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Apoptosis-gene expression in hematopoietic system: Normal and pathological conditions (Review)

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Abstract. Gene expression involving apoptosis in the hematopoietic system is reviewed. In normal and hematological disorders, Fas-Fas ligand and tumor necrosis factor-α-receptor interaction play a major role in enhancing apoptosis. On the other hand, bcl-2 or certain novel proteins (including FADD, RIP, TRADD and sentrin) prevent apoptosis. Apoptosis is involved in myelodysplastic syndrome and pathogenesis of leukemia. Expression of Fas antigen plays a role in negative regulation of hematopoiesis in the bone marrow as does interferon-γ.

Contents

1. Introduction
2. Fas and apoptosis
3. Interleukin-1ß converting enzyme (ICE) and related family (ICH)
4. Ceramide and candidates for second messenger
5. Other antiapoptotic signal transduction by hematopoietic growth factors
6. Bcl-2 as an antiapoptotic mechanism
7. p53 and other oncogenes on apoptosis
8. Apoptosis, transcriptional factors and endonuclease
9. The relationship between differentiation and apoptosis
10. Relationship between apoptosis and differentiation
11. Apoptosis and other hematological disorders
12. Comments

1. Introduction

Apoptosis is a major issue not only in immunology but also in hematology (1-6). Recently several Japanese scientists have contributed very important findings to apoptosis research. First, Tsujimoto *et al* reported the presence of *bcl*-2 gene rearrangement and immunoglobulin heavy chain in B cell follicular lymphoma, and its gene acts as a regulator of the apoptosis-preventive mechanism (7,8). The *bcl*-2 product is located in the nuclear or mitochondrial membrane, and prevents programmed cell death from oxidative stress. p53 has been found to regulate the expression or action of *bcl*-2 or *bax*. Yonehara, Trauth *et al* and Krammer *et al* independently found that one of their antibodies acts as a cell-killing factor which co-down-regulates the tumor necrosis factor-α-receptor (9-11). This antibody was used to clone the gene which encodes the Fas antigen which regulates programmed cell death (12). The ligand for this Fas antigen (Fas ligand), was cloned by Suda *et al* (13). The ligand acts as a killing factor for cytotoxic lymphocytes against a tumor (14), or as a regulating factor for eradication of the T cells (15,16). Krammer *et al* noted that APO-1 mediated double-positive $CD4^+/CD8^+$ T cell apoptosis in the thymus (11). The nerve growth factor (NGF) receptor family has also been well investigated, especially death domain and intra-cellular signal transduction. Interleukin-1ß converting enzyme (ICE) has been identified as an apoptosis-enhancing factor (17), which is also recognized as an important mechanism in hematopoietic cells. ICE is a homologue of Ced-3 (18), which has been identified as having apoptosis-inducing activity in *C. elegans* and is one of the serine protease family. Fas-associated programmed cell death in T cell line, has been studied in detail, but not human hematopoietic stem cell apoptosis. One reason may be the difficulty in harvesting the stem cells, and the other is the detection of apoptosis. Here we review the apoptosis-related gene expression in human hematopoietic cells in normal and pathological conditions.

2. Fas and apoptosis

Structure of human Fas antigen and ligand gene. Cheng *et al* have molecularly cloned the human chromosomal Fas gene using human Fas cDNA as a probe (19). The gene consists of nine exons and spans more than 26 kb. It contained consensus sequences for AP-1, GF-1, NY-Y, CP-2, EBP20, and c-*myb*.

The Fas ligand gene is located in chromosome 1q23 as shown by *in situ* hybridization, and has several transcriptional *cis*-regulatory elements such as SP-1, NF-κB, and IRF-1 (20).

Expression of Fas antigen in hematopoietic stem cells. Tomizuka *et al* demonstrated that apoptosis-related gene expression was analyzed in $CD34^+CD33^-$ Fas-cells from human

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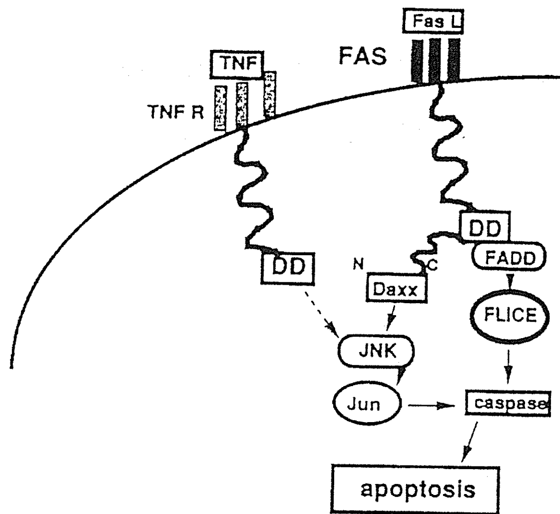

Figure 1. Schematic demonstration of Fas ligand-Fas or tumor necrosis factor (TNF) receptor system and their signal transduction involving death domain in apoptosis.

bone marrow cells, and we selected colonies or burst cells and analyzed them by RT-PCR. We did not observe the presence of Fas ligand mRNA, but bcl-2 is present in all cells fractionated by FACS (21,22).

The action of Fas ligand mimicking antibody and negative regulators on human stem cells. In the pathogenesis of aplastic anemia or myelodysplastic syndrome, the presence of negative regulators which may act on human stem cells has been suggested. Cytotoxic T lymphocytes is a candidate which may produce negatively acting cytokines. Interferon-γ inhibits hematopoietic colony formation (23).

The human CD34-positive ($CD34^+$) CD33-negative ($CD33^-$) fraction is one of the most primitive cells in the bone marrow. We first investigated the characterization of $CD34^{\pm}CD95^{\pm}$ cells from human bone marrow cells sorted by a fluorescence activated cell sorter (FACS), effects of hematopoietic inhibitory factors (tumor necrosis factor-α, interferon-γ, and Fas ligand mimicking antibody) on human colony formation including long term culture initiating cells (LTC-IC), and the apoptosis or anti-apoptosis related gene expression in $CD34^+CD33^-$ cells. We also analyzed the gene expression in the cells formed in the granulocyte-macrophage colony as well as erythroid colony.

Fas ligand mimicking antibody and partially purified Fas ligand (kindly provided by Dr S. Nagata) inhibited colony formation in methylcellulose culture. TNF acts via p75 TNF receptor in TNF cytotoxicity and inhibited colony formation (24,25).

Anti-Fas antibody induced a lethal effect in mice via fulminant hepatic failure (26).

Fas-mediated signal transduction and death domain. Fas antigen is a type 1 transmembrane protein which belongs to tumor necrosis factor/nerve growth factor (TNF/NGF) receptor family (Fig. 1). Fas antigen has a death domain in the intracellular region requiring transduction of the programmed cell death signal. The yeast two-hybrid system study showed that several interacting proteins have been identified. Tyrosine kinase is not involved in these molecules. Boldin *et al* noted the presence of a novel protein that interacts with the death domain of Fas/APO-1 (27). Kitson *et al* observed that tissue-restricted WSL-1 interacts specifically with TNFR1-associated molecule TRADD (28). By the same method using the yeast two-hybrid system the presence of FADD was identified, RIP, FAP, and FAF-1 (29). The sequence which shares the death domain has several homologues of tumor necrosis factor receptor type 1, such as TRADD for the TNF receptor (30). Hsu *et al* demonstrated TRADD-TRAF2 and TRADD-FADD interactions as two distinct TNF receptor 1 signal transductions (31). Boldin *et al* found a novel protease MACH involving MORT1/FADD interacting protease in Fas or TNF receptor-induced cell death (32). Muzio *et al* reported that FLICE, a novel FADD-homologue ICE/CED-3-like protease is recruited to Fas-inducing signals (33). Sentrin protects against Fas/ APO-1 and TNF-mediated cell death (34). Oshimi and Miyazaki revealed that the elevated cytosolic Ca^{2+} level induced the regulation of Fas-mediated DNA fragmentation and apoptotic morphologic changes (35). The human ubiquitin conjugating enzyme (UBC) was found to associate with Fas (CD95), and may play a role in Fas signal transduction (36).

3. Interleukin-1ß converting enzyme (ICE) and related family (ICH)

ICE has been cloned and described as a homologue of Ced-3 in *C. elegans*, which induces apoptosis in the mammalian fibroblasts when ICE is highly expressed (17). At least eight ICE homologues have been reported and they belong to the serine protease family (18). ICE is a converting enzyme which makes IL-1 from IL-1 precursor protein (pro-IL-1). Their nomenclature is not definite, but TX/ICH-2/ICErel II/RIC-2, CPP/Yama/Apopain, Mch2, and Mch3/ICE-LAP3/CMH-1 have been identified (37,38). Watari *et al* found that human hematopoietic progenitors such as $CD34^+CD45RA^{lo}CD71^{lo}$ produce IL-1ß themselves and showed the presence of ICE (39). In human myeloid leukemia U937 cells inhibitor of ICE prevents antitumor agent-induced apoptosis demonstrated by Mashima *et al* (40). The role of proteases other than serine proteases including ICH was extensively reviewed by Patel *et al* (41). Autocrine IL-1ß regulates both proliferation and apoptosis in thymoma cells, and at least in T lymphoma IL-1ß may play a role in apoptosis (42).

4. Ceramide and candidates for second messenger

Hannun and Obeid extensively reviewed the action of ceramide as an intracellular signal for apoptosis (43). In some cell lines, ionizing radiation acts on the cellular membrane to generate ceramide and initiate apoptosis (44). On the other hand, acid sphingomyelinase-deficient human lymphoblasts are defective in radiation-induced apoptosis (45), and this is direct evidence that ceramide acts on apoptosis. Ceramide mediates Fas-induced cytotoxicity (46).

5. Other antiapoptotic signal transduction by hematopoietic growth factors

In special conditions such as in IL-9/IL-9 receptor interaction, growth regulation by IL-9 requires a single tyrosine phosphorylation for STAT activation, and antiapoptotic activity (47). In the case of IL-3/GM-CSF receptors, Kinoshita *et al* noted that signaling suppresses apoptotic death in hematopoietic cells (48). Williams *et al* reported that hematopoietic colony stimulating factors promote cell survival and suppress apoptosis (49). Liu *et al* found the requirement for dATP and cytochrome c (50), using a cell-free extract apoptosis system, which may be useful to identify further new molecular events in the cytoplasm. Nuclear apoptosis has been reported under mitochondrial control (51).

6. Bcl-2 as an antiapoptotic mechanism

Bcl-2 protein acts on mitochondria to prevent apoptosis probably via suppression of oxidative stress (8). As a target molecule, protein kinase Raf-1 to mitochondria is a candidate (52). Bcl-2 molecule is an antiapoptotic marker in tonsillar plasma cells which are rescued from apoptosis by bone marrow fibroblasts (53). Yoshino *et al* noted the inverse expression of bcl-2 protein and Fas antigen in lymphoblasts in peripheral T and B lymphocytes (54). The level of *bcl*-2 expression determines the susceptibility to anti-Fas-mediated cell death in peripheral blood lymphocytes, monocytes and neutrophils (55). On the contrary, a bcl-2 homologue, bcl-x_L regulates cell death. Serine phosphorylase of death agonist, BAD binds to 14-3-3 not to bcl-x_L (56). Gibson *et al* demonstrated the presence of a novel member of the bcl-2 family, bal-w, which promotes cell survival (57).

7. p53 and other oncogenes in apoptosis

Mice deficient in wild-type p53 are more resistant to induction of apoptosis in hematopoietic cells (58). Absence of p53 induces immortalization of hematopoietic cells by the *myc* and *rag* oncogenes (59). p53 itself binds to BAX protein to regulate bcl-2 protein. Blandino *et al* showed that p53 modulates apoptosis of IL-3 derived cells (60). p53 mutants lose apoptosis but not cell-cycle arrest (61). Lotem *et al* considered that p53 controls cellular oxidative stress and apoptosis (62). Mutations of p53 or *ras* oncogene have been reported in patients with myelodysplastic syndrome as well as acute myelogenous leukemia, and p53 seems to be an important regulator of leukemogenesis as well as bcl-2 function (Fig. 2). Also p53-triggers LMP1 protein (63).

8. Apoptosis, transcriptional factors and endonuclease

Leukemia-associated E2A-HLF chimeric transcriptional factor reverses apoptosis (64), and in mice E2F-1 promotes apoptosis and suppresses proliferation (65). The study of transcriptional factors on apoptosis is not enough to document the regulation of apoptosis, but many transcriptional factors have been cloned from specific chromosomal abnormalities in the MDS or AML patients. In nuclear DNA degradation as a marker of apoptosis, endogenous deoxyribonuclease is activated (66).

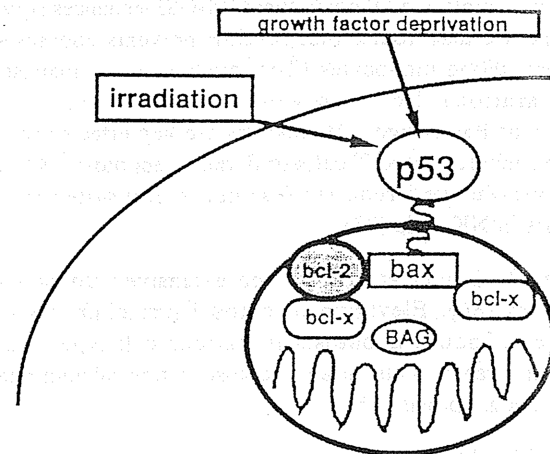

Figure 2. Interaction between p53 and *bcl*-2 gene in apoptosis.

9. The relationship between differentiation and apoptosis

Apoptosis is considered as one of the physiological processes necessary to reconstitute a new biological structure *in vivo*, and differentiation is considered as the mechanism which makes individual cells act on tumor killing or defense mechanism against exogenous pathogens. The relationship between the two processes is still not clear, but Cotter *et al* reviewed the necessity of cell death in the myeloid lineage, especially after killing bacteria (67). Tumor necrosis factor (TNF)-α is considered as a differentiation inducing factor (68) and a key regulatory molecule acting on hematopoietic stem cells. The physiological concentration of retinoic acid such as 10^{-8} M inhibits IL-6 induced macrophage differentiation and apoptosis in murine hematopoietic cell lines (69). Suppression of apoptosis is not sufficient to induce granulocyte differentiation (70). Terui *et al* found that the expression of differentiation-related phenotypes and apoptosis are independently regulated during myeloid differentiation (71).

Elimination of B cells by apoptosis. Bone marrow fibroblasts prevent apoptosis of bcl-2^+ tonsillar plasma cells (53). Macrophages mediate B cell deletion in mouse bone marrow by apoptosis (72). Elimination of self-reactive B cells are CD95-dependent upon interaction with $CD4^+$ T cells (73). Nishiuchi *et al* detected CD95 on immature B cells (74). CD40 engages and protects concurrently with antigen receptor, naive and memory human B cells from CD95-mediated apoptosis (75).

B cell apoptosis has been found to be induced by TH_0, but not TH_2, $CD4^+$ T cells (76). Phosphorylated CREB proteins induce *bcl*-2 expression and rescue the cell from apoptosis (77). Nerve growth factor is also an autocrine survival factor for memory B cells (78). In T cell apoptosis two members of the nerve growth factor (NGF) receptor family play a major role in apoptosis, but, in the case of B lymphocytes, NGF is a preventive factor for apoptosis (78).

T cells. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells (79,80).

Monocytes. Bratton *et al* noted that GM-CSF enhances survival (81), and we also found that M-CSF prevents apoptosis of peripheral blood monocytes (Tomizuka H, *et al*, manuscript in preparation). Bcl-2 prevents apoptosis even in the presence of Fas antigen. Monocytes are key effector cells to prime peripheral blood T cells to undergo apoptosis (82), and monocyte-derived reactive oxygen metabolites induce apoptosis in NK cells (83).

Neutrophils. Squier *et al* reviewed extensively apoptosis in leukocytes (84). Elevation of cytosolic calcium has been reported to induce apoptosis in various cell types, but in neutrophils transient elevation of cytosolic free calcium retards subsequent apoptosis (85).

Eosinophils. Matsumoto *et al* noted that anti-Fas antibody induced apoptosis in human eosinophils *in vitro* (86). Druilhe *et al* demonstrated that Fas antigen plays a major role in apoptosis in factor-deprived eosinophils (87). Fas antigen expression and activation may contribute to the resolution of inflammatory allergic reactions. The antiapoptotic effects of GM-CSF and IL-5 requires activation of both Lyn and Syk (88). Transforming growth factor ß abrogates the effects of IL-5 on eosinophils and induces their apoptosis (89).

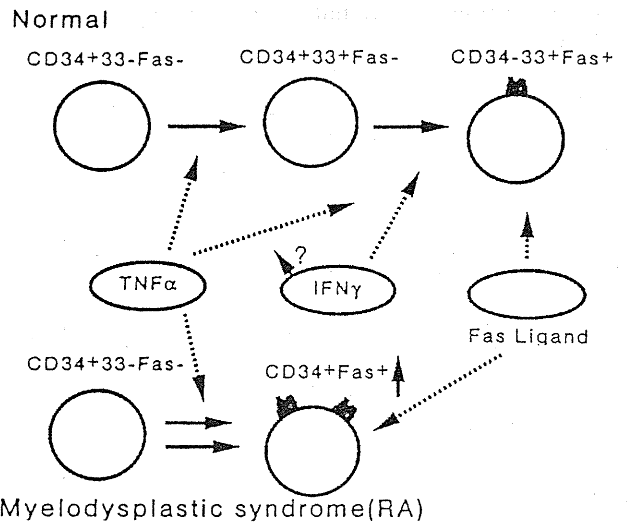

Figure 3. Sequential presentation of CD34, CD33, and Fas antigen in normal and myelodysplastic hematopoietic progenitors. The dotted arrows show negative regulation, and solid arrows positive or up regulation.

10. Relationship between apoptosis and differentiation

In myeloid lineage, CD33 expression precedes the expression of Fas antigen, and Terui *et al* demonstrated an independent pathway of apoptosis (71). Kelley *et al* showed that apoptosis derived from erythropoietin occurs during the G1 and S phases of the cell cycle without growth arrest (90).

Fas antigen expression in myelodysplastic syndrome (MDS). We reported higher Fas antigen (CD95) expression in refractory anemia of myelodysplasia (91). Yoshida *et al* proposed a paradix or paradigm for apoptosis in myelodysplasia (92). The presence of apoptosis in myelodysplasia has been considered as a working hypothesis. The cell kinetics study by Raza *et al* and programmed cell death in bone marrow biopsies of myelodysplasia revealed extensive apoptosis (93). Bone marrow biopsy samples showed apoptosis in stromal and hematopoietic cells of MDS (94). Recently the presence of apoptosis has been proved by morphologic and DNA ladder formation. Gersuk *et al* demonstrated increased expression of Fas antigen and Fas ligand in bone marrow CD34$^+$ cells from patients with MDS (95). Expression level of both surface molecules are heterogeneous, but the Fas system plays a major role in apoptosis of MDS CD34$^+$ cells. c-Myc expression is enhanced in MDS and *bcl*-2 expression is suppressed, and the relationship is inversed in AML (96). Anzai *et al* observed the presence of marked apoptosis of MDS-derived myelomonocytic leukemia cell line P39, and they propose that retinoic acid-induced differentiation model in this cell line is suitable for research (97). Kawabata *et al* found higher levels of Ca^{2+}-independent endonuclease activity in marrow non-adherent cells from patients with MDS and AML (98).

Fas antigen-Fas ligand in aplastic anemia. Philpot *et al* demonstrated increased apoptosis in aplastic anemia marrow progenitor cells (99). In aplastic anemia, it is quite difficult to see Fas-Fas ligand interaction even using small scale PCR technology, due to lack of cells. Although T cell clones have been established from some patients who suffer from aplastic anemia, we could not detect a significant level of Fas ligand mRNA by RT-PCR. We do not know whether the Fas-Fas ligand system is involved in this disease. Philpot *et al* demonstrated that bone marrow CD34$^+$ cells of patients with the aplastic anemia showed increased expression of Fas antigen (99). Zoumbos *et al* reported that circulating activated suppressor T cells is critical in aplastic anemia (100). Antigen recognition and target molecules by T cells need to be studied further. In immuno-suppressant responsive aplastic anemia one type of HLA molecule predicts a response to treatment (101). A particular type of HLA molecule might be the recognition site for T cells. In Fanconi anemia, p53-dependent radiation-induced apoptosis is altered (102). Yuan *et al* demonstrated that in ß-thalassemia apoptosis is accelerated in erythroid precursors (103). Apoptosis may explain the mechanism of ineffective hematopoiesis in several types of anemia (Fig. 3).

IRF-1 and pathogenesis of myelodysplastic syndrome and preleukemia. In one of the myelodysplastic syndromes, which has a chromosomal abnormality of 5q$^-$, IRF-1 gene was consistently deleted at one or both alleles in patients with leukemia and myelodysplastic syndromes characterized by 5q aberrations. Accelerated exon skipping of IRF-1 mRNA may cause the inactivation of IRF-1 and could contribute to the development of human hematopoietic malignancies (104). Tan *et al* reported that lysyl oxidase gene is the target of IRF-1, and it may play a role in tumor suppression (105). On the other hand, Tanaka *et al* showed that p53 suppresses the tumor together with IRF-1 in response to DNA damage by irradiation (106). Hematopoietic cells from mice deficient in wild-type p53 are more resistant to induction of apoptosis by DNA damaging agents (107), and p53 may play an important role in leukemogenesis or abnormality of apoptosis.

Fas antigen expression in leukemia. CD95 is analyzed by FACS and has been reported to be expressed in myeloid as well as lymphoid leukemia.

In acute promyelocytic leukemia, we investigated CD95 expression in leukemic CD33+ cells. CD95 expression changes markedly during all-trans retinoic acid (ATRA) treatment, and in complete remission, CD95 is expressed in CD33+ cells. CD95- leukemic cells expressed PML/RARα mRNA before complete remission, and proved to be CD95+ cells differentiated by ATRA from CD95- cells in CD33+ leukemic cells. The expression of CD95 can predict the responsiveness to ATRA. RA-resistant disease does not show any increase of CD95 expression during RA treatment (Tomizuka H, *et al*, manuscript in preparation).

In other types of acute myelocytic leukemia, CD95+ leukemic cases have a higher complete remission rate than CD95- cases (108). In myeloid leukemic cells hematopoietic cytokines inhibit apoptosis induced by transforming growth factor ß1 and cancer chemotherapy compounds (109). This finding may be useful to consider treatment for leukemia using apoptosis. Paclitaxel induced apoptosis in patients with acute leukemia treated in a phase I trial (110). Taxol derivatives and etoposide are good candidate apoptosis inducers of antineoplastic agents. In human leukemia cells which have glucocorticoid receptors, glucocorticoid caused apoptosis via the receptors (111). Protein kinase C inhibitor also induced apoptosis in human leukemia cell lines (112). ICE inhibitor affects on AML progenitor proliferation (113). Interferon-γ inhibits apoptosis in AML cells (114). Several cytokines or inhibitors can be candidates enhancing apoptosis associated with chemotherapeutic agents in leukemic cells.

CD95 (115) and IL-4 mediate apoptosis in human T-ALL (116). In T cell acute lymphocytic leukemia, they were resistant to Fas-induced apoptosis, which is determined by a bcl-2 independent anti-apoptotic program (117).

Ten of the 21 patients with B chronic lymphocytic leukemia and hairy cell leukemia had the Fas antigen before cultivation, and up-regulated Fas antigen expression after cultivation with γ-interferon (118). *In vitro,* in CLL cells apoptosis is prevented by human bone marrow stromal cells (119).

K562 cells, as a model of chronic myelogenous leukemia, are resistant to various stimulators of apoptosis. BCR-ABL maintains resistance of CML cells to apoptosis (120), as the mechanism with delay of G2/M transition after DNA damage is mediated (121). Antisense oligonucleotides corresponding to the translation starting point of bcr downregulate bcr-abl protein and renders it susceptible to induction of apoptosis by chemotherapeutic agents or serum deprivation. P210 bcr-abl interacts with the IL-3 receptor ßc subunit and constitutively induces tyrosine phosphorylation (122). Expression of a temperature sensitive v-abl protein reverses the effects of the antisense oligonucleotides (123).

In adult T-cell leukemia, the functional Fas antigen expresses and anti-Fas antibody induces apoptosis (124).

11. Apoptosis and other hematological disorders

In multiple myeloma, myeloma cells express Fas antigen/APO-1 (CD95) but only some are sensitive to anti-Fas antibody (125). Massaia *et al* demonstrated dysregulation of fas and *bcl*-2 expressed in T cells of myeloma patients (126). ATRA has been introduced to clinical trials in several cases in USA and in Japan (127). Myeloma cells cultured with ATRA showed apoptosis. However, hypercalcemia is the main problem when we consider the clinical application of ATRA in multiple myeloma. Ectopic *bcl*-2 expression and/or IL-6 mediated up-regulation of bcl-x_L prevent myeloma cell apoptosis (128).

Su *et al* demonstrated retinoic acid induced apoptosis and repressed MDR-expressed refractory T cell lymphoma (129). The TNF ligand superfamily is involved in the pathophysiology of malignant lymphoma (130). Beraus reported the expression of p53 and bcl-2 during the indolent phase of germinal center cell lymphoma (131). In diffuse large B-cell lymphoma bcl-2 and p53 expression was correlated to p53 expression (132). BTK mediates radiation-induced apoptosis in DT-40 lymphoma B cells (133). Tanaka *et al* found that in human serum Fas ligand is elevated in T lymphoblastic lymphoma (134), and Sato *et al* reported that a high level of soluble Fas ligand accompanied an aggressive nasal lymphoma (135), which is derived from natural killer cells.

CD95 ligand in graft rejection. CD95 ligand may play a role in graft reject or GVHD (136), and serum estimation of Fas ligand may contribute to pathophysiological understanding. Regulation of the Fas system may be a good candidate to control apoptosis in GVHD.

Mutations in Fas gene and autoimmune lymphoproliferative disorders. Watanabe-Fukunaga *et al* suggested that lymphoproliferation disorders in mice are caused by defects in Fas antigen (CD95) (137), and Takahashi *et al* reported that similar lymphoproliferative diseases in mice are caused by a point mutation with Fas ligand gene (138). The Canale-Smith syndrome is a childhood disorder characterized by lymphadenopathy and autoimmunity (139). Three novel Fas antigen gene mutations have been identified, all of which were heterozygous and predicted to impair signal transduction by Fas (140).

Human immunodeficiency virus and apoptosis. Human immunodeficiency virus (HIV) infection leads to the deletion of CD4+ T lymphocytes, and is preceded by a progressive loss of T cell mediated immunity (141). After the entry of HIV into T lymphocytes via CCKR5 fusin, T cell receptor mediated apoptosis of CD4+ T lymphocytes from HIV persons caused Fas-mediated cell death probably via ICE (142). This mechanism explains the loss of T cells and probably the presence of neutropenia in HIV patients. Accelerated apoptosis is present in neutrophils in HIV patients. The protease antagonist may be useful to prevent apoptosis of CD4+ T lymphocytes, and a clinical trial should be considered. Gene transfer using protease inhibitors or the preventive mechanism working for death domain in CD4+ T lymphocytes may be considered in the near future.

12. Comments

The Fas antigen-Fas ligand system has been found to play an important role in regulating T cell depletion in the

immune system in the thymus. The loss of Fas antigen expression explains the animal model of systemic lupus erythematosus, lpr mice. On the other hand, loss of Fas ligand regulation explains another animal model, gld mice. Both types of mice are good models of dysregulation of apoptosis. Canale-Smith syndrome is a human counterpart of lpr. However, we do not yet have a mouse model which may explain hematological disorders. Studies regarding the involvement of Fas antigen in human hematopoietic cells have been limited to lymphoid lineages. The Fas antigen is expressed on the cell surface of T cells, B cells, and monocytes. Recent development of FACS technology has enabled analysis in a very low percentage of cells such as hematopoietic stem cells. Among $CD34^+$ cells in human bone marrow, the presence of Fas antigen undergoes loss of proliferation activity. The expression of CD33 preceeds and afterwards expresses Fas antigen in myeloid differentiation. In a monocyte lineage the expression of Fas antigen does not always result in cell death, because of anti-apoptotic products such as bcl-2 protein in the cytoplasm, which prevent apoptosis. In the myelodysplastic syndrome, we found that Fas antigen expression was enhanced in the bone marrow cells, and apoptosis was detected by the TUNEL method. Gene rearrangement of interferon response factor-1 (IRF-1) has been observed in all patients with the myelo-dysplastic, $5q^-$ syndrome, known as a pre-leukemia syndrome. IRF-1 gene knock-out studies have revealed the dysregulation of apoptosis and leukemogenesis. In the normal tumor immunological system, tumorigenesis is prevented by the interferon system, and the IRF-1 gene is located in the site of chromosome 5q. In preleukemia and AML, the presence of apoptosis is proved in various ways, and the expression of Fas antigen (CD95) is functionally active and undergoes to apoptosis by anti-Fas antibody. For further details see the extensive review by Nagata (143).

The ICE family is now called caspase (143). The structure of bcl-x_L, like the ionic channel, functions as a regulator of permeability transition. We should study the mechanism and signal transduction of other pathways or substrates for caspases other than the tumor necrosis factor and Fas system. Several groups have been trying to determine the Fas-binding protein that activates apoptosis. Yang *et al* demonstrated using two hybrid screenings that Daxx can bind to the Fas death domain, and lacks a death domain itself (144). It activates the JNK (Jun N-terminal kinase) pathway. This finding might lead to a breakthough in further understanding apoptosis.

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Eur. Cytokine Netw., Vol. 9 n° 1, *March 1998*, 47-55

Interleukin-8 and monocyte chemotactic protein-1 production by a human glioblastoma cell line, T98G in coculture with monocytes: involvement of monocyte-derived interleukin-1alpha

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ABSTRACT. We have previously demonstrated that a human glioblastoma cell line, T98G cells, produced high levels of interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) when stimulated with IL-1 or tumor necrosis factor-α (TNF-α). In this study, we found that T98G cells are capable of producing large amounts of IL-8 and MCP-1 when cocultured with human peripheral blood monocytes or a monocytic cell line, U937 cells. Since it is possible that both glioblastoma cells and monocytes are capable of producing chemokines, we determined which type of cells actually produced IL-8 and MCP-1, by the fixation of one or the other cell type with 3% paraformaldehyde (PA). This procedure revealed that T98G cells were the main source and that PA-treated monocytes effectively stimulated IL-8 and MCP-1 production by T98G cells. Both IL-8 and MCP-1 gene expression and protein production by T98G cells were confirmed by northern blot as well as immunohistochemical staining methods. To analyze the molecules on human monocytes responsible for inducing IL-8 and MCP-1 by T98G cells, several antibodies (Abs) as well as IL-1 receptor antagonist (IL-1Ra) were tested. Anti-IL-1α Ab and IL-1Ra almost completely abolished the IL-8/MCP-1-inducing capacity of the PA-fixed monocytes, while no inhibition was obtained with anti-IL-1β, anti-TNF-α or Abs against CD11b/18, L-selectin or ICAM-1, indicating that membrane-associated IL-1α is involved in the IL-8/MCP-1 induction, while secreted IL-1α plays a major role in this cell-to-cell, *i.e.*, juxtacrine interaction in unfixed conditions.

Keywords: IL-8, MCP-1, glioblastoma, IL-1α, IL-1 receptor antagonist.

INTRODUCTION

Cytokines are important regulatory proteins that influence the growth and differentiation of normal and malignant glial cells [1-3]. It is well recognized that cytokines are produced not only by cells of the immune system, but also by astrocytes and microglial cells in the central nervous system (CNS), and these cytokines may have roles as growth factors in normal and pathological conditions affecting the brain responses including infectious diseases of the CNS, demyelinating diseases and brain tumors [2-4], or radiation-induced brain injury [5]. Receptors for cytokines such as IL-1 and TNF-α were demonstrated to be present in the hippocampus and in the choroid plexus [4], on astrocytes and microglial cells [4, 6-9]. IL-1 and TNF-α production and autocrine responses to these cytokines, particularly in the case of CNS tumors, seem to be important for the growth of glioma cells and the subsequent cascade of host reactions leading to cytokine production. It has been reported that not only proinflammatory cytokines such as IL-1 and TNF-α, but also CXC and CC chemokines including IL-8 and monocyte chemotactic protein-1 (MCP-1)/monocyte chemotactic and activating factor (MCAF) are produced in the CNS and CNS tumors in response to IL-1 or TNF-α [6, 10-14]. Locally produced chemokines are thus thought to have a role in attracting inflammatory cells to the brain region.

We [11, 12] and other groups [13-15] have previously determined that human astrocytoma and glioblastoma cell lines express high levels of IL-8 and MCP-1/MCAF mRNA in response to IL-1 or TNF-α. Since human vascular endothelial cells are capable of producing considerable quantities of IL-8 or MCP-1 during coculture with monocytes [16-18], we investigated whether human glioblastoma cell lines produce IL-8/MCP-1 when cocultured with human monocytes or monocytic cell lines. In addition, the molecules present on monocytes responsible for the induction of IL-8/MCP-1 were characterized.

MATERIALS AND METHODS

Cytokines and antibodies. Human rIL-1α, TNF-α, and IL-8 were kindly provided by Dainippon Pharmaceutical Co., Osaka, Japan. Peroxidase-conjugated swine anti-rabbit IgG was purchased from Dako Japan, Tokyo. Anti-IL-8 (WS-4) and anti-MCP-1 (#30C) mAb were produced and purified as described elsewhere [19, 20]. Murine rIL-1Ra was prepared and purified as described previously [21, 22]. Human rIL-1Ra was purchased from the Pepro Tech EC Ltd. (London, England). Neutralizing mAb against human IL-1α (clone 28) was described [23] and mAb against TNF-α (anti-PT-050; clone 14E/3) was obtained from Dainippon Pharmaceutical Co. These mAb, at a dose of 10 µg, could neutralize 10 ng rIL-1α or rTNF-α, respectively. Anti-human L-selectin (Dreg-2), anti-CD11b/18 (R15.7/Hy) and ICAM-1 (RR1/1.1.1) mAb were kindly provided by Dr. Robert Rothlein, Boehringer Ingelheim, USA.

Cell lines. A human glioblastoma cell line, T98G [11, 12] and a histiocytic cell line, U937 were obtained from the Japanese Cancer Research Resources Bank (JCRB, Kamiyoga, Tokyo). These cell lines were maintained in tissue culture dishes in RPMI 1640 medium (Nissui Seiyaku, Tokyo) supplemented with 5% heat-inactivated FCS, 2 mM glutamine, 100 units/ml penicillin G, and 100 µg/ml streptomycin.

Preparation of human peripheral blood monocytes. Peripheral blood mononuclear cells (MNC) were isolated from heparinized venous blood from healthy adult donors by Ficoll-Conray density gradient centrifugation. Monocytes were allowed to adhere to plastic dishes during a 2 hours incubation, washed twice with warmed RPMI medium and detached using a rubber policeman as described elsewhere [16, 17]. Alternatively, monocytes were separated by discontinuous Percoll-gradient sedimentation in order to avoid activation by adherence [24]. Removal of $CD3^+$ cells and enrichment of $CD14^+$ monocytes by a magnetic cell sorting device (Dynal, Veritas, Oslo, Norway) gave more than 90% esterase-positive cells.

IL-8 and MCP-1 detection by ELISA. T98G cells were grown in RPMI 1640 medium supplemented with 1% FCS. These cells were then incubated with monocytes or IL-1α (10 ng/ml) at 37° C for 24 hours. IL-8 and MCP-content of the culture supernatants were measured by ELISA as established previously [19, 20], using the combination of monoclonal and polyclonal Abs. The detection limits of IL-8 and MCP-1 ELISAs were 20 pg/ml and 40 pg/ml, respectively. For the MCP-1 ELISA, human rMCP-1 (Peprotech, London) was used as standard. IL-1α was measured by ELISA with a detection limit of 4-250 pg/ml (Biotrak, Amersham). All samples were assayed at least in duplicate.

Preparation of the cell membrane fraction. For the preparation of crude cell membranes, U937 cells (~ 7×10^8 cells) preincubated with PMA for 48 hours were disrupted by homogenization in buffer A (25 mM HEPES, 2 mM EDTA, 5 mM EGTA, 4 mM 2-ME, 1 mM PMSF, 100 µg/ml trypsin inhibitor, 10 µg/ml pepstatin and 500 µg/ml leupeptin). The homogenate was mixed with 0.66 M sucrose in buffer A and centrifuged at 1,000 rpm for 5 min to remove nuclei. Nuclei-free supernatant was mixed with 40% sucrose and centrifuged for 1 hour at 4° C at 100,000 x g for recovery of the crude membrane fraction. This crude membrane fraction was resuspended in 5 vol of buffer A and centrifuged for 1 hour at 4° C at 100,000 x g for further purification of the membrane fraction. The precipitate was suspended in buffer A, and the protein concentration was determined by the Bradford method using BSA as standard.

Fixation of cells. T98G or monocytes were washed with PBS and incubated with freshly prepared 3% paraformaldehyde (PA, Sigma) in PBS, pH 7.4 at 4° C, for 15 min, 1 hour or 4 hours, as described by Conlon *et al.* or Bailly *et al.* [25, 26]. The cells were washed twice in PBS (in particular experiments PBS containing 10% FCS) and then used for coculture experiments.

Total mRNA preparation and northern blot analysis. Total RNA was extracted from T98G cells or monocytes by a single-step guanidine isothiocyanate centrifugation method. Ten µg of total RNA was loaded onto a 1% agarose gel containing 7.5% formaldehyde and was blotted onto the GeneScreen (DuPont NEN). Membranes were hybridized with ^{32}P-labeled IL-8 cDNA or MCP-1 cDNA as described elsewhere [11, 12].

Immunohistochemistry and flow cytometry. Immunohistochemical staining with anti-IL-8 and anti-MCP-1 MAb was performed as described previously [16]. Briefly, T98G cells (5×10^5 cells/ml), monocytes (2.5×10^5) or the mixture were incubated on coverslips in culture plates (3.5 cm, NUNC). After incubation at 37° C for 18 hours, coverslips were rinsed with PBS and fixed with 4% PA for 10 min at room temperature. They were preincubated in 0.3% H_2O_2 in methanol for 20 min, and rinsed in 0.1% Triton X-100/PBS. The nonspecific binding sites were blocked with 10% normal rabbit serum, followed by the addition of anti-IL-8 (WS-4) or anti-MCP-1 (#30C) MAb. After incubation at 37° C for 2 hours, the coverslips were rinsed again in PBS, overlaid with biotinylated rabbit anti-mouse IgG, and incubated for 1 hour at 37° C. Coverslips were treated with streptavidin reagent (Histofin) for 30 min, rinsed in PBS, overlaid with 0.05% 2,3'-diamino-benzidine tetrahydrochloride in 0.05 M Tris-HCl buffer (pH 7.6) and 0.01% H_2O_2 for 5 min to allow color development, and then rinsed with distilled water. Methylene blue was used as a counterstain. Cell-associated IL-1α, IL-1β or TNF-α were stained with monoclonal anti-IL-1α, IL-1β or TNF-α antibodies as mentioned, followed by FITC-labeled rabbit anti-mouse IgG, and flow cytometry using FACS Calibur (Beckton-Dickinson).

RESULTS

Coculture of T98G and monocytes produces high levels of IL-8 and MCP-1. Since a human glioblastoma cell line, T98G produced IL-8 and MCP-1 in

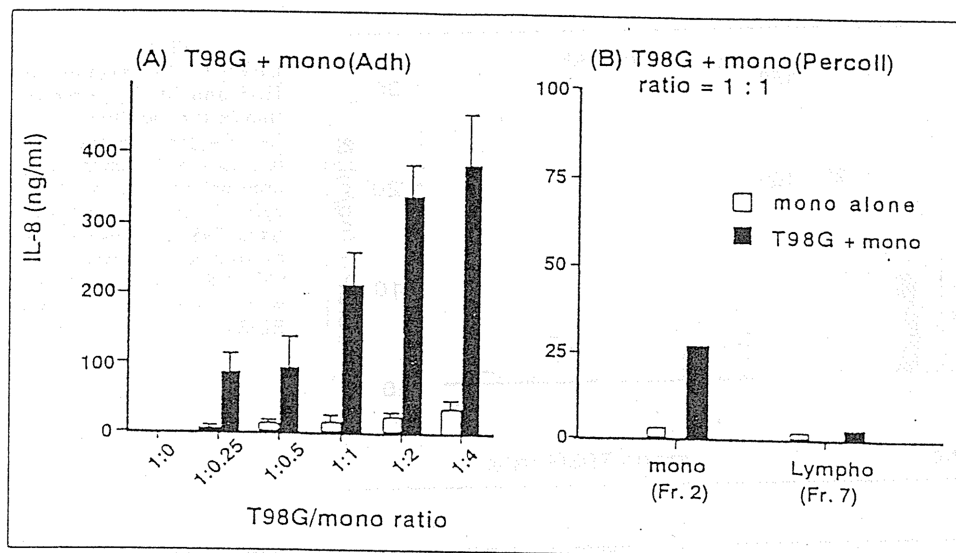

Figure 1
IL-8 induction in coculture of a human glioblastoma cell line, T98G cells, and peripheral blood monocytes (mono).
(A) Effect of increasing number of monocytes (0.25 to 4 x 10^5 cells) on IL-8 production from a fixed number of T98G cells (1 x 10^5 cells/well). Monocytes were separated by adherence to plastic. (B) Monocytes obtained by Percoll-gradient (Fr. 2), followed by the removal of $CD3^-$ T cells, were added to T98G cells (1 x 10^5 cells/well) at a ratio of 1:1. Lymphocyte population with less than 1% monocytes (Fr. 7). Experiments from two individuals are indicated. IL-8 in the 24 hours-culture supernatants was determined using ELISA.

response to IL-1 or TNF-α [11, 12], we wanted to determine whether T98G cells incubated with human peripheral blood monocytes produce IL-8 in culture supernatants over 24 hours. IL-8 production by a 1 x 10^5 cells/well of T98G cells increased in the presence of increasing numbers of monocytes with a T98G/monocyte while only minimal IL-8 production was detected in cultures of T98G or monocytes alone (Figure 1A and B). Monocytes were much more effective than lymphocytes in this coculture system (Figure 1B). It should be noted that maximal IL-8 production reached 500-1,000 ng/ml/24 hours with a T98G/monocyte ratio of 1: 4. This was higher than the stimulation induced by 5 to 10 ng/ml of IL-1α which was 100 to 200 ng/ml IL-8 for 24 hours (data not shown). These observations indicate that coculture with monocytes is very effective in inducing IL-8 production by T98G cells. Monocytes obtained by a discontinuous percoll-gradient stimulated 10 fold less IL-8 production (10 to 30 ng/ml at T98G/monocyte ratio of 1: 1) than plastic dish adherent monocytes (100 to 400 ng/ml) during 24 hours-culture period (Figure 1B) indicating that adherence activated monocytes are able to induce IL-8 production.

Identification of the major IL-8 producers in the coculture of T98G cells and monocytes. It is possible that either T98G cells or monocytes or both were capable to produce IL-8 in the coculture experiments. To clarify this point, T98G cells or monocytes were treated for 15 min with 3% PA and used as stimulator cells. While PA-treated monocytes themselves exhibited no IL-8 production, they effectively induced IL-8 production by T98G cells, although the magnitude was significantly less than that of untreated monocytes (Table 1). It should be noted that coculture of T98G cells and monocytes exhibited synergistic production of IL-8, as compared with one way stimulation using PA-fixed monocytes (*e.g.* 780 ng/ml *versus* 116.7 ng/ml, at a ratio of 1: 4). As shown in Figure 2A, PA-treated monocytes increased not only IL-8 but also MCP-1 production by T98G cells, whereas PA-treated T98G

Table 1
Effect of PA-treatment of monocytes and T98G cells on IL-8 production[a)]

Untreated monocytes	IL-8 (ng/ml)	Untreated monocytes + T98G	IL-8 (ng/ml)
0		0	< 1
0.5 x 10^5	< 1	0.5 x 10^5	315
1.0 x 10^5	5.5	1.0 x 10^5	421
2.0 x 10^5	13.2	2.0 x 10^5	502
4.0 x 10^5	25.0	4.0 x 10^5	780
PA-fixed monocytes	**IL-8 (ng/ml)**	**PA-fixed monocytes + T98G**	**IL-8 (ng/ml)**
0		0	< 1
0.5 x 10^5	< 1	0.5 x 10^5	6.4
1.0 x 10^5	< 1	1.0 x 10^5	18.4
2.0 x 10^5	< 1	2.0 x 10^5	61.1
4.0 x 10^5	< 1	4.0 x 10^5	116.7

[a)] T98G (1 x 10^5 cells) were cocultured with the indicated number of untreated or 3% PA-fixed (15 min) monocytes for 24 hours, and the IL-8 content in the cultured supernatants was determined by ELISA. Mean of two independent experiments is shown.

cells failed to stimulate more chemokine production than that produced by monocytes alone. Since it was reported that short PA-fixation time failed to prevent IL-1α leakage [18, 25, 26], we also tested whether a longer PA-fixation time, *i.e.* 4 hours-fixation and intensive washing with PBS containing 10% FCS, could induce IL-8 production. As shown in Table 2, 4 hours-fixation induced lower but nevertheless significant IL-8 production by T98G cells. An interesting observation was that the presence of a higher IL-1α content in the supernatants induced higher IL-8 production. It must be stressed that IL-8 induction by PA-fixed monocytes was lower than that induced by unfixed monocytes, indicating that coculture of viable T98G and monocytes provide the best conditions for IL-8 production. These results also indicate that monocytes are the source of IL-1α, whereas T98G cells were the main IL-8/MCP-1 producer cells in this coculture system.

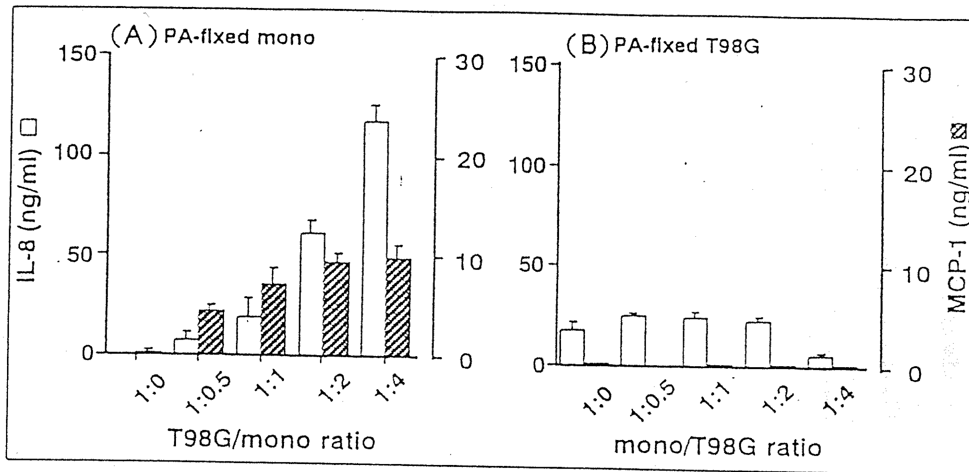

Figure 2
Effect of PA-fixation on IL-8 and MCP-1 production in the coculture.
(A) PA-fixed monocytes (0.5 to 4 x 10^5 cells) were used as stimulant of T98G cells (1 x 10^5). (B) PA-fixed T98G cells were used as stimulant of monocytes (1 x 10^5). IL-8 and MCP-1 were determined by ELISA.

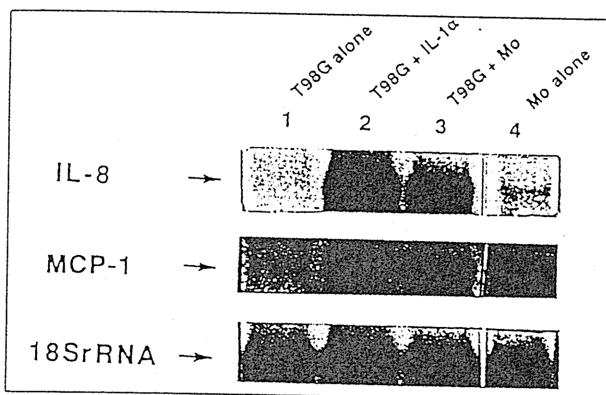

Figure 3
Expression of IL-8 mRNA in a coculture of T98G cells and monocytes.
Total RNA was extracted from T98G cells (lane 1) or monocytes alone (lane 4) cultured for 6 hours, and T98G cells incubated with IL-1α (5 ng/ml; lane 2), or with monocytes (at a T98G/mono ratio of 1/0.25; lane 3).

Table 2
Effect of PA-treatment on IL-8 production by T98G cells: effect of short and long PA-fixation periods[a]

T98G cells (1 x 10^5 cells)	Monocytes treatment (2 x 10^5 cells)	Presence of IL-1α[b] (ng/ml)	IL-8 production (ng/ml)
+	–	< 0.01	< 0.1
+	untreated	0.68	220 ± 12
+	PA-fixed 15 min	0.17	42 ± 2
±	PA-fixed 4 hours	0.13	31 ± 15
–	untreated	0.56	12 ± 4
–	PA-fixed 15 min	0.07	< 0.1
–	PA-fixed 4 hours	0.02	< 0.1

[a] T98G (1 x 10^5 cells) were cocultured for 24 hours with the indicated number of untreated or 3% PA-fixed (15 min or 4 hours) monocytes, and IL-8 production in the cultured supernatants was determined by ELISA. Mean of two independent experiments is shown.
[b] IL-1α content in the culture supernatants was determined by ELISA.

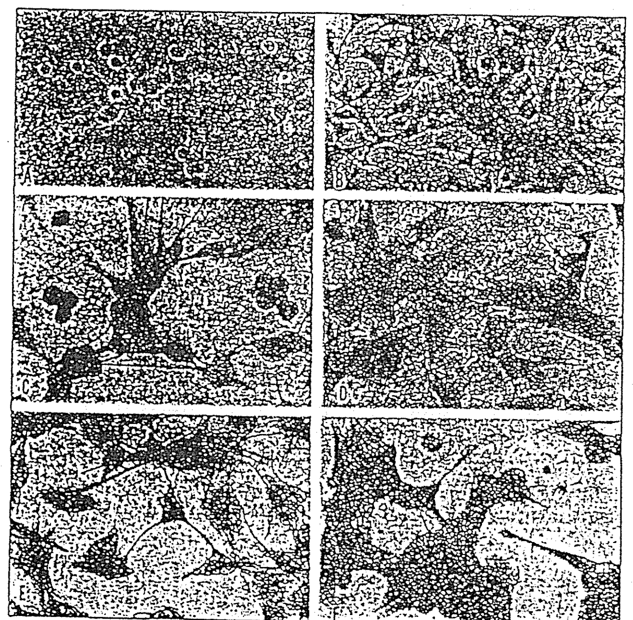

Figure 4
Detection of IL-8 and MCP-1-producing cells by immunohistochemical staining.
(A) monocytes alone, (B) T98G alone, (C) and (E) IL-1α-stimulated T98G, (D) and (F) T98G and monocytes cocultured for 18 hours. (A) to (D) were stained with anti-IL-8 Ab (10 µg/ml), (E) and (F) were stained with anti-MCP-1 Ab (5 µg/ml). Arrows in (D) and (F) mark monocytes. Magnification x 200.

Induction of IL-8/MCP-1 mRNA expression in the coculture system. We examined whether IL-8/MCP-1 mRNA expression was induced in the coculture system. As shown in Figure 3, both IL-1α-stimulated T98G cells and T98G cells cocultured with monocytes expressed high levels of IL-8/MCP-1 mRNA as shown by northern blotting, while no significant IL-8 mRNA (but minimal level of MCP-1 mRNA), was induced in unstimulated T98G or monocytes cultures, indicating that the induction of IL-8 and MCP-1 mRNA occurred probably at the transcriptional level.

Identification of IL-8/MCP-1 producing cells by immunohistochemical staining. Whether T98G or monocytes were the producers of IL-8/MCP-1 in this coculture system was evaluated by immunohistochemical staining. As shown in Figure 4, most T98G cells stimulated with IL-1α (10 ng/ml) for 18 hours were identified as IL-8- and MCP-1-producing cells as they are stained by specific antibodies against human IL-8

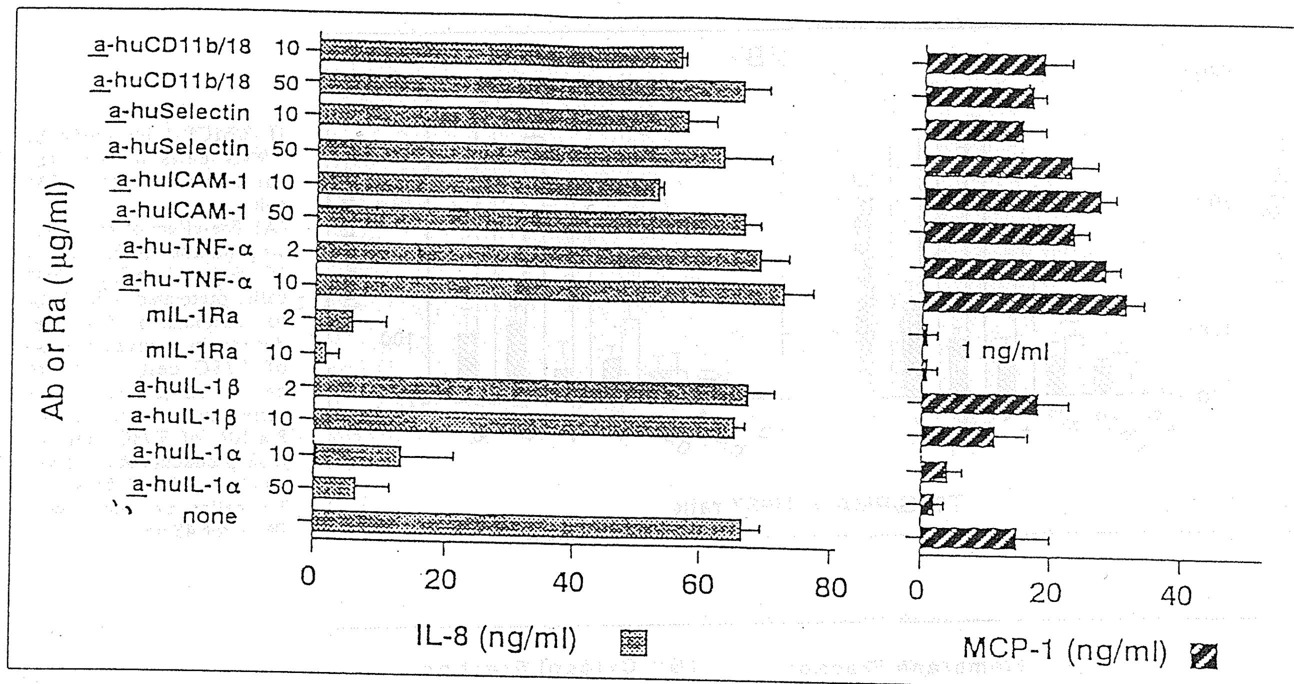

Figure 5
Effect of anti-cytokine Abs or IL-1Ra on IL-8/MCP-1 production.
T98G cells were cocultured with the PA-fixed monocytes. Each Ab or IL-1Ra at the indicated concentrations were added at the onset of the coculture of T98G cells and monocytes at a ratio of 1 to 1.5.

(WS-4) or MCP-1 (#30C) (Figure 4 C and E). Similarly, most T98G cells cocultured for 18 hours with a small number of monocytes (at a ratio of 1: 0.5) were significantly stained with the anti-IL-8 or MCP-1 Abs (Figure 4D and F), confirming that T98G cells are IL-8- and MCP-1-producing cells.

Characterization of the molecules involved in inducing IL-8 and MCP-1. It seems to be important to identify the molecules on the monocyte surface that are responsible for the induction of IL-8/MCP-1 by T98G cells. For this purpose, we employed PA-fixed monocytes as stimulator cells in order to avoid two-way stimulation. As shown in Figure 5, IL-8/MCP-1 production was not affected by the presence of anti-human adhesion molecules (anti-CD11b/18 Ab, anti-L-selectin Ab, or anti-ICAM-1 Ab) at doses of 10 to 50 μg/ml. Moreover, no significant reduction was observed by anti-TNF-α or anti-IL-1β Abs. However, either anti-IL-1α Ab (10 to 50 μg/ml) or murine IL-1Ra (2 to 10 μg/ml) almost completely abrogated IL-8/MCP-1 production by T98G cells in this coculture system. Examination of the dose response curve demonstrated that a 50% reduction of IL-8 production by anti-IL-1α Ab and mIL-1Ra was achieved with 1-2 μg/ml and 0.5-1 μg/ml, respectively. It should be noted that both human IL-1Ra as well as murine IL-1Ra were effective in inhibiting IL-8/MCP-1 production in this coculture system (data not shown). The above data indicate that membrane-bound IL-1α present on the PA-fixed monocytes is responsible for IL-8/MCP-1 induction by T98G cells. Although soluble IL-1α which might be shed from the PA-fixed cells, might also be effective, any IL-1α shed from the PA-fixed monocytes could be neutralized by anti-IL-1α Ab or by mIL-1Ra.

It should be mentioned that when unfixed monocytes and glioblastoma cells were cocultured, either anti-IL-1α or IL-1β Ab partially abrogated chemokine-inducing activity (data not shown), suggesting that IL-1β as well as IL-1α is also involved in this cell-to-cell interaction.

Participation of IL-1α in a monocytic cell line, U937, in the coculture system. In order to obtain a large amount of the monocyte-derived cell membrane fraction, we tested several human monocytic cell lines. A PMA-treated (but not untreated) U937 cell line could induce significant levels of IL-8/MCP-1 production by T98G cells, although the maximal level of U937-induced IL-8/MCP-1 production was lower than that induced by monocytes (Figure 6).

Preparations of membrane and cytosol fractions were both capable of stimulating IL-8/MCP-1 production as shown in Figure 7. Again, IL-8/MCP-1-inducing capacity was abrogated by the addition of anti-IL-1α Ab as well as by mIL-1Ra but not by anti-IL-1β Ab, indicating that the IL-8/MCP-1-inducing capacity of the membrane fraction was mostly ascribable to the IL-1α. Since the IL-8/MCP-1-inducing capacity in the cytosol fraction was not completely abrogated by the anti-IL-1α or mIL-1Ra, cytosolic molecules other than IL-1α may also be involved in the IL-8/MCP-1 induction. To analyze the involvement of soluble IL-1α in this stimulation, we determined the IL-1α contents in the coculture of T98G and U937 cells. As indicated in

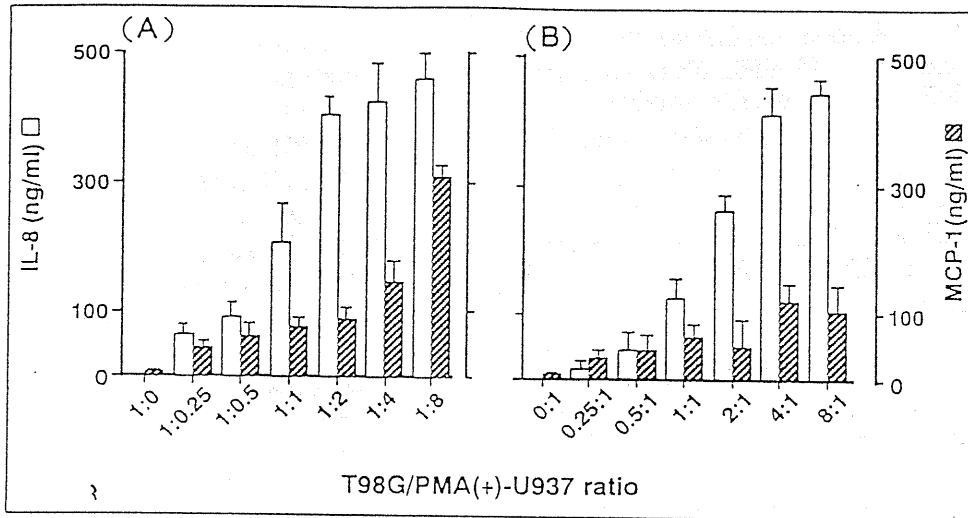

Figure 6
IL-8/MCP-1 induction by T98G cells during the interaction with U937 cells.
(A) The effect of an increasing number (0.25 to 8 x 10^5 cells) of unfixed U937 cells, pretreated with PMA for 48 hours, on IL-8 production by a given number of T98G cells (1 x 10^5 cells). (B) Effect of an increasing number (0.25 to 8 x 10^5) of T98G cells on IL-8 production by a given number of U937 cells (1 x 10^5 cells), pretreated with PMA for 48 hours.

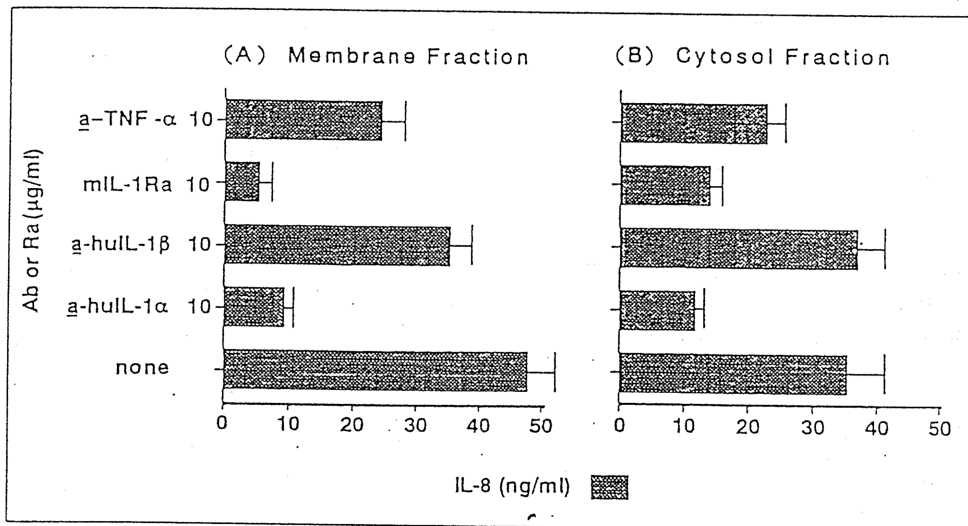

Figure 7
Effect of anti-cytokine Abs or IL-1Ra on IL-8/MCP-1 production in a coculture of T98G and U937 cell membrane (A) or cytosol fraction (30 μg/ml, respectively) (B).

Table 3
Effect of PA-treatment on IL-1α and IL-8 production by T98G and U937 cells[a)]

T98G treatment (1 x 10^5 cells)	U937 cells treatment (2 x 10^5 cells)	Presence of IL-1α (ng/ml)	IL-8 production (ng/ml)
untreated			
+	–	< 0.02	< 0.5
–	untreated	0.13	< 0.5
+	untreated	0.31	405 ± 18
+	PA-fixed 15 min	0.18	328 ± 23
+	PA-fixed 1 hour	0.05	156 ± 12
PA-fixed 1 hour			
+	–	< 0.02	< 0.5
+	untreated	0.10	36.1 ± 2.9
+	PA-fixed 15 min	0.06	< 0.6
+	PA-fixed 1 hour	0.05	< 0.5

[a)] T98G (1 x 10^5 cells) were cocultured with 2 x 10^5 untreated or 3% PA-fixed (15 min or 1 hour) PMA-stimulated U937 cells for 24 hours. IL-1α and IL-8 content in the cultured supernatants was determined by ELISA. Mean of two independent experiments is shown.
[b)] IL-1α content in the culture supernatants was determined by ELISA.

Table 3, 1 hour PA-fixed, PMA-stimulated U937 cells gave significant levels of IL-8 (156 ± 12 ng/ml) with low levels of IL-1α production (0.05 ng/ml) in culture supernatants, while a higher level of IL-1α (0.31 ng/ml) was produced in the coculture of both unfixed combinations, with increased IL-8 production (405 ± 18 ng/ml). Direct stimulation by 1 ng/ml rIL-1α generally gave < 50 ng/ml of IL-8 by T98G cells, detectable levels of IL-1α did not explain the entire IL-8-inducing activity in the culture supernatants, suggesting the combined effect of IL-1α and IL-1β in this interaction. PA-fixed T98G cells stimulated minimal IL-8 production by U937 cells (36.1 ± 2.9 ng/ml), confirming that the major source of IL-8 was ascribable to T98G cells. Finally, we attempted to detect whether monocytes or PMA-treated U937 cell line express IL-1 molecules on its surface. Only marginal IL-1α, but no IL-1β or TNF-α expression were seen by flow cytometry analysis (data not shown). We could not confirm, therefore, whether this subtle difference of IL-1α expression might affect the differential capacity of IL-8 induction or not.

DISCUSSION

We have demonstrated that coculture of a human glioblastoma cell line, T98G cells and monocytes produced high levels of IL-8 and MCP-1. Not only T98G cells, but also some other glioma cell lines including T430 and U373 cells, which were reported to produce IL-8 and MCP-1 [12], were also confirmed to produce these chemokines when cocultured with monocytes (data not shown). Although we previously reported that human umbilical cord vascular endothelial cells produced IL-8/MCP-1 as well as GM-CSF by coculture with monocytes [16, 17], we could not identify the molecules responsible for inducing these chemokines. It is likely that the adhesion of monocytes to cultured endothelial cells or extracellular matrix results in the induction of several cytokines genes, including those of TNF-α and CSF-1 [27, 28]. Even the adhesion of monocytes to Petri dishes induced the expression of IL-1β mRNA or proto-oncogenes such as c-fos and c-jun, in monocytes [29], suggesting that the adhesive interaction activates monocytes and macrophages. Actually, our observation that monocytes separated by a Percoll gradient induced lower IL-8 production by T98G cells than monocytes collected by plastic-adherence is consistent with the above observation. These experiments also indicated that T98G cells are the major sources of the CXC and CC chemokines in this coculture system.

T98G cells were stimulated to produce these chemokines by the membrane-associated IL-1α on the monocytes as well as secreted IL-1α in this cell-to-cell interaction. Of interest in this study may be that anti-IL-1α Ab and IL-1Ra effectively abrogated the IL-8/MCP-1-inducing capacity, indicating that membrane-bound IL-1α on the monocytes was responsible for this activity. This is consistent with the previous findings that membrane-bound IL-1α, but not IL-1β, is the biologically active IL-1, and that only IL-1α but not IL-1β, was present on the monocyte surface [18, 25, 26]. As pointed out by Bailly *et al.* [26] and Suttles *et al.* [30], IL-1 activity was detected even 48 to 96 hours after PA-fixation, indicating that both IL-1α and IL-1β are leaking from the fixed monocytes, although these authors used LPS-stimulated monocytes compared to our unstimulated monocytes. Although the PA-fixation was done for 4 hours, and was followed by thorough washing, up to 1 ng/ml of IL-1α was detected in the supernatants of the cultured PA-fixed monocytes. Therefore, it is possible that leaked or shed IL-1α, in addition to the membrane-bound IL-1α, may be involved in this interaction. The observation that the IL-8/MCP-1-inducing activity of the fixed monocytes was almost completely neutralized by anti-IL-1α Ab as well as IL-1Ra, indicated definitively the participation of IL-1α, either in a membrane-bound form or soluble form, in this juxtacrine interaction of monocytes and glioblastoma cells. Presumably, in the unfixed conditions of both viable cell-to-cell interaction, soluble forms of IL-1α and IL-1β play a major role in this interaction, membrane-associated IL-1α also being involved to some extent in this interaction.

We could not demonstrate direct evidence of the presence of IL-1α on the monocytes or on the PMA-stimulated U937 cell surface by flow cytometry analysis, although Zola *et al.* [31] previously demonstrated that CD14^{+} monocytes expressed a positive IL-1α staining profile. The discrepancy between their results and ours appears to be due to their use of a more sensitive method *i.e.*, a three-step staining method with three color staining rather than our, less sensitive, two-step staining method.

It is widely recognized that inflammatory cytokines are produced at the sites of the injury in CNS during the course of stroke, multiple sclerosis, AIDS, or brain neoplasm. These cytokines are presumably involved in signals promoting structural and functional responses to the injury [2-4]. TNF-α protein and mRNA have been detected in biopsy samples of glial brain tumors, including glioblastomas, anplastic astrocytomas and was particularly found in infiltrating macrophages and perivascular micrioglia. Quantitative evaluation revealed the positive correlation between TNF-α levels and infiltrating leukocytes [8]. At sites of radiation-induced, delayed brain injury harboring malignant gliomas, both TNF-α and IL-6 were detected particularly in infiltrating macrophages [5]. Furthermore, human CNS tumors including glioblastoma and astrocytoma express IL-8/MCP-1 *in vitro* [11-13], and *in vivo* [14, 15]. By the use of the reverse-transcription polymerase chain reaction, most glioblastomas have been shown to express significant IL-8/MCP-1 mRNA levels [11, 15], and the mRNA expression could be stimulated by IL-1 and TNF-α [11-13, 15].

IL-8 may attract inflammatoy cells into CNS tumors. Despite the chemotactic effects of IL-8 on neutrophils, these cells are not commonly found in glial tumors [6]. However, lymphocyte infiltrates are common, which was originally thought to be attributable to the T cell chemoattractant property of IL-8 [32], although this notion has not been confirmed in an *in vivo* study [33] and T cell chemotaxis is now considered to be largely attributable to CC chemokines including RANTES, MIP-1α/β [34], or the recently defined TARC [35]. IL-8 can markedly stimulate direct angiogenesis in the rat and rabbit cornea [36, 37], suggesting the possibility that IL-8 may also participate in glioma neovascularization, although conflicting evidence is also available [38].

MCP-1 mRNA expression and protein production have been attributed to glioma cells, endothelial cells and possibly infiltrating macrophages [4, 8, 39]. MCP-1 thus produced recruits monocytes/macrophages and enhances them to infiltrate into the glioma region, where MCP-1 activates monocytes/macrophages to suppress the growth of malignant brain tumor cells [40, 41]. Actually, it is reported that tumor cyst fluids of glioblastomas and astrocytomas were able to induce monocyte chemoattraction in an *in vitro* assay [15]. Interestingly, glioblastoma and neuroblastoma cells transfected with MCP-1 cDNA were significantly growth-suppressed by the cocultured monocytes (Morita *et al.* manuscript in preparation). Thus,

selective and controlled induction of chemokines at the tumor sites should be important for managing the successful recruitement and activation of monocytes as well as other effector cells.

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IL-13 as Well as IL-4 Induces Monocytes/Macrophages and a Monoblastic Cell Line (UG3) to Differentiate into Multinucleated Giant Cells in the Presence of M-CSF

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The formation of multinucleated giant cells (MGCs) from monocytes/macrophages is controlled by various cytokines whose crucial roles are not fully understood. In this study, we found that interleukin (IL)-13 as well as IL-4 induced peripheral blood monocytes (PBMs) and monoblastic cell line, UG3, to differentiate into MGCs in the presence of macrophage colony-stimulating factor (M-CSF), while IL-2, IL-7 or IL-10 did not. The presence of M-CSF was essential to this MGC formation, because IL-3 or granulocyte-macrophage colony-stimulating factor (GM-CSF) could not replace M-CSF. IL-4 and IL-13 have been known to inhibit the formation of osteoclast-like cells in the presence of stroma cells or osteoblastic cells. But in our system without stroma cells, IL-4 or IL-13 induced some of characteristics of osteoclasts such as tartrate-resistant acid phosphatase (TRAP) activity, vitronectin receptor (vit-R) expression and resorptive activity for hydroxyapatite, but not the expression of receptors for parathyroid hormone or calcitonin. These results suggest possible involvement of IL-4 and IL-13 in MGCs and osteoclasts development, and UG3 may be useful to further investigate the roles of IL-4 and IL-13 in the formation and physiology of MGCs, and the relationship between these MGCs and osteoclasts.
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IL-4 and IL-13 are secreted from Th2 lymphocytes (1), and have significant homology in protein sequence (2). IL-4 and IL-13 have anti-inflammatory activities for monocytes/macrophages such as suppression of the secretion of proinflammatory cytokines, growth factors and chemokines (1-3). IL-4 plays an important role in formation of MGCs from monocytes/macrophages in vivo (4) and in vitro (5-9). IL-13 also induces human monocyte clustering, and it has been hypothesized that IL-13 induces formation of MGCs (10). Recently, it was reported that IL-13 induces monocytes/macrophages to fuse independent of other growth factors (6).

In vivo, monocyte/macrophage-derived MGCs are observed as osteoclasts (11) or so-called foreign body type MGCs (FBMGCs) (12). Osteoclasts are the primary bone resorbing cells, expressing TRAP, a high level of carbonic anhydrase II, calcitonin receptor (cal-R) and vit-R (13). On the other hand, FBMGCs, recognized as macrophage polykaryons, are observed under conditions of hyperimmunity such as infection, rheumatoid arthritis, neoplasia and foreign body reaction against implants (14). FBMGCs may represent down regulation of chronic active inflammation, or participate in frustrated phagocytosis of foreign materials (12). But their precise roles are not known, because of difficulty of isolation and a lack of suitable models.

Previously, we established a human monoblastic cell line, UG3, which has the following features: (1) GM-CSF- or IL-3-dependent proliferation, (2) GM-CSF- or M-CSF-induced differentiation to mature macrophages, (3) M-CSF- and IL-4-induced differentiation to MGCs with hydroxyapatite resorbing activity and TRAP activity (9). In this study, we showed that IL-13 as well as IL-4 had an ability to induce UG3 cells and PBMs to differentiate into MGCs, exclusively in the presence of M-CSF, whereas IL-2, IL-7 or IL-10 did not have such ability. Although IL-4- or IL-13-induced MGCs were characterized as FBMGCs but not osteoclasts, IL-4 or IL-13 induce markers of osteoclasts such as TRAP activity, vit-R expression and resorbing activity for hydroxyapatite in the absence of stroma cells. UG3 responded to IL-4 and IL-13 in the same way as normal human PBMs on MGC formation, and could be a suitable model for studying monocytic differentiation into MGCs and osteoclasts.

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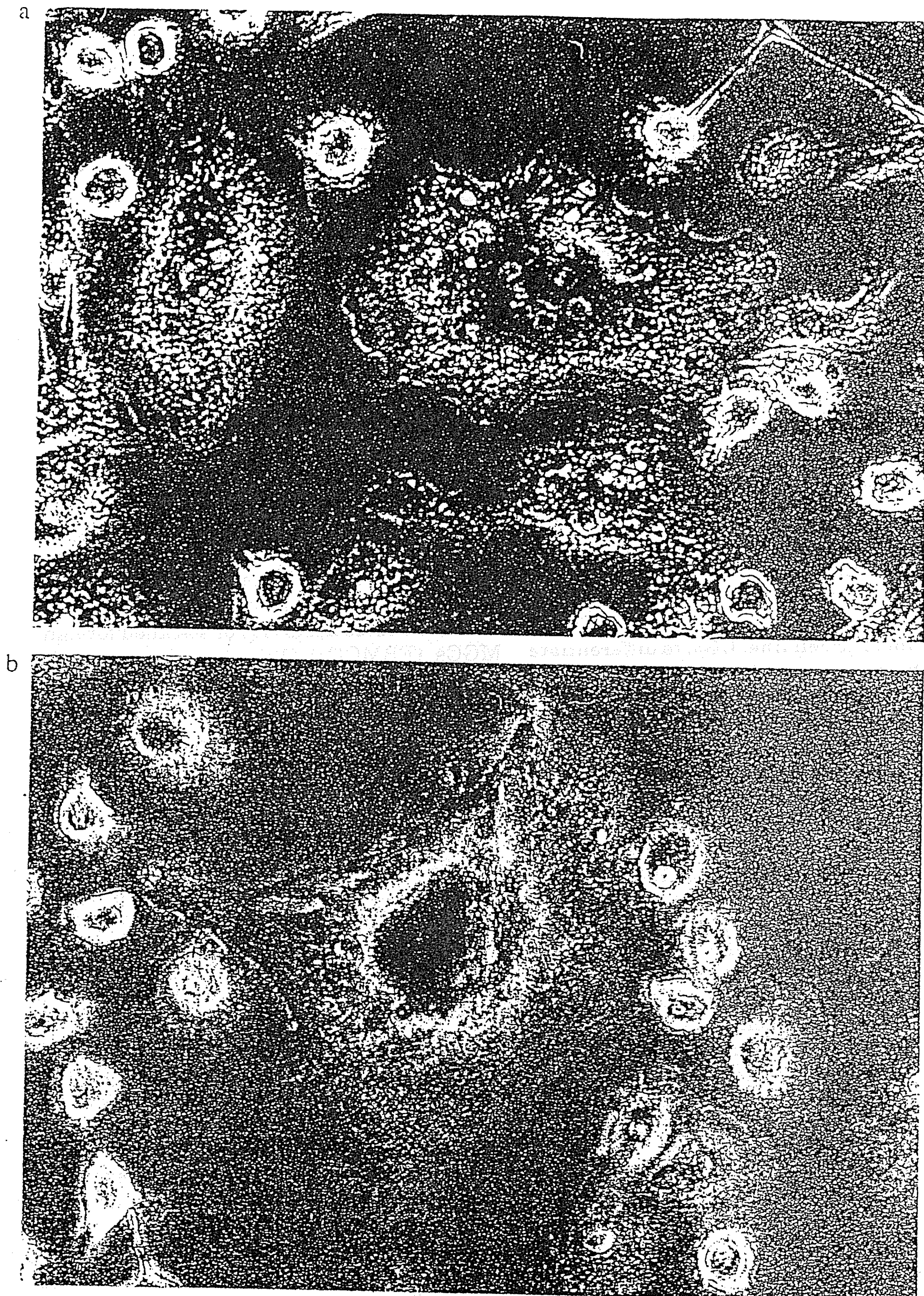

FIG. 1. MGCs stained for TRAP. M-CSF-preincubated UG3 cells were cultured for two weeks in the presence of 100 ng/ml M-CSF and (a) 10 ng/ml IL-4 or (b) 10 ng/ml IL-13, then stained for TRAP. Magnification: 75-fold. Histochemical analysis was performed on cells obtained from four independent cultures.