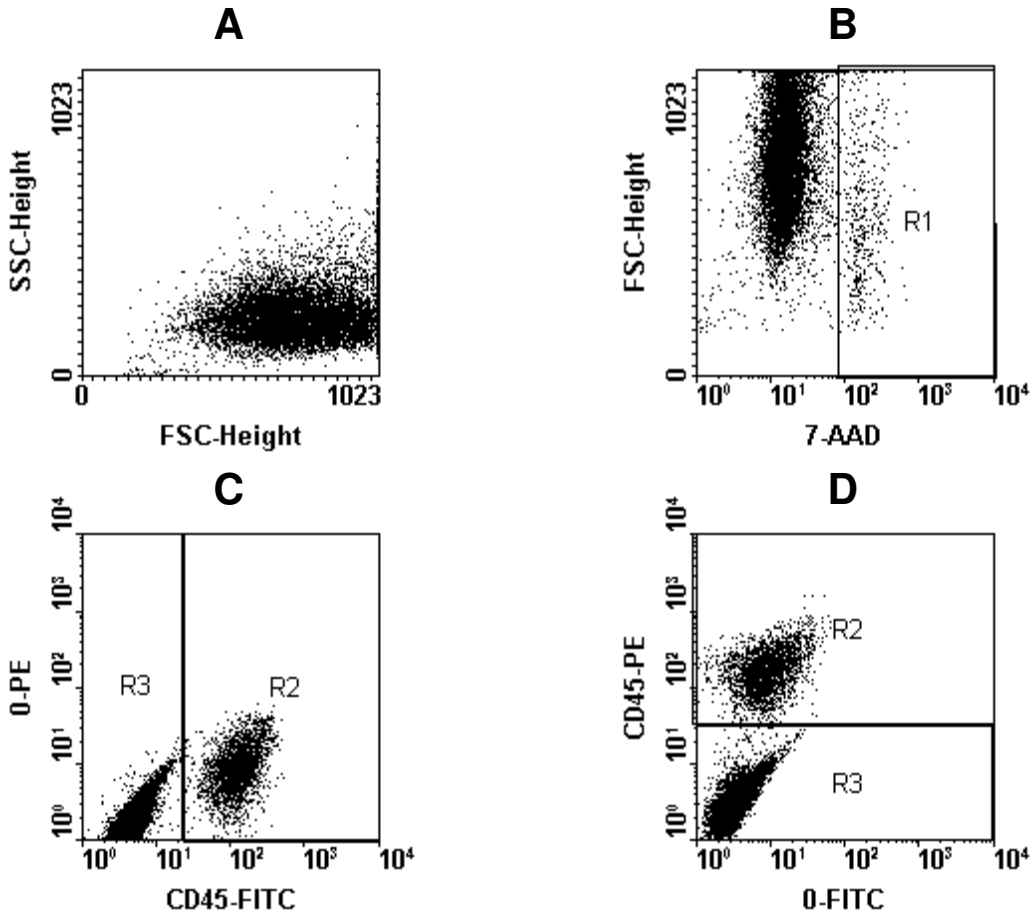


Figure 1: *Representative Dot-Plots of the primary cultures of rat marrow stromal cells. A: forward (FSC) vs. sideward scatter (SSC); B: 7-AAD positive cells (dead cells) marked as R1 were excluded from the evaluation; C and D: differentiation between both cell populations was achieved with CD45-PE or CD45-FITC staining. R2 represents CD45+ hematopoietic cells and R3 CD45- rat MSC.*

RESULTS

In a previous study we have shown that primary cultures (up to 7 days) of rat MSC always contain population of CD45+ HC (10-30 %)[20] if no other separation procedure than adherence to tissue culture plastic was used (Fig.1). No CD31 positive cells (including endothelial cells) were detected and according to optical observations, there were no preadipocytes in our cultures (data not shown). By 6-parameter flow cytometry we were able to determine the surface marker expression on HC and MSC separately without the need to mechanically separate the populations. Double staining with antibodies against CD45, a common leukocyte antigen, enabled us to distinguish between HC and MSC. The quantitative results are presented in Fig. 2. CD45- MSC expressed CD29, CD49e, CD54 and CD90 strongly (LME>10), CD44 and CD49a intermediate (5<LME<10) and CD49b, CD49c, CD61 and CD106 low (LME<5). They did not express the hematopoietic markers CD45, CD11a, and CD11b/c. We did not detect any expression of CD49d and CD51 or αV/β5 on MSC. CD45+ hematopoietic cells expressed CD11a, CD29, CD44, CD49a and CD90 low, CD49e and CD11b/c intermediate and strongly CD54.

Adhesion experiments (Figure 4) showed that CD45- MSC adhered well to tissue culture plastic in the presence of FBS, followed by adhesion to fibronectin. CD45- MSC adhered also well to collagen 4, collagen 1, and on vitronectin and weaker to laminin. CD45- MSC didn't adhere to BSA coated wells.[MSOffice38] On the contrary, CD45+ cells showed strong adhesion to BSA-coated wells and to tissue culture plastic in the presence of FBS. The adhesion of CD45+ HC to fibronectin was also high and surprisingly also adhesion to vitronectin. CD45+ cells showed, however, low adhesion to laminin, collagen 1 and collagen 4 (figures 4B-D).

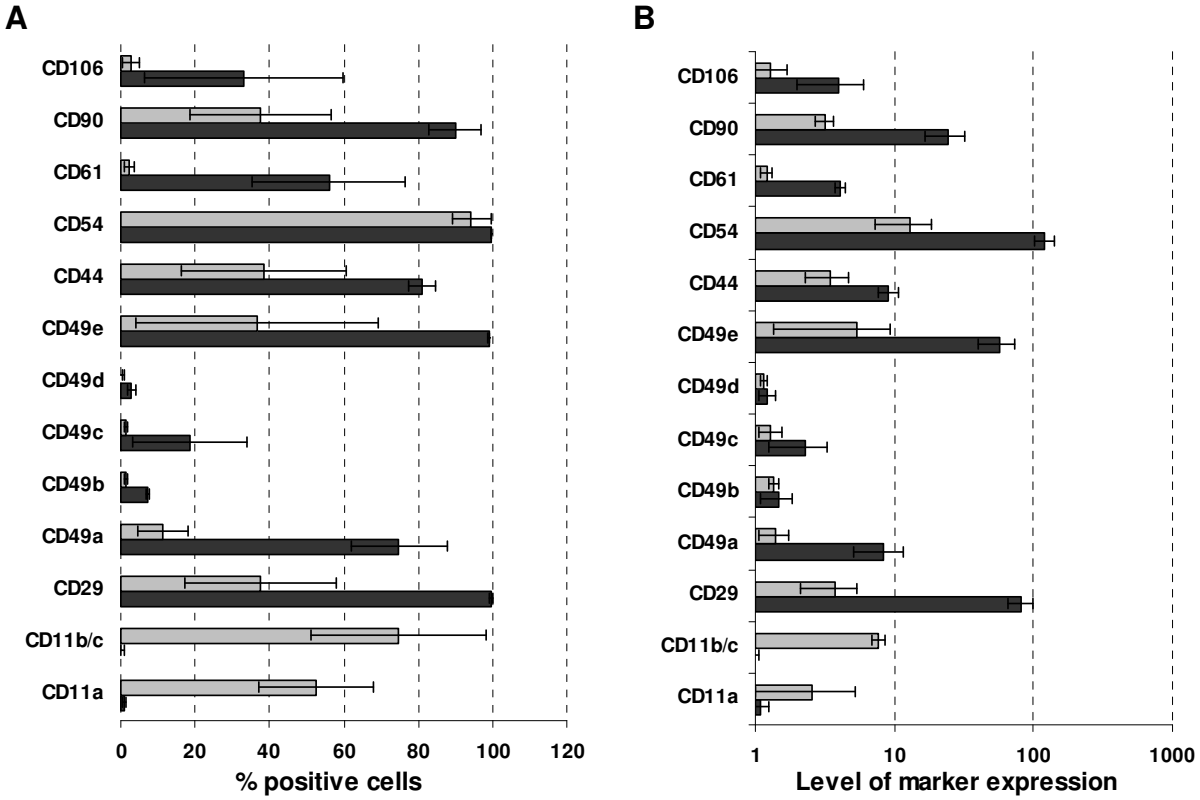


Figure 2: *Quantitative evaluation of flow cytometric measurements. Grey bars represent CD45+ cells and black bars represent CD45- MSC. Represented values are an average of 3 independent experiments ± standard deviation.*

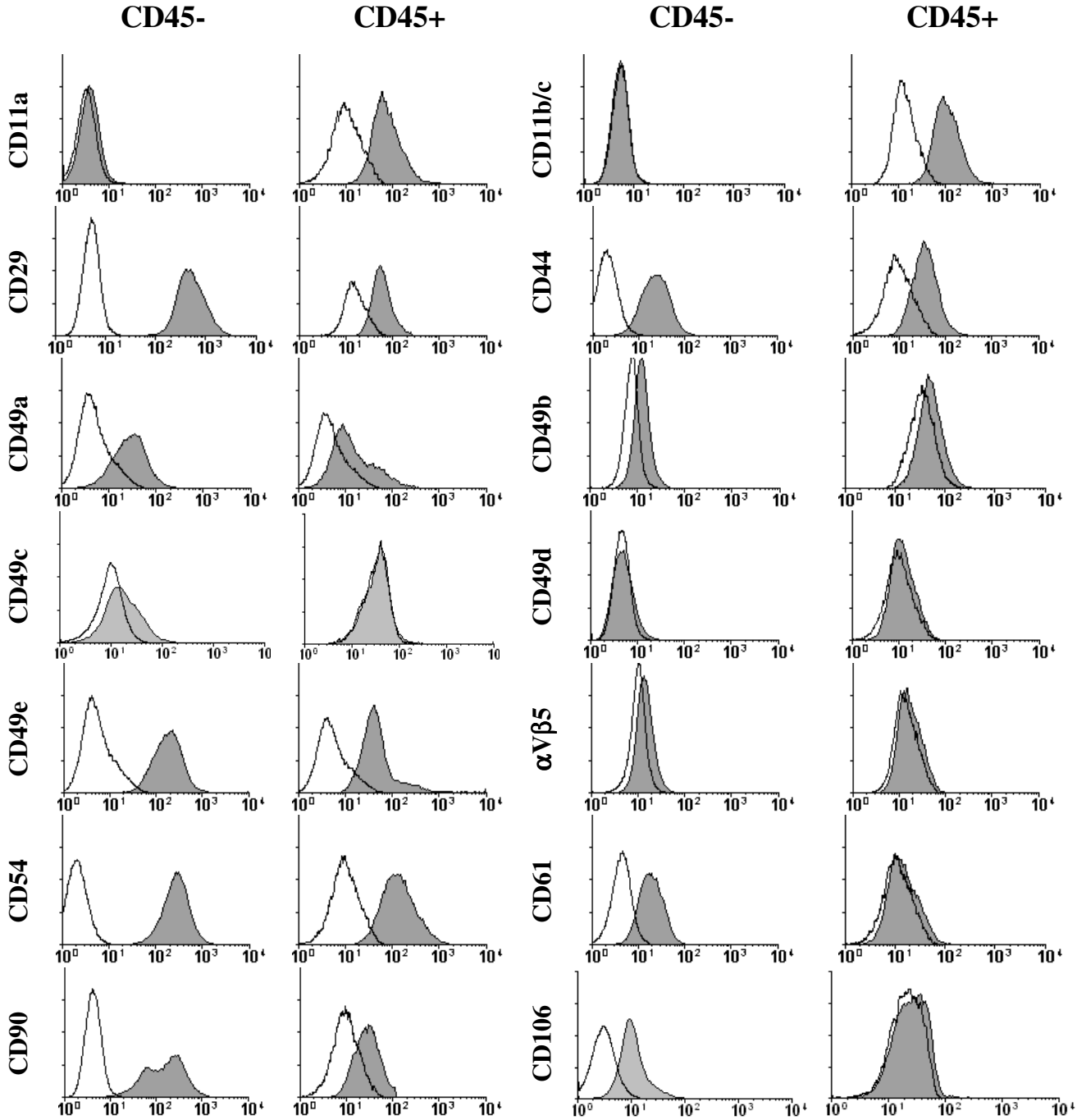
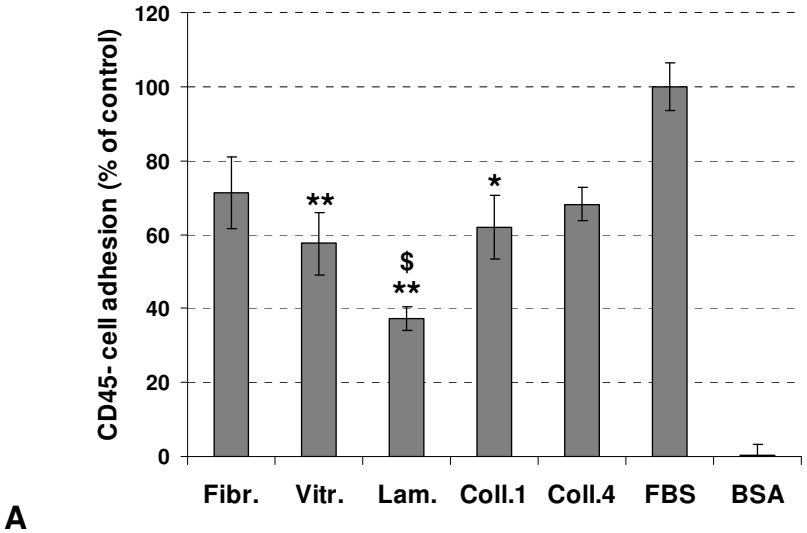
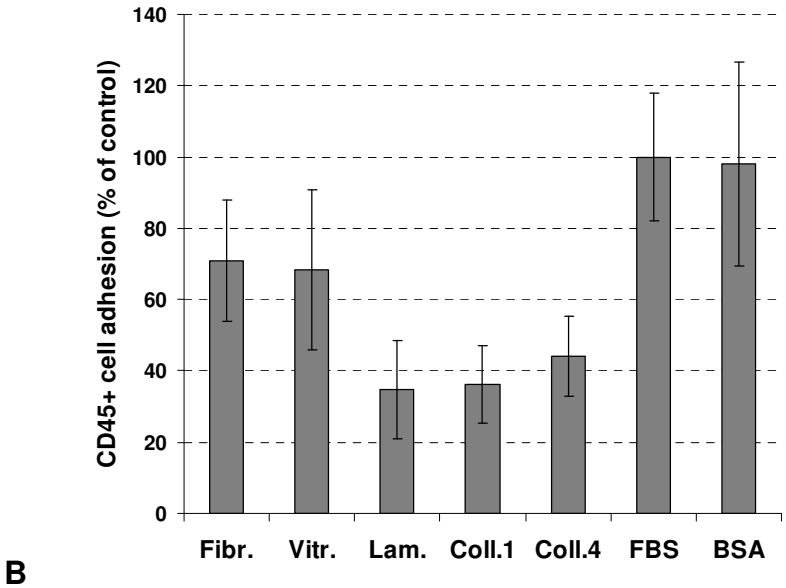


Figure 3: *Representative histograms of the marker expression. CD45+ and CD45- cells are separated on the basis of CD45 staining, as seen in Figure 1. Empty histograms represent fluorescent intensity of the isotype controls.*



A



B

Figure 4: *Adhesion of separated cell populations (CD45+ and CD45-) to extracellular matrix coated wells, as assessed with crystal violet absorbance measurements. Figure 4A shows the adhesion of CD45- MSC and 4B of CD45+ HC. The results are an average of 3 independent experiments performed in triplicate + standard error of the mean. Statistical significance as determined with Tukey-test is marked with * ($p < 0.05$) or ** ($p < 0.01$) compared to FBS and $ ($p < 0.05$) as compared to fibronectin. Fibr.: fibronectin, Vitr.: vitronectin, Lam.: laminin, Coll.1: collagen I, Coll.4: collagen IV, FBS: fetal bovine serum, BSA: bovine serum albumin.*

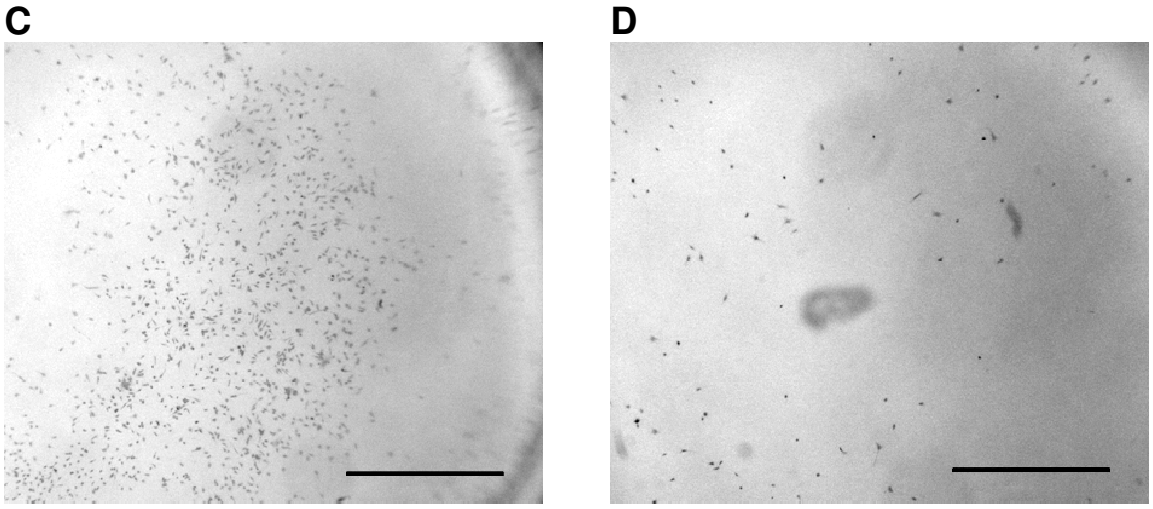


Figure 4, continued: *C, D: Representative photographs of the wells with CD45+ HC. Figure 4C shows the cell density on a vitronectin coated well, and the Figure 4D the cell density on a collagen I coated well. Bar = 1mm.*

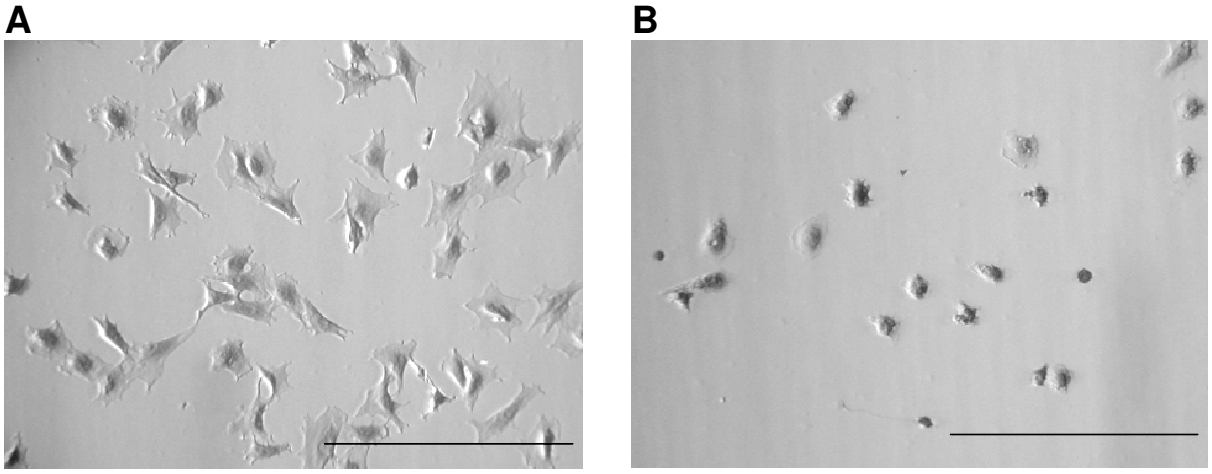


Figure 5: *Morphology of CD45- MSC (A) and CD45+ cells (B) after separation procedure. Cells were seeded for 3 hours on tissue culture polystyrene in medium supplemented with 10% FBS. Bar=250µm.*

DISCUSSION

It is known from studies on human and murine MSC, that primary cultures are often contaminated with hematopoietic cells of macrophage morphology and endothelial cells[9,22]. The presence of hematopoietic cells in primary cultures of rat marrow derived stromal cells has also been reported[23]. In our cultures of plastic-adherent rat bone marrow cells we detected varying proportions of CD45+ cells (10 - 30 %)[20]. Stem cell characteristics of rat MSC, concerning frequency of CFU-F and osteoprogenitors, have been already studied[12,18,23]. However, detailed surface marker characterization on early-passage-rat MSC is still missing.

Characteristic non-integrin adhesion receptors

CD45- cells from our cultures were positive for CD90, CD44 and CD106, being characteristic markers for MSC. It is known that CD106 interacts with CD49d/CD29 (α4β1 integrin, VLA-4) and α4β7 integrin playing an important role in leukocyte extravasation[24]. Through CD49d/CD29 CD106 also promotes early hematopoietic cell adhesion on human MSC[25].

CD44 was expressed by both CD45- MSC and CD45+ HC. The presence of CD44 on human MSC distinguishes them from CD44- bone marrow fibroblasts[26]. CD44 is also weakly expressed on osteoblasts, but stronger on osteocytes and osteoclasts. Its role in bone biology is still poorly known [4,27]. The receptor is important in cell adhesion to hyaluronate, laminin, collagen and[MSOffice39] fibronectin[4]. It is involved in leukocyte attachment and rolling on endothelial cells, homing to the peripheral lymph organs and sites of inflammation and leukocyte aggregation[28]. Similarly it was shown that CD90 interacts with CD11b/CD18 in leukocyte adhesion to endothelium[29]. The Ig superfamily glycoprotein CD90 is rat Thy-1 antigen, and a commonly stated marker for progenitor cell types[30,31]. It was highly expressed on our CD45- MSC, confirming their progenitor character and weakly or not on CD45+ HC suggesting the presence of more mature cells.

Another marker, known to be expressed by human MSC and osteoblasts is CD54 (ICAM-1)[32,33]. We detected high expression of this marker on both, CD45+ HC and CD45- MSC. In rat it was found on vascular endothelium in lymphoid tissues, thymic stromal cells, monocytes, macrophages, mast cells and weakly on peripheral lymphocytes[34,35]. CD54 is a ligand for the hematopoietic integrins CD11a/CD18 and CD11b/CD18, and, additionally, together with CD106, CD54 may play a role in the interactions of osteoclast precursors with stromal cells in the bone marrow microenvironment[4].

CD106, CD44, CD90 and CD54 can all promote cell-cell adhesion, and, whether expressed by CD45- MSC or CD45+ HC, might contribute to the secondary "adherence" of HC to the already adherent cells in our cultures. Leukocyte integrins (CD11a, CD11b and CD11c/CD18) in general appear to mediate the attachment that accompanies rolling and conversion of leukocytes from circulating cells to adherent tissue cells. By that leukocyte integrins are activated, and the endothelium expresses increased amounts of CD54[5]. Similar interactions have been suggested between MSC and hematopoietic stem cells in the bone marrow and in the culture, since the adhesion receptor pattern on MSC is comparable to that on endothelial cells[36].

β1- and β3 associated integrins on MSC and HC

CD29 is integrin subunit β1 and is expressed on the cell surface as a heterodimer with one of the distinct integrin α chains. With CD49a through CD49f (α1 through α6), it forms the VLA (very late antigen) complexes (VLA-1, etc.), which are adhesion receptors for various extracellular matrix proteins e.g. collagens, fibronectin, laminin, vitronectin, osteopontin. As a result, CD29 has a broad tissue distribution, including lymphocytes, endothelia, smooth muscle, and epithelia[37] and plays an important role in cellular processes, including embryogenesis and hematopoietic stem cell development[3]. Therefore it was expected that both types of cells found in our cultures expressed CD29, though CD45- cells expressed CD29 stronger than CD45+ cells.

Among the tested α subunits of CD29 complexes, we found CD49a (α1) to be stronger expressed on CD45- MSC than on CD45+ HC. CD49a/CD29 is a receptor for collagens 1, collagen 4 and laminin[38], and it promotes proliferation upon activation[39]. CD49a positive selection of human MSC from fresh bone marrow results in plastic-adherent CD45+ and CD45- colonies[40] correlating with our observations, that both, CD45+ and CD45- cells expressed this marker. CD49a is otherwise expressed on activated T cells, monocytes, smooth muscle cells and endothelial cells[41]. Another collagen receptor, CD49b/CD29, was only weakly expressed on CD45- MSC, whereas it was not expressed on CD45+ cells. CD49b/CD29 is a receptor for laminin and collagens 1 to 4[38]. In rat it is expressed on activated lymphocytes, epithelial cells and platelets[41]. CD49b is connected with matrix remodeling, collagenase gene expression in osteoclasts and regulating collagen type I and other osteogenic marker expression in osteoblasts[4,39]. We tested 2 lots of Ha1/29 antibodies and the level of CD49b integrin expression was low or negative by both lots (data shown for one lot only). The third collagen receptor CD49c/CD29 was expressed only on CD45- MSC and not on CD45+

HC. Almost all cultured cell lines express CD49c, as well as basal epidermal cells, epithelial cells, B lymphocytes, monocytes, platelets, hematopoietic progenitor cells, and early osteoblastic cells[4,46]. It binds fibronectin, collagen 1, laminin and some other proteins[38].

The fibronectin receptor CD49e/CD29 (α5β1) is an often studied integrin, with a proven role in extracellular fibronectin assembling[43]. We found CD49e to be strongly expressed on both cell populations, CD45+ and CD45-, which is in agreement with the broad cellular distribution of this integrin. CD49e is also strongly expressed by human MSC[33,36]. CD49e/CD29 binding appears to be necessary for osteoprogenitor cell survival and bone development[4].

Integrin subunit CD61 (β3) was detected only on CD45- MSC and not on CD45+ HC from our cultures. It associates with subunit CD51 (αV) to form the vitronectin receptor and with CD41 (αIIb) chain to form the gpIIb/IIIa complex[44]. Both receptors mediate adhesion to fibronectin, fibrinogen, vitronectin, trombospondin, von Willebrand factor, and CD51/CD61 also to thrombospondin, osteopontin and bone sialoprotein 1[38,44]. It was shown that CD51/CD61 also binds to endothelial CD31/PECAM[51]. In rat CD61 is expressed on platelets[46], megacaryocytes[47], T lymphocytes[48] and on granulocytes[49]. The importance of CD61 for bone formation can be assumed, since human MSC[33], as well as MSC derived osteoblasts and HC derived osteoclasts express this marker[3].

Characterization of plastic-adherent hematopoietic cells

Hematopoietic cells in our cultures were positive for other general leukocyte / myeloid markers like integrin subunits CD11b/c and CD11a. CD18 (β2) integrin forms a heterodimer with any of the three distinct CD11 α subunits (CD11a or αL, CD11b or αM, CD11c or αX) to form, respectively, LFA-1, Mac-1 and gp150,95[35]. Like CD45, CD11a/CD18 is also found on the majority of the leukocytes and mediates a variety of heterotypic and monotypic intercellular adhesions through interaction with CD54 and CD102[35]. Beside cell-cell adhesion, CD11b/CD18 mediates binding to fibrinogen and some other proteins[5]. The OX-42 (anti CD11b/c) antibody reacts with the CR3 complement (C3bi) receptor, found on most monocytes, granulocytes, macrophages and dendritic cells[34,35]. It appears to recognize a common epitope, shared by CD11b and CD11c (integrin αM and αX chains)[35], mature and immature cells. CD45+ HC were further negative for CD3, CD4, CD45RA, CD161a and a granulocyte marker[50], which lead us to assume that our CD45+ cells represent a mixed cell population of mature and immature cells of the myeloid lineage, including monocytes, macrophages and their progenitor cells[50].

Adhesion of MSC and hematopoetic cells (HC) to ECM

After isolation, cell attachment to culture surfaces *in vitro* is usually mediated by adhesion proteins contained in serum-supplemented medium. Beside unspecific adhesion to tissue culture polystyrene, the initial adhesion and spreading of osteoblastic and various other cell types depends primarily on adsorbed vitronectin and fibronectin[51], whereas hematopoietic cells adhere also on other serum proteins (e.g. serum IgG, von Willebrand factor[52]). Both cell populations found in our cultures expressed among ECM-binding integrins fibronectin-binding integrin CD49e/CD29 strongly and MSC additionally vitronectin-binding integrin subunit CD61. The expression of these integrins could be both, the reason for cell adherence or the consequence of culture conditions used in this study[51]. The adhesion experiments showed that CD45-, as well as CD45+ cells adhered strongly to tissue culture polystyrene in the presence of FBS.

The adherence of CD45- MSC correlated well with the level of distinct integrin expression, determined by flow cytometry. Among tested ECM coatings, CD45- cells adhered to fibronectin, followed by collagen IV, collagen I and vitronectin, similar as it was reported for human MSC[53].

Also CD45+ HC adhered in the correlation to their integrin expression. CD45+ cells showed only low expression of integrins alpha 1, alpha 2, and alpha 3, and consequently didn't adhere to a relevant extent to collagen I, collagen IV and laminin. Shen and Horbett[54] showed that blood monocytes adhere to a similar proportion to the uncoated tissue culture polystyrene (TCPS), or to TCPS coated with serum, fibronectin or BSA, which is comparable to our observations of CD45+ HC adhesion. However, due to the smaller size of CD45+ HC (Figure 6), we hit upon the assay detection limit, resulting from lower dye uptake per cell. Generally CD45+ HC adhered to a lesser extent than CD45- MSC. Additionally, the separation procedure might have reduced the HC adhesion. Therefore, no statistical significant differences in adhesion of CD45+ HC could be proved. Nevertheless, the results shown in Figure 4b could be confirmed with optical observations, as shown on representative photographs of CD45+ cells on vitronectin and collagen I coated wells (Fig. 4c and 4d). However, we detected no expression of CD61 by CD45+ HC, known to mediate the adhesion of HC to vitronectin[38]. Some additional studies would help us to precisely analyze vitronectin-receptor expression on HC, before we can exclude CD61- or CD51-involved adhesion to vitronectin.

The data on CD29- and CD61- associated integrin expression allow for the following assumptions. Whereas surfaces with immobilized vitronectin or fibronectin would not cause the adhesion of desired CD45- MSC only, but also adhesion of undesired CD45+ HC, we expected a more selective adhesion to collagen-coated surfaces mediated by collagen / laminin integrins, if CD29 integrin-independent binding mechanisms[55,56] remain negligible. Adhesion experiments confirmed our expectations, because HC didn't adhere to collagen I or IV. Therefore collagen I and IV could serve as selective substrates for MSC adhesion. However, it is necessary to keep in mind that HC do not only attach via ECM, but also by cell-cell interactions. Nevertheless, a decrease in HC adhesion to a substrate appears to be possible by a proper substrate choice, e.g. collagens.

CONCLUSION

In vitro studies on communication between MSC and HC can help us to better understand observed effects *in vitro* and maybe some *in vivo* circumstances in which cells normally function. For their maintenance the cells use integrins and other adhesion receptors to communicate with ECM and other cells. We found that some hematopoietic cells from the bone marrow remain adherent in primary rat MSC cultures. They may use different mechanisms than MSC to adhere, since they express a different pattern of adhesion molecules. Our adhesion experiments showed that collagen coated surfaces represent substitutes for selective CD45- MSC adhesion. Apart from that, in this study rat MSC were characterized by means of surface marker expression in an extend that brings the characterization of rat MSC a step closer to human and mouse MSC.

REFERENCES

(1) *Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet. 1970;3:393-403.*

(2) *Knight MA, Evans GR. Tissue engineering: progress and challenges. Plast Reconstr Surg. 2004;114:26E-37E.*

(3) *Schaffner P, Dard MM. Structure and function of RGD peptides involved in bone biology. Cell Mol Life Sci. 2003;60:119-132.*

(4) *Horton MA, Nesbitt SA, Bennett JH, Stenbeck G. Integrins and Other Cell Surface Attachment Molecules of Bone Cells. In: Bilezikian JB, Raisz LG, Rodan GA, eds. Principles of Bone Biology. San Diego, San Francisco, New York, Boston, London, Sydney, Tokio: Academic Press; 2002:265-286.*

(5) *Ruoslahti E. Integrins. J Clin Invest. 1991;87:1-5.*

(6) *Barry FP, Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. The International Journal of Biochemistry & Cell Biology. 2004;36:568-584.*

(7) *Deans RJ, Moseley AB. Mesenchymal stem cells: Biology and potential clinical uses. Experimental Hematology. 2000;28:875-884.*

(8) *Prockop D.J. Marrow stromal cells as stem cells for nonhematopoetic tissues. Science 276, 71-74. 1997.*

(9) *Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca RD et al. Multilineage Potential of Adult Human Mesenchymal Stem Cells . Science 284, 143-147. 1999.*

(10) *Gronthos S, Simmons PJ, Graves SE, Robey PG. Integrin-mediated interactions between human bone marrow stromal precursor cells and the extracellular matrix. Bone. 2001;28:174-181.*

(11) *Majumdar MK, Keane-Moore M, Buyaner D et al. Characterization and functionality of cell surface molecules on human mesenchymal stem cells. J Biomed Sci. 2003;10:228-241.*

(12) *Javazon EH, Colter DC, Schwarz EJ, Prockop DJ. Rat marrow stromal cells are more sensitive to plating density and expand more rapidly from single-cell-derived colonies than human marrow stromal cells. Stem Cells. 2001;19:219-225.*

(13) *ter Brugge PJ, Torensma R, De Ruijter JE, Figdor CG, Jansen JA. Modulation of integrin expression on rat bone marrow cells by substrates with different surface characteristics. Tissue Eng. 2002;8:615-626.*

(14) *Lieb E, Milz S, Vogel T et al. Effects of transforming growth factor beta1 on bonelike tissue formation in three-dimensional cell culture. I. Culture conditions and tissue formation. Tissue Eng. 2004;10:1399-1413.*

(15) *Crevensten G, Walsh AJ, Ananthakrishnan D et al. Intervertebral disc cell therapy for regeneration: mesenchymal stem cell implantation in rat intervertebral discs. Ann Biomed Eng. 2004;32:430-434.*

(16) *Neubauer M, Fischbach C, Bauer-Kreisel P et al. Basic fibroblast growth factor enhances PPARgamma ligand-induced adipogenesis of mesenchymal stem cells. FEBS Lett. 2004;577:277-283.*

(17) *ter Brugge PJ, Jansen JA. In vitro osteogenic differentiation of rat bone marrow cells subcultured with and without dexamethasone. Tissue Eng. 2002;8:321-331.*

(18) *Dobson KR, Reading L, Haberey M, Marine X, Scutt A. Centrifugal isolation of bone marrow from bone: an improved method for the recovery and quantitation of bone marrow osteoprogenitor cells from rat tibiae and femurae. Calcif Tissue Int. 1999;65:411-413.*

(19) *Diaz-Romero J, Gaillard JP, Grogan SP et al. Immunophenotypic analysis of human articular chondrocytes: Changes in surface markers associated with cell expansion in monolayer culture. J Cell Physiol. 2004.*

(20) *Ahmed N. VB, Rohde E, Strunk D, Schulz MB, Grifka J, Grässel S. CD45-positive cells of hematopoietic origin enhance chondrogenic marker gene expression in rat marrow stromal cells. Chapter 2.*

(21) *Wang QR, Wolf NS. Dissecting the hematopoietic microenvironment. VIII. Clonal isolation and identification of cell types in murine CFU-F colonies by limiting dilution. Exp Hematol. 1990;18:355-359.*

(22) *Herbertson A, Aubin JE. Cell sorting enriches osteogenic populations in rat bone marrow stromal cell cultures. Bone. 1997;21:491-500.*

(23) *Aubin JE. Osteoprogenitor cell frequency in rat bone marrow stromal populations: role for heterotypic cell-cell interactions in osteoblast differentiation. J Cell Biochem. 1999;72:396-410.*

(24) *Bevilacqua MP. Endothelial-leukocyte adhesion molecules. Annu Rev Immunol. 1993;11:767-804.*

(25) *Patrick CW, Jr., Juneja HS, Lee S, Schmalstieg FC, McIntire LV. Heterotypic adherence between human B-lymphoblastic and pre-B-lymphoblastic cells and marrow stromal cells is a biphasic event: integrin very late antigen-4 alpha mediates only the early phase of the heterotypic adhesion. Blood. 1995;85:168-178.*

(26) *Potian JA, Aviv H, Ponzio NM, Harrison JS, Rameshwar P. Veto-like activity of mesenchymal stem cells: functional discrimination between cellular responses to alloantigens and recall antigens. J Immunol. 2003;171:3426-3434.*

(27) *Reyes-Botella C, Montes MJ, Vallecillo-Capilla MF, Olivares EG, Ruiz C. Antigenic phenotype of cultured human osteoblast-like cells. Cell Physiol Biochem. 2002;12:359-364.*

(28) *Cuff C, Kothapalli D, Azonobi I, Chun S, Zhang Y, Belkin R et al. The Adhesion Receptor CD44 Promotes Aterosclerosis by Mediating Innflamatory cell Recruitment and Vascular Cell Activation. J.Clin.Invest. 108, 1031-1040. 2001.*

(29) *Wetzel A, Chavakis T, Preissner KT et al. Human Thy-1 (CD90) on activated endothelial cells is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). J Immunol. 2004;172:3850-3859.*

(30) *Lucas T, Krugluger W, Samorapoompichit P et al. Self-renewal, maturation, and differentiation of the rat myelomonocytic hematopoietic stem cell. FASEB J. 1999;13:263-272.*

(31) *Crook K, Hunt SV. Enrichment of early fetal-liver hemopoietic stem cells of the rat using monoclonal antibodies against the transferrin receptor, Thy-1, and MRC-OX82. Dev Immunol. 1996;4:235-246.*

(32) *Horton MA, Nesbitt SA, Benett JH, Stenbeck G. Integrins and Other Cell Surface Attachment Molecules of Bone Cells. Principles of Bone Biology. 2002:265-286.*

(33) *Minguell JJ, Erices A, Conget P. Mesenchymal Stem Cells. Experimental Biology and Medicine. 2001;226:507-520.*

(34) *Banuls MP, Alvarez A, Ferrero I, Zapata A, Ardavin C. Cell-surface marker analysis of rat thymic dendritic cells. Immunology. 1993;79:298-304.*

(35) *Tamatani T, Kotani M, Miyasaka M. Characterization of the rat leukocyte integrin, CD11/CD18, by the use of LFA-1 subunit-specific monoclonal antibodies. Eur J Immunol. 1991;21:627-633.*

(36) *Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res. 2004;95:9-20.*

(37) *Springer TA. Adhesion receptors of the immune system. Nature 346, 425-434. 1990.*

(38) *Anselme K. Osteoblast adhesion on biomaterials. Biomaterials. 2000;21:667-681.*

(39) *Heino J. The collagen receptor integrins have distinct ligand recognition and signaling functions. Matrix Biol. 2000;19:319-323.*

(40) *Deschaseaux F, Gindraux F, Saadi R et al. Direct selection of human bone marrow mesenchymal stem cells using an anti-CD49a antibody reveals their CD45med,low phenotype. Br J Haematol. 2003;122:506-517.*

(41) *Hemler ME, Huang C, Schwarz L. The VLA protein family. Characterization of five distinct cell surface heterodimers each with a common 130,000 molecular weight beta subunit. J Biol Chem. 1987;262:3300-3309.*

(42) *Takada Y, Murphy E, Pil P et al. Molecular cloning and expression of the cDNA for alpha 3 subunit of human alpha 3 beta 1 (VLA-3), an integrin receptor for fibronectin, laminin, and collagen. J Cell Biol. 1991;115:257-266.*

(43) *Magnusson MK, Mosher DF. Fibronectin: structure, assembly, and cardiovascular implications. Arterioscler Thromb Vasc Biol. 1998;18:1363-1370.*

(44) *Kieffer N, Phillips DR. Platelet membrane glycoproteins: functions in cellular interactions. Annu Rev Cell Biol. 1990;6:329-357.*

(45) *Piali L, Hammel P, Uherek C et al. CD31/PECAM-1 is a ligand for alpha v beta 3 integrin involved in adhesion of leukocytes to endothelium. J Cell Biol. 1995;130:451-460.*

(46) *Ashkar S, Weber GF, Panoutsakopoulou V et al. Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. Science. 2000;287:860-864.*

(47) *Miao D, Murant S, Scutt N, Genever P, Scutt A. Megakaryocyte-bone marrow stromal cell aggregates demonstrate increased colony formation and alkaline phosphatase expression in vitro. Tissue Eng. 2004;10:807-817.*

(48) *Moulder K, Roberts K, Shevach EM, Coligan JE. The mouse vitronectin receptor is a T cell activation antigen. J Exp Med. 1991;173:343-347.*

(49) *Frieser M, Hallmann R, Johansson S et al. Mouse polymorphonuclear granulocyte binding to extracellular matrix molecules involves beta 1 integrins. Eur J Immunol. 1996;26:3127-3136.*

(50) *Vogel B, Vogel T, Rohde E, Strunk D, Goepferich A, Schulz MB. Myeloid Cells Supress in vitro Osteogenic Differentiation of Rat Mesenchymal Progenitor Cells. Chapter 3.*

(51) *Wilson CJ, Clegg RE, Leavesley DI, Pearcy MJ. Mediation of biomaterial-cell interactions by adsorbed proteins: a review. Tissue Eng. 2005;11:1-18.*

(52) *Jenney CR, Anderson JM. Adsorbed serum proteins responsible for surface dependent human macrophage behavior. J Biomed Mater Res. 2000;49:435-447.*

(53) *Conget PA, Minguell JJ. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. J Cell Physiol. 1999;181:67-73.*

(54) *Shen M, Horbett TA. The effects of surface chemistry and adsorbed proteins on monocyte/macrophage adhesion to chemically modified polystyrene surfaces. J Biomed Mater Res. 2001;57:336-345.*

(55) *Koenigsmann M, Griffin JD, DiCarlo J, Cannistra SA. Myeloid and erythroid progenitor cells from normal bone marrow adhere to collagen type I. Blood. 1992;79:657-665.*

(56) *Garnotel R, Rittie L, Poitevin S et al. Human blood monocytes interact with type I collagen through alpha x beta 2 integrin (CD11c-CD18, gp150-95). J Immunol. 2000;164:5928-5934.*

CHAPTER 5

PILOT STUDY TO MODULATE INTEGRIN EXPRESSION ON RAT MARROW STROMAL CELLS

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INTRODUCTION

The objective of this pilot study was to find ways to modulate the expression of cell adhesion receptors in order to investigate if we can modulate and improve marrow stromal cell (MSC) adhesion to peptides and matrices used in tissue engineering. It is known that integrins and growth factor receptors "communicate" with each other. When integrins are clustered and activated by extracellular matrix (ECM) proteins, they also recruit growth factor receptors and can amplify the signaling effect of growth factors. This synergy is important in cell cycle progression and cell migration[1]. It has been reported that vice versa supplementation with certain cytokines and growth factors can change the integrin expression pattern[2,3]. Hence, we used this approach to modulate integrin expression in CD45-negative rat MSC. Transforming growth factor-β1 (TGF- β1) has already been successfully used for improvement of collagen 1 production in rat MSC cultures and was therefore considered as an interesting candidate for this study[4,5]. Furthermore, we evaluated the influence of platelet-derived growth factor (PDGF), for which it has been shown to enhance osteoprogenitor cell proliferation[6]. Atorvastatin, a lipid-lowering drug with osteoinductive properties[7], was also tested in this study.

It is known that with progression of differentiation cells can change their integrin expression pattern[1]. Along these lines, dexamethasone, which induces osteogenic differentiation, is known to cause changes in integrin expression[1]. Cultured osteoblasts, for example, express high levels of vitronectin integrins αVβ3 and αVβ5[1,8,9]. Their adhesion can therefore be enhanced by surface immobilization of cyclic RGD peptides (cRGDfK)[10] selective for these integrins. Many integrins show RGD-dependent binding, though some other binding sequences have been found and could be therefore used to enhance cell adhesion; e.g. DGEA and GER for integrin α2β1 and EILDV for α4β1 integrin[11].

Since the published data are derived from different methods of analysis, no truly useful information about modulating integrin expression during MSC osteogenesis is available. In addition to immunohistochemistry and gene expression analysis, flow cytometry can also be used. Rat MSC already express β3 integrin[12], but in contrast to osteoblasts, they do not adhere well to RGD-modified polymer films[10]; two possible reasons can be hypothesized: low integrin expression or insufficient ligand density[13]. The aim of this pilot study was to find out if the addition of growth factors and the supplementation with the differentiating medium can increase β3 integrin expression or change the expression of other integrins in a way that may be useful for selective surface modification of biomaterials.

MATERIALS AND METHODS

Suppliers

Fetal calf serum (Gibco, Invitrogen, UK; Lot No.: 40F7430K); Penicillin-Streptomycin (Gibco, Invitrogen, UK; 10,000 units / mL penicillin G sodium and 10,000 µg / mL streptomycin sulphate); L-glutamine (Sigma Aldrich, Germany; 200 mM); Trypsin-EDTA (Gibco, Invitrogen, UK; 0.25 % Trypsin and 1mM EDTA.4Na); Minimum essential medium Eagle, alpha modification (Sigma Aldrich, Germany; 10.1 g / L); simvastatin (MSD, Germany); atorvastatin (Pfizer, USA).

Cell culture

Rat bone marrow was isolated from the femur and tibia of 6- to 8-week-old (150-200 g) male Sprague-Dawley rats by the centrifugal method[14]. Freshly isolated bone marrow cells were resuspended in primary medium (10 % FCS, 1 % Penicillin/Streptomycin, 0.5 % L-glutamine and minimal essential medium, alpha modification). Cells obtained from one rat (2 tibias and 2 femurs) were seeded in four T150 flasks. The cells were cultured in a humidified atmosphere with 5 % CO_2 at 37°C and left to adhere 3 days before non-adherent cells were removed. Media was changed 3 times a week and when the cultures reached 80 - 100 % confluence, cells were detached from the surface with trypsin-EDTA and counted.

Modulation of integrin expression

After seven days, primary culture cells were depleted of CD45-positive cells by the immunomagnetic procedure (described in Chapter 4). CD45-negative cells were seeded at a density of 1 x 10^6 per T150 flask for 24 hours in primary media. Thereafter cells received complete differentiation media (primary medium supplemented with 50 mg / L ascorbic acid, 7 mM β-glycero-phosphate and 10 nM dexamethasone) supplemented with either growth factors or statins. Control cultures were grown in primary media. We added platelet-derived growth factor (PDGF-BB; 20 ng / mL, R&D systems, USA), transforming growth factor-beta1 (TGFβ1; 1 ng / mL; Tebu-Bio, Offenbach, Germany), and atorvastatin (5 µM, Pfizer, USA). On the 4th day, cells were detached with trypsin-EDTA and the expression of various integrins was determined by flow cytometry (see Chapter 4 for detailed description).

RESULTS

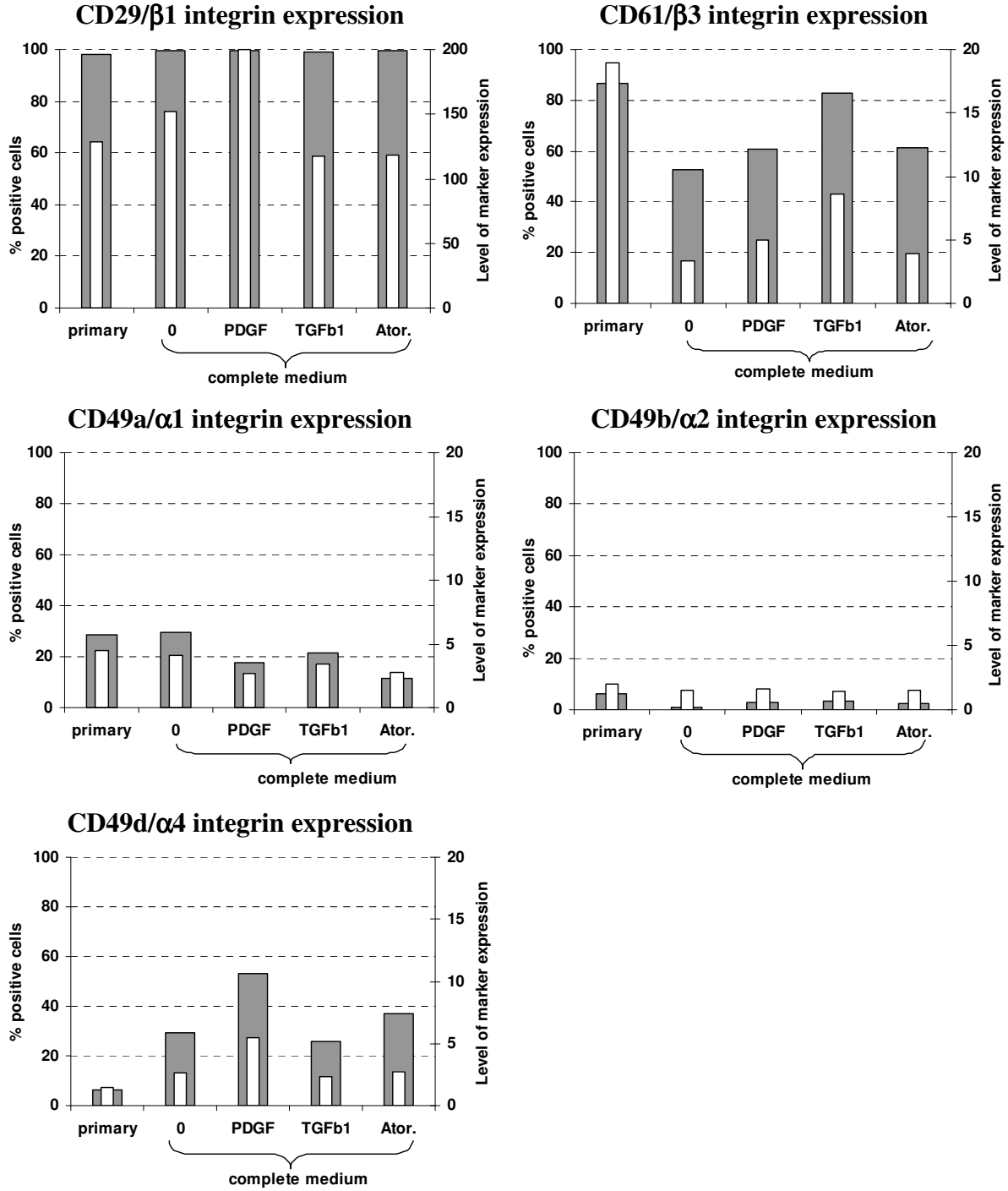

Figure 1: *Flow cytometric data on the influence of various growth factors and statin on MSC integrin expression after supplementation with PDGF (20 ng / mL), TGFβ1 (1 ng / mL), and atorvastatin (5 μM). Primary cultures of rat bone marrow cells were depleted of CD45+ cells (MACS); CD45- MSC were seeded in T150 flasks (1 x 10^6 cells / flask) and cultured for 4 days in supplemented media until analyzed. Gray bars: % positive cells; white bars: level of marker expression.*

Influence of PDGF, TGF-β1 and statins on integrin expression

None of the supplements had an influence on the expression of integrin β1 (CD29), and α2 (CD49b) as shown in Figure 1. The expression of α1 (CD49a) in all secondary cultures, was lower than the expression in primary cultures, which was 74.9 ± 13 %[12]. Supplementation of osteogenic medium with PDGF, TGFβ1 and atorvastatin caused a slight reduction of the α1 (CD49a) expression. All cells cultured in complete media showed increased α4 (CD49d) expression, compared to cells cultured in primary media. On the contrary, β3 (CD61) expression was lower in cultures with complete media than in primary media.

Influence of PDGF, TGF-β1 and statins on cell proliferation

Cell proliferation for all tested conditions was determined as well (Figure 2). We saw that complete media slowed down the proliferation, but the addition of PDGF caused even faster expansion than primary media.

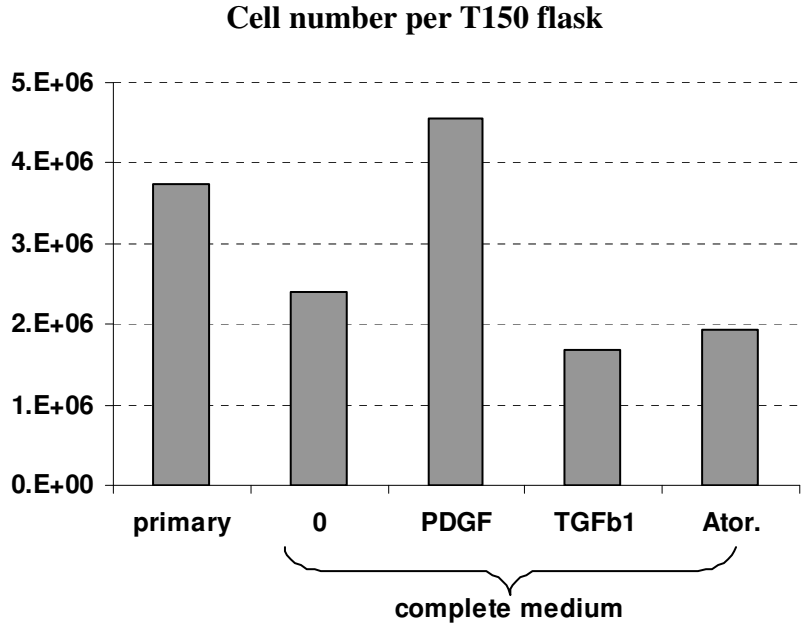

Figure 2: *Proliferation of CD45- MSC in primary medium or in complete medium supplemented with PDGF (20 ng / mL), TGFβ1 (1 ng / mL), atorvastatin (5 μM). After depletion of CD45+ cells, CD45- MSC were seeded in T150 flasks (1x10^6 cells / flask) and cultured for 4 days until analyzed.*

DISCUSSION

In this screening study, we tried to find ways to modulate the level of integrin expression for subsequent MSC immobilization on surfaces modified with integrin-specific peptides, starting with the finding that rat MSC in contrast to human osteoblasts showed only sparse adhesion to surfaces modified with cyclic RGD peptide (cRGDfK). This peptide is specific for the integrins αVβ3 and αVβ5[10]. The poor adhesion could be due to lower expression of specific integrins on rat MSC. Hence, we determined β3 (CD63) expression in primary cultures of rat MSC[12] (Chapter 4) and compared this to results obtained in this study. While CD45-negative cells from primary cultures contained 56.0 ± 20.5% CD61-positive cells, about 85% of the cells were positive for this integrin when further cultured in primary medium. Similar results were found in complete medium in the presence of TGF-β1, while less cells expressed this marker in complete medium alone or with PDGF and atorvastatin supplementation (Figure 1). Nevertheless, CD61 is expressed in human osteoblasts, as well as in osteoclasts[11] and its *in vitro* expression seems to rely on culture conditions, especially supplementation with serum, which includes CD61-binding protein, vitronectin[9]. It has been suggested that osteoblasts do not express integrin αVβ3 *in vivo*, but express it only under certain *in vitro* culture conditions[9]. It is known that some differentiated cells, like chondrocytes, lose their phenotype after isolation and 2-dimensional culturing (dedifferentiation). Therefore, higher expression of CD61 could correlate with lower differentiation grade of the cells in our experiments. Nevertheless, further studies could confirm these observations.

Interestingly, the expression of α4 (CD49d) was increased in the osteogenic media cultures, compared to primary and secondary cultures in proliferation media (Chapter 4). CD49d is expressed in osteoblasts[8] and could be, based on our observations, an early marker for osteogenic differentiation. The most interesting results were obtained when the cultures were treated with PDGF: (1) cells showed very high CD49d expression, and (2) the cell number was twice as high as without PDGF. The cell number in PDGF treated group was even higher than in the control group with primary medium (no osteogenic treatment). Although the data presented here are only preliminary, they show some interesting results, especially in the case of CD49d.

Concerning the expression of collagen integrins CD49a/α1 and CD49b/α2, opposing roles have been described; whereas α1 is connected with cell proliferation, α2 is coupled with

osteogenic differentiation[15]. We detected no significant differences in α2 expression compared to primary CD45- cells (Chapter 4). On the other hand, α1 expression was lower in all secondary cultures than in primary cultures (Chapter 4), without significant differences between groups. In some cases, the reduction of α1 expression could be connected with increased α4 expression (Figure 1), however studies have to be repeated to precisely evaluate these findings.

Overall, we conclude that the experimental setup to study differences in surface marker expression was appropriate. As discussed in previous chapters, the primary cultures were always contaminated with CD45-positive hematopoietic cells, which can influence MSC proliferation and differentiation[16]. We therefore isolated CD45-negative cells and cultured them further. This isolation also eliminated the need for simultaneous staining of hematopoietic cells for flow cytometry. It is known that high density culturing or 100% cell confluence also induces osteogenesis[17]. A single cell suspension from tightly packed cells, obtained using trypsin-EDTA or Accutase® (PAA, Pasching, Austia), yielded results not significantly different from primary culture cells (data not shown). We speculate that using detachment methods designed for less confluent monolayer cultures resulted in the detachment of cells from the periphery only, which are probably less differentiated than cells from the center of bone nodules[17]. We therefore decided to grow the cells at lower cell density and only for a short time, to prevent cell-cell contacts and to avoid cell-aggregate formation.

Integrins play an important role in cell biology, since each integrin has a specific effect on cell proliferation and differentiation. The role of integrins differs from cell type to cell type. Here we demonstrated how the integrin expression pattern could be modulated with changes in culture media and then detected by flow cytometry. Future studies that monitor integrin expression, cell adhesion, proliferation and differentiation simultaneously will give us further insight into how to regulate integrin expression and the impact of integrin expression level changes on cells in culture.

REFERENCES

(1) *Siebers MC, ter Brugge PJ, Walboomers XF, Jansen JA. Integrins as linker proteins between osteoblasts and bone replacing materials. A critical review. Biomaterials. 2005;26:137-146.*

(2) *Nesti LJ, Caterson EJ, Wang M, Chang R, Chapovsky F, Hoek JB et al. TGF-beta1 calcium signaling increases alpha 5 integrin expression in osteoblasts. J Orth Res 20, 1042-1049. 2002.)*

(3) *Creyghton WM, Waard-Siebinga I, Danen EH et al. Cytokine-mediated modulation of integrin, ICAM-1 and CD44 expression on human uveal melanoma cells in vitro. Melanoma Res. 1995;5:235-242.*

(4) *Lieb E, Milz S, Vogel T et al. Effects of transforming growth factor beta1 on bonelike tissue formation in three-dimensional cell culture. I. Culture conditions and tissue formation. Tissue Eng. 2004;10:1399-1413.*

(5) *Lieb E, Vogel T, Milz S, Dauner M, Schulz MB. Effects of transforming growth factor beta1 on bonelike tissue formation in three-dimensional cell culture. II: Osteoblastic differentiation. Tissue Eng. 2004;10:1414-1425.*

(6) *Caplan AI, Reuben D, Haynesworth SE. Cell-based tissue engineering therapies: the influence of whole body physiology. Adv Drug Deliv Rev. 1998;33:3-14.*

(7) *Mundy G, Garret R, Harris S, Chan J, Chen D, Rossini G et al. Stimulation of bone formation in vitro and in rodents by statins. Science 286, 1946-1949. 1999.*

(8) *Anselme K. Osteoblast adhesion on biomaterials. Biomaterials 21, 667-681. 2000.)*

(9) *Wilson CJ, Clegg RE, Leavesley DI, Pearcy MJ. Mediation of biomaterial-cell interactions by adsorbed proteins: a review. Tissue Eng. 2005;11:1-18.*

(10) *Lieb E, Hacker M, Tessmar J et al. Mediating specific cell adhesion to low-adhesive diblock copolymers by instant modification with cyclic RGD peptides. Biomaterials. 2005;26:2333-2341.*

(11) *Horton MA, Nesbitt SA, Bennett JH, Stenbeck G. Integrins and Other Cell Surface Attachment Molecules of Bone Cells. In: Bilezikian JB, Raisz LG, Rodan GA, eds. Principles of Bone Biology. San Diego, San Francisco, New York, Boston, London, Sydney, Tokio: Academic Press; 2002:265-286.*

(12) *Vogel B, Vogel T, Rohde E, Strunk D, Göpferich A, Schulz MB. Phenotype characterisation of plastic-adherent rat bone marrow cells. 2006. Unpublished Work*

(13) *Griffith LG, Naughton G. Tissue engineering-current challanges and expanding opportunities. Science. 2002;295:1009-1016.*

(14) *Dobson KR et al. Centrifugal isolation of bone marrow from bone: an improved method for the recovery and quantitation of bone marrow osteoprogenitor cells from rat tibiae and femurae. Calcif Tissue Int. 1999;65:411-413.*

(15) *Heino J. The collagen receptor integrins have distinct ligand recognition and signaling functions. Matrix Biol. 2000;19:319-323.*

(16) *Friedenstein AJ, Latzinik NV, Gorskaya YuF, Luria EA, Moskvina IL. Bone marrow stromal colony formation requires stimulation by haemopoietic cells. Bone Miner 18[3], 199-213. 1992.*

(17) Aubin JE. Osteoprogenitor cell frequency in rat bone marrow stromal populations: role for heterotypic cell-cell interactions in osteoblast differentiation. J Cell Biochem. 1999;72:396-410.

CHAPTER 6

TRACING MSC PHENOTYPE: FROM BONE MARROW TO SEVERAL PASSAGES

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INTRODUCTION

The rat was one of the first animals from which mesenchymal stem / progenitor cells (MSC) were isolated. Despite of this, detailed phenotype characterization of rat MSC is still not complete. The frequency of colony-forming unit fibroblasts (CFU-F) has been determined in primary and secondary cultures of rat bone marrow cells[1], which are known to be contaminated with adherent endothelial and hematopoietic cells (HC)[2,3]. Surface marker characterization was performed on cells after 5 passages[4], because HC are lost from cultures after multiple passages and that clonogenic cells are enriched with rapid passaging. The problem with characterizing MSC is that they are usually described as cultured cells that can change their phenotype with extensive subculturing. Cultured cells express some typical markers like vitronectin receptor-integrin $\alpha V\beta 3$ and/or $\alpha 3$ integrin subunit[5,6]. Markers such as these are likely the result of culturing the cells in the presence of fetal bovine serum (FBS) containing vitronectin and fibronectin, which mediate cell adhesion to the culture surface[5,6].

The standard protocol for rat MSC isolation uses whole bone marrow isolated from femurs and tibias of an animal. Until recently, a flushing method was used to flush the bone marrow from bones using a syringe and media[7]. Dobson et al.[8] showed that bone marrow could be easily isolated by centrifuging the bones, with the CFU-F yield increased 100 % compared to the flushing method. We compared both methods and our results confirmed published data (data not shown). Furthermore, contamination with hematopoietic cells was not elevated using the centrifugal method (data not shown).

Since bone tissue engineering requires several dozens of millions of cells for 3-dimensional experiments, enrichment and expansion of MSC in the shortest amount of time is needed. As observed in our previous study (Chapter 2), contamination with HC cannot be diminished by simply varying adherence time after cell isolation. One successful alternative for isolation of a pure MSC population is immunomagnetic depletion of HC using magnetic-associated cell sorting (MACS). However, this method can be expensive if routinely used, especially if large numbers of cells need to be separated. Therefore, isolation of MSC from cultures earlier than day 7, such as day 3, could be more cost-effective. Another option for MSC enrichment would be subculturing over several passages. However, cell culture and cell adhesion are both known to alter antigen expression[9]. For studies on the adhesive properties of MSC, the integrin expression pattern of cells used for the experiments needs to be unchanged and to this end we examined the surface marker expression of adherent cells after 3 days in culture, cells of the first passage and of cells passaged for four times.

Most of the commercially available antibodies for flow cytometry were developed for the characterization of hematopoietic cells, which are most commonly isolated from blood or bone marrow. Freshly isolated cells, however, have different characteristics than cultured cells; they are on average smaller, and can be divided into granulocyte, monocyte and lymphocyte fractions based upon size and granularity (Figure 1, left panel). Freshly isolated blood or bone marrow cells are also not typically adherent, whereas when analyzing cultured cells, adherent cells need to be first detached from the surface using enzymatic or other methods, which can "damage" surface antigens. Hematopoietic cell surface marker expression is better characterized than that of cultured MSC, therefore freshly isolated bone marrow cells were used as a positive control to determine the optimal concentration of antibodies to use in our flow cytometry experiments for MSC characterization. The phenotype of rat MSC freshly isolated by the indirect selection method is still largely unknown[9].

Rat bone marrow is often analyzed to evaluate preclinical and toxicological effects of diverse therapies on the hematopoietic system[10]. Microscopic evaluation of rat bone marrow (smear) shows that the HC ratio in normal bone marrow between myeloid and erythroid cells is between 1.07 and 1.93. Variations in this ratio can indicate abnormalities in hematopoiesis[10]. Rat megakaryocytes are the largest hematopoietic cells, with a frequency of 0.40 - 0.77 % of total nucleated cells in rat bone marrow[10]. Reported percentages of erythroid precursors in rat bone marrow vary from 20 % to almost 40 % of nucleated cells, whereas myeloid/granulocyte precursors comprise 33.6 - 52%. Monocytes and their precursors are present in low numbers in healthy rat bone marrow, in contrast to lymphocytes, which comprise 7.34 to 21.10 % of total nucleated cells[10]. Non-hematopoietic cells in the bone marrow include mast cells (1 %), osteoblasts and osteoclasts, as well as endothelial cells[10]. These data are based on cell morphology and could be improved with differential cell staining using antibodies against some typical rat cell antigenes. An overview of rat marker expression is presented in Table 1 (source: BD Pharmingen). Flow cytometry, however, could enable to get more precise data, because more cells could be evaluated in the shortest time. In our case, the characterization of rat hematopoietic cells was inevitable, because they share the same microenvironment with MSC in the bone marrow, as well as *in vitro*, and, hence, could play an important role in MSC biology.

This chapter includes:

1. Characterization of freshly isolated rat bone marrow cells including evaluation of percentages of hematopoietic subclasses and estimation of MSC-like cell frequency.
2. Comparison of CD45-negative cells in adherent populations harvested on day 3, day 7 and several-times passaged cells (MSC phenotype tracing).

MATERIALS AND METHODS

Suppliers

Gibco, Invitrogen, UK: Fetal calf serum, FCS (Lot No.: 40F7430K); Penicillin-Streptomycin (10,000 units / mL penicillin G sodium and 10,000 µg / mL streptomycin sulphate); Trypsin-EDTA (0.25 % Trypsin and 1 mM EDTA.4Na); Sigma Aldrich, Germany: L-glutamine (200 mM); Minimum essential medium Eagle, alpha modification (α-MEM; 10.1 g / L); 7-Aminoactinomycin D (7-AAD); Corning, Netherlands: tissue culture flasks.

Cell isolation and cell culture

Rat bone marrow was isolated from the femur and tibia of six-week-old male Sprague-Dawley rats (Institut für Labortierkunde und -genetik Himberg, Medical University Vienna, Austria) by the method described in Dobson et al.[8]. Briefly, femurs and tibias were removed under aseptic conditions and cleaned of any muscle and tendon tissue. The bones were cut in the middle and the parts distal from knee were discarded. The epiphyses were pierced with an 18 gauge syringe and bones placed in Eppendorf tubes with a broad piece of a pipette tip inserted to hold the bone. The bones were centrifuged for 1 minute at 400 g and bone marrow cells were resuspended in medium, collected using a 23 gauge syringe and centrifuged (5 min at 1350 rpm).

For the analysis of the bone marrow, freshly isolated bone marrow cells were incubated with lysing buffer (0.8 % NH_4Cl, pH 7.3) at room temperature for 5 minutes to lyse the erythrocytes[5,6], followed by two wash steps with PBS. For cell culture, freshly isolated bone marrow cells were resuspended in primary medium (10 % FCS, 1 % Penicillin / Streptomycin, 0.5 % L-glutamine and α-MEM). Cells obtained from 1 rat (2 tibias and 2 femurs) were seeded in two 150 cm^2 (3-day adherence only) or four 150 cm^2 (3-day adherence and proliferation) flasks and were cultured in a humidified atmosphere with 5 % CO_2 at 37°C. After adhesion for 3 days, nonadherent cells were removed and the adherent fraction washed twice with 16 mL PBS per flask to remove spare attached cells. Cells were further cultured with media changes 3 times a week.

Passage: On day 3 or 7 after cell isolation, the media was removed from the flasks, flasks were flushed with 16 mL / flask PBS, and 5 mL / flask 0.2 % trypsin-EDTA was added. The cells were left to detach for 5 minutes at 37°C. Trypsin was deactivated by addition of 25 mL primary medium. For the analysis of passaged cells (e.g. passage 4), day 7 cells were subcultured with or without depletion of CD45+ hematopoietic cells (HC) for several passages.

Single-cell suspensions were passed through a 40μm cell strainer, centrifuged, resuspended in PBS, counted using a hemacytometer and analyzed with flow cytometry.

Flow cytometry

The cell suspension was first incubated for 15 minutes at 4°C with 10% v/v sheep serum and then for 30 minutes at 4°C with antibodies against rat CD markers or with corresponding isotype controls (Table 1). Cells were washed with PBS containing sodium azide and bovine serum albumin and resuspended in the same buffer before measurements. Just prior to making measurements, 20 % v/v 7-AAD (20 μg / mL) was added to each sample for 3 minutes to stain the dead cells, which were excluded from the evaluation. For cell staining with antibody against CD49c, the cells were fixed and permeabilized using a Fix&Perm® kit (An der Grub, Kaumberg, Austria) according to the manufacturer's instructions. When staining required incubations with anti-mouse secondary antibodies, anti-CD45 was added at the end, following a blocking step with normal mouse serum.

All measurements were preformed on a FACSCalibur® instrument and data acquired with CellQuest software (both BD Biosciences, USA). Measurements were evaluated with WinMDI 2.8 Software (free access on http://facs.scripps.edu/software.html). A minimum of $1x10^4$ viable cells was acquired per data set. Positive cells were defined as having a measured fluorescence greater than that of 99.5 % of the cells stained with each associated isotype control. A population was considered positive for a surface marker when the percentage of positive cells for that surface marker was equal to or greater than 5 %[11].

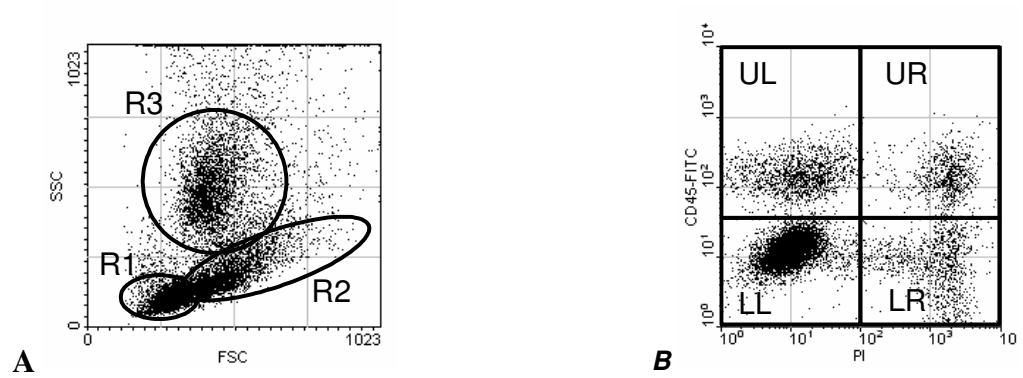


Figure 1: A: *FSC-SSC or size-granularity dot-plot of freshly isolated rat bone marrow cells and the distribution of lymphocyte- (R1), monocyte- (R2), and granulocyte-like fraction (R3);* ***B:*** *propidium iodine (PI) staining of dead cells detected in 3rd fluorescence channel. LL quadrant contains cells negative for both types of fluorescence. The UR quadrant contains cells positive for both types of fluorescence. UL and LR are positive for one fluorescence and negative for the other one.*

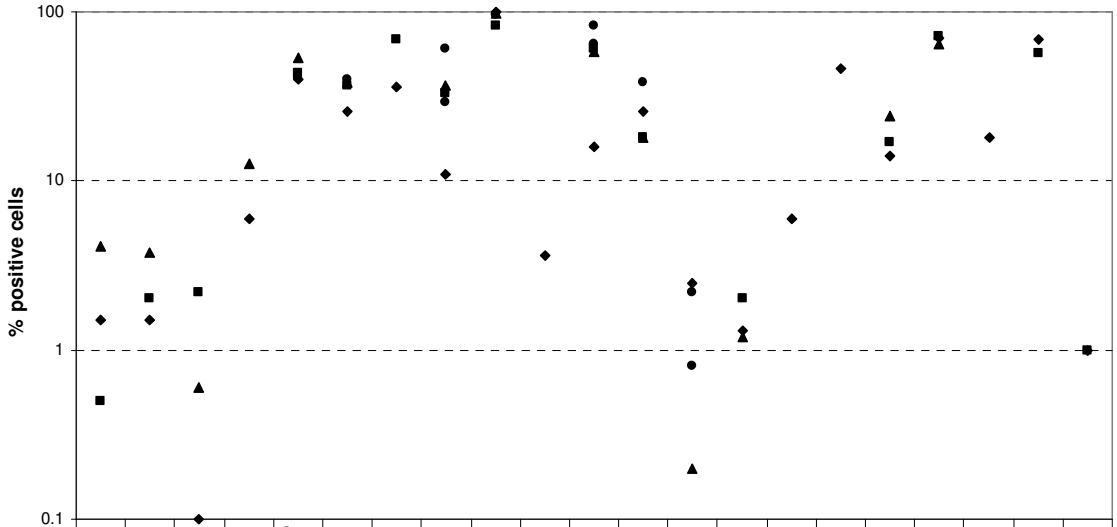
Table 1: *List of antibodies used for flow cytometric characterization of rat cells.*

CD	Other name	Sp.	Clone	Ig class	Label	Producer	Cat.Nu.	NK	GRAN	T LY	MONO	ENDO	MEGA	B LY	DEND	ERY
CD3	TCR	Ms	G4.18	IgG3 k	PE	BD	554833	ND	ND	Most/Dev	ND	ND	ND	ND	ND	ND
CD4		Ms	OX-38	IgG2a k	FITC	BD	554843	ND	ND	Sub/Dev	Most	ND	ND	ND	Sub	ND
CD8b		Ms	341	IgG1	FITC	BD	554973	ND	ND	Sub/Dev	Sub/Act	ND	ND	ND	Unkn	ND
CD11a	αL integrin	Ms	WT.1	IgG2a k	PE	BD	550972	Most	Most	Most	Most/Dev	ND	Most	Most	Most	MD
CD11b/c	αMX integrin	Ms	OX-42	IgG2a k	FITC	BD	554861	Unkn	Most/De	ND	Most/Dev	Unkn	Unkn	ND	Sub/Act	ND
CD18	β2 integrin	Ms	WT.3	IgG1 k	-	BD	554977	Most	Most	Most	Most	Unkn	Most	Most	Most	ND
CD29	β1 integrin	Ham	Ha2/5	IgM k	FITC	BD	555005	Unkn	Sub	Most	Most	Most	Most	Most	Unkn	Unkn
CD31	PECAM-1	Ms	TLD-3A12	IgG1	FITC	Acris	SM272F	Unkn	Unkn	Sub	Unkn	Most	Most	Most	Unkn	Unkn
CD44	H-CAM	Ms	OX-50	IgG1	PE	Acris	SM273R	Most	Most	Most/Act	Most	Unkn	Unkn	Sub/Act	Unkn	Unkn
CD45	LCA	Ms	MRC OX-	IgG1	PE	Acris	SM274R	Most	Most	Most	Most	ND	Unkn	Most	Most	ND
CD45RA		Ms	OX-33	IgG1 k	PE	BD	551402	Unkn	ND	ND	ND	ND	Unkn	Most	Unkn	ND
CD49a	α1 integrin	Ham	Ha31/8	IgG2 l	-	BD	555001	Unkn	Unkn	Sub/Act	Most	Sub	Unkn	ND	Unkn	ND
CD49b	α2 integrin	Ham	Ha1/29	IgG2 l	FITC	BD	554999	Sub	Unkn	Sub/Act	Unkn	Unkn	Most	Most/Act	Unkn	Unkn
CD49c	α3 integrin	Ms	42	IgG1	-	BD	611044	Unkn	Unkn	Unkn	Most	Unkn	Unkn	Most	Unkn	Unkn
CD49d	α4 integrin	Ms	TA-2	IgG1	FITC	Serotec	MCA1383F	Unkn	Sub	Most	Most	Unkn	Unkn	Most	Unkn	Unkn
CD49e	α5 integrin	Ham	HMα5-1	IgG1 k	-	BD	553350	Unkn	Sub	ND	Unkn	Most	Unkn	Sub	Unkn	Unkn
CD51	αV integrin	Ms	21	IgG1	-	BD	611012	Unkn	Unkn	Unkn	Unkn	Unkn	Unkn	Unkn	Unkn	Unkn
aVb5 (CD51)	αVβ5 integr	Ms	P1F6	IgG1	FITC	Chemicon	MAB1961F									
CD54	ICAM-1	Ms	1A29	IgG1 k	PE	BD	554970	Unkn	Sub	Most/Act	Most	Most/Act	Unkn	Most/Act	Most	Unkn
CD61	β3 integrin	Ham	2C9.G2	IgG1 k	PE	BD	553347	ND	ND	ND	ND	Most/Act	Most	Unkn	Unkn	Unkn
CD90	Thy-1	Ms	OX-7	IgG1 k	FITC	BD	554897	Unkn	Sub	Sub/Dev	Unkn	Sub	Unkn	Sub/Dev	Sub	Unkn
CD106	VCAM-1	Ms	MR106	IgG1 k	PE	BD	559229	Unkn	ND	ND	Unkn	Sub/Act	Unkn	ND	Sub	Unkn
CD161a	NKR-P1A	Ms	10/78	IgG1 k	FITC	BD	555008	Unkn	Unkn	Most/Actt	Most	Unkn	Unknkn	Unkn	Most	Unkn
Granulocyte		Ms	HIS48	IgM	FITC	BD	554907	Sub	Most/Act	ND	Sub	Unkn	Unkn	ND	Unkn	Unkn
Ham IgG	2nd step	Ms	G70/204	IgG1 k	PE	BD	554056									
Ms IgG	2nd step	Go			APC	BD	550826									
Mineral oil	isotype	Ms	MOPC-	IgG1 k	-	BD	557273									
unknown	isotype	Ms	MOPC-21	IgG1 k	FITC	BD	555748									
Unknown	isotype	Ms		IgG1	PE	CB	CBL600P									
TNP-KLH	isotype	Ms	G155-178	IgG2a k	FITC	BD	553456									
TNP	isotype	Ms	G155-178	IgG2a k	PE	BD	555574									
TNP-KLH	isotype	Ham	A19-3	IgG1 k	-	BD	553969									
TNP-KLH	isotype	Ham	A19-3	IgG1 k	PE	BD	553972									
KLH	isotype	Ham	He4/8	IgG2 l	-	BD	553962									
KLH	isotype	Ham	He4/8	IgG2 l	FITC	BD	553964									
TNP-KLH	isotype	Ham	G235-1	IgM	FITC	BD	553960									

Sp.: species; Ms: mouse; Ham: hamster; Go: goat;
PE: phycoerythrin;
FITC: fluorescein isothiocyanate
APC: allophycocyanin
BD: Pharmingen
CB: Cymbus Biotechnology
NK: natural kiler cells
Gran: granulocytes
T LY: T lymphocytes
Mono: monocytes
Endo: endothelial cells
Mega: megakaryocytes/Platelets
BLY: B lymphocytes
Dend: Dendritic cells
Ery: Erythrocytes
Most: on most cells
Sub: on subset of cells
ND: not detected
Unkn: unknown

RESULTS

Freshly isolated bone marrow cells



	CD3	CD4	CD8b	CD 161a	Granulocyte	CD11a	CD11b/c	CD18	CD29	CD44	CD45	CD45RA	CD49a	CD49b	CD49c	CD49d	CD49e	CD54	CD61	CD90	CD 106
◆ measurement 1	1.5	1.5	0.1	6	40	26	36	11	99	3.6	16	26	2.5	1.29	6	46	14	70	18	68	1
■ measurement 2	0.5	2	2.2	0	43	36.7	68	33	83		61	18	0	2			17	72	0	57	1
▲ measurement 3	4.1	3.8	0.6	12.8	53	38		37	98		58	18	0.2	1.2			24	64	0		
● measurement 4					41	40		61	95		83	38	2.2								
● measurement 5								29			65		0.8								

Figure 2: *Flow cytometric evaluation of rat bone marrow. Measurements to determine % positive cells for distinct markers were repeated up to 5 times. Ten thousand events were collected for every measurement.*

To determine the appropriate antibody concentration for flow cytometric applications, rat bone marrow and spleen cells were used, since it is known that hematopoietic cells express a number of different surface markers. Data on spleen cells are not presented. Additionally, rat bone marrow subpopulations were determined. Single measurement results are presented in Figure 2. Approximately 60 % of the bone marrow cells expressed CD45. Not all CD45+ cells co-expressed hematopoietic integrins (CD11a, b/c) (data not shown). Most cells of the granulocyte fraction (high SSC) expressed CD11a, 11b/c and CD18 (Figure 3). Cells positive for the granulocyte marker were found in the granulocyte fraction.

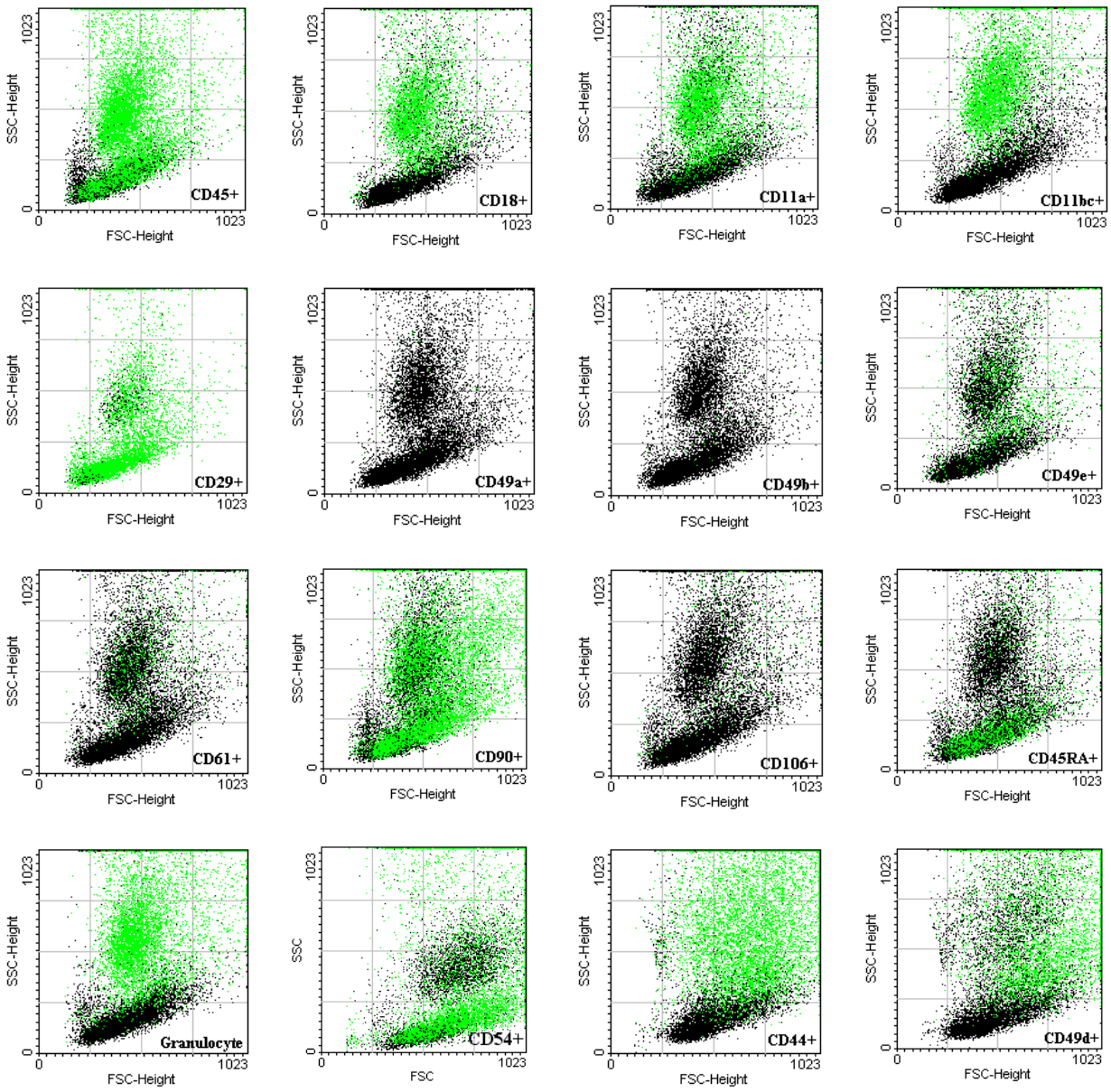


Figure 3: *Flow cytometric rat bone marrow analysis: expression of single markers (green) on FSC-SSC dot-plots. The whole bone marrow was subjected to an erythrocyte lysis, therefore erythrocytes are not present in the dot-plots.*

Almost all cells from bone marrow expressed CD29 and around 70 % expressed CD54 (ICAM-1; Figure 2). Stem cell marker CD90 was also highly expressed (57 – 68 %), as was CD49d (46 %). CD45RA+ cells comprised 18 – 38 % of bone marrow nucleated cells, whereas other more mature hematopoietic markers (CD3, CD4, CD8b, CD161a) comprised < 10 %. The presence of cells positive for MSC markers CD49a and CD106 in the bone marrow was below 2.5 % and their expression as well as size and granularity were broadly distributed. Fewer than 2 % of the cells expressed CD49b, 6 % expressed CD49c, and 14-24 % of the cells expressed CD49e. The

distribution of rat bone marrow cells positive for distinct markers is presented in FSC-SSC dot-plots in Figure 3.

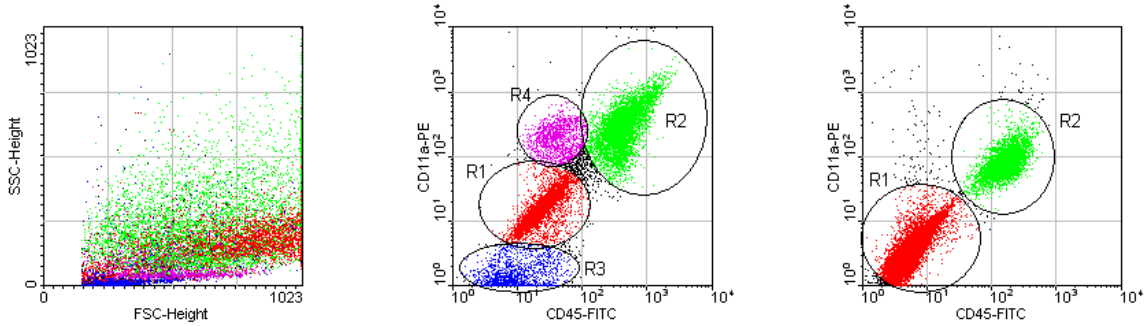


Figure 4: *Distribution of plastic-adherent cell populations. Left and middle plots show "day 3" adherent cells and right plot shows "day 7" cells. R1: CD45- MSC; R2: CD45+ HC; R3 and R4 are two additional populations detected on day 3. The size and distribution of all populations are shown in the left FSC vs. SSC dot-plot, where the low granularity and size of R3 and R4 are evident.*

Cultured bone marrow cells: day 3, day 7 and passage 4

After detailed characterization of plastic-adherent cells after one week of expansion (Chapter 3), we tried to detect similar patterns of surface marker expression (CD45- / CD11a- / CD11bc- and CD29+ / CD54+ / CD90+ / CD44+ / CD106+ / CD61+ / CD49a+ / CD49b low / CD49c low / CD49e+) in adherent cells passaged on day three after isolation and in cells passaged four times. On day 7 we observed two main cell populations: CD45- MSC (80 %) and CD45+ cells (20 %). Interestingly, cells detached on day 3 contained two additional populations (Figure 4); based on FSC and SSC, we observed small non-granulated cells (31-52 %); one of these was CD45low and co-expressed CD11a (R4 population; Figure 4, middle plot). The other small population was negative for all hematopoietic markers (R3 population; Figure 4, middle plot). The CD45- population detected on day 3 and on day 7 (Figure 4, R1; green) expressed MSC-specific markers and was therefore described as MSC. MSC showed a constant phenotype on day 3, day 7 and after several passages, as seen in Figure 5. On day 3, CD61 expression was slightly lower than on day 7. Also on day 3, a hematopoietic cell population with surface marker expression similar to that observed on day 7 could be detected, comprising the majority of the larger cells on day 3. Whereas on day 7, the ratio of the two major populations, CD45- MSC and CD45+ HC, was approximately 4 : 1, on day 3, the ratio between these two populations was 0.7 ± 0.2 : 1 (mean of n = 4 ± sd).

One way to enhance the MSC yield is the expansion over several passages. However, the phenotype of the several-times passaged cells should be similar to the phenotype of often studied primary culture MSC to ensure comparable results. Therefore, for the characterization of cells passaged for four times, the primary cultures (day 7) were depleted of CD45+ cells and CD45- MSC seeded at the density of 1,000 cells cm^2. The proliferation kinetics was not affected by passaging, as shown in Figure 6. After 4 passages, surface marker expression was determined. No significant differences were observed in MSC phenotype compared to primary culture MSC, with the exception of CD106, which was lower at higher passage.

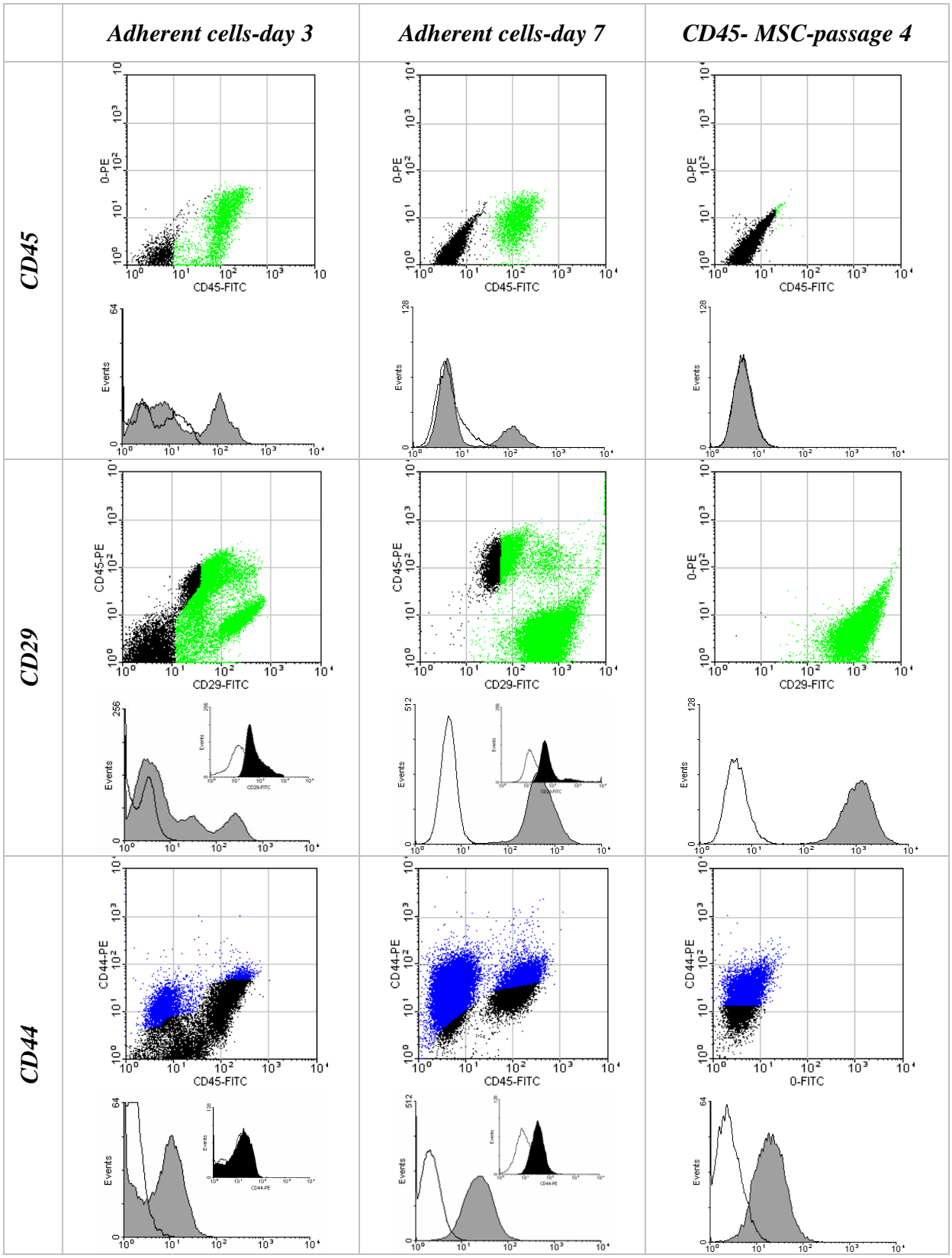


***Figure 5**: Comparison of surface marker expression in rat plastic-adherent cells during cultivation. Dot–plots show the distribution of adherent cells positive for a distinct marker (stained blue or green). The histograms show the distribution of the CD45- population (gray) separately from CD45+ population (black).*

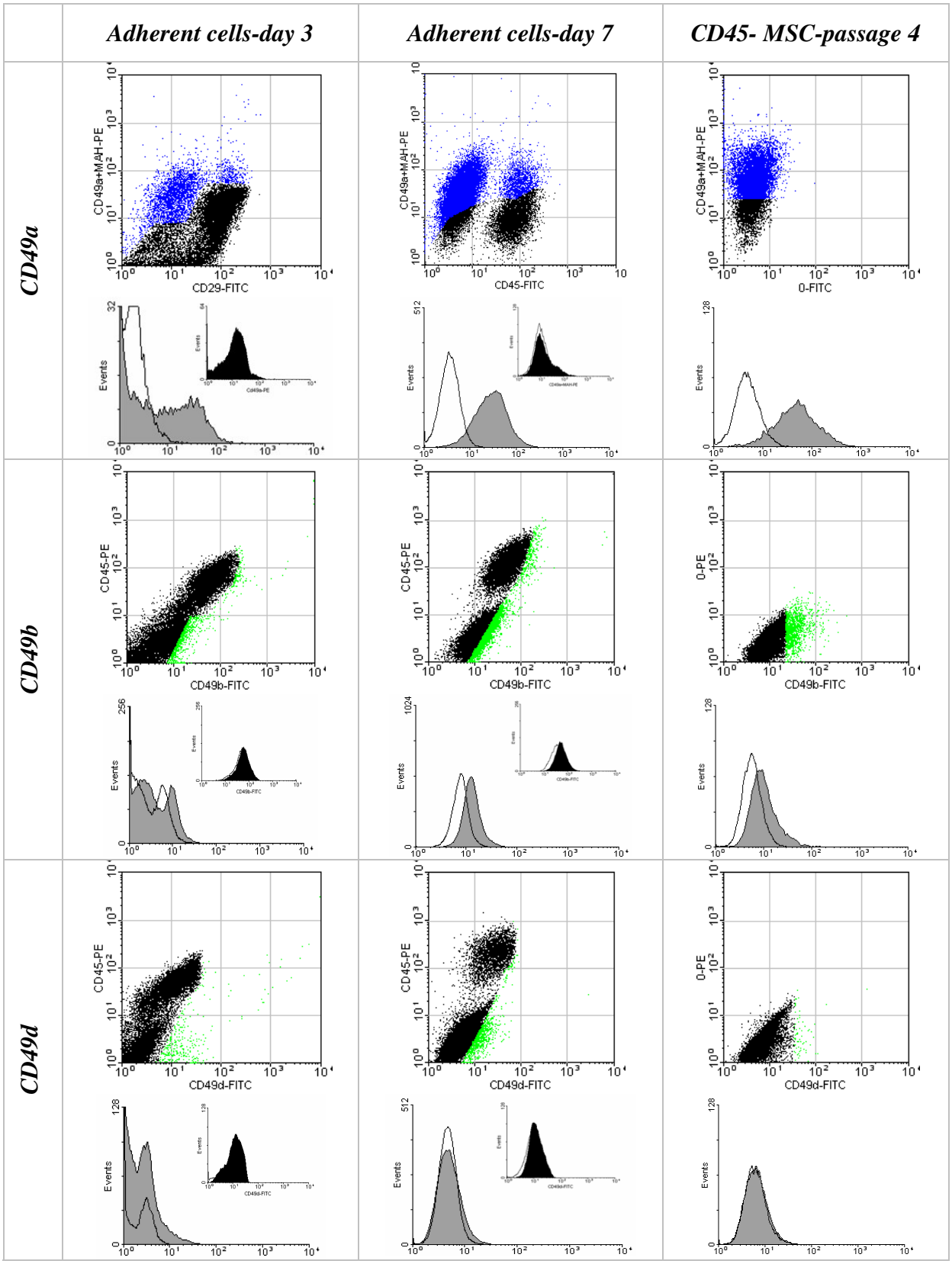


***Figure 5, continued**: Comparison of surface marker expression on rat plastic-adherent cells during cultivation. Dot–plots show the distribution of adherent cells positive for distinct marker (stained blue or green). The histograms show the distribution on CD45- population (gray) separately from CD45+ population (black).*

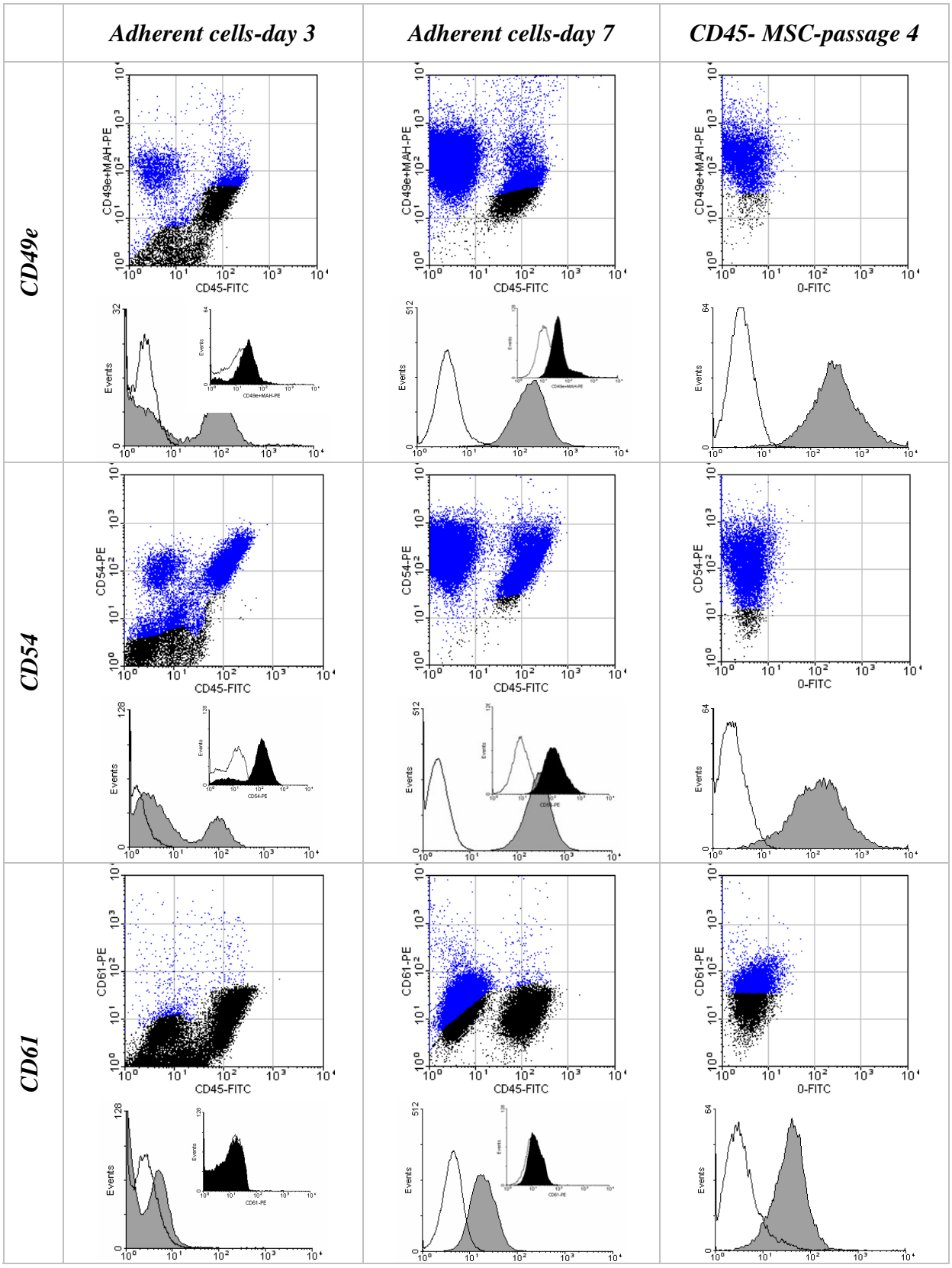


***Figure 5, continued**: Comparison of surface marker expression on rat plastic-adherent cells during cultivation. Dot –plots show the distribution of adherent cells positive for a distinct marker (stained blue or green). The histograms show the distribution on CD45- population (gray) separately from CD45+ population (black).*

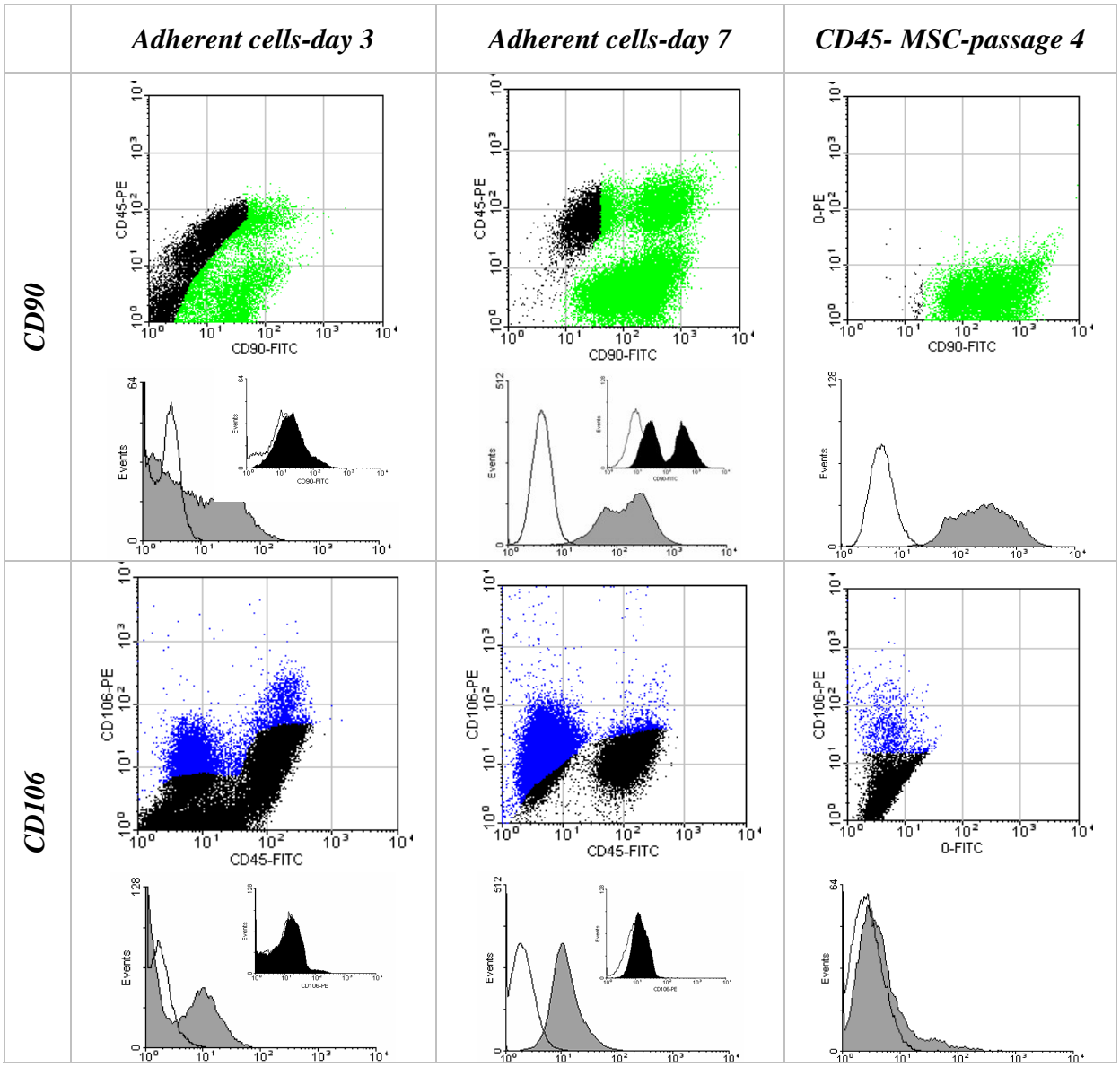


***Figure 5, continued**: Comparison of surface marker expression on rat plastic-adherent cells during cultivation. Dot–plots show the distribution of adherent cells positive for a distinct marker (stained blue or green). The histograms show the distribution on CD45- population (gray) separately from CD45+ population (black).*

DISCUSSION

Cell yield

For optimal cell yield, several parameters, ranging from cell isolation to the culture conditions, needed to be optimized. It appears that the length of time from bone dissection to bone marrow isolation with centrifugation plays a crucial role, as does the temperature of the media: the length of time from animal sacrifice to placing the cell suspension into flasks has to be as short as possible and the media temperature should be kept at 37°C. The centrifugal method of bone marrow isolation was successfully established, resulting in a much higher cell yield than obtained by the standard "flushing" method. On day 3, single adhered cells could be observed, some of which might have begun forming colonies already. Bone marrow cell seeding density was high enough to enable the cells to enhance each other's proliferation, resulting in rushed fibroblast-colony formation and cell proliferation. Cells from a single colony remain close, sometimes even in cell-cell contact, forming multi-layered colonies. This makes it difficult to determine the confluence of the whole flask. Nevertheless, the proliferation capacity seems not to be influenced by the high cell density at the end of the 7-day primary cultures, as seen in Figure 6 showing continuous cell proliferation up to passage 4.

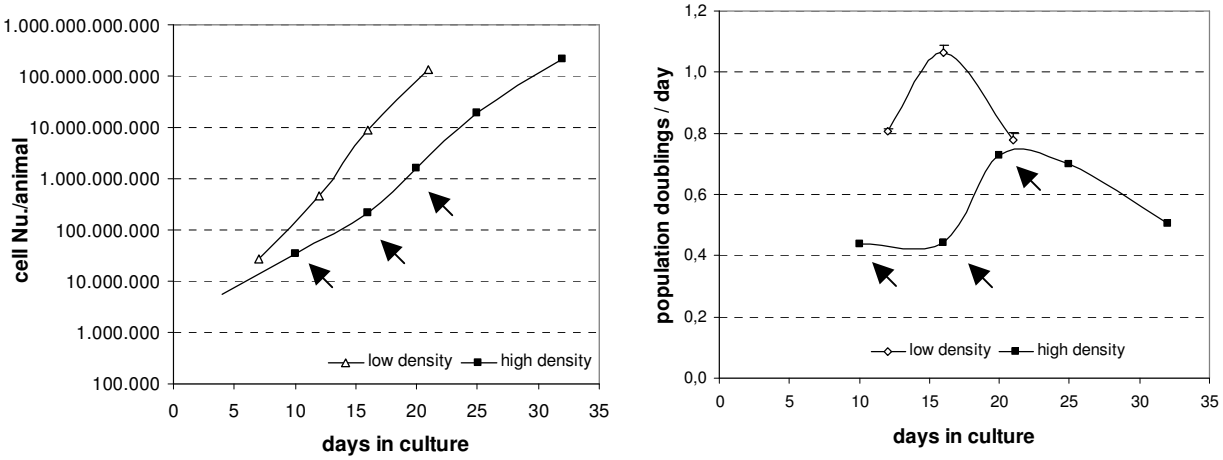


Figure 6: *Proliferation curves of rat MSC (high density (arrows): 6,667 cells / cm^2; low density (without arrows): 1,000 cells / cm^2). Low density curve is obtained from CD45-depleted MSC and the high density curve is from cells that have not undergone separation (HC are probably lost within first 2 passages).*

Freshly isolated bone marrow cells

Whereas whole bone marrow cells are seeded in flasks, flow cytometry was performed with cells after erythrocyte (Ery) lysis. Nevertheless, some Ery were still in the samples, observed as cells with low SSC and FSC and negative for CD45[12] (Figure 3). CD45 is higher expressed in mature cells (e.g. spleen cells, shown in our preliminary studies) and therefore detected in lower levels in bone marrow known to be a source of hematopoietic progenitor cells. However, CD45 expression levels in rat hematopoietic stem cells (HSC) are still not well characterized. We observed an even distribution of CD45+ cells in FSC-SSC dot-plots, as seen in Figure 3. Hematopoietic integrins CD18/CD11a and CD18/CD11bc were expressed on bone marrow cells in lower proportions than CD45. All subunits, CD11a, CD11bc and CD18, were strongly expressed on granulated cells, and CD11a/CD18 was also expressed on the lymphocyte and monocyte fractions as seen in Figure 3. The hematopoietic integrins are in general present on all rat hematopoietic cell lineages, except on Ery[12]; CD11bc is also not detected on lymphocytes[12]. This correlates with our observations of positive-cell distributions, but not with our measured percentages: we detected 26 – 40 % CD11a+ cells and 11 - 61% CD18+ cells, but 36 – 68 % CD11bc+ cells. Additional multi-color staining needs to be done to evaluate co-expression of these integrins. Nevertheless, cells positive for those markers dominated the bone marrow granulocyte fraction, which was additionally positive for the granulocyte marker (Figure 3). Granulocyte marker is known to be expressed not only on granulocytes but also on Ery progenitors, CD45- erythroblasts[12]. We detected 18 - 38% CD45RA+ cells, known to be B lymphocytes, distributed in the lymphocyte fraction of bone marrow cells. T lymphocytes, identified by CD3, CD4 and CD8b expression (Table 1), accounted for fewer than 5 % of the cells, suggesting that B lymphocytes were the predominant cell type in the bone marrow lymphocyte population. The amount of lymphocytes that we detected correlated well with smear evaluations, where 7 – 21 % lymphocytes are usually found[10]. Literature data[10] mention 34 - 52% granulocyte progenitors in the rat bone marrow which together with lymphocytes, comprise a major fraction (up to 73 %) of bone marrow nucleated cells. Our results showed 40 - 53% granulocyte+ cells, which corresponded only to the granulocyte precursor amount[10]. Whereas the same antibody stains Ery progenitor also, we were not able to distinguish between granulocytes and Ery progenitors. The latter are reported to be present in a high proportion in the rat bone marrow as well[10]. Ery progenitors could be destroyed by the lysis and therefore could not be detected quantitatively.

CD29+, CD90+ and CD54+ cells were distributed throughout all cell fractions in the FSC-SSC dot-plot (Figure 3). 57 – 68 % of the cells expressed stem cell marker CD90 and were present in both the granulocyte (Ery progenitors) and monocyte fractions, in addition to the lymphocyte fraction, where the true stem cells are expected to be found. Megakaryocyte-platelet marker CD61 was co-expressed on granulocyte-positive cells (data not shown). Determined proportions of CD61+ cells varied, which could be a consequence of strong nonspecific antibody labeling observed with isotype control.

To date, only a few surface antigenic markers have been described that have been used in the purification of MSC (CFU-F) from the bone marrow (Stro-1, CD49a, D7-FIB, BMPRIA, LNGFR)[14]. The markers commonly claimed to be specific for cultured MSC, such as CD29 and CD44, have, in fact, broad cell reactivity and, hence, are unsuitable for the detection of MSC *in vivo*. Whereas the typical MSC markers, CD29, CD90, CD54, and CD44, were present on many cell types from the bone marrow (Table 1, Figure 3), other, more specific markers like CD49a and CD106 were present in very low percentages (< 2.5 %), less than the amount of CD45- cells (42 – 83 %). Therefore CD49a and CD106 may be more suitable markers for the estimation of MSC-like cells. For the exact determination of fresh bone marrow MSC phenotype, at least 10,000 MSC-like cells needed to be examined, meaning that at least 4 x 10^5 bone marrow cells need to be evaluated for every measurement. Boiret et al.[15] enriched CFU-F from human bone marrow using CD49a or CD73-positive selection and CD45+ cell depletion. However, Deschaseaux et al.[9] and Jones et al.[16] obtained human MSC-enriched populations (CD49a+ or D7-FIB+ cells) in a CD45low fraction, suggesting that CD45+ cell depletion from the fresh bone marrow might reduce the presence of MSC-like cells also.

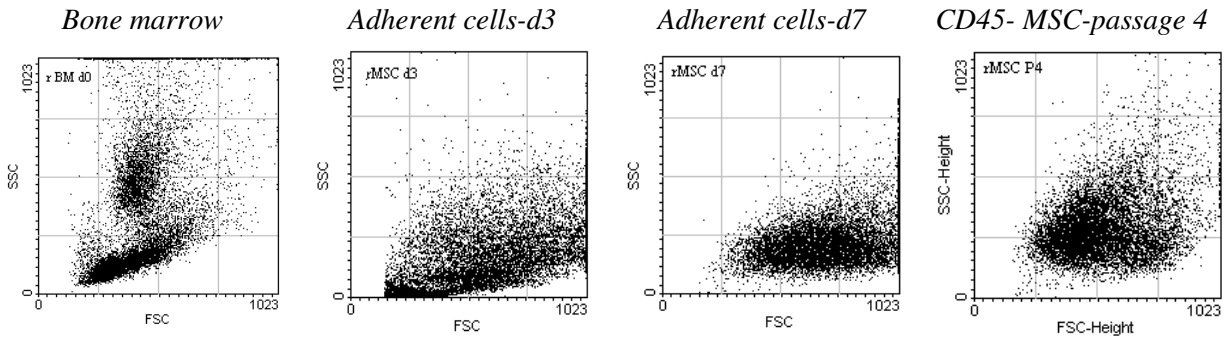


Figure 7: *Comparison of studied populations: Size (FSC) and granularity (SSC) distribution.*

Cultured plastic-adherent rat bone marrow cells

Using standard culture conditions for MSC proliferation, positive-selected human MSC (CFU-F) continuously express D7-FIB, CD13, CD10 and CD105, but lose the expression of Stro-1, LNGFR, and HLA-DR[16]. When cultured, CD45 and CD34 are also lost, but CD49a+ is retained[9]. Rat MSC have been phenotypically characterized after several passages[4] or as primary cultures of plastic-adherent bone marrow cells[17,18]. We wanted to compare the expression of surface markers on early bone marrow cultured cells (3 days and 7 days) with cells passaged for 4 passages. In Figure 7, the SSC-FSC dot-plots of bone marrow and cultured cells are compared. Although the parameters used for the measurements were unique for each population, a difference in average cell size could be observed; freshly isolated cells are much smaller than cultured cells (bone marrow vs. adherent cells-d3), which grow larger with each passage. With adherent cells, the FSC attenuation needed to be reduced to observe the whole population. Small non-granulated cells from d3 (low SSC and FSC) most likely correspond to the lymphocyte fraction of fresh bone marrow. The differences in cell size and auto-fluorescence required population-specific instrument settings, therefore also some deviations in population location in fluorescence dot-plots (Figure 5) could be observed.

Primary cultures of adherent bone marrow cells, cultured until sub-confluence (7 days), contained two main cell populations, as resolved with CD45 expression; CD45+ cells and CD45- cells expressing MSC-like markers (CD29, CD49a, CD49e, CD61, CD90, CD44, CD106). In adherent cells passaged 3 days after isolation, a population with a similar surface marker profile to the CD45- MSC fraction from day 7 could be observed. This fraction is marked as R1 in Figure 4 and comprised 23.5 ± 3.9 % (average of n = 4 ± sd) of cells passaged on day 3. Estimated MSC-like cell frequency is therefore 1.3% of nucleated bone marrow cells, based on cell counts performed on day 3 and after isolation (Table 1), under the assumption that in 3 days after isolation cells did not proliferate. This percentage is higher than the published frequency of CFU-F[19] and indicates that probably not all cells which express MSC markers are “true” stem cells or CFU-F. On day 3, a hematopoietic population similar to the CD45+ population detected on day 7 (Figure 4, R2) was observed, in addition to two other populations with low SSC and FSC. One population (Figure 4, R4), CD45low/CD11a+, could be megakaryocytes/platelets; a similar population was weakly positive for CD61, confirming this assumption (data not shown). Since megakaryocytes/platelets are primarily nonadherent, these cells could reside in the flasks after incomplete removal of nonadherent cells before analysis. The fourth population found on day

3 (Figure 4, R3) did not show significant expression of any of tested markers. Nevertheless, these cells could be small, rapidly self-renewing stem cells, an MSC subpopulation, described in low cell density cultures with low FSC and SSC[4,20,21]. However, for more detailed characterization of all populations, including the two additional populations found on day 3, many more cells have to be evaluated, separated and further cultured. However, our culture conditions favor the expansion of larger cells, particularly MSC-like cells, as illustrated by the increased MSC proportion comparing day 3 and day 7.

The expression of CD61 on CD45- MSC was higher on day 7 than on day 3, which confirms the connection of CD61 expression with *in vitro* culture conditions in the presence of FBS. CD61 expression did not change, however, from passage 1 to passage 4. In contrast, CD106 expression was lower in passaged cells than in primary culture cells (Figure 3).

MSC show limited in vitro proliferation capacity, which can be prolonged with low density culturing and special culture conditions. Figure 7 shows that, when seeded at 1,000 cells / cm^2, CD45- cells did not change their proliferative capacity with time (low density curve), maintaining 0.8 - 1 population doublings / day (PD / day). The second, high density curve was obtained by replating unseparated cells, at a density of 6,667 cells / cm^2 for the first three times, followed by 1,000 cells / cm^2. The low initial doubling rate (0.4 PD / day) could be due to high seeding density or due to the presence of hematopoietic cells, which probably did not proliferate in the culture and therefore disappeared from the MSC cultures within the first few passages[22]. Nevertheless, high density subculturing resulted in a lower PD / day and lower cell numbers compared to low density culturing.

Passaged cells (CD45-, passage four, low density) did not differ significantly from first passage CD45- MSC (Figure 3, day 7) with regard to surface marker expression (integrins and other markers). For successful tissue engineering applications, however, extensive-expanded MSC used should beside constant surface marker expression retain their differentiation potential. Ter Brugge et al. [23] showed that rat MSC lose osteogenic potential dramatically from passage 1 to passage 2. However, they did not discuss cell density at replating and confluence at the passage. It would be therefore important to determine the dependence of CD45- MSC differentiation on the passage number when the cells are proliferated at low densities, preventing the formation of dense colonies; the passages should be therefore performed at least twice a week.

CONCLUSION

This chapter shows that the culture conditions used favor the expansion of CD45- cells with MSC-like surface marker expression. Whereas less than 1.3 % cells from the bone marrow nucleated cells adhere within 3-days and show MSC-like phenotype, these cells proliferate rapidly and could be maintained in the culture over 30 days without changes in their proliferation kinetics. Four-times passaged cells showed similar phenotype to primary cultured CD45- cells (day 7), which are usually used for tissue engineering studies using rat cells. However, for tissue engineering applications also the differentiation capacity of passaged cells should remain unchanged. The contaminating hematopoietic cells found in primary cultures on day seven showed similar phenotype to cells found in the granulocyte and monocyte fraction of the fresh bone marrow. These cells could be partly eliminated by density gradient centrifugation. As we tried to determine the phenotype of freshly isolated MSC, we first analyzed adherent cells detached 3 days after isolation. With the exception of lower CD61 expression on day 3, CD45- MSC showed similar phenotype on day 3 as on day 7. The attempt to detect these cells in the fresh bone marrow showed that the expression of markers like CD49a and CD106 could indicate the presence of MSC-like cells. Additionally, at least 4 x 10^5 bone marrow cells need to be evaluated for every measurement. After the determination of MSC-like cell phenotype in the bone marrow, the strategies to isolate MSC by direct or indirect immunolabeling could be discussed.

REFERENCES

(1) *Aubin J. Osteoprogenitor Cell Frequency in Rat Bone Marrow Stromal Populations: Role of Heterotypic Cell-Cell Interactions in Osteoblast Differentiation. J Cell Biochem. 1999;72:396-410.*

(2) *Wang QR, Wolf NS. Dissecting the hematopoietic microenvironment. VIII. Clonal isolation and identification of cell types in murine CFU-F colonies by limiting dilution. Exp Hematol. 1990;18:355-359.*

(3) *Herbertson A, Aubin JE. Cell sorting enriches osteogenic populations in rat bone marrow stromal cell cultures. Bone. 1997;21:491-500.*

(4) *Javazon EH, Colter DC, Schwarz EJ, Prockop DJ. Rat marrow stromal cells are more sensitive to plating density and expand more rapidly from single-cell-derived colonies than human marrow stromal cells. Stem Cells. 2001;19:219-225.*

(5) *Takada Y, Murphy E, Pil P et al. Molecular cloning and expression of the cDNA for alpha 3 subunit of human alpha 3 beta 1 (VLA-3), an integrin receptor for fibronectin, laminin, and collagen. J Cell Biol. 1991;115:257-266.*

(6) *Wilson CJ, Clegg RE, Leavesley DI, Pearcy MJ. Mediation of biomaterial-cell interactions by adsorbed proteins: a review. Tissue Eng. 2005;11:1-18.*

(7) *Ishaug SL, Crane GM, Miller MJ et al. Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. J Biomed Mater Res. 1997;36:17-28.*

(8) *Dobson KR, Reading L, Haberey M, Marine X, Scutt A. Centrifugal isolation of bone marrow from bone: an improved method for the recovery and quantitation of bone marrow osteoprogenitor cells from rat tibiae and femurae. Calcif Tissue Int. 1999;65:411-413.*

(9) *Deschaseaux F, Gindraux F, Saadi R et al. Direct selection of human bone marrow mesenchymal stem cells using an anti-CD49a antibody reveals their CD45med,low phenotype. Br J Haematol. 2003;122:506-517.*

(10) *Provencher Bolliger A. Cytologic evaluation of bone marrow in rats: indications, methods and normal morphology. Vet Clin Pathol. 33, 58-67. 2004.*

(11) *Diaz-Romero J, Gaillard JP, Grogan SP et al. Immunophenotypic analysis of human articular chondrocytes: Changes in surface markers associated with cell expansion in monolayer culture. J Cell Physiol. 2004.*

(12) *BD Pharmingen. Technical Data Sheet. 2004. Ref Type: Catalog*

(13) *Dahlke MH, Lauth OS, Jäger MD, Roeseler T, Timott K, Jackobs S et al. In vivo depletion of hematopoietic stem cells in the rat by an anti-CD45 (RT7) antibody. Blood 99, 3566-3572. 2002.*

(14) *Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells. 2001;19:180-192.*

(15) *Boiret N, Rapatel C, Veyrat-Masson R et al. Characterization of nonexpanded mesenchymal progenitor cells from normal adult human bone marrow. Exp Hematol. 2005;33:219-225.*

(16) *Jones EA, Kinsey SE, English A, Jones RA, Straszynski L, Meredith DM et al. Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. Arthritis & Rheumatism 46[12], 3349-3360. 2002.*

(17) *Vogel B, Vogel T, Rohde E, Strunk D, Göpferich A, Schulz MB. Towards selective adhesion of rat marrow stromal cells: Phenotype characterisation and adhesive properties of plastic-adherent rat bone marrow cells. Chapter 4.*

(18) *ter Brugge PJ, Torensma R, De Ruijter JE, Figdor CG, Jansen JA. Modulation of integrin expression on rat bone marrow cells by substrates with different surface characteristics. Tissue Eng. 2002;8:615-626.*

(19) *Aubin JE. Regulation of Osteoblast Formation and Function. Rev Endocr Metab Disord. 2001;2:81-94.*

(20) *Colter DC, Sekiya I, Prockop DJ. Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. PNAS 98[14], 7841-7845. 2001.*

(21) *Colter D, Class R, DiGirolamo CM, Prockop DJ. Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. PNAS 97[7], 3213-3218. 2000.*

(22) *Prockop D.J. Marrow stromal cells as stem cells for nonhematopoetic tissues. Science 276, 71-74. 1997.*

(23) *ter Brugge PJ, Jansen JA. In vitro osteogenic differentiation of rat bone marrow cells subcultured with and without dexamethasone. Tissue Eng. 2002;8:321-331.*

CHAPTER 7

THE INFLUENCE OF SIMVASTATIN AND ATORVASTATIN ON OSTEOGENIC DIFFERENTIATION OF RAT MARROW STROMAL CELLS

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INTRODUCTION

In vitro bone tissue engineering utilizes three-dimensional cell carriers of metal alloys, hydroxyapatite or tricalciumphosphate ceramics, demineralized natural bone, or synthetic materials (e.g. poly-lactic acid, poly-lactic-co-glycolic acid) seeded with osteoblastic cells of different maturity grades from marrow stromal cells (MSC) to osteobasts[1]. However, the developing tissue suffers from insufficient differentiation and production of mineralized extracellular matrix (ECM)[2]. Therefore, growth factors like transforming growth factor β1 (TGF-β1), bone morphogenic protein-2 (BMP-2), and platelet derived growth factor (PDGF) have been more or less successfully used to overcome the sparse ECM production by several different mechanisms, like increased collagen I production, enhanced osteogenic differentiation, and enhanced proliferation, respectively[3]. Recent studies have suggested that statins, which are well established lipid-lowering drugs, influence bone turnover by stimulating bone formation and inhibiting bone resorption[4]. Several statins, including lovastatin, simvastatin, mevastatin, and fluvastatin, have been shown to increase bone formation in bone explant cultures. The mechanism of action bases on inhibition of 3-hydroxy-3-glutaryl-coenzyme A (HMG-CoA) reductase and blocking of the mevalonate pathway[4,5] (Figure 1). Nitrogen-containing bisphosphonate drugs also inhibit the mevalonate pathway, preventing the production of the isoprenoids and protein prenylation (Figure 1), which result in inhibition of osteoclast formation and function[5]. The proposed mechanism by which statins stimulate bone formation involves an increase in the expression and synthesis of bone morphogenic protein 2 (BMP-2)[6-9], which can be completely inhibited when adding mevalonate[10]. It was shown that lovastatin, simvastatin, fluvastatin, and mevastatin can increase new bone formation approximately 2- to 3-fold, an increase comparable to that seen after treatment with BMP-2 and fibroblast growth factor-1 (FGF-1)[8].

Rat MSC are suitable cells for bone tissue engineering, because they are easily accessible and can differentiate *in vitro* to the osteoblastic phenotype after dexamethasone supplementation[11,12]. Moreover, ascorbic acid and β-glycero-phosphate provide the chemical substrates for osteogenic matrix production[12]. MSC are a heterogenous cell population, including multipotential mesenchymal stem / progenitor cells, which can differentiate into osteoblasts, adipocytes, chondrocytes, myocytes, tenocytes, and cells of other mesenchymal tissues[13-15]. Supplementation with varying agents can result in differentiation to discrete cell types, e.g. the addition of dexamethasone is required for *in vitro* osteogenesis and adipogenesis, whereas supplementation with statins favors differentiation into the osteogenic

lineage, while inhibiting adipogenesis[16]. Beside *in vivo* studies on statin effects on net bone formation, *in vitro* studies are limited more or less to the osteoblastic or mesenchymal cell lines. There are insufficient data[17,18] on *in vitro* effects of statins on the osteogenesis of primary cultures of immature osteoprogenitors like MSC. Therefore, we investigated the influence of simvastatin and atorvastatin (Figure 2) supplementation on rat MSC cultures, in order to evaluate their potential for *in vitro* tissue engineering. Whereas simvastatin is often used as a statin standard control, atorvastatin is believed to show even better effects *in vivo* due to better distribution to bone tissue[9]. The effect of three different concentrations of both statins on cell proliferation, alkaline phosphatase (ALP) and calcium accumulation was investigated.

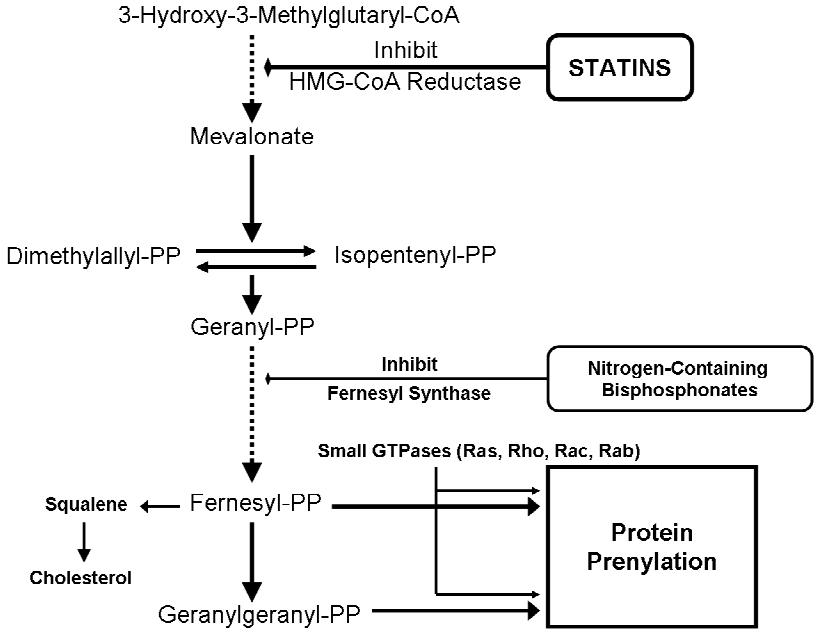


***Figure 1**: Inhibition of the mevalonate pathway by statins results in inhibition of protein prenylation and consequential lost of protein function.(from Bauer D.C.[19])*

MATERIALS AND METHODS

Suppliers

Fetal calf serum (Gibco, Invitrogen, UK; Lot No.: 40F7430K); Penicillin-Streptomycin (Gibco, Invitrogen, UK; 10,000 units / mL penicillin G sodium and 10,000 µg / mL streptomycin sulphate); L-glutamine (Sigma Aldrich, Germany; 200 mM); Trypsin-EDTA (Gibco, Invitrogen, UK; 0.25 % Trypsin and 1 mM EDTA.4Na); Minimum essential medium Eagle, alpha modification (Sigma Aldrich, Germany; 10.1 g / L); simvastatin (MSD, Germany); atorvastatin (Pfizer, USA).

Cell culture

Rat bone marrow was isolated from the femur and tibia of 6-8 weeks old (150-200g) male Sprague-Dawley rats using the centrifugal method[20]. Freshly isolated bone marrow cells were resuspended in primary medium (10 % FCS, 1 % Penicillin / Streptomycin, 0.5 % L-glutamine and minimal essential medium, alpha modification). Cells obtained from a single rat (2 tibias and 2 femur) were seeded in 4 x T150 flasks. The cells were cultured in humidified atmosphere with 5 % CO_2 at 37°C and left to adhere for three days before non-adherent cells were removed. Medium was changed three times a week and when the cultures reached 80 – 100 % confluence, they were detached from the surface with trypsin-EDTA and counted.

For the differentiation the cells were plated in 12-well plates at a density of 50,000 cells / cm^2 in primary medium for 24 hours. On day 1 the cells received complete medium (primary medium supplemented with 50 mg / L ascorbic acid, 2.16 g / L β-glycero-phosphate and 10^{-8} M dexamethasone). The medium was changed every 2 - 3 days and the samples were taken at different time points for the assays. Wells were washed with PBS and frozen at –20°C until all the samples were collected.

During the differentiation phase, the cultures received statins continuously with every media change. We tested three concentrations for each statin and the groups were:

Control, without statin: C,

Atorvastatin or simvastatin in 0.5 µM concentration: A05 or S05,

Atorvastatin or simvastatin in 1 µM concentration: A1 or S1,

Atorvastatin or simvastin in 5 µM concentration: A5 or S5.

Cell number

A fluorimetric assay[21] was performed to measure the total amount of DNA and, subsequently, to determine the cell number. The samples were thawed and the cells scraped off the surface and dispersed in PBS. After digestion with a papain solution for 18 h at 60°C, the released DNA was quantified using Hoechst 33258 dye.

Alkaline Phosphatase (ALP)

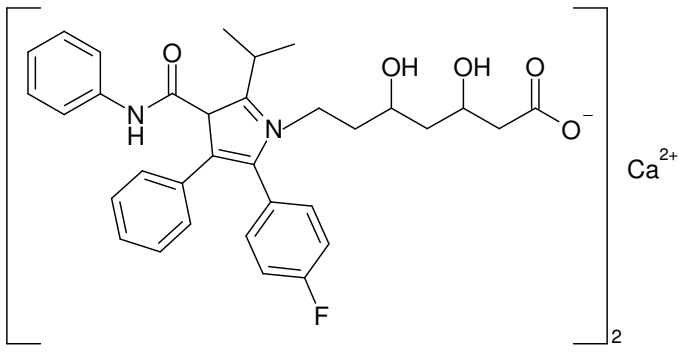
Cells were thawed and kept on ice during preparation of the samples. Cells were scraped, dispersed in TRIS buffer (1M Trisma base (Sigma, Germany), pH 8) and sonicated. A kinetics-based assay was performed to determine the enzymatic activity (RANDOX Kit, Randox Laboratories Ltd., Crumlin, UK) by measuring the absorbance of released product at 405 nm.

Von Kossa staining

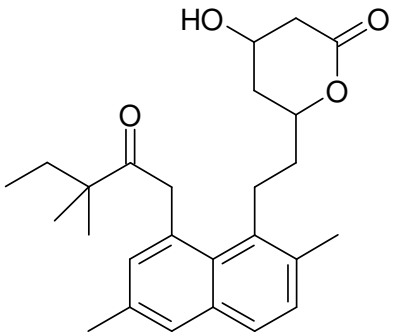
After 18 days of osteogenic differentiation, wells were rinsed with PBS and the cells fixed with 10 % neutral buffered formalin. Samples were incubated with 5 % aqueous silver nitrate solution, exposed to natural light for 30 min, and counterstained with 0.5 % safranin O. ImageJ was used to evaluate the % area positively stained for calcium.

Statistical analysis

Results are an average of 4 experiments ± standard deviation, unless otherwise stated. The level of significance was determined by Tukey test.



Atorvastatin calcium



Simvastatin

Figure 2: *Structural formula of atorvastatin (left) calcium salt and simvastatin (right).*

RESULTS

Influence of statins on proliferation in osteogenic medium

Statins were supplemented in three different concentrations with every media change, 3-times a week during the differentiation phase. Figure 3 shows the influence of statins on cell number during osteogenic differentiation. The addition of either simvastatin or atorvastatin in the investigated dose range decreased cell number after day 10 compared to the control. As early as day 10, cultures supplemented with 0.5 µM and 1 µM simvastatin showed significantly lower ($p < 0.01$) cell numbers compared to the control. The influence of the same concentrations of atorvastatin and simvastatin on cell number indicated that simvastatin is a stronger inhibitor of proliferation than atorvastatin, as seen on days 10 and 14. Both statins caused cell death and declining cell numbers after day 10, compared to the control, in which the cell number over the examined period was more or less constant. In the group treated with the highest dose of simvastatin (5 µM), detachment of cells from the wells and cell death were already evident after the first treatment. By day 10, all of the cells from this group had detached from the surfaces and therefore no further evaluation was possible. On day 14 we observed a decrease in cell number with increasing concentrations of both statins.

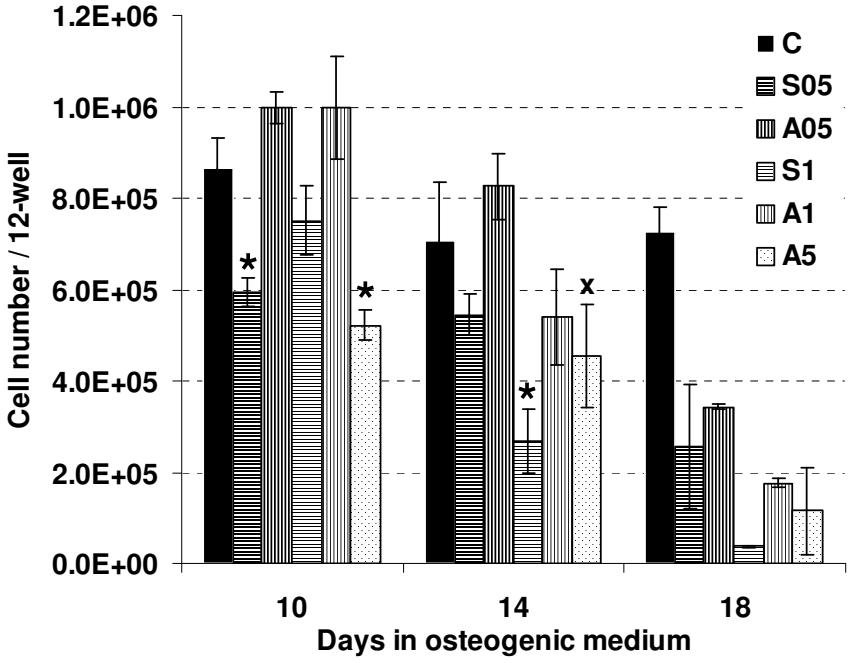


***Figure 3**: Proliferation in osteogenic medium. C: control without statin; S: simvastatin; A: atorvastatin; 05: 0,5 µM; 1: 1 µM and 5: 5 µM concentration.*
*Significant difference compared to C: * : $p < 0.01$ and x: $p < 0.05$.*

Influence of statins on osteogenic differentiation

The extent of osteogenic differentiation was assessed by determining an alkaline phosphatase (ALP) time profile, von Kossa staining of calcium deposition, and its quantification using the image analysis software. ALP time profile was assessed between days 10 and 18. As shown in Figure 4A, maximum ALP was determined on day 10. The lower proliferation after simvastatin addition was accompanied by significantly higher ALP activity per cell (Figure 4B) in both remaining simvastatin groups, compared to the control. The 1 µM simvastatin group showed higher ALP activity per cell than all groups supplemented with atorvastatin (Figure 4B). Interestingly, atorvastatin supplementation did not result in significant increases in the ALP activity per cell, compared to the control.

Mineralization (calcium phosphate deposition) was determined by von Kossa staining of the cultures after 18 days in osteogenic medium (Figures 5 and 6). We observed the detachment of cell layers, which then formed coils, in groups with simvastatin (Figure 5, S05 and S1), most likely resulting from extensive collagen matrix production and cross-linking. In the remaining simvastatin-treated wells showing no detachment, the mineralization was more extensive than in the control group (Figure 6). Groups supplemented with atorvastatin showed a slight dose-dependent increase in mineralization (Figure 6).

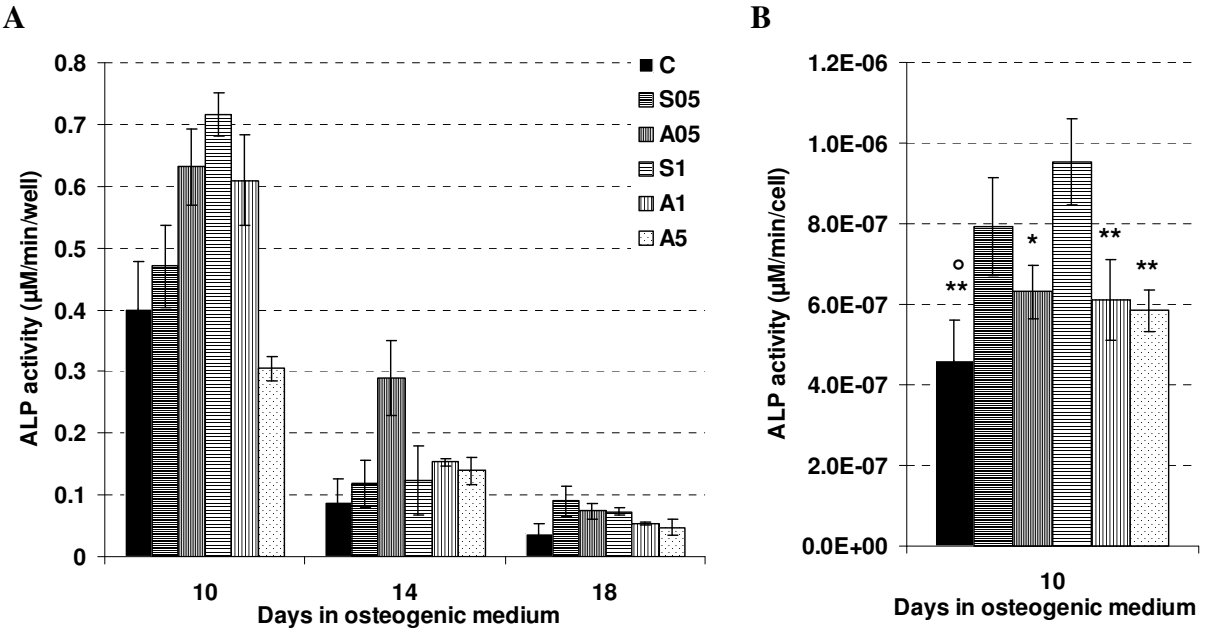


***Figure 4**: Alkaline phosphatase activity. A: time profile, per well; B: day 10, per cell.*
*The ALP activity was the highest on day 10 for the time points that we considered (A). Significant differences are marked as ** when $p < 0.01$ compared to S1, * when $p < 0.05$ compared to S05, and ° when $p < 0.01$ compared to S05.*

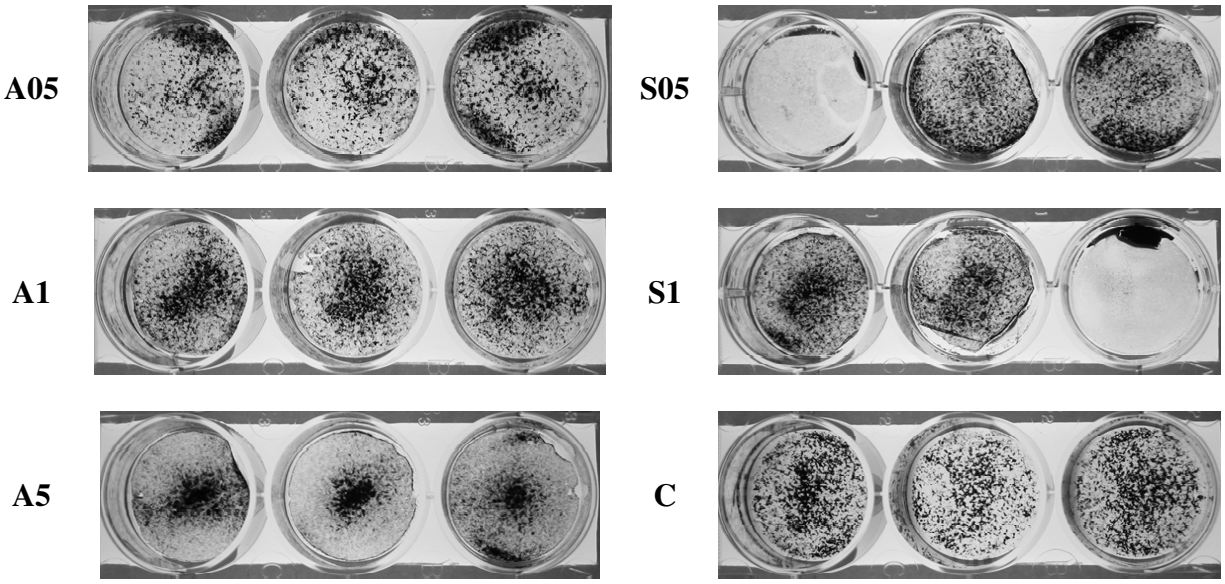


Figure 5: *Von Kossa silver nitrate staining of calcium deposition on day 18.*

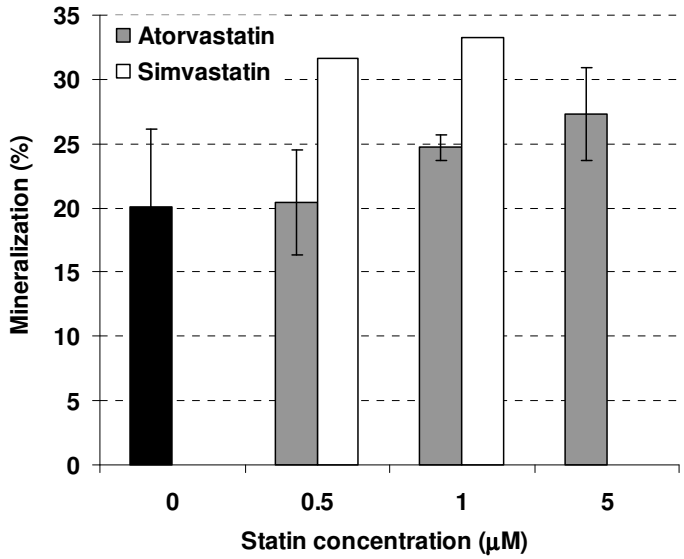


Figure 6: *Von Kossa silver nitrate staining: percentage of mineralized area after 18 days in osteogenic medium. Columns and error bars represent mean of (n = 2 or 3) ± standard deviation (only for n = 3).*

DISCUSSION

The osteogenic differentiation of marrow stromal cells (MSC) is a continuous process, triggered by dexamethasone, which induces MSC to differentiate into osteoprogenitors, which still proliferate. Osteoprogenitors begin to express alkaline phosphatase, an early osteogenic marker and large amounts of collagen I, which slowly mineralizes. With progressing maturation, the proliferation ceases as the cells develop into mature osteoblasts. Only mature osteoblasts secrete the late differentiation marker osteocalcin. Osteoblasts embedded in matrix become osteocytes or die by apoptosis[22]. Therefore, for a large part of osteoblasts differentiation is physiologically accompanied by cell death. Bone tissue engineering utilizing 3-dimensional cell carriers suffers from insufficient matrix production and distribution, caused in part by a lack in growth factor supply[23,24]. However, osteogenic growth factors are expensive and prone to cause side effects. Therefore, we tested the potential osteoinductive agents, simvastatin and atorvastatin, known as well tolerated lipid-lowering drugs, to determine which dosages may be appropriate for *in vitro* tissue engineering.

Our results show that cell proliferation up to day 10 was not inhibited by 0.5 and 1 µM atorvastatin, whereas a negative influence was found at the 5 µM dose. Simvastatin inhibited proliferation or caused cell death in all tested concentrations, with 5 µM being the most devastating. With increasing culture time, the negative effect on cell number was even more evident, suggesting that continuing treatment with statins over the entire differentiation period is not desired, but it might be beneficial during the early differentiation period. Mundy et al.[8] reported that 5 µM statin supplementation is the optimal concentration for rat calvaria osteoblast explant cultures, even resulting in increased osteoblast proliferation. Using more immature rat primary bone marrow cells, Maeda et al.[17] showed an osteoinductive effect of simvastatin at concentrations even below 1 µM. However, he and some other groups exposed the cultures to statins only for a couple days (3 - 7 days), in which period our cultures also show only the positive effects. Most of the studies used cell lines and exposed the cells to the statins for shorter time periods and analyzed the cultures immediately thereafter[6,17,18]. Only Baek et al.[25] reported a negative effect of simvastatin on the proliferation of human MSC after more than 10 day treatment, which could, together with our observations, indicate that (1) immature MSC are more sensitive to simvastatin supplementation than more mature cells and (2) the prolonged exposure to statins (more than 7 days) seem to have a negative effect on cell number.

On the other hand, we observed heightened alkaline phosphatase activity on day 10 in statin-supplemented groups compared to the control group, especially in simvastatin treated groups. This indicates that statins positively influenced the early phase of differentiation. Statin addition

only in the early differentiation phase might be sufficient, because it has been proposed that statins produce prolonged biological effects after only brief exposure[9]. In our study, statins also increased the mineralization. Unfortunately, the assessment of mineralization was performed only on day 18, where all groups treated with statins exhibited lower cell numbers. In other words, statins probably improved differentiation of rat MSC and mineralization, which was concealed by the toxic effects of prolonged exposure to statins.

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REFERENCES

(1) *Mauney JR, Volloch V, Kaplan DL. Role of adult mesenchymal stem cells in bone tissue engineering applications: current status and future prospects. Tissue Eng. 2005;11:787-802.*

(2) *Griffith LG, Naughton G. Tissue engineering-current challanges and expanding opportunities. Science. 2002;295:1009-1016.*

(3) *Vogel T. Improvement of matrix formation and tissue distribution for in vitro bone tissie engineering. 2007.*

(4) *Edwards CJ, Russel RGG, Spector TT. Statins and bone: Myth or reality? Calcif Tissue Int. 69, 63-66. 2001.)*

(5) *Staal A, Frith JC, French MH, Swartz J, Güngör T, Harrity TW et al. The Ability of Statins to Inhibit Bone Resorption is Directly Related to their Inhibitory Effect on HMG-CoA Reductase Activity. J Bone Miner Res 18, 88-96. 2003.*

(6) *Sugiyama M, Kodama T, Konishi K, Abe K, Asami S, Oikawa S. Compactin and Simvastatin, but not Pravastatin induce bone morphogenic protein-2 in human osteosarcoma cells. Biochem.Biophys.Res.Comm. 271, 688-692. 2000.*

(7) *Ohnaka K, Shimoda S, Nawata H, Shimokawa H, Kaibuchi K, Iwamoto Y et al. Pitavastatin enhanced BMP-2 and Osteocalcin expression by inhibition of Rho-associated kinase in human osteoblasts. Biochem.Biophys.Res.Comm. 287, 337-342. 2001.*

(8) *Mundy G, Garret R, Harris S, Chan J, Chen D, Rossini G et al. Stimulation of bone formation in vitro and in rodents by statins. Science 286, 1946-1949. 1999.*

(9) *Mundy GR. Statins and their potential for osteoporosis. Bone 29[6], 495-497. 2001.*

(10) *Werner N, Nickenig G, Laufs U. Pleiotropic effects of HMG-CoA reductase inhibitors. Basic Res.Cardiol. 97, 105-116. 2002.*

(11) *Aubin J, Triffit JT. Mesenchymal Stem Cells and Osteoblast Differentiation. In: Bilezikian JB, Raisz LG, Rodan GA, eds. Principles of Bone Biology.1. San Diego;San Francisco;New York;Boston;London;Sydney;Tokyo: Academic Press; 2002:59-81.*

(12) *Peter SJ, Liang CR, Kim DJ, Widmer MS, Mikos AG. Osteoblastic phenotype of rat marrow stromal cells cultured in the presence of dexamethasone, beta-glycerolphosphate, and L-ascorbic acid. J Cell Biochem. 1998;71:55-62.*

(13) *Deans RJ, Moseley AB. Mesenchymal stem cells: Biology and potential clinical uses. Experimental Hematology. 2000;28:875-884.*

(14) *Minguell JJ, Erices A, Conget P. Mesenchymal Stem Cells. Experimental Biology and Medicine. 2001;226:507-520.*

(15) *Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells. 2001;19:180-192.*

(16) *Li X, Ciu Q, Kao C, Wang GJ, Ballin G. Lovastatin inhibits adipogenic and stimulates osteogenic differentiation by supressing PPARy2 and increasing Cbfa1/Runx2 expression in bone marrow mesenchymal cell cultures. Bone 33, 652-692. 2003.*

(17) *Maeda T, Matsunama A, Kawane T, Horiuchi N. Simvastatin promotes osteoblast differentiation and mineralisation in MC3T3-E1 cells. Biochem.Biophys.Res.Comm. 280, 874-877. 2001.*

(18) *Song C, Guo.Z., Ma Q, Chen Z, Liu Z, Jia H et al. Simvastatin induces osteoblastic differentiation and inhibits adipocytic differentiation in mouse bone marrow stromal cells. Biochem.Biophys.Res.Comm. 308, 458-462. 2003. Ref Type: Journal (Full)*

(19) *Bauer DC. HMG CoA reductase inhibitors and the skeleton: a comprehensive review. Osteoporos Int. 2003;14:273-282.*

(20) *Dobson KR RLHMMXSA. Centrifugal isolation of bone marrow from bone: an improved method for the recovery and quantitation of bone marrow osteoprogenitor cells from rat tibiae and femurae. Calcif Tissue Int. 1999;65:411-413.*

(21) *Kim YJ, Sah RL, Doong JY, Grodzinsky AJ. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. Anal Biochem. 1988;174:168-176.*

(22) *Aubin JE. Regulation of Osteoblast Formation and Function. Rev Endocr Metab Disord. 2001;2:81-94.*

(23) *Freed LE, Vunjak-Novakovic G. Culture of organized cell communities. Adv Drug Deliv Rev. 1998;33:15-30.*

(24) *Sittinger M, Bujila J, Rotter N et al. Tissue engineering and autologous transplant formation: practical approaches with resorbable biomaterials and new cell culture techniques. Biomaterials. 1996;17:237-242.*

(25) *Baek KH, Lee WY, Oh KW et al. The effect of simvastatin on the proliferation and differentiation of human bone marrow stromal cells. J Korean Med Sci. 2005;20:438-444.*

CHAPTER 8

SUMMARY
&
CONCLUSION

This thesis considered aspects of cells and biomaterials required for effective tissue engineering of bone. Along these lines, we investigated cell characteristics decisive for the control of cell-biomaterial interactions and adhesion to three-dimensional carriers. While previous and cooperative work within group[1,2] concerning this subject dealt with the control of protein adsorption to biodegradable PEG-PLAs and mechanisms of surface modification, this thesis focused on cell characteristics required for selective cell adhesion.

Bone marrow stromal cells are a promising source of osteoprogenitor cells and can easily be obtained by bone marrow aspiration in humans or by bone centrifugation in rodents followed by cell selection based on adherence to tissue culture plastic. We characterized the populations found in the adherent cell fraction of the rat bone marrow. We found that adherent cells included marrow stromal cells (MSC), which are a source of the progenitors of mesenchymal cell types, and hematopoietic cells (HC). To determine if the HC fraction had positive or negative effects on osteogenic differentiation, we separated MSC and HC immunomagnetically and combined them in controlled and varied proportions. Because we found that co-culturing the MSC with HC hindered MSC differentiation, we focused on enrichment strategies for MSC. We first varied the adhesion time of bone marrow cells after isolation. In the second step we focused on selective adhesion of MSC to the extracellular matrix (ECM) proteins in the presence of HC. A rational approach to selective cell adhesion is the characterization of the ECM receptor expression pattern, i.e. integrin expression on the cell surface. We therefore investigated the integrin expression on MSC and HC depending on the time in culture. Moreover, we initiated investigations into the induced modification of integrin expression following the addition of growth factors. Since bone formation *in vitro* is often a problem in tissue regeneration, we finally explored the effects of statins, known to be beneficial for bone homeostasis *in vivo*, on osteogenic differentiation.

This work based on marrow stromal cells of rat origin. The rat model bears several advantages over the human model. First, rat cells are easily available, show lower interindividual variations than the human system, and provide immuncompetent animal defect models. Plastic-adherent rat bone marrow cells have been used for bone, cartilage, and fat tissue engineering studies[1,3,4], since they provide a population enriched for MSC. Since human and mouse MSC have been already phenotypically characterized, but less data were available on rat MSC, our first aim was to determine surface marker expression on *in vitro* expanded rat MSC. Focusing on the characterization of this population, we detected the presence of significant amounts (10 – 40 %) of CD45+ HC in primary cultures. In **Chapter 2** the adherence time after cell isolation was varied in order to reduce HC contamination.

Whereas the percentage of HC could not be significantly reduced, the adherence time of 3 - 4 days lead to significant higher cell yields than the adherence time of 1 - 2 days. This may be due to the adherence time itself allowing for more cells to adhere or due to the presence of non-adherent cells, known to contain platelets and megakaryocytes, which can induce adherent-cell proliferation[5]. Both mechanisms likely contributed to the improved MSC yield after prolonged initial adherence time. This is an important fact, since the cell number is crucial for tissue engineering applications. Therefore, we decided to allow bone marrow cells to adhere for 3 days after isolation, before non-adherent cells were removed. The adherent cells were further cultured until day 7 after isolation. Endothelial cells, which have also been found in primary MSC cultures[6], were not present in our cultures (**Chapter 4**).

The experiments presented in **Chapter 3** investigated the need for selective adhesion of MSC to the biomaterials and for the effect of contaminating HC. We studied the influence of CD45+ HC, from the primary MSC cultures on osteogenic differentiation. We first compared the ALP and calcium accumulation of populations containing different percentages of CD45+ cells and CD45- cells, keeping the total (CD45+ and CD45-) cell seeding density constant. Differentiation in pure CD45- cultures was strongly improved compared to the mixed cultures. With increasing proportions of HC (CD45+), ALP activity and calcium accumulation decreased. To exclude an effect of variable CD45- MSC seeding density in the first experiment, some additional scenarios were tested. Each of these experiments confirmed the negative effect of HC on MSC differentiation to the osteogenic lineage, while MSC seeding density and the separation procedure had no significant influence on differentiation (**Chapter 3**).

MSC dilution with dermal fibroblasts has been shown to cause similar suppression of osteogenesis, probably mediated by reduced MSC homotypic cell-cell contacts[7]. To examine whether this was also true for the effect of HC on osteogenic differentiation, we investigated the effects in trans-well inserts, where we spatially separated HC from MSC. In these systems HC can only interact with MSC by soluble factors (cytokines), but not by cell-cell contacts. Osteogenic gene expression was significantly higher in MSC cultured in the absence of HC than in the presence of CD45+ HC: The specific osteoblast marker osteocalcin was fourty-times higher expressed in monocultured MSC. This proves that plastic-adherent CD45+ cells secrete some soluble factors that suppress MSC osteogenic differentiation. These factors may be identified by screening studies on protein or gene level and confirmed by exogenous supplementation to the MSC culture. Some candidates are discussed in **Chapter 3**. In addition

to the tissue engineering applications, these results may have relevance for the understanding the "protective" mechanisms against spontaneous MSC differentiation in the bone marrow[8,9].

In order to enrich MSC in the presence of HC, we characterized the integrin expression of both populations to find selective receptors for ECM adhesion. Multi-parameter flow cytometry enabled us to characterize both cell populations found in the primary cultures of adherent rat bone marrow cells without the need for mechanical separation of the cells, as presented in **Chapter 4**. We found that the majority of adherent bone marrow cells (70 – 90 %) were CD45- / CD11bc- / CD31- / CD90+ / CD44+ / CD106+ / CD29+, expressing markers characteristic for MSC[10,11]. The same cells co-expressed integrin subunits CD49a, CD49b, CD49c, CD49e, and CD61, receptors for ECM molecules such as collagen I-IV, fibronectin, and vitronectin. In contrast, CD45+ HC expressed hematopoietic integrins like CD11a, CD11bc, and CD18, which are implicated in cell-cell interactions. HC also expressed the ECM integrins CD49e and low CD49a, which could, in turn, cause HC adherence to fibronectin or collagen, if activated. The adhesion of MSC and HC to the ECM-coated wells showed that MSC adherence corresponded to specific integrin expression: fibronectin > collagen IV, collagen I > vitronectin > laminin. CD45+ HC adhered strongly in the presence of FBS and on fibronectin and vitronectin-coated surfaces, but not to collagen or laminin. Moreover, HC exert strong non-specific adhesion to the TC plastic, as seen with BSA coated wells. Their adhesion to vitronectin could not be explained with the expression of vitronectin-specific integrin CD61, but with the possible involvement of non-specific adhesion or other mechanisms. On the other hand, collagen-coated substrates would enable the selective *in vitro* adhesion of MSC only (**Chapter 4**), at least in serum-free media, where fibronectin and vitronectin are absent. In **Chapter 5** an approach involving growth factor effects to modulate integrin expression for the enhancement of MSC adhesion to the modified surfaces is presented.

In **Chapter 6** further methods to enrich MSC for tissue engineering applications are proposed. Immunomagnetic depletion of CD45+ HC from the primary cultures (7 days) is possible, but separation of earlier cultures could be more cost-effective. The efficacy of MSC isolation directly from the bone marrow is debatable, due to their low frequency. We saw that the rat MSC phenotype was not greatly affected by cell culturing between day 3 and the fourth passage, indicating that multiple passages could be utilized for MSC enrichment. However, a potential loss of osteogenic differentiation capacity after each subculture should be considered.

As *in vitro* bone tissue engineering with rat MSC suffers from poor tissue production, which may be caused by insufficient cell differentiation, growth factors and osteoinductive drugs have been tested to overcome the problem. Mundy at al.[12] reported in 1999 that statins, lipid-lowering drugs, enhance bone formation *in vivo* and *in vitro* by rodents. We tested the effect of continuous supplementation of simvastatin and atorvastatin on the MSC differentiation phase (**Chapter 7**). We observed a dose-dependent increase in ALP and calcium accumulation, but a devastating effect on cell number after exposure to high concentrations. Furthermore, all investigated concentrations strongly decreased the cell numbers when applied for more than 10 days. Additional studies could confirm whether only brief exposure to statins could bring the desired effects: improved osteogenic differentiation without cell death.

To summarize the thesis, we showed the importance of methods like flow cytometry for characterizing heterogenous cell populations yielded both *in vitro* and *in vivo*. We showed that the contaminating cell populations (CD45+ cells) could be a source of sample variation between experiments, because the degree of contamination is also a significant variable. Furthermore, an immunomagnetic separation technique (e.g. MACS®) was successfully adopted for tissue engineering applications. While the hematopoietic stem cell microenvironment is widely studied, there is increasing interest in the MSC microenvironment (factors, cells); this thesis contributes to that growing body of knowledge. Finally, ways to induce selective MSC adhesion in the presence of contaminating HC are described; after the analysis of adhesion receptor expression, the receptor activity was tested in experiments on cell adhesion to the ECM-coated substrates.

REFERENCES

(1) *Lieb E, Tessmar J, Hacker M et al. Poly(D,L-lactic acid)-poly(ethylene glycol)-monomethyl ether diblock copolymers control adhesion and osteoblastic differentiation of marrow stromal cells. Tissue Eng. 2003;9:71-84.*

(2) *Lieb E, Hacker M, Tessmar J et al. Mediating specific cell adhesion to low-adhesive diblock copolymers by instant modification with cyclic RGD peptides. Biomaterials. 2005;26:2333-2341.*

(3) *Lennon DP, Haynesworth SE, Young RG, Dennis JE, Caplan AI. A chemically defined medium supports in vitro proliferation and maintains the osteochondral potential of rat marrow-derived mesenchymal stem cells. Exp Cell Res. 1995;219:211-222.*

(4) *Neubauer M, Fischbach C, Bauer-Kreisel P et al. Basic fibroblast growth factor enhances PPARgamma ligand-induced adipogenesis of mesenchymal stem cells. FEBS Lett. 2004;577:277-283.*

(5) *Friedenstein AJ, Latzinik NV, Gorskaya YuF, Luria EA, Moskvina IL. Bone marrow stromal colony formation requires stimulation by haemopoietic cells. Bone Miner 18[3], 199-213. 1992.*

(6) *Wang QR, Wolf NS. Dissecting the hematopoietic microenvironment. VIII. Clonal isolation and identification of cell types in murine CFU-F colonies by limiting dilution. Exp Hematol. 1990;18:355-359.*

(7) *Lennon DP, Haynesworth SE, Arm DM, Baber MA, Caplan AI. Dilution of human mesenchymal stem cells with dermal fibroblasts and the effects on in vitro and in vivo osteochondrogenesis. Dev Dyn. 2000;219:50-62.*

(8) *Erices A, Conget P, Rojas C, Minguell JJ. gp130 Activation by Soluble Interleukin-6 Receptor/Interleukin-6 Enhances Osteoblastic Differentiation of Human Bona Marrow-Derived Mesenchymal Stem Cells. Experimantal cell research. 2002;280:24-32.*

(9) *Caplan AI, Reuben D, Haynesworth SE. Cell-based tissue engineering therapies: the influence of whole body physiology. Adv Drug Deliv Rev. 1998;33:3-14.*

(10) *Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca RD et al. Multilineage Potential of Adult Human Mesenchymal Stem Cells . Science 284, 143-147. 1999.*

(11) *Deans RJ, Moseley AB. Mesenchymal stem cells: Biology and potential clinical uses. Experimental Hematology. 2000;28:875-884.*

(12) *Mundy G, Garret R, Harris S, Chan J, Chen D, Rossini G et al. Stimulation of bone formation in vitro and in rodents by statins. Science 286, 1946-1949. 1999.*

APPENDIX

ABBREVIATIONS

7-AAD	7-aminoactinomycin D, DNA dye
α-MEM	Minimum Essential Medium Eagle, alpha modification
Ab	antibody
Ag	antigene
ALP	alkaline phosphatase
APC	allophycocyanin
BMP	bone morphogenic protein
BMPR	receptor for BMP
BSA	bovine serum albumin
Cbfa-1	core binding factor-1, bone transcription factor
CD	clusters of differentiation
	(cluster designation of monoclonal antibodies)
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylene-diamino-tetraacetic acid
EGF	endothelial growth factor
ESC	embryonic stem cells
FACS	fluorescence-activated cell sorting
FC	flow cytometry
FBS	fetal bovine serum
FGF	fibroblast growth factor
FITC	fluorescein isothyocyanate
FSC	forward scatter (FC parameter describing cell size)
HC	hematopoietic cells
HSC	hematopoietic stem cell
Ig	immunoglobulin
IGF	insulin-like growth factor
IL	interleukin
LME	level of marker expression (FC parameter)
LNGFR	low affinity nerve growth factor receptor
MACS	magnetic-activated cell sorting; trade name of Miltenyi Biotec
MAPC	multipotent adult progenitor cells

MFI	mean fluorescence intensity
MSC	marrow stromal cells, exceptionally: mesenchymal stem / progenitor cells
PBS	phosphate buffered saline, buffer
PD	population doublings
PDGF	platelet-derived growth factor
PE	phycoerythrin
PGA	poly-glycolic acid
PLA	poly-lactic acid
PLGA	poly-lactic-co-glycolic acid
PI	propidium iodine, DNA dye
RGD	peptide sequence: arginine-glycine-aspartic acid
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
sd	standard deviation
SCT	stem cell therapy
SEM	standard error of the mean
SSC	sideward scatter (FC parameter describing cell granularity)
TC	tissue culture
TCPS	tissue culture polystyrene
TE	tissue engineering
TGF	transforming growth factor
VEGF	vascular endothelial growth factor

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LIST OF PUBLICATIONS

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Kristl J., Volk B., Gašperlin M., Šentjurc M., Jurkovič P. 2003. Effect of colloidal carriers on ascorbyl palmitate stability. *Eur. J. Pharm. Sci., 19(4): 181-189.*

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Volk B., Vogel T., Rohde E., Strunk D., Göpferich A., Schulz M.B. Flow Cytometric Analysis of Plastic-Adherent rat bone marrow cells. 2004. TESI and ETES Joint Meeting, Lausanne, Schwitzerland in DPhG Jahrestagung, Regensburg, Germany.

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Volk B., Kristl J., Šentjurc M. Location of amphiphilic spin probe in colloid drug carrier systems studied by EPR. 2002. CRS Day Wienna, Austria.

Vogel T., Vogel B., Hacker M., Göpferich A., Schulz M.B. Approaches to solve the problem of uneven tissue distribution on constructs in tissue engineering of bone. 2005 ETES Munich, Germany.

Vogel T., Hacker M., Vogel B., Mörath M., Schönbacher K., Spreitzer A., Malli R., Schulz M.B. A microparticular controlled release system for VEGF designed to improve vascularisation of engineered bone tissue. 2005 PharmaSciFair Nice, France.

Vogel T., Lieb E., Volk B., Hacker M., Göpferich A., Schulz M.B. Dose-dependent Effects of TGF-β1 Supplementation on Bone Formation in 3-D Culture of Rat Marrow Stromal Cells. 2004. Strategies in Tissue Engineering, Würzburg, Germany.

Ahmed N., Volk B., Schulz M.B., Grifka J. and Grässel S. Rat mesenchymal stem cells isolation and chondrogenic differentiation in a novel coculture system. 2004. Strategies in Tissue Engineering, Würzburg, Germany.

Schüssele A., Volk B., Mayr H., Blunk T., Schulz M.B., Goepferich A. 2005. Surface modification of hydroxyapatite ceramic to modulate cell adhesion and improve tissue generation. 2005. 9th conference on ceramics, cells and tissues: chemistry and microstructure, Forli, Italy,.

Schüssele A., Volk B., Deisinger U., Skofitsch G., Schulz M. B., Göpferich A.. PEGylation of Hydroxyapatite-Ceramics by reductive amination. 2004. Strategies in Tissue Engineering, Würzburg, Germany.

Schüssele A., Volk B., Deisinger U., Stenzel F., Mueller R., Göpferich A., Schulz M.B. PEG grafted inorganic surfaces for the control of stem cell adhesion. 2004. Proc. Int. Meeting on Pharm., Biopharm. and Pharm. Technol., Nürnberg, Germany.

Rohde E., Malischnik C., Thaler D., Volk B., Schulz M., Linkesch W., Lanzer G., Strunk D. Human lineage-negative blood for regenerative SCT? 2004. Int. Hematol. Conference, Barcelona, Spain.

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