Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 4008–4011, July 1983
Cell Biology

Renal mesangial cell cultures as a model for study of erythropoietin production

(glomerulus/hypoxia)

ARMIN KURTZ*, WOLFGANG JELKMANN*, FRED SINOWATZ†, AND CHRISTIAN BAUER*

*Institut für Physiologie and †Institut für Anatomie, Universität Regensburg, Postfach 397, D-8400 Regensburg, Federal Republic of Germany

Communicated by Robert E. Forster, March 28, 1983

ABSTRACT **Mesangial cells derived from isolated glomeruli of rat kidney were grown as homogeneous cell lines in culture. They released, into the culture medium, erythropoietin that had free terminal galactosyl residues and was therefore not active *in vivo*. The production of erythropoietin by these cells was significantly enhanced by either lowering the Po_2 in the incubation atmosphere or by adding cobalt chloride to the culture medium. Therefore, mesangial cells in culture may be considered as an *in vitro* system in which the regulation of erythropoietin production can be studied under well-defined conditions.**

Erythropoietin (Ep) is a sialoglycoprotein that enhances the growth of erythroid precursor cells. In response to hypoxia, an increase in the plasma Ep level can be observed. Because this reaction is mainly triggered by the kidney, various attempts have been made to localize the site of Ep generation in this organ. Most reports of both *in vivo* and *in vitro* investigations have implicated the renal cortex (cf. refs. 1 and 2) and, in particular, glomerular cells (1, 3–5) as the site of production of Ep. However, studies on the regulation of Ep biosynthesis on a cellular level are not easy to perform because of the complexity of the glomerulus and its immediate vicinity, the juxtaglomerular cells.

With this in mind, we sought to develop a cell culture system in which the biosynthesis of Ep can be studied under conditions that are much better defined than in the intact organ. We show here that renal glomerular cells, which were clearly identified to be of mesangial origin, in culture produce Ep that has free galactosyl residues. The Ep production of these cells could be enhanced by hypoxia and cobalt chloride which are well-known stimuli of Ep formation *in vivo*. Therefore, the present culture system can be regarded as a useful model for the study of the hypoxia-induced increase of Ep synthesis.

MATERIALS AND METHODS

The preparation of isolated glomeruli from rat kidneys and subculture of glomerular outgrowths were done as described (5). Cells subcultured 3 weeks after the first inoculation were used. The cells were grown in RPMI 1640 (Boehringer Mannheim) supplemented with 10% fetal bovine serum (Boehringer Mannheim), penicillin at 100 units/ml, streptomycin at 10 μg/ml, and bovine insulin (Sigma) at 0.66 unit/ml. The tissue culture flasks (Falcon) were incubated at 37°C in a humidified atmosphere in incubators controlled for O_2 and CO_2 (Heraeus, Hanau, Federal Republic of Germany). In some instances fetal bovine serum was replaced by delipidated and deionized (6) bovine serum albumin. The cells in culture were tested for fibroblast contamination by maintaining them in RPMI 1640 containing dialyzed fetal bovine serum and *d*-valine substituted for *l*-valine, a condition that leads to a rapid detachment of fibroblasts from the disk (7).

Immunofluorescence Procedures. Knowledge of the types of intermediate filaments of the cytoskeleton is an important tool for the assessment of the origin of a given cell type (8). To evaluate the nature of the subunit proteins of the intermediate filaments, the cells were grown on coverslips and fixed in methanol at −10°C for 5 min. They were stained for desmin, vimentin, and prekeratin with the respective fluorescent antibodies exactly as described by Osborn *et al.* (8, 9).

Transmission Electron Microscopy. Cultured cells were fixed in 0.1 M cacodylate buffer (pH 7.4) containing 1% formaldehyde and 1.25% glutaraldehyde, postfixed in 1% cacodylate-buffered OsO_4, dehydrated in ethanol, and embedded as described (10). Ultrathin sections were stained with uranyl acetate and lead citrate.

Receptors for Immunoglobulin and Complement. To identify Fc and C3 receptors, the mesangial cells from three passages were incubated with ox erythrocyte–antibody complexes. The incubation and evaluation of rosette formation was done according to Kalden *et al.* (11).

Ep Assay. The Ep activity of the culture medium was determined with the fetal mouse liver cell test (12) and a serum-free incubation mixture (6). In some experiments, bone marrow cells from adult mice were used for the test. The total volume of one test was 600 μl including 60 μl of the sample to be tested for Ep activity. Standard Ep (human urinary Ep standardized against International Reference Preparation B) was kindly provided by the National Institutes of Health. All unknown samples from the culture medium were chromatographed on DEAE-cellulose at pH 4.5 (13) prior to their use in the test. Only material that bound to DEAE-cellulose was used in the test. This was done in order to remove insulin which stimulates the growth of late erythroid precursor cells (CFU-e) independently of Ep (14). Samples treated in this way did not contain insulin as evaluated by radioimmunoassay. All samples were concentrated at least 10-fold on Amicon YM-10 membranes prior to their use in the assay. Some samples also were tested in the *in vivo* bioassay for Ep that uses polycythemic mice as described (15).

The erythropoietic activity from the culture medium was further characterized with respect to the following criteria: (*i*) dose–response curve in comparison with human standard urinary Ep; (*ii*) effect of anti-Ep raised in rabbits against pure human urinary Ep (16) (this anti-Ep was generously donated by J. W. Fisher; the Ep–anti-Ep complex in the samples was precipitated as described (17); (*iii*) heat resistance (100°C for 5 min at pH 5.5) (18); (*iv*) binding to DEAE-cellulose at acidic pH (13); the column

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Ep, erythropoietin; WGL, wheat germ lectin; RCL, *Ricinus communis* lectin; MCCM, mesangial cell-conditioned medium; CFU-e, late erythroid precursor cells.

was operated with 0.05 M acetate buffer (pH 4.5) and bound material was eluted with 0.1 M Na_2HPO_4/0.5 M NaCl; (*v*) apparent molecular weight on a calibrated Sephadex G-150 column operated with 10 mM Hepes/0.15 M NaCl, pH 7.3; (*vi*) binding to wheat germ lectin (WGL) coupled to Sepharose 6 MB (Pharmacia); and (*vii*) binding to agarose-bound *Ricinus communis* lectin I (RCL I) (Vector, Burlingame, CA).

Chromatography on the lectins was done as described by Spivak *et al.* (19). Material that bound to WGL and RCL I was eluted with 0.01 M diacetylchitobiose (Sigma) and 0.5 M galactose (Sigma), respectively. All experiments described for the culture medium were also done with rat Ep and rat asialo-Ep. Rat Ep was partially purified by heat treatment (18) of plasma from rats exposed to hypobaric hypoxia. The asialo derivative of rat Ep was made by acid hydrolysis (20).

RESULTS

Characterization of the Cells in Culture. Subcultures obtained at day 21 from glomerular outgrowths have been considered to be of mesangial origin (21, 22). To identify the cells used in the present culture experiments, some cellular characteristics were evaluated. The cells were spindle shaped and grew in multilayers. Transmission electron microscopy showed an indented nucleus (Fig. 1) as well as numerous bundles of small filaments with attachment bodies. With respect to these features, the cells were completely homogeneous. Immunofluorescence microscopy of cells treated with labeled antibodies against the subunit proteins of intermediate filaments (8, 9) showed that all cells were vimentin positive, about 70% were desmin positive, and none was prekeratin positive. The distribution pattern of the intermediate filaments was unchanged when the cells were kept for 2 weeks in medium containing *d*-valine instead of *l*-valine. Furthermore, no C3 or Fc receptors could be detected in the present cell culture.

Erythropoietic Activity in the Culture Medium. The mesangial cell conditioned medium (MCCM) obtained from day 21 subcultures of renal glomerular cultures stimulated the growth of late erythroid precursor cells (CFU-e) from both fetal mouse livers and adult bone marrow in a serum-free culture system. It should be pointed out here that this erythropoietic activity was found in a total of 10 independent glomerulus-derived cell cultures. Preincubation of MCCM with anti-Ep antiserum resulted in a complete loss of the stimulation of colony formation from CFU-e. In order to test the reactivity of the anti-Ep antiserum against rat Ep, its effect on the Ep activity from kidneys of hypoxic rats (23) was determined in the exhypoxic mouse bioassay (15). It was found that the biological activity of this Ep was no longer detectable after incubation with anti-Ep. In no instance did MCCM produce a significant enhancement of the ^{59}Fe incorporation in polycythemic mice, despite the fact that the erythropoietic activity in the concentrated medium was well above the detectable level (50 milliunits/ml) of the *in vivo* assay.

To obtain more information about the nature of the erythropoietic factor present in the MCCM, it was further characterized as summarized in Table 1.

Factors Influencing Formation of the Erythropoietic Activity by Cultured Mesangial Cells. Fig. 2 shows the relationship between the erythropoietic activity of MCCM and the partial

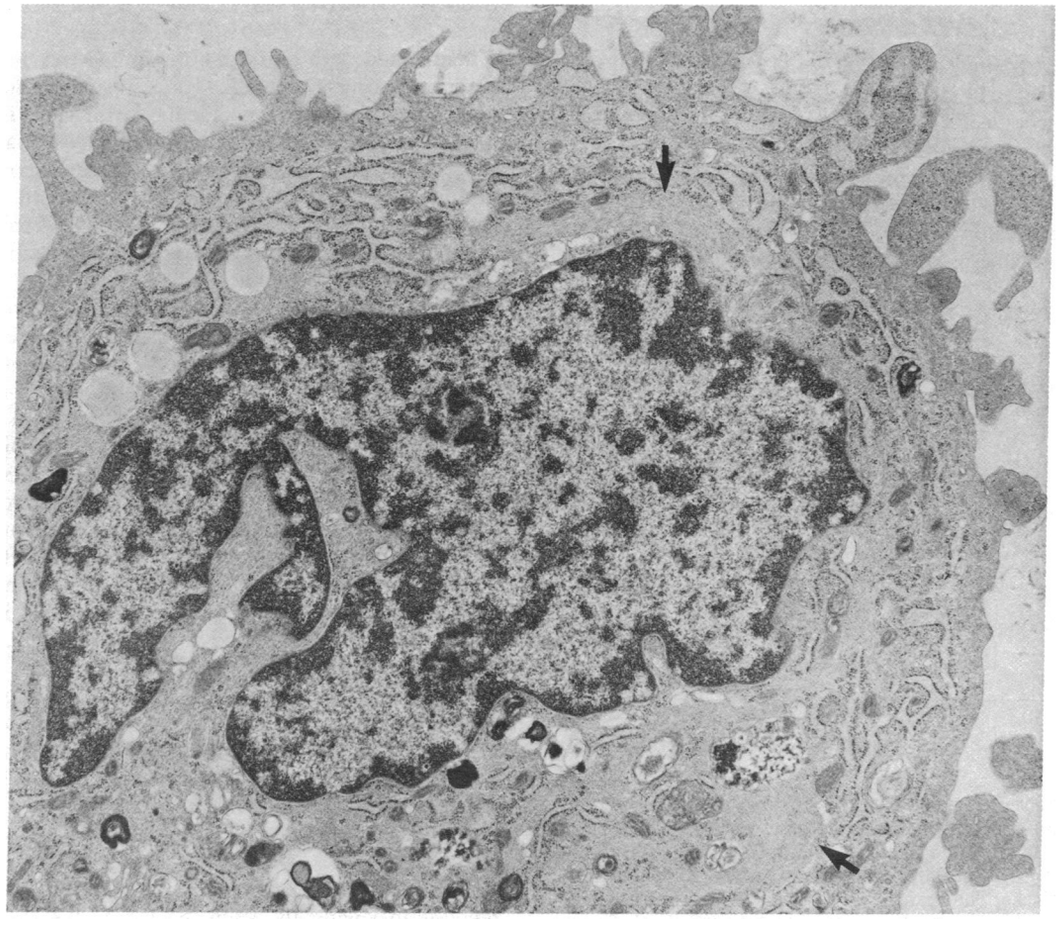

FIG. 1. Transmission electron microscopy of cultured mesangial cell. Arrows indicate bundles of microfilaments. (×12,600.)

Table 1. Biological and physicochemical properties of erythropoietic factor in MCCM and comparison with rat sialo-Ep and asialo-Ep

Property	MCCM	Asialo-Ep	Sialo-Ep
In vivo activity (exhypoxic mouse assay)	−	−	+
In vitro activity (FMLC assay)	+	+	+
Effect of anti-Ep antiserum	+	+	+
% activity bound to DEAE-cellulose at pH 4.5	75	25	100
% activity bound to WGL	97	95	100
% activity bound to RCL	95	98	0
% activity recovered after heat treatment	70	50	80
Apparent M_r on Sephadex G-150	60,000	55,000	60,000
Comparison of dose–response curves with standard Ep curve	Not different*	Not different*	Not different*

FMLC, fetal mouse liver cell.
*$0.5 < 2P < 0.6$.

pressure of oxygen in the incubation atmosphere. The erythropoietic activity was calculated by comparison with the effects of a standard Ep preparation in the assay. The erythropoietic activity increased exponentially as the partial pressure of oxygen was decreased. Addition of 10 μM $CoCl_2$ to the cell culture at 20% O_2 for 3 days induced an increase of the erythropoietic activity (mean ± SEM) in the MCCM from 1.6 ± 0.6 milliunits/ml (n = 5) to 5.9 ± 1.3 (n = 3).

To obtain information as to whether the formation of the erythropoietic activity in the MCCM was dependent on the presence of serum, purified bovine serum albumin (6) was substituted for fetal bovine serum in the culture medium. Cells kept for 3 days under these conditions at 2% O_2 also generated erythropoietic activity that bound to DEAE-cellulose at pH 4.5.

DISCUSSION

Identification of the Cells in Culture. The cells had an indented nucleus (Fig. 1) and many bundles of small filaments with attachment bodies. These features are also found in glomerular and extraglomerular mesangial cells *in situ* (24, 25). Glomerular epithelial cells, on the other hand, typically bear C3 receptors both *in vivo* and in cell culture (26). The cultured cells had no C3 receptors and had no prekeratin, which is evidence for the absence of contaminating glomerular epithelial cells (8, 9). Furthermore, glomerular mesangial cells are considered to be modified smooth muscle cells (25, 27), which contract after stimulation with angiotensin II (28, 29). The distribution of the different classes of intermediate filaments (desmin and vimentin) in the cell cultures we used was similar to the one found in smooth muscle cell cultures of vascular origin (30). In addition, Bachmann *et al.* (31) have recently demonstrated the coexistence of vimentin and desmin only in glomerular mesangial cells of intact kidneys but not in other glomerular cell types. These results as well as the other cellular characteristics described above led us to conclude that the erythropoietic activity in the culture medium was produced by mesangial cells.

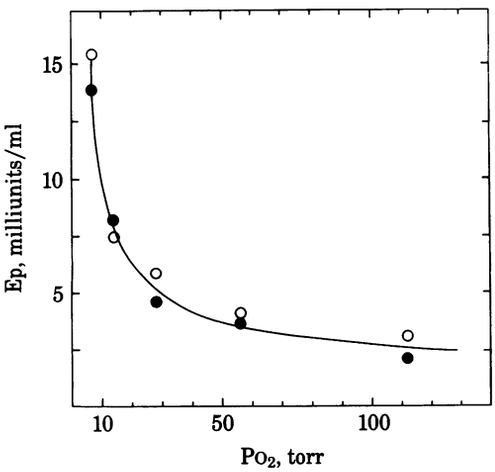

FIG. 2. Relationship between Ep activity in the culture medium and the partial pressure of oxygen in the incubation atmosphere. Each flask, which contained 10^7 cells in 15 ml of medium, was incubated at the various Po_2 values for 3 days. Data represent Ep activity in the medium pooled from 10 flasks. The change in Po_2 was achieved by either increasing (○) or decreasing (●) the oxygen concentration in the incubation atmosphere in two independent sets of experiments.

Characterization of the Erythropoietic Activity. The present study shows that mesangial cells in culture generated an erythropoietic activity that enhanced formation of CFU-e colonies in fetal and adult mouse erythroid tissue. The activity could be neutralized with anti-Ep but was inactive in the polycythemic mouse assay for Ep. These results indicate that the erythropoietic activity was not identical with native Ep but shared antigenic sites with it. Comparison of physical and chemical properties of native Ep and the erythropoietic activity revealed only one important difference: the binding to RCL I. The erythropoietic activity bound to RCL whereas native Ep does not (ref. 19; Table 1). Binding to RCL requires free galactosyl residues at the carbohydrate portion of glycoproteins. A well-known derivative of the native Ep that also has free galactosyl residues is asialo-Ep. Ep that bears either a decreased number of sialic acid residues (32) or is completely desialated (20) is not active *in vivo* because it is rapidly cleared from the circulation.

From the physical and chemical properties of the erythropoietic activity summarized in Table 1 we conclude that mesangial cells in culture generate Ep that is not fully sialated. Why this Ep carries a decreased number of sialic acid units is not known. Incubation of native Ep with the cell cultures for 3 days did not result in any loss of its activity in the polycythemic mouse assay. This indicated that no significant neuraminidase activity was present in the MCCM. Furthermore, omission of the antibiotics over a 20-day period or incubation of the cells with *N*-acetylmannosamine, which is considered to be a fairly specific precursor for sialic acids in glycoproteins (33), did not restore the *in vivo* activity of the Ep derived from mesangial cells.

It should be pointed out that the erythropoietic activity was also found in culture media that contained no fetal bovine serum. This result suggests that Ep was synthesized by the mesangial cells and not generated by an interaction between cells and a serum factor.

The rate of Ep production by mesangial cells was found to increase exponentially when the Po_2 in the incubation atmosphere was decreased (Fig. 2). Furthermore, cobalt, a well-

known stimulus for Ep production *in vivo* (34), also enhanced Ep production in mesangial cell cultures. These findings indicate that the stimulus-sensing site and the Ep formation site are located within the same cell.

Our results are consistent with observations made by other investigators. Fisher and co-workers, utilizing the indirect fluorescent antibody technique with anti-Ep, found intense fluorescence in the glomerular tuft of hypoxic human and animal kidneys (1). Furthermore, Burlington *et al.* (3) found *in vivo* active Ep in long-term cultures of goat glomeruli. The cell type they described in their cultures grew stacking and overlapping but the exact nature of these cells was not confirmed. Recently, McCully *et al.* (35) reported mesangial hyperplasia and erythrocytosis in rats after intrarenal injection of nickel subsulfide. They concluded that mesangial cells are implicated in Ep production. Our results, obtained in a well-defined culture system, support this view.

We are greatly indebted to Dr. J. W. Fisher (New Orleans) for providing us with anti-Ep and to Dr. Mary Osborn (Göttingen) for the immunofluorescence staining of the intermediate filaments. We are also grateful to Dr. W. Leibold (Giessen) for his help with the determination of the Fc and C3 receptors. The skilled technical assistance of Rita Spangler and Luise Rauch is gratefully acknowledged. We thank Lydia Schmid for her secretarial help. This work was supported financially by the Sonderforschungsbereich 43 of the Deutsche Forschungsgemeinschaft.

1. Fisher, J. W. & Busuttil, R. W. (1977) in *Kidney Hormones*, ed. Fisher, J. W. (Academic, London), Vol. 2, pp. 165–185.
2. Ogle, J. W., Lange, R. D. & Dunn, C. D. R. (1978) *In Vitro* **14**, 945–950.
3. Burlington, H., Cronkite, E. P., Reincke, U. & Zanjani, E. D. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3547–3550.
4. Gruber, D. F., Zucali, J. R., Wlekliniski, J., La Russa, V. & Miran, E. A. (1977) *Exp. Hematol. (Copenhagen)* **5**, 399–407.
5. Kurtz, A., Jelkmann, W. & Bauer, C. (1982) *FEBS Lett.* **137**, 129–132.
6. Iscove, N. N., Guilbert, L. J. & Weymann, C. (1980) *Exp. Cell Res.* **126**, 121–126.
7. Gilbert, S. F. & Migeon, B. R. (1975) *Cell* **5**, 11–17.
8. Osborn, M., Geisler, N., Shaw, G., Sharp, G. & Weber, K. (1982) *Cold Spring Harbor Symp. Quant. Biol.* **46**, 413–429.
9. Osborn, M. & Weber, K. (1982) *Methods Cell Biol.* **24**, 97–132.
10. Spurr, A. V. (1969) *J. Ultrastruct. Res.* **26**, 31–43.
11. Kalden, J. R., Peter, H. H., Roubin, R. & Cesarini, J. P. (1977) *Eur. J. Immunol.* **8**, 537–543.
12. Rich, I. & Kubanek, B. (1980) *J. Embryol. Exp. Morphol.* **58**, 143–155.
13. Goldwasser, E., White, W. F. & Taylor, K. B. (1962) *Biochim. Biophys. Acta* **64**, 487–496.
14. Kurtz, A., Jelkmann, W. & Bauer, C. (1983) *Br. J. Haematol.* **53**, 311–316.
15. Cotes, P. M. & Bangham, D. R. (1961) *Nature (London)* **191**, 1065–1067.
16. Rege, A. B., Brookins, J. & Fisher, J. W. (1982) *J. Lab. Clin. Med.* **100**, 829–843.
17. Jelkmann, W., Brookins, J. & Fisher, J. W. (1979) *Proc. Soc. Exp. Biol. Med.* **162**, 65–70.
18. Erslev, A. J., Caro, J., Kansu, E. & Silver, R. (1980) *Br. J. Haematol.* **45**, 65–72.
19. Spivak, J. L., Small, D., Shaper, J. H. & Hollenberg, M. D. (1978) *Blood* **52**, 1178–1188.
20. Goldwasser, E., Kung, C. & Eliason, J. (1974) *J. Biol. Chem.* **249**, 4202–4206.
21. Foidart, J. B., Dechenne, C. A., Mahieu, P., Creutz, C. E. & de Mey, J. (1979) *Invest. Cell Pathol.* **2**, 15–26.
22. Dunn, M. J., Petrulis, A. S., Scharschmidt, L. S., Jim, K. & Hassid, A. (1982) in *Biochemistry of Kidney Function*, ed. Morel, F. (Elsevier, Amsterdam), pp. 3–12.
23. Jelkmann, W. & Bauer, C. (1981) *Pflügers Arch.* **392**, 34–39.
24. Latta, H. (1973) in *Handbook of Physiology. Renal Physiology*, eds. Orloff, J. & Berliner, R. W. (American Physiological Society, Washington, DC), Sect. 8, pp. 1–29.
25. Barajas, L. (1979) *Am. J. Physiol.* **237**, F333–F343.
26. Kreisberg, J. I., Hoover, R. L. & Karnovsky, M. J. (1978) *Kidney Int.* **14**, 21–30.
27. Burkholder, P. M. (1982) *Lab. Invest.* **46**, 239–241.
28. Ausiello, D. A., Kreisberg, J. I., Roy, C. & Karnovsky, M. J. (1980) *J. Clin. Invest.* **65**, 754–760.
29. Mahieu, P. R., Foidart, J. B., Dubois, C. H., Dechenne, C. A. & Deheneffe, J. (1980) *Invest. Cell Pathol.* **3**, 121–128.
30. Travo, P., Weber, K. & Osborn, M. (1982) *Exp. Cell. Res.* **139**, 87–94.
31. Bachmann, S., Kriz, W., Kuhn, S. & Franke, W. W. (1983) *Histochemistry* **77**, 365–394.
32. Lukowsky, W. A. & Painter, R. H. (1972) *Can. J. Biochem.* **50**, 909–917.
33. Bennet, G. & O'Shaughnessy, D. (1981) *J. Cell Biol.* **88**, 1–15.
34. Goldwasser, E., Jacobson, L. O., Fried, W. & Plzak, L. F. (1958) *Blood* **13**, 55–60.
35. McCully, K. S., Rinehimer, L. A., Gillies, C. G., Hopfer, S. M. & Sunderman, F. W. (1982) *Virchows Arch. A* **394**, 207–220.