Apoptosis in the APO-1 System

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Cell-surface molecules are crucial in lymphocyte growth control. Such molecules may function as receptors for growth-stimulating cytokines or may be associated with receptors and transmit signals essential for growth regulation. Receptor blockade or removal of the stimulating cytokines may lead to decreased lymphocyte growth (Duke and Cohen 1986). Withdrawal of interleukins slows human lymphocyte growth and finally leads to the characteristic form of "programmed cell death" or apoptosis. Apoptosis is the most common form of eukaryotic cell death and occurs in embryogenesis, metamorphosis, tissue atrophy, and tumor regression. It is also induced by cytotoxic T lymphocytes and natural killer cells, by cytokines like tumor necrosis factor (TNF) and lymphotoxin (LT), and by glucocorticoids. The most characteristic signs of apoptosis are segmentation of the nucleus, condensation of the cytoplasm, membrane blebbing, and DNA fragmentation into multimers of about 180 bp (called a DNA ladder) (see Kerr and Harmon, this volume). To analyze mechanisms of lymphocyte growth control and to interfere with the replication of lymphoid tumor cells, we raised monoclonal antibodies against cell-surface molecules involved in these processes. Monoclonal antibodies were usually tested and selected by

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virtue of their binding to cell-surface antigens of test cells. Our aim was to define reactive monoclonal antibodies by functional assays, namely by abrogation of growth of malignant test cells in vitro. Monoclonal antibodies were raised against the human B-lymphoblast cell line SKW6.4. One monoclonal antibody, anti-APO-1, showed the strongest functional activity and reacted with an antigen (APO-1) of ~50 kD on a set of activated human lymphocytes, on malignant human lymphocyte lines, and on some patient-derived leukemic cells. Anti-APO-1 was of the IgG3/κ isotype and had a high affinity of $K_D = 1.9 \times 10^{-10}$. Despite many cell fusions undertaken in our laboratory, the hybridoma with anti-APO-1 activity has remained the only one in about 25,000 tested. Nanogram quantities of anti-APO-1 completely blocked proliferation of cells bearing APO-1 in vitro in a manner characteristic of apoptosis (Fig. 1). Cell death was preceded by changes in cell morphology and fragmentation of DNA. This process was distinct from antibody- and complement-dependent cell lysis and was mediated by the antibody alone (Trauth et al. 1989; Krammer 1989; Krammer et al. 1989; Köhler et al. 1990).

Purification of the APO-1 Antigen

It was important to further characterize the APO-1 molecule with the aim of learning more about its function. Therefore, we purified the APO-1 antigen from membranes of SKW6.4 cells. The purified APO-1 antigen was found to be a glycoprotein with apparent M_r of approximately 50,000, with about 8000 of the M_r accounted for by sugars. Purified APO-1 blocked anti-APO-1-induced apoptosis of SKW6.4 cells in vitro, proving its serological identity with the APO-1-membrane antigen. Large quantities of the APO-1 antigen enabled us to obtain a sequence of the APO-1 protein. A computer search revealed that APO-1 was a new cell-surface antigen. Motifs in the APO-1 sequence may provide us with a clue to the as yet elusive physiological function of the antigen.

The APO-1-mediated Signal

Induction of apoptosis was mediated by anti-APO-1 alone and was complement-independent. Nevertheless, the $F(ab')_2$ frag-

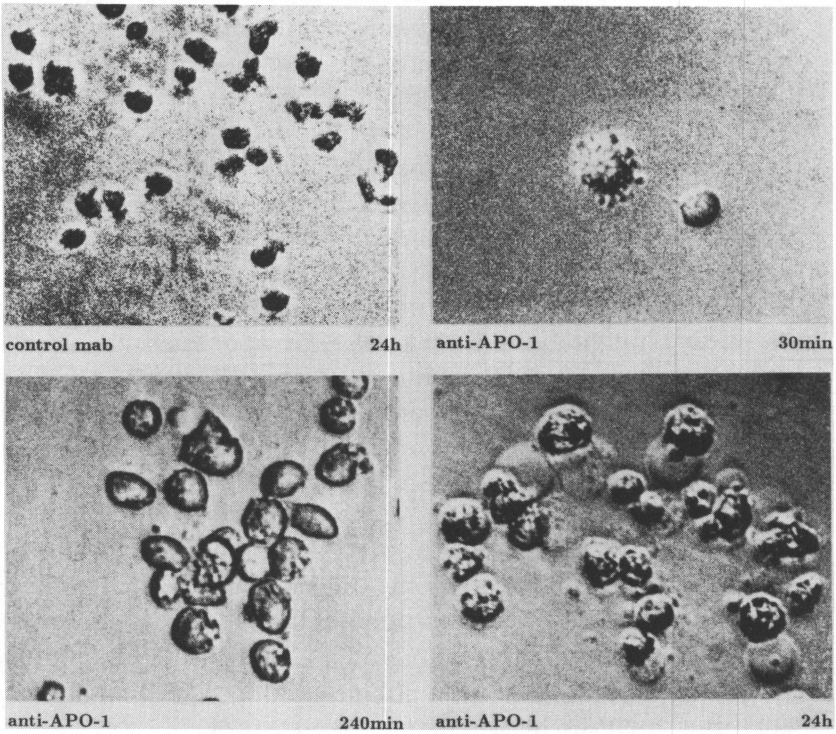

FIGURE 1 Induction of apoptosis of SKW6.4 cells by anti-APO-1. The time of in vitro induction with control monoclonal antibody or anti-APO-1 (1 μg/ml) is indicated.

ment of the IgG3 anti-APO-1 did not induce apoptosis. When cross-linked, however, by $F(ab')_2$ sheep anti-mouse Ig antibodies, apoptosis was observed. To further study the role of the Fc region of anti-APO-1, we isolated antibody class switch variants from the IgG3 anti-APO-1-secreting hybridoma cell line. We obtained anti-APO-1 antibodies of the IgG3, IgG1, IgG2b, IgG2a, and IgA isotypes. These antibodies showed the following effects: (1) a different degree of induction of apoptosis of SKW6.4 cells occurred in the following order: IgG3, IgG1, IgG2a, IgA, IgG2b. (2) Cross-linking of the less effective class switch variant IgG2b anti-APO-1 by Protein A showed the same degree of growth inhibition as IgG3 anti-APO-1. These results suggested that induction of apoptosis was dependent

on cross-linking of the APO-1 cell-surface antigen. IgG3 anti-APO-1 bound to the cell surface might have self-aggregating capacity via Fc-Fc interactions or bind to Fc receptors and therefore efficiently cross-link the APO-1 antigen. IgG2b anti-APO-1 might show fewer Fc-Fc interactions, be a less efficient cross-linker, and therefore be less effective in induction of apoptosis. Cross-linking of APO-1 on the cell membrane may be essential for APO-1-mediated signal transduction across the membrane.

We also asked whether internalization of APO-1 and/or anti-APO-1 might be a prerequisite for apoptosis in our system. The following experiments suggested that this is not the case. We chemically coupled anti-APO-1 to silica beads several times larger than cells and incubated SKW6.4 cells with these beads. We found that bead-coupled anti-APO-1 was an efficient inducer of apoptosis in SKW6.4 cells. These results reinforce our assumption that the APO-1 antigen may produce a genuine transmembrane signal, the nature of which remains to be investigated. These results also prompted us to develop systems that might allow us to study the anti-APO-1 apoptosis process in molecular terms. Thus, we looked for cellular systems that might be informative in this respect.

Selection of Cell Variants That Express the APO-1 Antigen but Are Resistant to Anti-APO-1-induced Apoptosis

After screening a large panel of human B- and T-cell lines, we found that expression of the APO-1 antigen is a prerequisite, although not sufficient by itself for anti-APO-1-induced cell death. Thus, we identified several strongly APO-1$^+$ cell lines resistant to anti-APO-1-induced apoptosis. To study this phenomenon further, we selected several cell variants that differed in the sensitivity to anti-APO-1. The B-cell line SKW6.4 (sIgM$^+$, APO-1$^+$, sensitive to 2 ng/ml anti-APO-1) was cultured with increasing amounts of anti-APO-1 for about 1 year. We obtained a stable variant that expressed the APO-1 antigen but was resistant to at least 50 μg/ml anti-APO-1. In addition, the T-cell line CCRF was cloned under limiting dilution conditions. Replica cultures of subclones were screened for susceptibility to anti-APO-1. Two subclones were selected that both expressed the APO-1 antigen but differed in sensitivity to anti-

APO-1 at least by a factor of 1000. It is conceivable that the mechanism of resistance to apoptosis in SKW6.4 and CCRF variant cells is different. In any case, however, this pair of cell lines shows very clearly that two requirements for anti-APO-1-induced apoptosis are important: the cell-surface expression of the APO-1 antigen and an intact apoptosis signal pathway. We presume that these findings may be of great future relevance to the putative use of the apoptosis concept in tumor therapy.

Apoptosis in Human T Lymphocytes

Another informative set of cells with respect to the APO-1-mediated signal of apoptosis are normal human T lymphocytes. Although we have data suggesting that, in contrast to resting B cells, activated B cells also undergo anti-APO-1-mediated apoptosis, in this paper, we focus primarily on T cells. The majority of normal human resting T lymphocytes do not express the APO-1 antigen. After activation, however, both the $CD4^+$ and $CD8^+$ subpopulations of T cells become positive for the APO-1 antigen. Although no significant difference in the amount of APO-1^+ T cells and in the epitope density of APO-1 antigens between T cells early (e.g., 1 day) or late (e.g., 6 days) after activation was observed, apoptosis was only induced by anti-APO-1 in the latter cell population. Hence, the susceptibility for induction of apoptosis in activated T lymphocytes is dependent on the stage of differentiation of these cells. A comparison of the set of APO-1^+ T cells early or late after activation might help to elucidate the enigma of "death genes" involved in anti-APO-1-mediated apoptosis. In addition, this phenomenon might help to understand in molecular terms the elimination of peripheral T cells at the cessation of an immune response.

Anti-APO-1-mediated Tumor Regression

As discussed above, anti-APO-1 induced apoptosis in various T- and B-cell lines in vitro. This result led us to test the anti-APO-1 efficiency in an experimental tumor system in vivo (Fig. 2). The human B-lymphoma line BJAB was chosen for these in vivo experiments. Xenografts of this line in nu/nu mice were

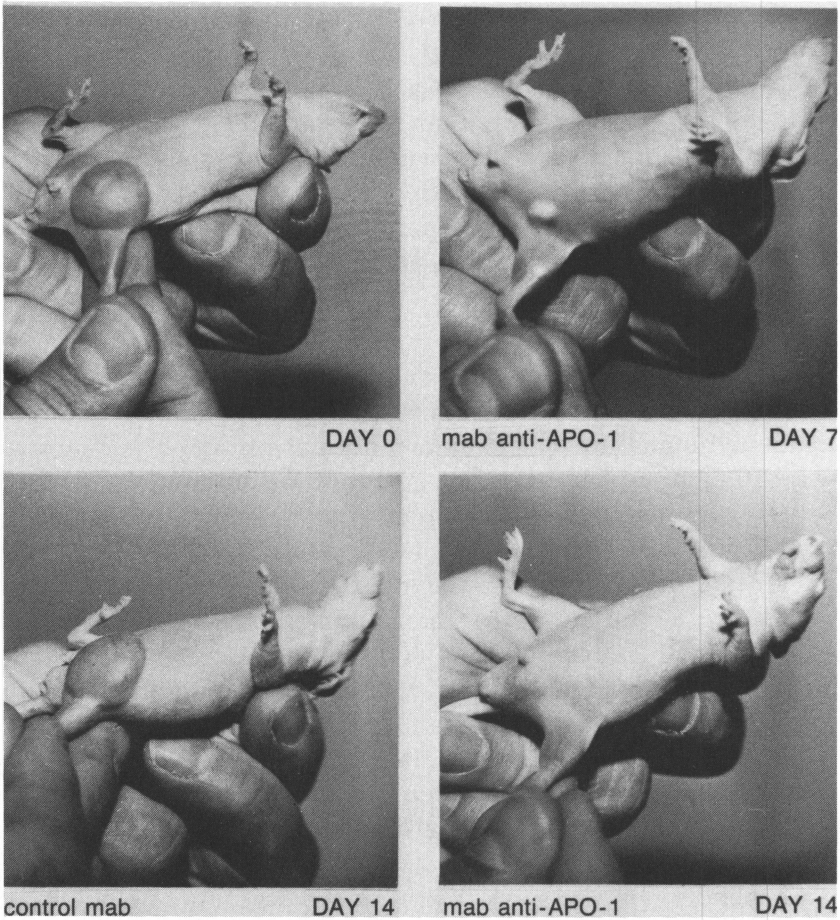

FIGURE 2 Anti-APO-1-mediated tumor regression of BJAB lymphoblastoid tumor xenotransplants in nu/nu mice. The pictures show prototype mice from each group. nu/nu mice with human BJAB lymphoblastoid tumors ~1.5–2.5 cm in diameter (day 0) were i.v. injected with 500 μg isotype matched control monoclonal antibody or anti-APO-1 (IgG3/κ) on day 0. Mice with tumors were photographed 7 and 14 days after monoclonal antibody injection.

previously shown to accumulate radiolabeled monoclonal antibodies only in the outer layer of the tumor, whereas central areas of nodules were virtually inaccessible. Using anti-APO-1 in BJAB-bearing nu/nu mice, we asked three questions: (1) Is anti-APO-1 as effective in vivo as in vitro? (2) Does anti-APO-1 affect the whole tumor despite preferential accumulation in

the periphery? (3) Does anti-APO-1-mediated tumor cell death in vivo alter the accessibility barriers of the BJAB tumor? The results were clear-cut. Anti-APO-1 antibodies, like all other antibodies tested, accumulated exclusively in the periphery of nodules even if up to 500 μg of antibody was injected per mouse. Nevertheless, established tumors ~1.5–2.5 cm in diameter regressed in 10/11 nude mice within a few days. Histological thin sections performed before complete tumor regression showed that as in vitro, anti-APO-1 also induced apoptosis in vivo. The action of the antibody, however, did not result in a disturbance of the accessibility barrier. We concluded from these experiments that tumors may be efficiently tackled by monoclonal antibodies, particularly anti-APO-1, despite restriction of accessibility, provided the cytolytic activity of the antibody is high and the residence time of the antibody in the tumor is long enough to "melt down" the tumor nodules from the outside (Trauth et al. 1989). In addition, the outcome of these experiments suggested that anti-APO-1-induced apoptosis is a valid concept worth testing for tumor treatment in a clinical situation, provided putative systemic toxicity of the antibody can be controlled.

One important result should be mentioned at this point. In preliminary experiments, we tested the in vivo therapeutic efficiency of anti-APO-1 on large SKW6.4 tumors. In vitro anti-APO-1-sensitive (S) and -resistant (R) SKW6.4 cells both expressing APO-1 on the cell surface were grown to tumors of about 2 cm in diameter in SCID mice. Anti-APO-1 treatment of these animals resulted in complete tumor regression of the $SKW6.4^S$ tumors only. Animals with $SKW6.4^R$ tumors were killed by the tumor. These results suggested that two requirements for anti-APO-1-mediated tumor regression by induction of apoptosis also exist in vivo: (1) expression of the APO-1 antigen *and* (2) an intact apoptosis signal pathway. As already stated, these results may have far-reaching implications for therapy using rational intervention strategies in the clinic.

Preclinical Applications of Apoptosis in the APO-1 System

The above in vivo experiments prompted us to test APO-1 expression in various tumor systems and to test in vitro induc-

tion of apoptosis in malignancies that may be candidates for future anti-APO-1 treatment in the clinic.

Expression of the APO-1 antigen on acute lymphoblastic leukemia cells. In T-acute lymphoblastic leukemia (ALL), APO-1 is expressed constitutively, especially in cases corresponding to stages of very early T-cell differentiation. Cells of the common ALL phenotype representing the malignant precursors of B cells weakly express APO-1 in a minority of cases. However, in these cells, APO-1 expression is induced in vitro by phorbol myristate acetate (PMA) and cytokines such as IL-4. In addition, the constitutive expression of APO-1 on pre-T-ALL cells is modulated by mitogens and cytokines. The APO-1 antigen may therefore be of importance for growth regulation in malignant lymphocytes and may also serve a function in the development of normal precursor cells. In addition, APO-1-positive malignant cells may be a new subgroup of ALL and may be a target for APO-1-directed therapeutic approaches in vitro and in vivo using the anti-APO-1 antibody.

Anti-APO-1 antibody-mediated apoptosis in adult T-cell leukemia. We have described that the APO-1 antigen is expressed on activated T cells and that sensitivity to induction of apoptosis by anti-APO-1 is acquired during long-term culture of activated T cells in the presence of IL-2. Since adult T-cell leukemia (ATL) cells are the transformed counterpart of mature T lymphocytes, we were interested to see whether these cells express the APO-1 antigen and whether they are sensitive to growth inhibition and induction of apoptosis by anti-APO-1. Expression of the antigen and sensitivity to the induction of cell death by anti-APO-1 were studied in human T-cell lines transformed by human leukemia virus type 1 (HTLV-1) and in cultured cells from patients with ATL. APO-1 was strongly expressed on both types of cells, and incubation of the cells with anti-APO-1 resulted in inhibition of proliferation and apoptosis. Induction of apoptosis may therefore be a therapeutic tool in HTLV-1-associated malignant disorders (Debatin et al. 1990).

Expression of the APO-1 phenotype in Burkitt's lymphoma cell lines correlates with a phenotype shift to a lymphoblastoid phe-

notype. We had previously found that APO-1 was also expressed on normal activated B cells (Trauth et al. 1989). Furthermore, a small subset of follicle center B cells residing at a location in which maturation, proliferation, and elimination by apoptosis of B cells takes place had been shown by immunohistochemistry to be APO-1$^+$. Therefore, we tested whether malignant counterparts of such germinal center B cells, Burkitt's lymphoma (BL) cells, expressed APO-1 and were sensitive to anti-APO-1-induced apoptosis. Taking together the evaluation of a large number of tests of BL cells and BL lines phenotypically resembling in vivo BL and cell lines showing a phenotype of Epstein-Barr virus-positive lymphoblastoid cells (LCL), the following results were obtained. BL cells directly isolated from tumor biopsies were APO-1$^-$. BL type cell lines were APO-1$^-$, and LCL type cell lines were APO-1$^+$. Cells of the BL/LCL phenotype showed a heterogeneous APO-1$^+$ pattern. Some but not all cells of the APO-1$^+$ phenotype were sensitive to anti-APO-1-induced apoptosis. The phenotypic shift of BL cell lines may correlate with the one in B-cell activation. Therefore, these cell lines may represent a useful system to study APO-1 expression and function in B cells.

Expression of the APO-1 antigen on glioblastoma cell lines and their susceptibility to apoptosis. To assess the potential usefulness of anti-APO-1 for therapy in other tumor systems, we also tested human glioblastoma cell lines for expression of the APO-1 antigen and susceptibility to anti-APO-1-induced apoptosis. Most cell lines expressed APO-1 at least at a low level. Some cell lines showed growth inhibition and apoptosis if incubated with anti-APO-1. Thus, although APO-1 was expressed on most cell lines tested, only a few responded to anti-APO-1. Subcloning a partially responsive cell line yielded APO-1$^+$, anti-APO-1-sensitive and APO-1$^+$, anti-APO-1-resistant subclones. The data in this cellular system, therefore, stress again that expression of the APO-1 antigen and an intact apoptosis signal pathway are necessary for successful anti-APO-1-mediated apoptosis. Presently, we are investigating which parameters determine the susceptibility of such clones to induction of apoptosis, and whether local anti-APO-1 therapy might be considered in such a disease where survival

after relapse is short and no therapeutic possibilities exist.

APO-1 expression in colorectal carcinomas correlates with poor prognosis. All above data on various malignant cells show a common trait. APO-1 expression on the same type of tumor varies. In addition, similar variability is observed as to susceptibility to anti-APO-1-induced apoptosis on APO-1$^+$ malignant cells. Tumors are either sensitive, resistant, or composed of sensitive and resistant cells. This observation also extends to sarcomas and mammary carcinomas not extensively discussed here. Although the physiological function of APO-1 is still unclear, one may speculate that the observed heterogeneity is meaningful for the biology of the tumor and thus also for the clinical course of the malignant disease. These considerations led us to investigate APO-1 expression on colorectal carcinomas and to correlate our findings with the clinical parameters of this malignant disease.

By means of immunohistochemistry, we found that APO-1 is expressed in normal colon epithelium. In a minor fraction of colon adenomas and in 39.6% of colorectal carcinomas, however, APO-1 expression was diminished. In 48.3% of carcinomas, predominantly of the nonmucinous type, APO-1 was completely abrogated. The normal level of APO-1 expression in carcinoma was correlated with the mucinous type ($p<0.0001$). Reduced or lost antigen expression was more frequent in carcinomas localized in the rectum ($p<0.0001$). In a group of 149 patients who had undergone potentially curative surgery for colorectal carcinoma, the physiological level of APO-1 expression was correlated with a shorter survival after relapse ($p = 0.031$) and with an increased risk of tumor-related death ($p = 0.051$) (P. Möller et al., in prep.). This suggested that the APO-1 antigen is important for signals in growth control of normal and malignant cells. Thus, APO-1 may confer growth advantage to malignant cells and determine the grade of malignancy. Furthermore, this first set of clinical data underscores the importance of APO-1 testing and correlation with patient histories in other malignancies. This applies particularly to those in which heterogeneous APO-1 expression is already observed. It would not be surprising if the APO-1 antigen also constituted a valuable prognostic parameter in such diseases.

DISCUSSION AND OUTLOOK

We showed that anti-APO-1 specifically blocked growth and triggered programmed cell death, apoptosis, of a set of activated normal lymphocytes and cells from malignant lymphoid and nonlymphoid lines after binding to the cell-surface protein antigen APO-1. The APO-1 antigen does not seem to be part of the TNF receptor complex, since its representation on the surface of various cells does not correspond to the distribution of TNF receptors; i.e., macrophage cell lines tested so far are APO-1$^-$. Nevertheless, it will be important to test whether various apoptosis pathways such as the one triggered by TNF and anti-APO-1 have common features.

Apoptosis is found in all tissues and also in cells from lower organisms. It is conceivable, therefore, that several distinct cell-surface antigens with a different tissue distribution are involved in the induction of apoptosis. Elucidation of the structure of APO-1, its possible connection to the cytoskeleton, and the molecular events following anti-APO-1 binding may resolve some of these issues.

Since APO-1 is expressed on mature activated lymphocytes, additional experiments will be needed to determine whether the antigen might play a role in the down-regulation of the immune response and be involved in selection and elimination of lymphocytes. It has previously been shown that LT, TNF, and killer cells with their effector molecules induce apoptotic cell death. Because anti-APO-1 also induces apoptosis, a number of possibilities might be considered for the physiological role of the APO-1 antigen. APO-1 might be a receptor for cytotoxic molecules or for autocrine growth factors. Alternatively, it could be a molecule essential for vertical or lateral growth signal transduction. Thus, anti-APO-1 might trigger receptors for lytic molecules or block receptors for growth signals. Putative signals given by APO-1 may remain an enigma until the structure of the antigen reveals its secrets. In any case, the elucidation of the APO-1-mediated apoptosis pathway will constitute a challenge for our research and will provide a basis for the development of a rational intervention strategy in various diseases, particularly cancer.

Our data also have clinical relevance. Anti-APO-1 may be useful as a diagnostic tool to define subsets of normal and malignant lymphocytes and other tumor types. In addition, induction of apoptosis may have implications for antitumor therapy. Antibodies have frequently been used as heteroconjugates with toxins or drugs to destroy tumor cells. Our data, however, show that monoclonal antibodies alone can be lethal to target cells, provided these cells express APO-1 and have an intact apoptosis pathway. Anti-APO-1 might, therefore, be considered for ex vivo or in vivo therapy, under conditions where reactivity with vital normal cells can be excluded or tolerated. Thus, in the immediate future, careful toxicity studies in SCID mice reconstituted with a human immune system, in primates, and in patients will be necessary.

It is easily imagined that a successful putative anti-APO-1 therapy might go beyond a therapy of cancer and might involve elimination by apoptosis, e.g., of activated lymphocytes in autoimmune diseases. It should also be considered that apoptosis may be involved in the pathomechanism of the elimination of T-helper lymphocytes in AIDS, a process that is still largely not understood. In this context, we tested the presence of APO-1$^+$ lymphocytes and of anti-APO-1 autoantibodies in AIDS. We found the number of APO-1$^+$ cells increased in HIV$^+$ donors. In addition, in the serum of HIV$^+$ donors, anti-APO-1 autoantibodies were detected. These findings may suggest a role for apoptosis in the depletion of T cells in AIDS and clearly warrant further studies.

Finally, the molecular investigation of cell death induced by anti-APO-1 might lead to a general understanding of apoptosis. In this case, the use of modified or normal physiological ligands to the cell-surface antigen initiating apoptosis or of chemicals interfering with the apoptotic signal might be envisaged.

Taken together, the APO-1 apoptosis system might help to find "death genes" and clarify whether death occurs in steps, is a single-hit event, or can be reversed once its initial signals are triggered. Thus, the investigation of apoptosis shows that essential questions of death are linked and can be as exciting as the essential questions of life.

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