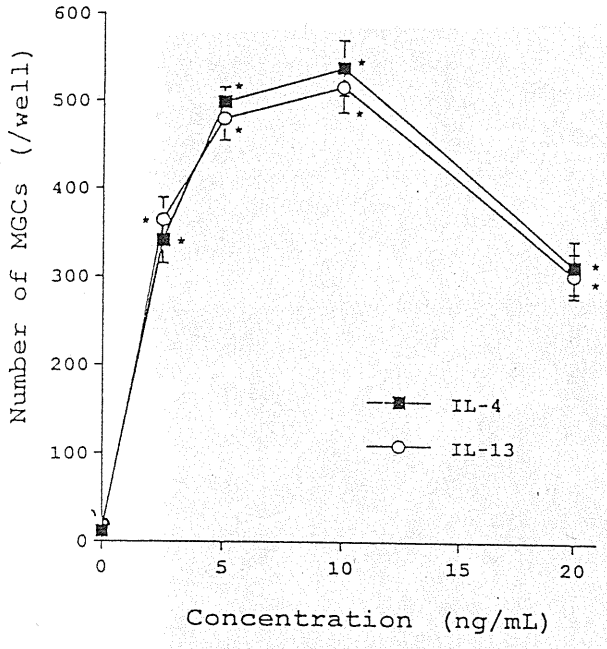


FIG. 2. Dose response effect of IL-4 and IL-13 on the generation of MGCs from UG3 cells. UG3 cells (3×10^5/ml) were cultured in 48-well culture plates with 100 ng/ml M-CSF in the presence or absence of various concentrations of IL-4 or IL-13. Error bars indicate standard deviation of cell numbers. *;$P<0.01$ compared with the number of MGC with M-CSF alone. Results are representative of three independent experiments.

MATERIALS AND METHODS

Cytokines. Recombinant human IL-3 was provided by Kirin Brewery Co., Ltd., Tokyo, Japan. Recombinant human M-CSF was provided by Morinaga Milk Industry Inc., Tokyo, Japan. Recombinant human GM-CSF, IL-2, IL-4 and IL-7 were purchased from Genzyme Corporation, Cambridge, MA. Recombinant human IL-10 and IL-13 were purchased from R&D Systems, Inc., Minneapolis, MN.

Cell preparation. PBMs were obtained from the peripheral blood of healthy volunteers after obtaining informed consent. Mononuclear cells were separated by Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden, d=1.077) density gradient centrifugation at 400g for 30 minutes. The interphase containing mononuclear cells was harvested, then washed twice with phosphate-buffered saline (PBS) and once with fresh Iscove's modified Dulbecco's medium (IMDM) (GIBCO BRL, Gaithersburg, MD). The cells were cultured in IMDM supplemented with 5% fetal calf serum (FCS) (CSL Limited, Victoria, Australia) at 37°C in an atmosphere of 5% CO_2 in air for two hours. Adherent cells were harvested with trypsin-EDTA and washed twice with PBS and once with IMDM with 5% FCS as mature monocytes.

UG3 cells were maintained at a density of 2.5×10^5/ml in IMDM supplemented with 5% FCS and IL-3 (5 ng/ml) at 37°C in an atmosphere of 5% CO_2 in air. The cells were washed twice with PBS, then moved to IMDM containing 5% FCS and 100 ng/ml M-CSF. After a 10-day preincubation in the presence of M-CSF, the cells were harvested with trypsin-EDTA and washed twice with PBS and once with IMDM supplemented with 5% FCS. The PBMs or M-CSF-preincubated UG3 cells were cultured in new dishes in IMDM containing 5% FCS and 100 ng/ml M-CSF with or without IL-2 (100 U/ml), IL-4 (10 ng/ml), IL-7 (1 ng/ml), IL-10 (10 ng/ml) or IL-13 (10 ng/ml).

Assay for TRAP activity of the cells. After a 2-week culture with or without various cytokine combinations in 48-well culture plates, the cells were stained for TRAP with a commercially available kit (Sigma Diagnostics, St Louis, MO) according to the manufacturer's instructions. Cells containing at least 3 nuclei were considered MGCs.

Detection of PTH-R, cal-R and vit-R expression in MGCs. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to detect PTH-R or cal-R messenger RNA (mRNA). Primers used were; PTH-Rs; 5'-AGGACGCTGTGCTCTACTCT-3', PTH-Ras; 5'-AGCTCTGACACTGACCCACA-3', cal-Rs; 5'-GCTTCTTTGTTGCGACCATC-3', cal-Ras; 5'-CTGGGAGGATGGAGAATACT-3'. Complementary DNA was synthesized by reverse transcription in a 80 μl reaction mixture containing 1 μg total cellular RNA, 150 μg/ml random hexanucleotide and 50 U reverse transcriptase (Seikagaku Co., Tokyo, Japan). A volume of 0.5 μl cDNA reaction mixture or control template was amplified in the presence of 1 U Thermus aquaticus DNA polymerase (Takara, Shiga, Japan), 25 mmol/l dNTP and 10 mmol/l of each specific primer in a total volume of 20 μl, on a Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT). One PCR cycle consisted of denaturation at 95°C for 1 minute, primer annealing at 62°C for 1 minute, and extension at 72°C for 2 minutes, and this cycle was repeated 40 times. An aliquot of 6 μl of each PCR product was electrophoresed in 3% agarose (NuSieve, FMC BioProducts, Rockland, ME), and stained with ethidium bromide. For detection of vit-R, immunostaining was performed using monoclonal antibody 23c6 (ENDOGEN, Woburn, MA) and Histofine detection system (Nichirei Co. Ltd., Tokyo, Japan) according to the manufacture's instructions.

Analysis of hydroxyapatite-resorption activity. The cells which were prepared as described in "Cell preparation" were seeded on plastic slides covered with hydroxyapatite (Osteologic, Millenium

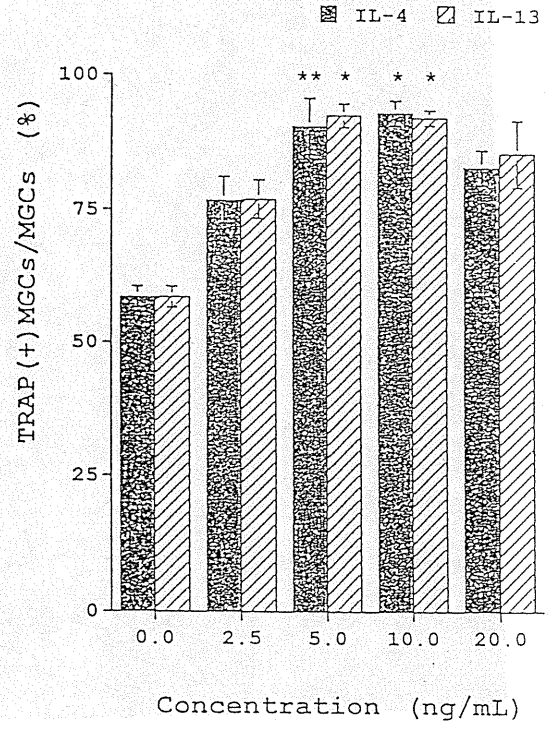


FIG. 3. Dose response effect of IL-4 and IL-13 on the percentage of TRAP positive MGCs in all MGCs. UG3 cells (3×10^5/ml) were cultured in 48-well culture plates with 100 ng/ml M-CSF in the presence or absence of various concentrations of IL-4 or IL-13. Error bars indicate standard deviation of percentage. *;$P<0.01$, **;$P<0.05$ compared with the percentage in the presence of M-CSF alone. Results are representative of three independent experiments.

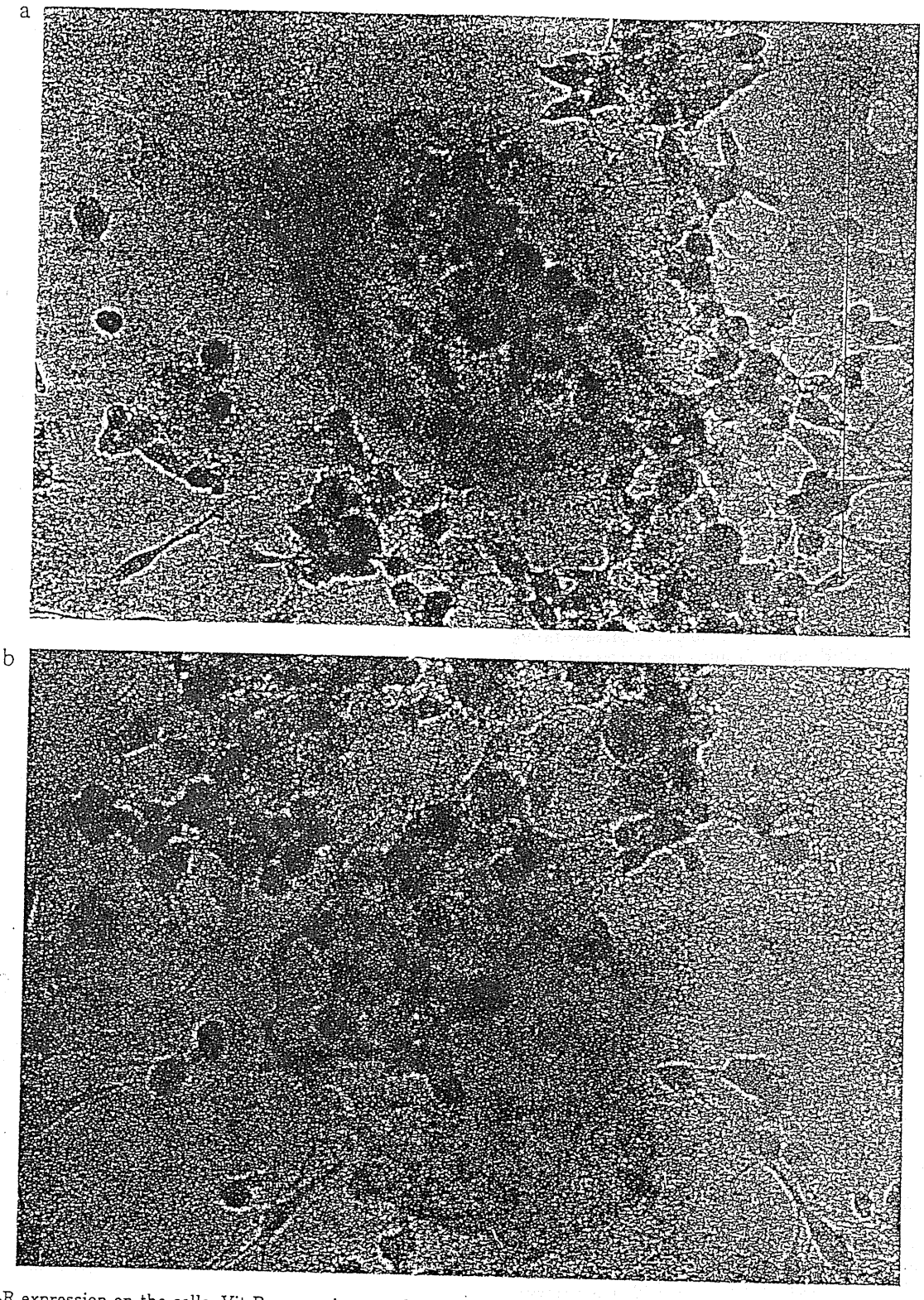


FIG. 4. Vit-R expression on the cells. Vit-R expression was determined by immunohistochemistry on UG3 cells cultured with (a); 100 ng/ml M-CSF and 10 ng/ml IL-4, (b); 100 ng/ml M-CSF and 10 ng/ml IL-13 or (c); 100 ng/ml M-CSF alone for two weeks. Magnification: 200-fold. Histochemical analysis was performed with samples taken from three independent cultures.

C

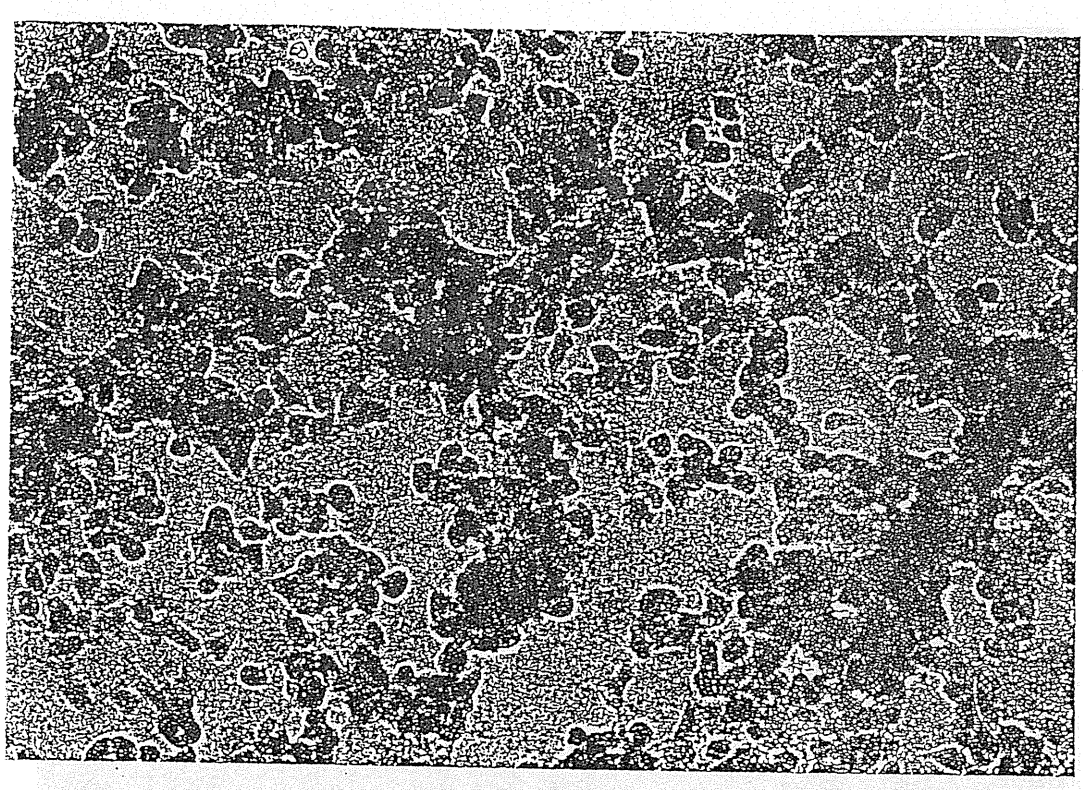


FIG. 4—*Continued*

Biologix Inc., Ontario, Canada). After a 2-week culture, the cells were removed and subjected to von Kossa staining (15).

Statistical analyses. Statistical analyses were performed with paired t-tests.

RESULTS

Morphology and TRAP activity of IL-4- or IL-13-induced MGCs in the presence of M-CSF. UG3 cells, preincubated in the presence of 100 ng/ml M-CSF for 10 days, formed MGCs with TRAP activity after a 2-week culture in IMDM with 5% FCS supplemented with combinations of M-CSF (100 ng/ml), and IL-4 (10 ng/ml) or IL-13 (10 ng/ml) (Fig. 1a or 1b, respectively). Both the MGCs induced by IL-4 and IL-13 in the presence of M-CSF were similar in morphology. PBMs also formed MGCs with TRAP activity after a 2-week culture with IL-4 or IL-13 in the presence of M-CSF (data not shown). The morphology of MGCs derived from UG3 cells and PBMs was the same. PBMs and UG3 cells did not form MGCs after incubation with IL-2 (100 U/ml), IL-7 (1 ng/ml) or IL-10 (10 ng/ml) in the presence of M-CSF (100 ng/ml) (data not shown). IL-4 (10 ng/ml) or IL-13 (10 ng/ml) induced UG3 cells and PBMs to aggregate but not to form MGCs without M-CSF, even when IL-3 (5 ng/ml) or GM-CSF (1 ng/ml) were added in the culture medium instead of M-CSF (data not shown).

Dose-dependent effects of IL-4 and IL-13. MGC formation from UG3 was maximum when the concentration of IL-4 or IL-13 was 10 ng/ml (Fig. 2). The percentage of TRAP-positive MGCs reached plateau at 5 ng/ml of IL-4 or IL-13 (Fig. 3). Based on these results, all the following experiments were conducted at the concentration of 10 ng/ml of IL-4 or IL-13.

Expression of cal-R, PTH-R and vit-R. MGCs differentiated from UG3 cells stained positive for vit-R when cultured with combinations of M-CSF (100 ng/ml) and either of IL-4 (10 ng/ml) (Fig. 4a) or IL-13 (10 ng/ml) (Fig. 4b). On the other hand, UG3 cells cultured in the presence of M-CSF (100 ng/ml) alone were negative for vit-R (Fig. 4c). Normal PBMs were also positive for vit-R when cultured with M-CSF and either of IL-4 or IL-13, but were negative when cultured with M-CSF alone (data not shown). No messenger RNA for cal-R or PTH-R was detected by RT-PCR in MGCs cultured with any of the combinations of cytokines from UG3 cells or PBMs (data not shown).

Hydroxyapatite resorption activity of MGCs. In the presence of M-CSF (100 ng/ml) and IL-4 (10 ng/ml) (Fig. 5a) or M-CSF (100 ng/ml) and IL-13 (10 ng/ml) (Fig. 5b), some of the MGCs differentiated from UG3 cells cultured in Osteologic slide flasks resorbed hydroxyapatite, while none of those cultured with M-CSF (100 ng/ml) alone did (Fig. 5c). UG3 cells cultured with IL-4 or IL-13 did not resorb hydroxyapatite in the presence of IL-3 or GM-CSF instead of M-CSF (data

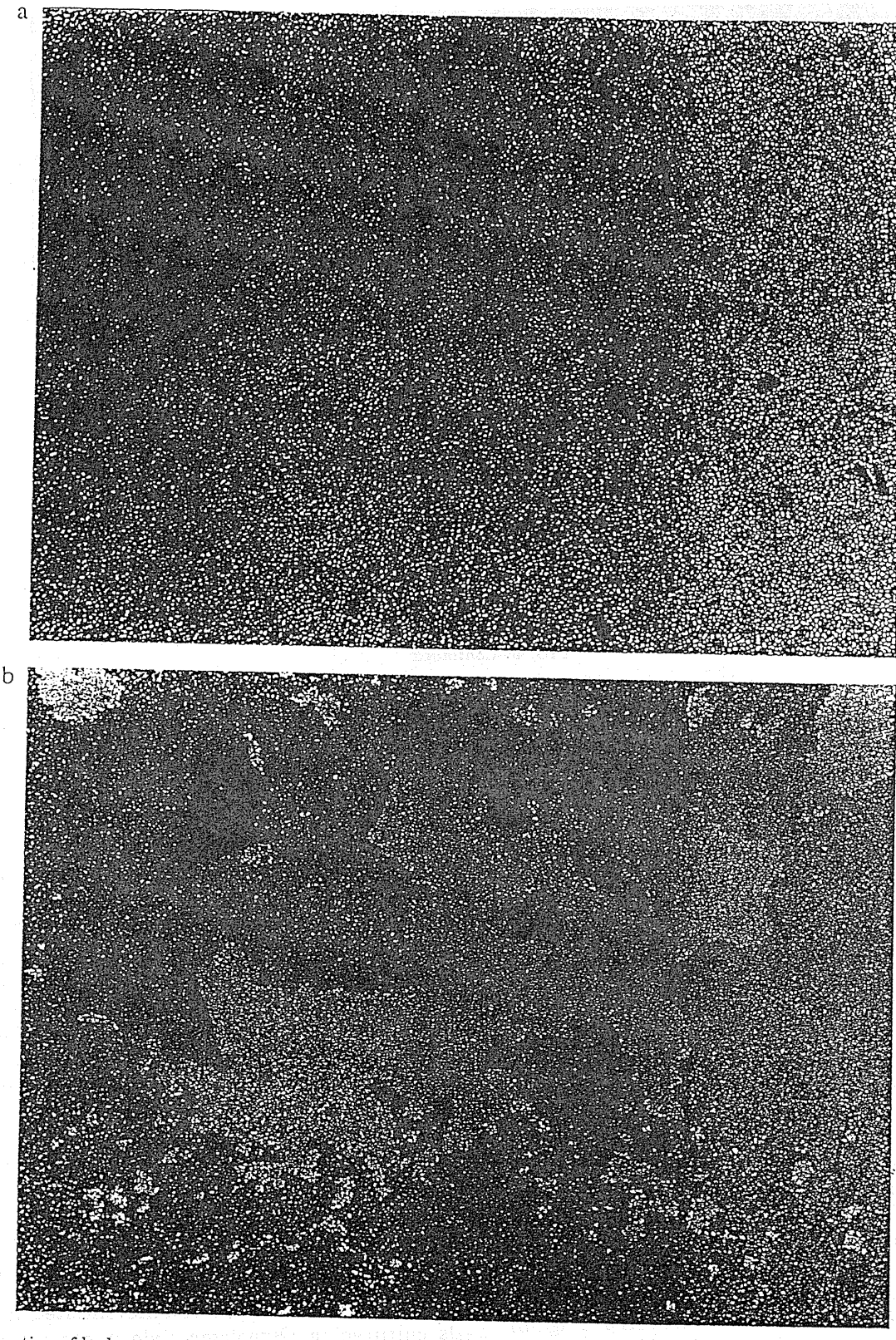


FIG. 5. Resorption of hydroxyapatite by MGCs exposed to M-CSF and IL-4 or IL-13. Hydroxyapatite was stained black by von Kossa staining. UG3 cells had pericellular clear areas resulting from hydroxyapatite resorption when cultured in the presence of (a) 100 ng/ml M-CSF and 10 ng/ml IL-4 or (b) 100 ng/ml M-CSF and 10 ng/ml IL-13, while those cultured in the presence of (c) 100 ng/ml M-CSF alone did not. Magnification: 50-fold. Analysis was performed with cells obtained from three independent experiments.

FIG. 5—*Continued*

not shown). Normal PBMs behaved in the same way as UG3 (data not shown).

DISCUSSION

In the present study, we showed that both IL-4 and IL-13 induced UG3 cells as well as PBMs to differentiate into MGCs, exclusively in the presence of M-CSF. With regard to MGC formation, some investigators reported that IL-4 alone was enough to induce formation of MGCs from rat microglia (16) and human monocytes/macrophages (17), but others reported that other growth factors were needed for MGC formation from human monocytes/macrophages (7-9). Akagawa reported that a combination of GM-CSF and IL-4 did not induce MGC formation (5), but McNally and McInnes reported that IL-3 and GM-CSF stimulate IL-4-induced MGC formation (7, 17). Recently, DeFife reported that IL-13 also induced human monocytes/macrophages to form MGCs in the absence of other growth factors (6). In the present study, both IL-4 and IL-13 induced UG3 cells and PBMs to differentiate into MGCs in the presence of M-CSF, while they did not in the presence of GM-CSF or IL-3 instead of M-CSF. Our results suggest that the presence of M-CSF is essential to IL-4- and IL-13-induced MGC formation from monocytes/macrophages. The discrepancy in the results may reflect different cell source or culture conditions.

Previous studies demonstrated that IL-2, IL-4, IL-7, IL-9, IL-13 and IL-15 share the common gamma chain of IL-2 receptor (2, 18). Interestingly, IL-2, IL-7 and IL-10 did not show activities to form MGCs from either UG3 cells or PBMs, whereas IL-4 and IL-13 did. It can be postulated that the signal transduction pathway of IL-2/IL-7 in MGC formation from monocytes/macrophages is different from that of IL-4/IL-13 as recently reported for lymphoid cells (19) and for osteoblast-like cells (20).

Intercellular adhesion molecule-1 (ICAM-1)/leukocyte function-associated antigen-1 (LFA-1) (21), macrophage mannose receptor (MMR) (23) and class II major histocompatibility complex (MHC) (24) are suspected to be essential in IL-4-induced MGC formation from monocytes/macrophages. DeFife reported that IL-13 acts independently of IL-4 on MGC formation (6). Although the alpha chain of IL-4 receptor is a component of IL-13 receptor (24), the presence of another possible IL-13 receptor component was also suggested (25). The details of IL-4- or IL-13-induced MGC formation have yet to be elucidated, and suitable models are needed to further investigate the mechanism of IL-4- or IL-13-induced MGC formation.

Expression of cal-R and/or PTH-R and bone-resorbing activity are essential characteristics to define osteoclasts (13). Because of the lack of mRNA of

cal-R or PTH-R, our MGCs induced by IL-4 or IL-13 in the presence of M-CSF could not be considered as osteoclasts. Takahashi reported that direct contact with osteoblastic cells is essential for monocytes to differentiate into osteoclasts (26), and other reports showed that IL-4 and IL-13 inhibited the formation of TRAP-positive MGCs in the culture of whole bone marrow cells or coculture of bone marrow cells with osteoblastic cells (20, 27). In our study, IL-4- and IL-13-induced MGCs displayed TRAP activity, vit-R expression and hydroxyapatite resorbing activity without coculturing with stroma cells. Furthermore, IL-4 and IL-13 enhanced TRAP-positivity of these MGCs (Fig. 2, 3). Although these IL-4- and IL-13-induced MGCs in the presence of M-CSF lack cal-R and PTH-R, and therefore are distinct from osteoclasts, these MGCs might have already been committed to osteoclasts as reported by Pandey et al (28) and Quinn et al (29). These results suggest possible initiation by IL-4 and IL-13 in osteoclasts development from monocytes/macrophages.

UG3 is the first human cell line which forms MGCs induced by IL-4 and IL-13 in the same way as normal human PBMs, and is a useful tool with which to further investigate the function and signal transduction of IL-4 and IL-13 in monocytes/macrophages including mechanisms of formation of MGCs, physiology of MGCs and the relationship between these MGCs and osteoclasts.

REFERENCES

1. de Waal Malefyt, R., Figdor, C. G., Huijbens, R., Mohan Peterson, S., Bennett, B., Culpepper, J., Dang, W., Zurawski, G., and de Vries, J. E. (1993) *J. Immunol.* 151, 6370–6381.
2. Zurawski, S. M., Vega, F., Jr., Huyghe, B., and Zurawski, G. (1993) *Embo J.* 12, 2663–2670.
3. Yano, S., Yanagawa, H., Nishioka, Y., Mukaida, N., Matsushima, K., and Sone, S. (1996) *J. Immunol.* 157, 2660–2665.
4. Kao, W. J., McNally, A. K., Hiltner, A., and Anderson, J. M. (1995) *J. Biomed. Mater. Res.* 29, 1267–1275.
5. Akagawa, K. S., Takasuka, N., Nozaki, Y., Komuro, I., Azuma, M., Ueda, M., Naito, M., and Takahashi, K. (1996) *Blood* 88, 4029–4039.
6. DeFife, K. M., Jenney, C. R., McNally, A. K., Colton, E., and Anderson, J. M. (1997) *J. Immunol.* 158, 3385–3390.
7. McInnes, A., and Rennick, D. M. (1988) *J. Exp. Med.* 167, 598–611.
8. Takashima, T., Ohnishi, K., Tsuyuguchi, I., and Kishimoto, S. (1993) *J. Immunol.* 150, 3002–3010.
9. Ikeda, T., Sasaki, K., Ikeda, K., Yamaoka, G., Kawanishi, K., Kawachi, Y., Uchida, T., Takahara, J., and Irino, S. (1998) *Blood* 91, 4543–4553.
10. McKenzie, A. N., Culpepper, J. A., de Waal Malefyt, R., Briere, F., Punnonen, J., Aversa, G., Sato, A., Dang, W., Cocks, B. G., Menon, S., de Vries, J. E., Banchereau, J., and Zurawski, G. (1993) *Proc. Natl. Acad. Sci. USA* 90, 3735–3739.
11. Takahashi, N., Udagawa, N., Tanaka, S., Murakami, H., Owan, I., Tamura, T., and Suda, T. (1994) *Dev. Biol.* 163, 212–221.
12. Zhao, Q., Topham, N., Anderson, J. M., Hiltner, A., Lodoen, G., and Payet, C. R. (1991) *J. Biomed. Mater. Res.* 25, 177–183.
13. Suda, T., Udagawa, N., Nakamura, I., Miyaura, C., and Takahashi, N. (1995) *Bone* 17, 87s–91s.
14. Chambers, T. J., and Spector, W. G. (1982) *Immunobiology* 161, 283–289.
15. Von Kossa, J. (1901) *Beitr. Anat.* 29, 163.
16. Lee, T. T., Martin, F. C., and Merrill, J. E. (1993) *Glia* 8, 51–61.
17. McNally, A. K., and Anderson, J. M. (1995) *Am. J. Pathol.* 147, 1487–1499.
18. Russell, S. M., Keegan, A. D., Harada, N., Nakamura, Y., Noguchi, M., Leland, P., Friedmann, M. C., Miyajima, A., Puri, R. K., Paul, W. E., and Leonard, W. J. (1993) *Science* 262, 1880–1883.
19. Smerz Bertling, C., and Duschl, A. (1995) *J. Biol. Chem.* 270, 966–970.
20. Onoe, Y., Miyaura, C., Kaminakayashiki, T., Nagai, Y., Noguchi, K., Chen, Q. R., Seo, H., Ohta, H., Nozawa, S., Kudo, I., and Suda, T. (1996) *J. Immunol.* 156, 758–764.
21. Kazazi, F., Chang, J., Lopez, A., Vadas, M., and Cunningham, A. L. (1994) *J. Gen. Virol.* 75, 2795–2802.
22. Stein, M., Keshav, S., Harris, N., and Gordon, S. (1992) *J. Exp. Med.* 176, 287–292.
23. Orentas, R. J., Reinlib, L., and Hildreth, J. E. (1992) *J. Leukocyte Biol.* 51, 199–209.
24. Hilton, D. J., Zhang, J. G., Metcalf, D., Alexander, W. S., Nicola, N. A., and Willson, T. A. (1996) *Proc. Natl. Acad. Sci. USA* 93, 497–501.
25. Vita, N., Lefort, S., Laurent, P., Caput, D., and Ferrara, P. (1995) *J. Biol. Chem.* 270, 3512–3517.
26. Takahashi, N., Akatsu, T., Udagawa, N., Sasaki, T., Yamaguchi, A., Moseley, J. M., Martin, T. J., and Suda, T. (1988) *Endocrinology* 123, 2600–2602.
27. Riancho, J. A., Zarrabeitia, M. T., Mundy, G. R., Yoneda, T., and Gonzalez Macias, J. (1993) *J. Bone Miner. Res.* 8, 1337–1344.
28. Pandey, R., Quinn, J., Joyner, C., Murray, D. W., Triffitt, J. T., and Athanasou, N. A. (1996) *Ann. Rheum. Dis.* 55, 388–395.
29. Quinn, J. M., Sabokbar, A., and Athanasou, N. A. (1996) *J. Pathol.* 179, 106–111.

RAPID COMMUNICATION

Activated Endothelial Cells Induce Apoptosis in Leukemic Cells by Endothelial Interleukin-8

By Yasuhito Terui, Masayuki Ikeda, Hiroshi Tomizuka, Tadashi Kasahara, Tetsuya Ohtsuki, Masaya Uwai, Masaki Mori, Takehito Itoh, Miyuki Tanaka, Muneo Yamada, Seiichi Shimamura, Yukihito Ishizaka, Kazuma Ikeda, Keiya Ozawa, Yasusada Miura, and Kiyohiko Hatake

Tumor cells are eradicated by several systems, including Fas ligand-Fas and tumor necrosis factor (TNF)-tumor necrosis factor receptor (TNFR). In the previous study, we purified an apoptosis-inducing factor (AIF) to homogeneity from a medium conditioned by PDBu-treated HL-60 cells. N-terminal sequence analysis showed that AIF is identical to endothelial interleukin-8 (IL-8). A novel apoptosis system, in which endothelial cells participate via endothelial IL-8 release, is identified here. Human umbilical vein cells (VE cells) produce and secrete IL-8 by stimulation of IL-1α and TNF-α. Endothelial IL-8, which is secreted from VE cells by stimulation of IL-1α and TNF-α, induces apoptosis in myelogenous leukemia cell line K562 cells. Monocyte-derived IL-8 could not induce apoptosis in K562 cells. Moreover, interaction between VE cells and K562 cells induces the release of endothelial IL-8 from VE cells, and the attached K562 cells undergo apoptosis. Moreover, interactions between VE cell and other cell lines, such as HL-60, U937, Jurkat, and Daudi, induce the secretion of endothelial IL-8 and the induction of apoptosis in cell lines. Endothelial IL-8 significantly inhibits tumor growth of intraperitoneal and subcutaneous tumor mass of K562 cells and induces apoptosis in their cells in vivo. Endothelial IL-8 plays an important role in apoptosis involving endothelial cells, which may provide us with a new therapy for hematological malignancies.

APOPTOSIS IS AN ACTIVE process in which various types of cells are selectively deleted during embryonic development and in the adult multicellular organism under certain physiologic conditions.[1] Cell death occurs during the development and regulation of the immune system, leading to deletion of self-reactive T and B lymphocytes, regulation of immunologic memory, and lysis of target cells by cytotoxic T lymphocytes and natural killer (NK) cells.[2-4] Alteration of the genes that control apoptosis may lead to a variety of diseases such as autoimmune, malignant clonal growth, neurodegenerative diseases, as well as prolonged survival of cells during latent viral infection.[5] Cells with induced apoptosis appear shrunken, with condensed or fragmented nuclei. Apoptotic cells have fragmented DNA, with DNA ladders in electrophoresis.[6,7]

Tumor cells have systems to protect themselves from induction of apoptosis in various physiologic conditions.[8-10] Alteration of bcl-2 or bcl-x, well-known as antiapoptosis genes, is observed in many tumor cells in leukemia, lymphoma, and other types of cancers.[11,12] Overexpression of these genes in cancer cells leads to partial or complete resistance to several conditions such as the presence of chemotherapeutic agents and irradiation.[13,14] Leukemic cells are known to undergo apoptosis under several conditions, and the basic strategy of leukemia therapy is the induction of apoptosis.[15] Chemotherapeutic agents such as etoposide and all-*trans* retinoic acid can induce apoptosis, killing leukemic cells.[15] Because cytosine arabinoside can reduce expression of bcl-2 or bcl-x in leukemic cells, their lifespan is shortened by induction of apoptosis.[16,17]

Some mechanisms induce apoptosis against tumor cells and leukemic cells, removing them from the body. Fas ligand and TNF-α, which physiologically exist in the body, are also inducers of apoptosis in leukemic cells and other cells.[18,19] It has been reported that signals for some cytokines might select induction of apoptosis or proliferation in target cells,[20] but no cytokine was able to induce apoptosis in leukemic cells and other tumor cells. Moreover, tumor cells are directly attacked by cytotoxic T cells, NK cells, and macrophages, inducing apoptosis.[21,22] For example, cytotoxic T cells and NK cells attach to tumor cells and release perforin. A serine protease, granzyme B, is transferred into the cytosol of the target cells, and then apoptosis is induced.[23,24] It has been reported that endothelial cells are also associated with inhibition of tumor cell invasion,[25] but the mechanism is not clear.

The human myelogenous leukemia cell line HL-60[26] can be induced to differentiate into monocyte/macrophage lineage by phorbol esters, undergoing apoptosis.[27] In the previous study, we have purified an apoptosis-inducing factor (AIF) to homogene ity from a medium conditioned by PDBu-treated HL-60 cells.[28] N-terminal sequence analysis showed that AIF is identical to endothelial interleukin-8 (IL-8). Human recombinant endothelial IL-8 induces apoptosis in most leukemic cell lines, such as K562, HL-60, Jurkat, KG-1, U937, and THP-1, but monocyte-derived IL-8 does not. Endothelial IL-8, which has added five amino acids to the N terminus of monocyte-derived IL-8, is active in the induction of apoptosis, but its action against tumor cells in vivo has never been clarified.

To investigate the biological significance of endothelial IL-8,

From the Division of Hematology, Department of Internal Medicine, Jichi Medical School, Kawachi-gun, Tochigi, Japan; the Biochemical Research Laboratory, Morinaga Milk Industry Co Ltd, Zama City, Kanagawa, Japan; and the Department of Biochemistry, Kyoritsu College of Pharmacy, Minato-ku, Tokyo, Japan.

Submitted May 18, 1998; accepted July 28, 1998.

Supported by a grant-in-aid from the Ministry of Education, Science and Culture of Japan; the Research on Advanced Medical Technology; the Japanese Foundation for Multidisciplinary Treatment of Cancer; and Jichi Medical School Young Investigator Award.

Address reprint requests to Kiyohiko Hatake, MD, PhD, Division of Hematology, Department of Internal Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-machi, Kawachi-gun, Tochigi 329-04, Japan; e-mail: kiyohiko@jichi.ac.jp.

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. section 1734 solely to indicate this fact.

0006-4971/98/9208-005$3.00/0

we observed the antitumor effect of endothelial cells in vitro and the antitumor effect of endothelial IL-8 in vivo.

MATERIALS AND METHODS

Reagents. Lipopolysaccharide (LPS) was obtained from Sigma (St Louis, MO). Recombinant human IL-1α, recombinant human tumor necrosis factor-α (TNF-α), recombinant human interferon-γ (IFN-γ), and antihuman monoclonal IL-8 antibody were purchased from R & D Systems Inc (Minneapolis, MN). Recombinant human endothelial and monocyte-derived IL-8 were purchased from Genzyme (Cambridge, MA).

Cell lines and cell culture. A human chronic myelogenous leukemia cell line, K562,[29] was obtained from ATCC (Rockville, MD) and was maintained in GIT medium (Wako, Tokyo, Japan).[30] Human myelogenous leukemic cell line HL-60, human monocytic leukemia cell line U937, human T-cell leukemia cell line Jurkat, and human myeloma cell line Daudi were also obtained from ATCC. Human umbilical venous cells (VE cells) were purchased from Cell Systems Co (Kirkland, WA) and were maintained in CS-C serum-free medium. Normal human monocytes were isolated in a nascent state from the peripheral blood of healthy volunteers, as described previously.[31] In brief, collected mononuclear cells were resuspended in phosphate-buffered saline (PBS) and then incubated onto an MSP-P plate (JIMRO, Gunma, Japan) for 1 hour at 37°C. Adherent cells (monocyte; >90% purity) were collected and were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc, Gaithersburg, MD). Monocytes (1×10^6 cells/mL) and human umbilical venous cells (VE cells, confluent) were cultured with or without LPS (500 ng/mL), IL-1α (10 U/mL), TNF-α (10 ng/mL), IFN-γ (200 U/mL), and macrophage colony-stimulating factor (M-CSF; 100 ng/mL) in 24-well plates. Monocytes or VE cells was also cocultured with K562 cells with or without the factors listed above in 24-well plates with culture chambers (Intercell (Kurabo, Osaka).

Detection of IL-8. For detection of IL-8 in culture media, an enzyme-linked immunosorbent assay (ELISA) system for human IL-8 (Amersham, Arlington Heights, IL) was used.[32] In brief, endothelial cells or monocytes were cultured with various factors and the supernatants were collected by centrifugation at 15,000 rpm after 2 days. Samples were then applied to the wells of a microtiter plate coated with a specific monoclonal antibody for IL-8. After washing away any unbound sample proteins, an enzyme-linked polyclonal antibody specific for IL-8 was added to the wells and allowed to bind to the IL-8 that was bound during the incubation. After a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color-developed in proportion to the amount of IL-8. Absorbance at 450 nm was determined by spectrophotometer (Bio-Rad, Hercules, CA). For detection of intracytoplasmic IL-8 in endothelial cells cultured with or without K562 cells, immunofluorescent staining for intracellular IL-8 (CytoStain kit; PharMingen, San Diego, CA) was performed. In brief, endothelial cells cultured with or without K562 cells for 3 days were fixed and permeabilized by Cytofix/Cytoperm solution and Perm/Wash solution. After washing, fixed/permeabilized cells were stained by fluorochrome-conjugated antihuman IL-8 antibody. After washing, flow cytometric analysis was performed by FACScan (Becton Dickinson, Mountain View, CA).

MTT assay. K562 cells were seeded at 1×10^5 cells/mL onto confluent human umbilical venous cells in CS-C medium. After 3 days, K562 cells were collected and the ca pacity to reduce 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenylte trazolium bromide (MTT; Sigma) was determined.[33] After adding 10 μL of MTT solution (5 mg/mL MTT in PBS), the preparation was incubated at 37°C for 4 hours. Cells with MTT for mazan were dissolved in 0.04 N HCl in 2-propanol, and color absorbance was measured at 595 nm by microplate reader (Bio-Rad).

TUNEL assay. Additionally, residual cells were incubated with digoxigenin-dUTP terminaldeoxynucleotidyl transferase mixture and subsequently were stained with peroxidase-conjugated antibody to digoxigenin (Apop Tag PLUS; Oncor, Gaithersburg, MD),[34] counterstained with 1% methyl green in sodium acetate (pH 4.0), and mounted. Specimens were examined and photographed with a microscope. The percentage of apoptotic cells was determined by microscopically counting more than 200 cells. Statistical analysis was performed using the Student's *t*-test.

In vivo experiments. Male Balb/c nu/nu mice were purchased from Japan Charles River and were age-matched (5 weeks of age) at the onset of each experiment. Nude mice were inoculated with 10^6 K562 cells into the peritoneal space. Intraperitoneal tumor masses of K562 cells were injected daily with recombinant human endothelial IL-8 or monocyte-derived IL-8 for 2 days. As a control, saline was injected. Intraperitoneal cells were collected daily by washing with 5 mL PBS. Cell counting, Wright-Giemsa staining, and TUNEL assay were also performed. Mice were injected with 5×10^5 viable K562 cells by subcutaneous injection in a midline ventral position in a total volume of 0.1 mL PBS. Test mice bearing subcutaneously established K562 tumors (confirmed after 4 days of inoculation) were injected daily (for 11 days) into tumor with endothelial IL-8 or monocyte-derived IL-8 in a total volume of 0.1 mL saline. As a control, saline and TNF-α were injected. Tumor size was calculated using the formula described by Kyriazis et al,[35] as follows: tumor volume = width^2 × length × 0.4.

Tumors were resected in toto, fixed in 10% neutral formalin solution (Sigma), embedded in paraffin, sectioned at 4 mm, and stained with hematoxylin and eosin or with TUNEL assay. The injected dose of IL-8 was 100 ng per mouse per day. The dose of TNF-α was 200 U per mouse. Statistical analysis was performed using the Student's *t*-test.

RESULTS

Secretion of IL-8 from monocytes and endothelial cells by various factors. IL-8 takes several forms of various lengths that are produced by monocytes and endothelial cells. To examine by which factors monocytes and endothelial cells are induced to produce IL-8, possible inducers such as LPS, IL-1α, TNF-α, IFN-γ, and M-CSF were added to culture media of monocytes and endothelial cells (Fig 1). Concentrations of IL-8 in medium were then measured by an ELISA system after 2 days. When monocytes were stimulated with LPS, TNF-α, IFN-γ, and M-CSF, concentrations of IL-8 in culture medium significantly increased from 7.7 ± 1.2 ng/mL to 56.7 ± 4.5 ng/mL, 30.8 ± 3.6 ng/mL, 26.9 ± 3.0 ng/mL, and 19.4 ± 2.5 ng/mL, respectively. On the other hand, when LPS, IL-1α, and TNF-α were added to culture media of endothelial cells, concentrations of IL-8 in culture medium increased from 1.1 ± 1.0 ng/mL to 12.8 ± 2.3 ng/mL, 70.1 ± 10.6 ng/mL, and 61.5 ± 7.0 ng/mL, respectively.

Secretion of IL-8 from endothelial cells by attachment to K562 cells. To investigate the effect of interaction between monocytes or endothelial cells and K562 cells on secretion of IL-8, monocytes or human umbilical endothelial cells (VE cells) were cultured with or without human leukemic cell line K562 cells for 2 days (Fig 2A). Concentrations of IL-8 in conditioned media did not increase significantly when monocytes were cultured with K562 cells. When VE cells were cultured with K562 cells, the concentration of IL-8 in culture medium increased from 2.1 ± 1.1 ng/mL to 75.1 ± 5.8 ng/mL as compared with that in culture medium of only VE cells. IL-8 in medium of K562 cells alone was 0.6 ± 0.3 ng/mL. Moreover, we observed a change in intracytoplasmic IL-8 of VE cells

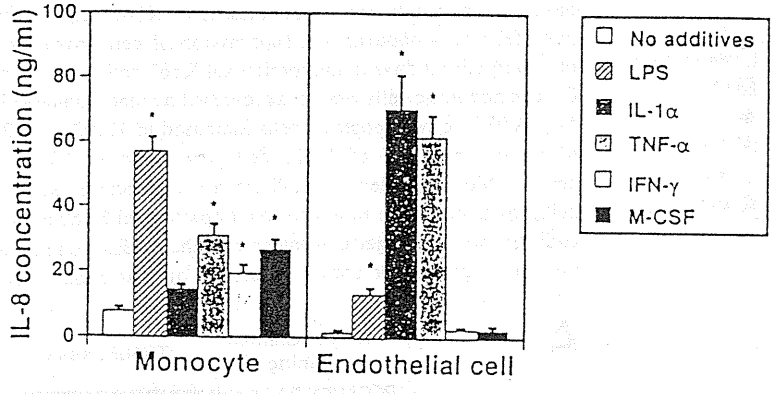


Fig 1. Effect of various cytokines on production of IL-8 from monocytes and endothelial cells. Monocytes (1 × 10^6 cells/mL) and human umbilical venous cells (VE cells, confluent) were cultured with or without LPS (500 ng/mL), IL-1α (10 U/mL), TNF-α (10 ng/mL), IFN-γ (200 U/mL), and M-CSF (100 ng/mL) in 24-well plates. After 2 days, supernatants were collected and an ELISA system for IL-8 was performed. Columns represent the mean ± SD (bar) of three independent experiments. Statistical analysis was performed using the Student's *t*-test. $*P < .01$.

during interaction between VE cells and K562 cells using immunofluorescent staining by flow cytometric analysis to examine whether IL-8 is produced in VE cells (Fig 2B). The percentage of intracellular IL-8–positive VE cells was 22.7% ± 5.3% during culture of VE cells alone. When VE cells were cocultured with K562 cells for 2 days, the percentage of intracellular IL-8–positive VE cells increased to 55.5% ± 7.0%. These results suggest that VE cells can produce and secrete IL-8 during interaction with K562 cells but that monocytes cannot. Moreover, we examined concentrations of IL-8 when other cell lines such as HL-60, U937, Jurkat, and Daudi were cocultured with VE cells. When VE cells were cultured with HL-60, U937, Jurkat, or Daudi cells, concentration of IL-8 in culture medium increased from 2.1 ± 1.1 ng/mL to 74.2 ± 2.0 ng/mL, 71.2 ± 2.4 ng/mL, 48.1 ± 3.3 ng/mL, or 44.6 ± 3.9 ng/mL as compared with that in culture medium of only VE cells, respectively.

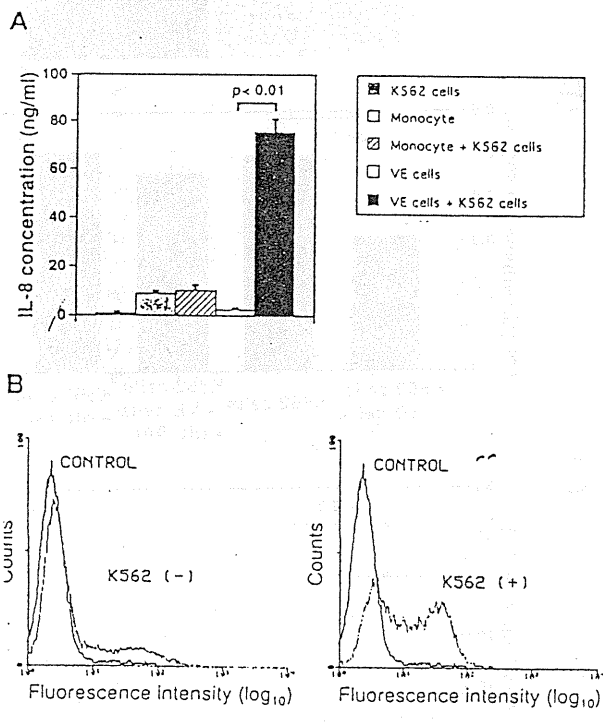


Fig 2. Effect of interaction between either monocytes or endothelial cells and K562 cells on secretion of IL-8 from endothelial cells. Monocytes (1 × 10^6 cells/mL) and human umbilical venous cells (VE cells, confluent) were cultured with or without K562 cells (5 × 10^4 cells/mL) for 2 days, and supernatants were then collected. (A) ELISA system for IL-8 was performed. Columns show the means of three independent experiments. Statistical analysis was performed using the Student's *t*-test. (B) When VE cells were cocultured with (right) or without (left) K562 cells, the remaining VE cells, after removal of K562 cells, were collected and expression of intracytoplasmic IL-8 was examined as described in Materials and Methods. Purified mouse Ig G1 was used as a control antibody (CONTROL). The vertical axis indicates the frequency of fluorescence-positive cells. Intracytoplasmic IL-8 density is depicted on the horizontal axis. The representative data from three independent experiments are shown.

Endothelial IL-8 secreted by various factors induces apoptosis in K562 cells. To examine whether IL-8 secreted from endothelial cells by several factors induces apoptosis in K562 cells, K562 cells in Intercell-insert were separately cultured with VE cells stimulated with various factors. K562 cells in Intercell were collected after 2 days, and MTT and TUNEL assays were performed (Fig 3). When K562 cells were cultured with various factors such as LPS, IL-1α, TNF-α, IFN-γ, and M-CSF, growth of K562 cells was not suppressed as measured by MTT assay (Fig 3A). However, when endothelial cells were cultured with IL-1α or TNF-α, growth of K562 cells was significantly suppressed to 74.2% or 74.6% as compared with control (no additives), respectively (Fig 3A). Moreover, when VE cells were cultured with IL-1α or TNF-α, the percentage of apoptotic cells in K562 cells increased from 1.2% ± 0.1% to 25.6% ± 2.5% or 25.3% ± 1.7%, respectively (Fig 3B). This effect was blocked by monoclonal anti–IL-8 antibody (data not shown). Our interpretation was that interaction between endothelial cells and K562 cells during separate culture by Intercell could neither inhibit cell growth nor induce apoptosis against K562 cells (Fig 3A and B). This suggests that endothelial IL-8, secreted from endothelial cells by various factors, induces apoptosis in K562 cells.

Interaction between endothelial cells and K562 cells induces apoptosis in K562 cells. To identify the biological significance of endothelial IL-8, we examined the effect of interaction between normal human venous endothelial cells (VE cells) and K562 cells on the inhibition of cell growth and the induction of apoptosis (Fig 4). When K562 cells were attached onto VE cells, growth of K562 cells was suppressed at 62.0% as compared with K562 cell culture alone (Fig 4A and B), and 32.0% ± 3.1% of K562 cells underwent apoptosis (Fig 4A and C). Because the concentration of IL-8 in coculture medium of

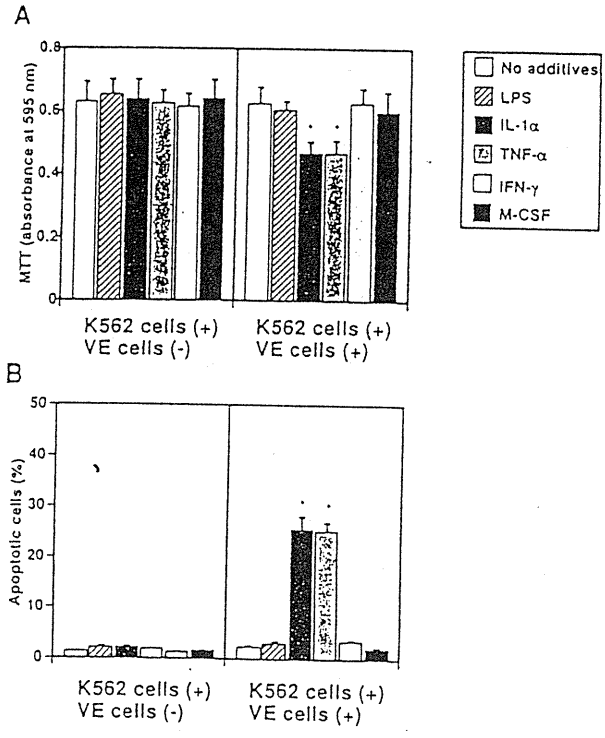


Fig 3. Effect of IL-8 from endothelial cells on induction of apoptosis in K562 cells. K562 cells alone (5 × 10^4 cells/mL) or human umbilical venous cells (VE cells, confluent) and K562 cells (5 × 10^4 cells/mL) were cultured with or without LPS (500 ng/mL), IL-1α (10 U/mL), TNF-α (10 U/mL), IFN-γ (200 U/mL), and M-CSF (100 ng/mL) in 24-well plates. Coculture of VE cells and K562 cells was mediated by Intercell. After 2 days, MTT assay (A) and TUNEL assay (B) were performed. Columns show the means of three independent experiments. Statistical analysis was performed using the Student's *t*-test. $^*P < .01$.

VE cells and K562 cells increased to 75.1 ng/mL from 2.1 ng/mL in medium of VE cells alone (Fig 2), we examined whether the effects of the interaction between VE cells and K562 cells on cell growth inhibition and apoptosis are blocked by anti–IL-8 antibody. When anti–IL-8 antibody was added to coculture media of VE cells and K562 cells, MTT reducing activity recovered to that of K562 cell culture alone (Fig 4B). Moreover, the percentage of apoptotic cells decreased to 7.8% ± 0.6% when anti–IL-8 antibody was added to coculture media of VE cells and K562 cells (Fig 4C). This result suggests that the inhibition of K562 cell growth and the induction of apoptosis were blocked by anti–IL-8 antibody. Western blot analysis also showed that endothelial IL-8 was detected in the culture medium of VE cells and K562 cells (data not shown). Moreover, when HL-60 cells, U937 cells, Jurkat cells, or Daudi cells were attached onto VE cells, 34.7% ± 3.1%, 31.0% ± 3.6%, 24.3% ± 4.0%, or 20.7% ± 3.1% of cells underwent apoptosis, respectively. These findings indicate that endothelial cells can induce apoptosis in the attached K562 cells or other cell lines by releasing endothelial IL-8.

Endothelial IL-8 suppresses cell growth of K562 cells and induces apoptosis in vivo. Therefore, we investigated whether endothelial IL-8 can induce apoptosis or suppress cell growth in leukemic cells in vivo. Endothelial IL-8 was injected daily for 2 days into intraperitoneal tumor masses of K562 cells in nude mice (Fig 5). Apoptosis and suppression of cell growth were observed after 2 days in intraperitoneal K562 cells (Fig 5B and C), and apoptotic cells were phagocytosed by macrophages (Fig 5A). After 2 days, apoptotic cells increased to 12.8% ± 2.2%. Moreover, numbers of K562 cells decreased to 33.3% of control. Monocyte-derived IL-8 did not significantly suppress cell growth or induce apoptosis in intraperitoneal K562 cells. In addition, we investigated whether endothelial IL-8 could suppress cell growth or induce apoptosis against subcutaneous

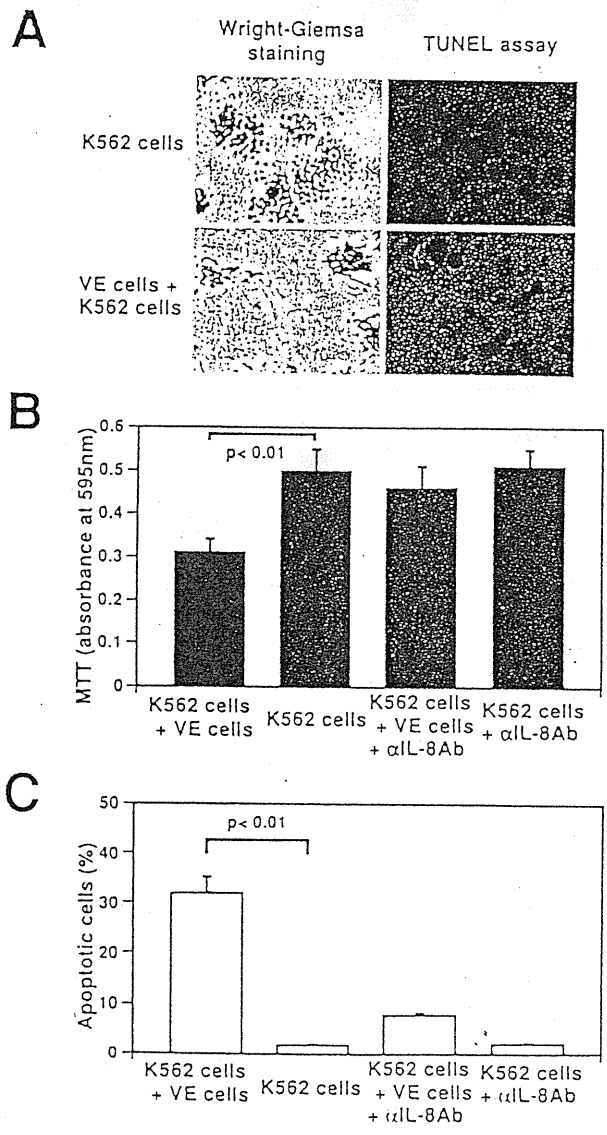


Fig 4. Effect of interaction between human venous endothelial cells (VE cells) and K562 cells on secretion of endothelial IL-8 and apoptosis. K562 cells (5 × 10^4 cells) were seeded with or without confluent VE cells in 24-well plates. Cell culture was boosted with or without anti–IL-8 antibody (5 μg/mL) every 24 hours. After 2 days, Wright-Giemsa staining was performed in 24-well plates (A). K562 cells and supernatants were collected, and MTT assay (B), TUNEL assay (C), and ELISA for IL-8 were performed. For (A), original magnification × 60. Data shown come from three independent experiments. Statistical analysis was performed using the Student's *t*-test.

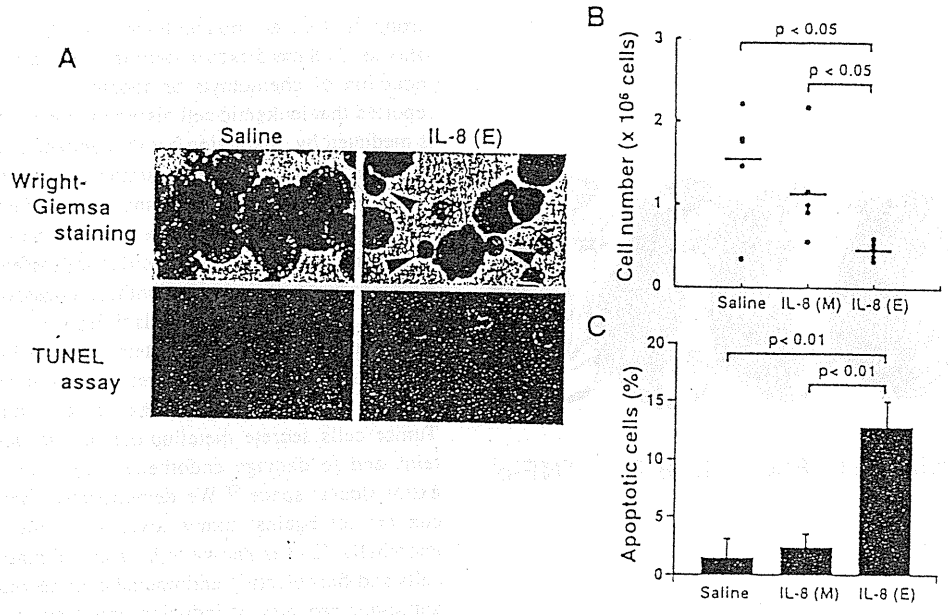


Fig 5. Inhibition of cell growth and induction of apoptosis on treatment of endothelial IL-8 in nude mice. The peritoneal space of nude mice (5 examined) were inoculated with K562 cells, and agents such as saline, endothelial IL-8 (IL-8 [E]), and monocyte-derived IL-8 (IL-8 [M]) were injected daily as described in Materials and Methods. (A) Morphology (Wright-Giemsa staining) and apoptotic cells (TUNEL assay) of intraperitoneal cells were collected. Arrows indicate the apoptotic cells. Original magnification × 160 and × 80. (B) Inhibition of cell growth of K562 cells by endothelial IL-8. Horizontal bars show the means. (C) The percentage of apoptotic cells was determined microscopically by counting more than 200 cells in situ on staining slides. Columns represent the means ± SD (bar) of three independent experiments. Statistical analysis was performed using the Student's *t*-test.

K562 cells (Fig 6). Endothelial IL-8 was then injected daily from day 4 to day 11 into subcutaneous K562 cell tumors established in nude mice, and the antitumor and the apoptosis-inducible effects of endothelial IL-8 were examined. Mice showed a visible response to endothelial IL-8 intratumor inoculations characterized by apoptosis (Fig 6A). Therefore, tumor size of endothelial IL-8 treatment decreased in 52.2% of control (saline; Fig 6B). Tumor size of TNF-α treatment decreased in 41.3% of control (Fig 6B). On the other hand, monocyte-derived IL-8 treatment did not either induce apoptosis or suppress cell growth against subcutaneous K562 cells (Fig 6).

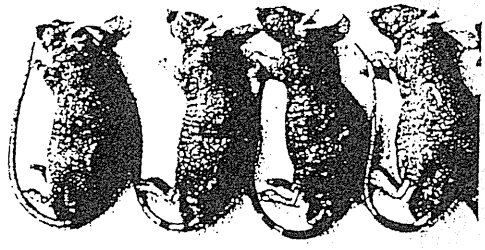
Antitumor effect of endothelial IL-8 is due to induction of apoptosis in K562 cells. To investigate whether the antitumor effect of endothelial IL-8 is due to induction of apoptosis, pathological examination was performed by hematoxylin-eosin staining and TUNEL staining (Fig 7). Histologically, subcutaneous K562 tumors that responded to either endothelial IL-8 or TNF-α (10 mice of each were examined) generally displayed homogenous central necrosis with intratumor bleeding (Fig 7H and K). Within the viable tumor tissue, many tumor cells became smaller than control cells and showed either condensation or fragmentation of nuclei (Fig 7B and H). Neutrophil and lymphocyte infiltrations were unremarkable in both groups. Control K562 tumors (saline- and monocyte-derived IL-8 groups) displayed little or no tumor necrosis (Fig 7A and D), and their cells had no change in cell size or nuclei (Fig 7B and E). Tumor sections were stained with TUNEL assay specific for apoptotic cells. TUNEL assay showed that in 45.2% or 47.8% of K562 tumor cells inoculated with either endothelial IL-8 or TNF-α, apoptosis was induced (Fig 7I and L). However, control tumor cells showed little apoptosis (Fig 7C and F).

DISCUSSION

In this study, we observed that endothelial cells were able to secrete endothelial IL-8 after stimulation with IL-1α and TNF-α and that endothelial IL-8 was able to induce apoptosis in leukemic cells. Moreover, endothelial cells that attached to leukemic cells secreted IL-8, and endothelial IL-8 induced apoptosis in leukemic cells. In the previous study, we purified an apoptosis-inducing factor derived from differentiated HL-60 cells.[28] This apoptosis-inducing factor is identical to endothelial IL-8. Human recombinant endothelial IL-8 is able to induce apoptosis in most leukemic cell lines, but monocyte-derived IL-8 is not.

IL-8 was originally isolated from culture supernatants of stimulated human monocytes and identified as a protein of 72 amino acids.[36,37] The open reading frame of the IL-8 cDNA encodes for 99 amino acids,[38] and the mature form is processed further at the N terminus, yielding several biologically active truncation analogs.[39-41] The occurrence of N-terminal variants depends on cell type and culture conditions. Of the two major forms, the 72-amino acid form (monocyte-derived IL-8; SAKELRC. . .) predominates in cultures of monocytes and macrophages,[40] and the 77-amino acid form (endothelial IL-8; AVLPRSAKELRC. . .) prodominates in cultures of tissue cells such as endothelial cells[42] and fibroblasts.[43] Endothelial IL-8 has five extra N-terminal amino acids lacking in monocyte-derived IL-8. Because endothelial IL-8 is converted to monocyte-derived IL-8 by serine proteases such as thrombin,[44] monocyte-

A



Saline IL-8(M) IL-8(E) TNF-α

B

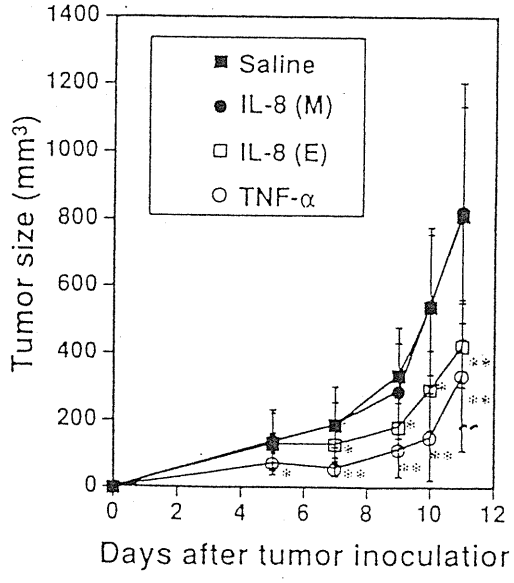


Fig 6. Endothelial IL-8 suppressed growth of subcutaneous K562 cell tumors. The subepiderms of nude mice (10 examined per group) were inoculated K562 cells, and agents were injected with endothelial IL-8 (IL-8 [E]), monocyte-derived IL-8 (IL-8 [M]), saline, and TNF-α daily as described in Materials and Methods. As controls, monocyte-derived IL-8, saline, and TNF-α were used. Data shown come from 10 nude mice. Statistical analysis was performed using the Student's *t*-test. In (B), $*P < .05$ and $**P < .005$. (A) Photograph of suppression of subcutaneous K562 tumor by endothelial IL-8 (IL-8 [E]). As controls, saline, monocyte-derived IL-8 (IL-8 [M]), and TNF-α were used.

derived IL-8 exists mainly in plasma.[45] Many previous reports have demonstrated that monocyte-derived IL-8 is an inflammatory chemoattractant for neutrophils and that several types of cells, such as monocytes, lymphocytes, and fibroblasts, produce IL-8 after stimulation with various agents.[46] On the other hand, the chemotactic activity of endothelial IL-8 is one twentieth as strong as that of monocyte-derived IL-8. The difference of whether IL-8 has 5 extra N-terminal amino acids may decide the induction of chemotaxis or apoptosis. Moreover, it has been reported that leukemic cell dissemination to extravascular space is mediated by interaction between leukemic cells and endothelial cells.[47] Our study demonstrated that endothelial IL-8 plays an important role in the antitumor action of endothelial cells.

Recently, it has been demonstrated that vascular cells are important participants in antitumor host defense. Vascular cells, which express nitric oxide synthase in response to IFN-γ and TNF-α, can kill leukemic cells.[48] However, the mechanism of the antitumor host defense system associated with vascular cells has not been clarified. Many researchers have discussed angiogenesis for tumor vascularization and tumor metastasis.[49] Tumor cells secrete metalloproteinase to destroy matrix proteins and to damage endothelial cells, and then they invade extravascular space.[50] We demonstrated that endothelial IL-8 can protect against tumor invasion in this system. Because endothelial IL-8 is known to be secreted mainly by endothelial cells and fibroblasts,[51] endothelial cells secreting IL-8 have the antitumor property of inducing apoptosis in contacting leukemic cells like other antitumor cells, such as macrophages and NK cells. This is a novel function of endothelial cells involving tumor cell eradication in the body, mainly via endothelial IL-8. Endothelial IL-8 may have an important role in induction of apoptosis in tumor cells in the blood stream when they anchor to endothelial cells.

Surprisingly, in our in vivo experiment, endothelial IL-8 inhibited growth of K562 cell tumors in the same manner as TNF-α. We demonstrated that TNF-α did not directly suppress cell growth or induce apoptosis in K562 cells, but that TNF-α allows endothelial cells to secrete IL-8 and can indirectly kill leukemic cells. It has been reported that TNF-α modulates expression of various biological molecules in endothelial cells.[52] The in vivo effect of TNF-α on killing tumor cells may be explained by both a direct death signal mediated through its receptor and the indirect release of biological modulators such as endothelial IL-8 from endothelial cells. Therefore, it is possible that the antitumor effect of TNF-α in vivo may be mediated by secretion of endothelial IL-8 from intratumor endothelial cells. However, angiogenesis for tumor vascularization will aid tumor progression, and tumor cells produce and secrete some endogenous regulators such as endostatin and angiostatin.[53,54] In this study, injected TNF-α acted on endothelial cells growing in tumors, and endothelial IL-8 might then be released from endothelial cells. There are two possible roles of IL-8 from endothelial cells in host defense against tumor cells. First, endothelial cells will secrete endothelial IL-8 when tumor cells in the vessels contact endothelial cells, and then the anchored tumor cells may be directly killed by endothelial IL-8. Second, when the growing endothelial cells in tumors are stimulated with some cytokines such as TNF-α, they will secrete endothelial IL-8 and induce apoptosis.

If we know how to stimulate release or production of endothelial IL-8, we may be able to develop new methods of treatment. In 10 of 14 clinical cases, we observed that apoptosis was significantly induced in fresh leukemic cells by endothelial IL-8 in vitro. Endothelial IL-8, with five extra N-terminal amino acids, can induce apoptosis, but monocyte-derived IL-8, which

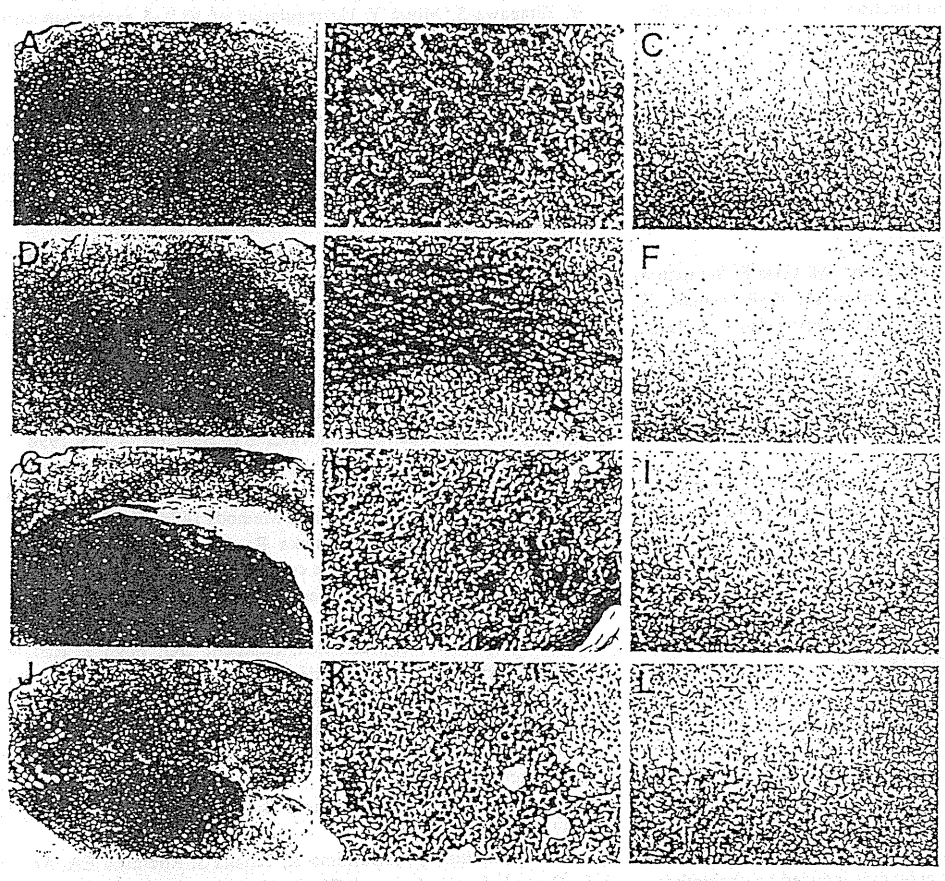


Fig 7. Microscopic morphology of progressive and regressing subcutaneous K562 tumors. Balb/c nu/nu mice were injected subcutaneously with K562 cells and were subsequently injected with saline (A through C), monocyte-derived IL-8 (D through F), endothelial IL-8 (G through I), and TNF-α (J through L). Tumors were removed in toto, and hematoxylin and eosin staining (A, B, D, E, G, H, J, and K) or TUNEL assay (C, F, I, and L) was performed after 8 days. Original magnification × 5 for (A), (D), (G), and (J); × 20 for (B), (C), (E), (F), (H), (I), and (K).

lacks them, cannot do so. Because most IL-8 in blood plasma exists in the form of monocyte-derived IL-8[45] and does not induce apoptosis in leukemic cells,[28] this phenomenon will be important in leukemia therapy.

VP-16 is well-known to be an anticancer agent and an inducer of apoptosis in leukemic cells. When leukemic cells were treated with both 0.1 μmol/L VP-16 and 20 ng/mL endothelial IL-8 for 48 hours, apoptotic cells increased as compared with the treatment of only 0.1 μmol/L VP-16 (data not shown). This result suggests that endothelial IL-8 can enhance the effect of VP-16 on the induction of apoptosis and may therefore be clinically promising in combination with VP-16.

Understanding of the receptor for induction of apoptosis will be important to further research on chemokines, and we are currently investigating receptor(s) and apoptosis-signaling for them in our system. Anti–IL-8 receptor antibody or IL-8 receptor antagonist, or specific binding protein(s) for endothelial IL-8, should be characterized. About 20% of target cells (K562 cells) undergo apoptosis by endothelial IL-8; therefore, we need to have more susceptible cells as targets, using subclones of the cells. In a preliminary experiment, we separated whole K562 cells into three fractions of cell cycle-phases, ie, G0/G1-phase, S-phase, and G2/M-phase fractions by counterflow centrifugal elutriation system,[30] and then examined the susceptibility of K562 cells in each cell-cycle phase to apoptosis induced by endothelial IL-8. This experiment demonstrated that susceptibility to apoptosis is higher in the G0/G1 phase of cell cycle than in the S and G2/M phases in K562 cells (data not shown). In our experiment, proliferation can be less changed but apoptosis markedly increased (Fig 3A). We showed bcl-2–overexpressing cells in any cell cycle phases lost susceptibility to apoptosis.[30] Moreover, thymocyte apoptosis by methylprednisolone and etoposide is independent of proliferation.[55] This indicates that the antiapoptotic genes-expressing cells escaped from endothelial IL-8–induced apoptosis can proliferate.

In conclusion, endothelial cells involving a novel apoptotic system have been identified. In the mechanism of this system, endothelial IL-8 release plays an important role. It is not only an inflammatory mediator but also an apoptosis-inducing factor in leukemic cells.

ACKNOWLEDGMENT

The authors thank S. Kurokawa, H. Ishikawa, and T. Ishida for technical assistance.

REFERENCES

1. Raff MC: Social controls on cell survival and cell death. Nature 356:397, 1992

2. Cohen JJ, Duke RC, Fadok VA, Sellins KS: Apoptosis and programmed cell death in immunity. Annu Rev Immnol 10:267, 1992

3. Green DR, Scott DW: Activation-induced apoptosis in lymphocytes. Curr Opin Immunol 6:476, 1994

4. Griffith TS, Ferguson TA: The role of FasL-induced apoptosis in immune privilege. Immunol Today 18:240, 1997

5. Thompson CB: Apoptosis in pathogenesis and treatment of disease. Science 267:1456, 1995

6. Kerr JFR, Wyllie AH, Currie AR: Apoptosis: A basic biological

phenomenon with wild-ranging implications in tissuekinetics. Br J Cancer 26:239, 1972

7. Wyllie AH, Kerr JFR, Currie AR: Cell death: The significance of apoptosis. Int Rev Cytol 68:251, 1990

8. Mannick JB, Miao XQ, Stamler JS: Nitric oxide inhibits Fas-induced apoptosis. J Biol Chem 272:24125, 1997

9. Rokhlin OW, Hostager BS, Bishop GA, Sidorenko SP, Glover RA, Gudkov VA, Cohen MB: Dominant nature of the resistance to Fas-and tumor necrosis factor-alpha-mediated apoptosis in human prostatic carcinoma cell lines. Cancer Res 57:3941, 1997

10. Ohtsu M, Sakai N, Fujita N, Kashiwagi M, Gasa S, Shimizu S, Eguchi S, Tsujimoto Y, Sakiyama Y, Kobayashi K, Kuzumaki K: Inhibition of apoptosis by the actin-regulatory protein gelsolin. EMBO J 16:4650, 1997

11. Krajewski S, Krajewska M, Ehrmann J, Sikorska M, Lach B, Chatten J, Reed JC: Immunohistochemical analysis of Bcl-2, Bcl-X, Mcl-1, and Bax in tumors of central and peripheral nervous system origin. Am J Pathol 150:805, 1997

12. Pallis M, Zhu YM, Russell NH: Bcl-x(L) is heterogenously expressed by acute myeloblastic leukemia cells and is associated with autonomous growth in vitro and with P-glycoprotein expression. Leukemia 11:945, 1997

13. Kim CN, Wang X, Huang Y, Ibrado AM, Liu L, Fang G, Bhalla K: Overexpression of Bcl-X(L) inhibits Ara-C-induced mitochondrial loss of cytochrome c and other perturbations that activate the molecular cascade of apoptosis. Cancer Res 57:3115, 1997

14. Simonian PL, Grillot DA, Nunez G: Bcl-2 and Bcl-XL can differentially block chemotherapy-induced cell death. Blood 90:1208, 1997

15. Fisher DE: Apoptosis in cancer therapy: Crossing the threshold. Cell 78:539, 1994

16. Decaudin D, Geley S, Hirsch T, Castedo M, Marchetti P, Macho A, Kofler R, Kroemer G: Bcl-2 and Bcl-XL antagonize the mitochondrial dysfunction preceding nuclear apoptosis induced by chemotherapeutic agents. Cancer Res 57:62, 1997

17. Benito A, Silva M, Grillot D, Nunez G, Fernandez LJL: Apoptosis induced by erythroid differentiation of human leukemia cell lines is inhibited by Bcl-XL. Blood 87:3837, 1994

18. Takahashi T, Tanaka M, Brannan CI, Jenkins NA, Copeland NG, Suda T, Nagata S: Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. Cell 76:969, 1994

19. Heller RA, Kronke M: Tumor necrosis factor receptor-mediated signaling pathways. J Cell Biol 126:5, 1994

20. Miyazaki T, Liu ZJ, Kawahara A, Minami Y, Yamada K, Tsujimoto Y, Barsoumian EL, Permutter RM, Taniguchi T: Three distinct IL-2 signaling pathways mediated by bcl-2, c-myc, and lck cooperate in hematopoietic cell proliferation. Cell 81:223, 1995

21. Walker PR, Saas P, Dietrich PY: Role of Fas ligand (CD95L) in immune escape: The tumor cell strikes back. J Immunol 158:4521, 1997

22. Whiteside TL, Chikamatsu K, Nagashima S, Okada K: Antitumor effects of cytolytic T lymphocytes (CTL) and natural killer (NK) cells in head and neck cancer. Anticancer Res 16:2357, 1996

23. Heusel JW, Wesselschmidt RL, Shresta S, Russell JH, Ley TJ: Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. Cell 76:977, 1994

24. Sarin A, Williams MS, Alexander MMA, Berzofsky JA, Zacharchuk CM, Henkart PA: Target cell lysis by CTL granule exocytosis is independent of ICE/Ced-3 family proteases. Immunity 6:209, 1997

25. Sternlicht MD, Barsky SH: The myoepithelial defense: A host defense against cancer. Med Hypotheses 48:37, 1997

26. Collins SJ, Gallo RC, Gallagher RE: Continuous growth and differentiation of human myeloid leukemic cells in suspension culture. Nature 270:347, 1977

27. Terui Y, Furukawa Y, Sakai T, Kikuchi J, Sugahara H, Kanakura Y, Kitagawa S, Miura Y: Up-regulation of VLA-5 expression during monocytic differentiation and its role in negative control of the survival of peripheral blood monocytes. J Immunol 156:1981, 1996

28. Terui Y, Ikeda M, Tomizuka H, Kasahara T, Ohtsuki T, Uwai M, Mori M, Itoh T, Tanaka M, Yamada M, Shimamura S, Miura Y, Hatake K Identification of a novel apoptosis-inducing factor derived from leukemic cells: Endothelial interleukin-8, but not monocyte-derived, induces apoptosis in leukemic cells. Biochem Biophys Res Commun 243:407, 1998

29. Lozzio CB, Lozzio BB: Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood 45:321, 1975

30. Terui Y, Furukawa Y, Kikuchi J, Saito M: Apoptosis during HL-60 cell differentiation is closely related to a G0/G1 cell cycle arrest. J-Cell Physiol 164:74, 1995

31. Hashimoto S, Yoda M, Yamada M, Yanai N, Kawashima T, Motoyoshi K: Macrophage colony-stimulating factor induces interleukin-8 production in human monocytes. Exp Hematol 24:123, 1996

32. Ko Y, Mukaida N, Panyutich A, Voitenok NN, Matsushima K, Kawai T, Kasahara T: A sensitive enzyme-linked immunosorbent assay for human interleukin-8. J Immunol Methods 149:227, 1992

33. Efferth T, Fabry U, Osieka R: Apoptosis and resistance to daunorubicin in human leukemic cells. Leukemia 11:1180, 1997

34. Williams GT: Programmed cell death: Apoptosis and oncogenesis. Cell 65:1097, 1991

35. Kyriazis AP, Kyriazis AA, Scarpelli DG, Fogh J, Rao MS, Lepera R: Human pancreatic adenocarcinoma line Capan-1 in tissue culture and the nude mouse: Morphologic, biologic, and biochemical characteristics. Am J Pathol 106:250, 1982

36. Walz A, Peveri P, Aschauer H, Baggiolini M: Purification and amino acid sequence of NAF, a novel neutrophil-activating factor produced by monocytes. Biochem Biophys Res Commun 149:755, 1987

37. Yoshimura T, Matsushima K, Tanaka S, Robinson EA, Appella E, Oppenheim JJ, Leonard EJ: Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. Proc Natl Acad Sci USA 84:9233, 1987

38. Schmid J, Weissmann C: Induction of mRNA for a serine protease and a β-thromboglobulin-like protein in mitogen-stimulated human leukocytes. J Immunol 139:250, 1987

39. Yoshimura T, Robinson EA, Appella E, Matsushima K, Showalter SD, Skeel A, Leonard EJ: Three forms of monocyte-derived neutrophil chemotactic factor (MDNCF) distinguished by different length of the amino-terminal sequence. Mol Immunol 26:87, 1989

40. Lindley I, Aschauer H, Seifert JM, Lam C, Brunowsky W, Kownatzki E, Thelen M, Peveri P, Dewald B, von Tscharner V, Walz A, Baggiolini M: Synthesis and expression in Escherichia coli of the gene encoding monocyte-derived neutrophil-activating factor: Biological equivalence between natural and recombinant neutrophil-activating factor. Proc Natl Acad Sci USA 85:9199, 1988

41. Van Damme J, Van Beeumen J, Conings R, Decock B, Billiau A: Purification of granulocyte chemotactic peptide/interleukin-8 reveals N-terminal sequence heterogeneity similar to that of beta-thromboglobulin. Eur J Biochem 181:337, 1989

42. Gimbrone MA Jr, Obin MS, Brock AF, Luis EA, Hass PE, Hébert CA, Yip YK, Leung DW, Lowe DG, Kohr WJ, Darbonne WC, Bechtol KB, Baker JB: Endothelial interleukin-8: A novel inhibitor of leukocyte-endothelial interactions. Science 246:1601, 1989

43. Schröder J-M, Sticherling M, Henneicke HH, Preissner WC, Christophers E: IL-1α or tumor necrosis factor-α stimulate release of three NAP-1/IL-8-related neutrophil chemotactic proteins in human dermal fibroblasts. J Immunol 144:2223, 1990

44. Leavell KJ, Peterson MW, Gross TJ: Human neutrophil elastase abolishes interleukin-8 chemotactic activity. J Leukoc Biol 61:361, 1997

45. Baggiolini M, Dewald B, Moser B: Interleukin-8 and related chemokines-CXC and CC chemokines. Adv Immunol 55:97, 1993

46. Van Damme J: Interleukin-8, in Thomson A (ed): The Cytokine Handbook. London, UK, Academic, 1994, p 185

47. Azzarelli B, Easterling K, Norton JA: Leukemic cell-endothelial cell interactions in leukemic cell dissemination. Lab Invest 60:45, 1989

48. Geng Y-J, Hellstrand K, Wennmalm A, Hansson GK: Apoptotic death of human leukemic cells induced by vascular expressing nitric oxide synthesis in response to γ-interferon and tumor necrosis factor-α. Cancer Res 56:866, 1996

49. Folkman J, Ingber D: Angiogenesis. J Biol Chem 267:10931, 1992

50. Thorgeirsson UP, Lindsay CK, Cottam DW, Gomez DE: Tumor invasion, proteolysis, and angiogenesis. J Neurooncol 18:89, 1993

51. Baggiolini M, Walz A, Kunkel SL: Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. J Clin Invest 84:1045, 1989

52. Mantovani A, Bussolino F, Introna M: Cytokine regulation of endothelial cell function: From molecular level to the bedside. Immunol Today 18:231, 1997

53. O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J: Endostatin: An endogenous inhibitor of angiogenesis and tumor growth. Cell 88:277, 1997

54. Dong Z, Kumar R, Yang X, Fidler IJ: Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma. Cell 88:801, 1997

55. Evans DL, Tiby M, Dive C: Differential sensitivity to the induction of apoptosis by cisplatin in proliferating and quiescent immature rat thymocyte is independent of the levels of drug accumulation and DNA adduct formation. Cancer Res 54:1596, 1994

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 243, 407–411 (1998)
ARTICLE NO. RC988103

Identification of a Novel Apoptosis-Inducing Factor Derived from Leukemic Cells: Endothelial Interleukin-8, but Not Monocyte-Derived, Induces Apoptosis in Leukemic Cells

Yasuhito Terui,* Masayuki Ikeda,† Hiroshi Tomizuka,* Tadashi Kasahara,‡ Tetsuya Ohtsuki,* Masaya Uwai,* Masaki Mori,* Takehito Itoh,† Miyuki Tanaka,† Muneo Yamada,† Seiichi Shimamura,† Yasusada Miura,* and Kiyohiko Hatake*,[1]

*Division of Hematology, Department of Internal Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-machi, Kawachi-gun, Tochigi 329-04, Japan; †Biochemical Research Laboratory, Morinaga Milk Industry Co. Ltd., 5-1-83 Higashihara, Zama City, Kanagawa 228, Japan; and ‡Division of Biochemistry, Kyouritsu Pharmaceutic College, 1-5-30 Sibakouen, Minato-ku, Tokyo 105, Japan

Received December 25, 1997

The human myelogenous leukemia cell line HL-60, treated with phorbol 12, 13-dibutyrate (PDBu), produces apoptosis-inducing factors (AIFs) in leukemic cells. We have purified AIF against leukemic cell line K562 as target cells, and N-terminal amino acid sequencing analysis revealed that this purified protein is identical to endothelial cell-derived interleukin-8 ([(Ala)-IL-8]$_{77}$). In Western blot analysis of supernatants of PDBu-treated HL-60 cells,only [(Ala)-IL-8]$_{77}$ was detected. Moreover, recombinant human [(Ala)-IL-8]$_{77}$ induced apoptosis in leukemic cell lines such as K562, HL-60, KG-1, U937, THP-1 and Jurkat, but monocyte-derived IL-8 ([(Ser)-IL-8]$_{72}$) did not. Therefore [(Ala)-IL-8]$_{77}$ plays an important role in inducing apoptosis against leukemic cells and may lead to a new therapy for leukemia. © 1998 Academic Press

Apoptosis plays an important role in cancer therapy today. Most leukemic cells are well known to undergo apoptosis by several events, and the basic strategy of leukemia therapy is the induction of apoptosis (1). For example, many chemotherapeutic agents such as cytosine arabinoside and VP-16 induce apoptosis and kill leukemic cells (1). On the other hand, Fas ligand and TNF-α, which physiologically exist in the body, are also inducers of apoptosis in leukemic cells and other cells (2, 3). It has been reported that a signal for some cytokines might select induction of apoptosis or proliferation in target cells (4), but no cytokine could induce apoptosis in leukemic cells or other tumor cells.

The human myelogenous leukemia cell line HL-60 (5) can be induced to differentiate into monocyte/macrophage lineage by phorbol esters such as phorbol 12, 13-dibutyrate (PDBu), and undergoes apoptosis (6). TNF-α and interleukin-6 (IL-6) have previously been identified as differentiation-inducing factors (DIFs) in this system by other researchers (7, 8), who reported that their addition to media could induce differ entiation of leukemic cells. However, apoptosis-inducing factors (AIFs) have not yet been identified in this system. Identification of physiological factors could develop an understanding of the onset of leukemia and help identify new therapies.

Here we purified an apoptosis-inducing factor (AIF) to homogeneity from a medium conditioned by PDBu-treated HL-60 cells, as described. Activity of this AIF was assayed by measuring MTT dye in the erythroblastic leukemia cell line K562 (9), since dead cells such as apoptotic cells do not have MTT reducing activity.

MATERIALS AND METHODS

Cell lines and reagents. K562 (chronic myelogenous leukemia), HL-60 (acute myelogenous leukemia), THP-1 (acute monocytic leukemia), and Jurkat (T cell leukemia) were purchased from American Type Culture Collection. NB4 (acute promyelocytic leukemia) was a kind gift from Dr. Michel Lanotte. These cell lines were maintained in GIT medium (Wako, Tokyo, Japan), and were resuspended at 1 $\times$ 10^5 cells/ml in HamF12/DMEM (Gibco) containing 10% GIT medium in experimental attempts to induce apoptosis. Human recombinant endothelial IL-8, monocyte-derived IL-8, and mouse monoclonl anti-human IL-8 antibody were purchased from R & D system.

Purification and sequencing of an apoptosis-inducing factor. HL-60 cells were resuspended at 2 $\times$ 10^5 cells/ml in HamF12/DMEM

[1] To whom all correspondence should be addressed. E-mail: kiyohiko@jichi.ac.jp. Fax: 0285-44-5258.

0006-291X/98 $25.00

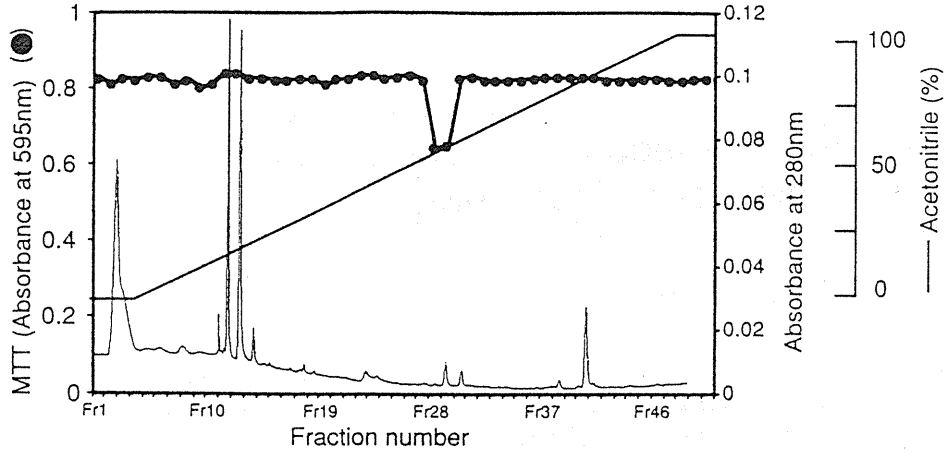


FIG. 1. Reverse-phase HPLC and N-terminal sequence of apoptosis-inducing factor (AIF). The pool of active fraction eluted from hydroxyapatite HPLC was applied to the reverse-phase column, μBondasphere (Waters) using a high performance liquid chromatography (HPLC) system. The column was equilibrated with water containing 0.1% trifluoroacetic acid and eluted through a 100-min linear time gradient with 100% acetonitrile. The flow rate was maintained at 1 ml/min.

(Gibco) containing 10% GIT medium (Wako) and PDBu (Sigma) 50 nM and incubated at 37 °C for 3 days. After 3 days, the supernatant was collected and precipitated in ammonium sulfate. The preparation was then purified further in a process including gel filtration high performance liquid chro matography (HPLC) and hydroxyapatite HPLC. Additionally, the pool of active fraction eluted from hydroxyapatite column was applied to reverse-phase HPLC. The purified AIF activity was eluted in approximately 50% acetonitrile in reverse-phase HPLC as a single peak coincident with AIF activity. The N-terminal sequence of native protein was determined by Edman degradation on an automated Applied Biosystems 473A sequencer (Applied Biosystems). The active fraction, which was eluted in approximately 50% acetonitrile in reverse-phase HPLC, was assayed by human IL-8 ELISA system (Amersham). The concentration of IL-8 in the active fraction was obtained from a standard curve.

Detection of apoptosis by MTT assay and TUNNEL assay. K562 cells were seeded at 1×10^5 cells/ml in HamF12/Dulbecco's modified Eagle's medium (DMEM) with 10% GIT medium and recombinant human IL-8 (Genzyme) was added to media at various concentrations. After 2 days, the capacity to reduce 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) was determined. After adding 10 μl of MTT solution (5 mg/ml MTT in phosphate buffered saline), the preparation was incubated at 37 °C for 4 hours. Cells with MTT formazan were dissolved in 0.04 N HCl in 2- propanol, and color absorbance was measured at 595 nm by microplate reader (Bio-Rad). Additionally, residual cells were incubated with digoxigenin-dUTP terminaldeoxynucleotide transferase mixture and subsequently stained with peroxidase-conjugated antibody to digoxigenin (Apop Tag PLUS), counterstained with 1% methyl green in sodium acetate (pH 4.0), and mounted. Specimens were examined and photographed with a microscope. Statistical analysis was performed by Student's t-test.

Immunoprecipitation and Western blot analysis. Supernatants (1.2 ml) of PDBu-treated HL-60 cells were collected at the indicated time, and rec-protein G Sepharose 4B (Zymed, CA) was added. After incubation for 1 hour at room temparature, the supernatants were collected, and then mixed polyclonal human anti-IL-8 antibody (IgG, R & D system, Inc., MN). After incubation for 1 hour at room temperature, protein G Sepharose 4B was added. Samples were centrifugated at 5,000 rpm for 5 min, and then precipitations were collected, following by washing twice with phosphate buffered saline. The precipitations were suspended with SDS-polyacrylamide sample buffer. Moreover, Western blot analysis was performed using polyclonal human anti-IL-8 antibody (IgG, Santa Cruz Biotecnology) as indicated in ECL system (Amersham).

A

Apoptosis-inducing factor	AVLPRXAKELRXQXIKTYXK
Endothelial cell-derived IL-8 [(Ala)-IL-8]$_{77}$	AVLPRSAKELRCQCIKTYSK
Monocyte-derived IL-8 [(Ser)-IL-8]$_{72}$	SAKELRCQCIKTYSK

B

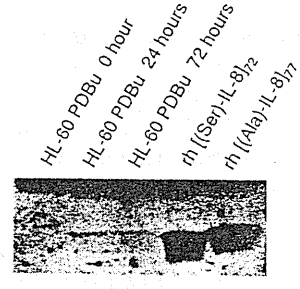


FIG. 2. (A) N-terminal sequence of this fraction with AIF activity. X shows an undetermined amino acid sequence. (B) Detection of [(Ala)-IL-8]$_{77}$ in conditioned media of PDBu-treated HL-60 cells by immunoprecipitation and Western blot analysis. As standards, recombinant human endothelial IL-8 (rh[(Ala)-IL-8]$_{77}$) and recombinant human monocyte-derived IL-8 (rh[(Ser)-IL-8]$_{72}$) were used.

RESULTS AND DISCUSSION

HL-60 cells were cultured at 2×10^5 cells/ml with 50 nM PDBu for 72 hours and differentiated into monocyte/macrophage lineage. We ob served that K562 cells were dead and induced apoptosis when the su pernatant from PDBu-treated HL-60 cells was added to culture media of K562 cells (data not shown). After the removal of cells and debris, the supernatant was precipitated by 95% ammonium sulfate and then concentrated. The preparation was then purified further in the process, including gel filtration high performance liquid chromatography (HPLC) and hydroxyapatite HPLC. Additionally, the pool of active fraction eluted from hydroxyapatite column was applied to reverse-phase HPLC. The purified AIF activity was eluted in approximately 50% acetonitrile in reverse-phase HPLC as a single peak coincident with AIF activity (Fig. 1). We obtained specific activity of 1×10^5 U per mg of protein (1U AIF inhibited 50% of maximum reduction as indicated by MTT reducing ability) and a final purification of 400-fold, calculated from the first supernatant concentration. The overall yield was 0.05%.

N-terminal sequence analysis of AIF showed a striking homology with the N-terminal sequence of human IL-8 derived from endothelial cells ($[(Ala)\text{-}IL\text{-}8]_{77}$) (10) (Fig. 2A). The purified AIF was detected with anti-IL-8 antibody on an enzyme-linked immunosorbent assay (ELISA), and the fraction with AIF contained 12 ng/ml IL-8 as measured. Immunoprecipitation and Western blot analysis revealed that $[(Ala)\text{-}IL\text{-}8]_{77}$ was contained in conditioned media of PDBu-treated HL-60 cells from 24 hours, but $[(Ser)\text{-}IL\text{-}8]_{72}$ was not (Fig. 2B). These results further indicate that AIF is identical to $[(Ala)\text{-}IL\text{-}8]_{77}$. For this reason we examined the apoptosis-inducing activity of recombinant human $[(Ala)\text{-}IL\text{-}8]_{77}$.

First, we examined whether $[(Ala)\text{-}IL\text{-}8]_{77}$ would induce apoptosis in K562 cells with characteristics the same as those of AIF. The concen tration of $[(Ala)\text{-}IL\text{-}8]_{77}$ required to suppress cell growth of K562 cells was 5 ng/ml, and the maximal concentration was 20 ng/ml. At an $[(Ala)\text{-}IL\text{-}8]_{77}$ concentration of 20 ng/ml, cell growth of K562 cells was inhibited at 80.0% of control (no additives) after 48 hours (Fig. 3A). To investigate whether apoptosis is induced by $[(Ala)\text{-}IL\text{-}8]_{77}$ in this system, we assessed the induction of apoptosis with TUNEL assay. With $[(Ala)\text{-}IL\text{-}8]_{77}$, apoptotic cells were significantly increased in $12.0 \pm 0.3\%$ of K562 cells after 48 hours (Fig. 3, B, C and D). The neutralizing monoclonal antibody against human IL-8 completely blocked both the suppression of cell growth and the induction of apoptosis by $[(Ala)\text{-}IL\text{-}8]_{77}$ (Fig. 3, A and D). It has been reported that there are molecules of IL-8 differ in length (11, 12). Since monocyte-derived IL-8 ($[(Ser)\text{-}IL\text{-}8]_{72}$) exists throughout in the body (11, 12), we investigated whether $[(Ser)\text{-}IL\text{-}8]_{72}$ can inhibitcell growth and induce apoptosis. $[(Ser)\text{-}IL\text{-}8]_{72}$, in that it lacks 5 extra N-terminal amino acids of $[(Ala)\text{-}IL\text{-}8]_{77}$ (Fig. 2A), could neither suppress cell growth nor induce apoptosis (Fig. 3, A and D). These results indicate that $[(Ala)\text{-}IL\text{-}8]_{77}$ can induce apoptosis in K562 cells because there are 5 extra N-terminal amino acids with biological activity. To investigate whether apoptosis induced by $[(Ala)\text{-}IL\text{-}8]_{77}$ is mediated by FasL-Fas or TNF-α-TNFR systems, we examined the effect of anti-Fas and anti-TNF-α receptor antibodies on apoptosis induced by $[(Ala)\text{-}IL\text{-}8]_{77}$. Apoptosis induced by $[(Ala)\text{-}IL\text{-}8]_{77}$ could not be blocked by anti-Fas antibody and

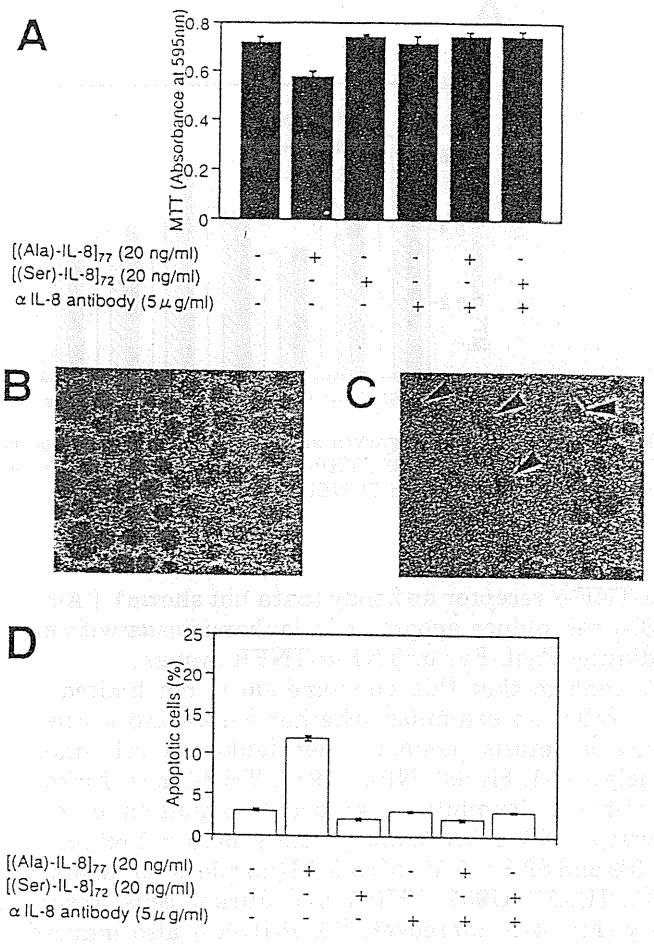


FIG. 3. Inhibition of cell growth and induction of apoptosis on K562 cells by endothelial cell-derived IL-8 ($[(Ala)\text{-}IL\text{-}8]_{77}$). (A) K562 cells ($1 \times 10^5$ cells/ml) were cultured with and without 20 ng/ml IL-8 (Genzyme) at 37 °C for 48 hours in HamF12/DMEM medium, and MTT assay was performed. Monoclonal antibody against human IL-8 (5 μg/ml) (R & D Systems) was added to culture medium. (B and C) K562 cells (1×10^5 cells/ml) were cultured with (C) or without (B) 20 ng/ml $[(Ala)\text{-}IL\text{-}8]_{77}$ at 37 °C. After 48 hours, apoptotic cells were detected by *in situ* staining with Apop Tag PLUS (Oncor), which gives a dark contrast insoluble precipitate indicative of genomic fragmentation, as described. Arrows indicate apoptotic cells. Magnification: × 160. (D) The percentage of apoptotic cells was determined microscopically by counting more than 200 cells on *in situ*-staining slides. Data shown come from three independent experiments. Standard deviations are shown by horizontal bars.

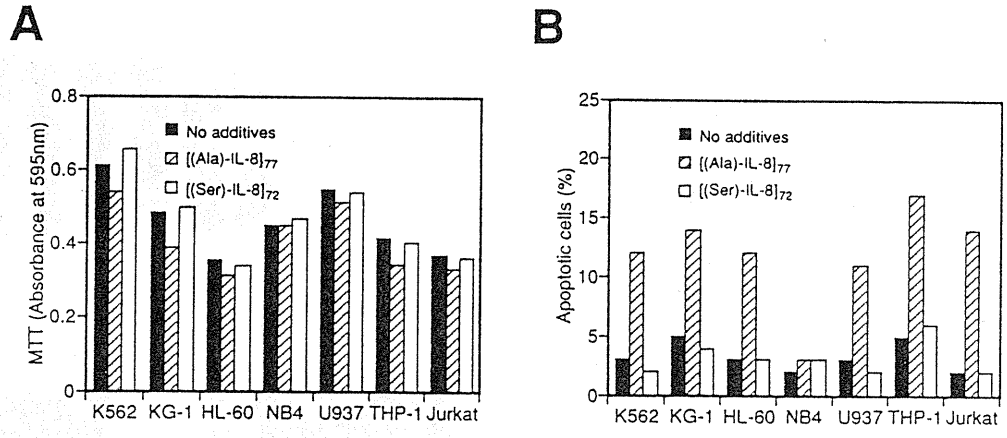


FIG. 4. Inhibition of cell growth and induction of apoptosis on various leukemic cell lines by $[(Ala)\text{-}IL\text{-}8]_{77}$, but not by $[(Ser)\text{-}IL\text{-}8]_{72}$. K562, KG-1, HL-60, NB4, U937, THP-1 and Jurkat cells (1×10^5 cells/ml) were cultured with and without 20 ng/ml IL-8 at 37 °C for 48 hours, and MTT assay (A) and TUNEL assay (B) were performed.

anti-TNF-α receptor antibody (data not shown). $[(Ala)\text{-}IL\text{-}8]_{77}$ can induce apoptosis in leukemic cells without mediating FasL-Fas or TNF-α-TNFR systems.

To confirm that this phenomenon is not limited to K562 cells, we examined whether $[(Ala)\text{-}IL\text{-}8]_{77}$ could induce apoptosis against other leukemia cell lines, namely KG-1, HL-60, NB4, U937, THP-1 and Jurkat. $[(Ala)\text{-}IL\text{-}8]_{77}$ exhibited significant inhibition of cell growth at 80.7 ± 1.0%, 88.8 ± 2.0%, 84.0 ± 1.6%, 83.1 ± 3.3% and 89.8 ± 1.1% of control (no additives) against KG-1, HL-60, U937, THP-1 and Jurkat cells, respectively (Fig. 4A). Moreover, $[(Ala)\text{-}IL\text{-}8]_{77}$ also induced apoptosis at 14 ± 0.6%, 12 ± 1.0%, 11 ± 0.6%, 17 ± 1.2% and 14 ± 1.0% against KG-1, HL-60, U937, THP-1 and Jurkat cells, respectively (Fig. 4B). However, $[(Ala)\text{-}IL\text{-}8]_{77}$ could neither inhibit cell growth nor induce apoptosis in NB4 cells (Fig. 4). $[(Ser)\text{-}IL\text{-}8]_{72}$ did not have the ability either to inhibit cell growth or to induce apoptosis in other leukemic cell lines similar to K562 cells (Fig. 4). This result suggests that $[(Ala)\text{-}IL\text{-}8]_{77}$ can suppress cell growth and induce apoptosis in most leukemic cell lines.

IL-8 is well-known to be a potent mediator of the inflammatory response (13, 14). As indicated in our experiments, $[(Ala)\text{-}IL\text{-}8]_{77}$ has an important role in leukemic cell suppression, i.e., induction of apoptosis and inhibition of cell growth. Although it has previously been reported that MCP-1 and γIP-10 can act as anti-tumor factors (15, 16), our group found that $[(Ala)\text{-}IL\text{-}8]_{77}$ could act on leukemic cells as an apoptosis-inducing factor. When leukemic cells are treated with retinoic acid, interleukin-1β, γ interferon and TNF-α, they produce and secrete IL-8 (17, 18). Similarly, other tumor cell lines, such as colon cancer cell line HT-29 and neuroblastoma cell line SK-N-SH, could also produce and secrete IL-8, and undergo apoptosis during treatment withTNF-α, Fas ligand, and retinoic acid (19, 20). Our results indicate that secretion of IL-8 from these cells could not only cause chemo taxis and activation of leukocytes, but might also induce apoptosis in the secreting cells. Since most IL-8 in blood plasma exists in a form of $[(Ser)\text{-}IL\text{-}8]_{72}$ (21), and $[(Ser)\text{-}IL\text{-}8]_{72}$ does not induce apoptosis in leukemic cells, this phenomenon will be important in leukemia therapy.

The Duffy antigen receptor for chemokines (DARC) is a promiscuous chemokine receptor and a binding protein for the malarial parasite *Plasmodium vivax.* (22). Moreover, it has been recently discovered that chemokine receptors are cofactors for HIV entry into CD4-positive lympho cytes, and that CXC and CC chemokines have anti-HIV effects (23, 24). In addition to these findings, our results suggest that one of the chemokines and the receptor might participate in many infectious and oncogenic diseases, and that they might be helpful to the understanding and treatment of those diseases in the future. Although chemokine receptors are divided into four major classes, based on ligand preference, the receptor(s) for chemokines inducing apoptosis on leukemic cells is not yet known. CXC chemokine receptor (CXCR2) (25) for ELR (Glu-Leu-Arg) -CXC chemokines including IL-8, CC chemokine receptor 2 (CCR2) (26) for MCP-1, and CCR5 (27) for MIP-1α and RANTES are members of the shared receptor subfamily that is one of the four classes. These chemokine receptors might play an important role in the induction of apoptosis by chemokines. Understanding of the receptor for induction of apoptosis will be important to further research for chemokines, and we are currently investigating receptor(s) and apoptosis-signalling for them.

In conclusion, $[(Ala)\text{-}IL\text{-}8]_{77}$ is not only an inflammatory mediator but also an apoptosis-inducing factor in leukemic cells. However, $[(Ala)\text{-}IL\text{-}8]_{77}$ is very important

in the induction of apoptosis and the suppression of tumor cell growth, suggesting that further investigation of this IL-8 should yield new cancer therapies.

ACKNOWLEDGMENTS

We thank S. Kurokawa, K. Sato, T. Ishida for technical assistance. This research was funded by a grant-in-aid from the Ministry of Education, Science and Culture of Japan, the Japanese Foundation for Multidisciplinary Treatment of Cancer, and the Jichi Medical School Research Fund.

REFERENCES

1. Fisher, D. E. (1994) *Cell* **78**, 539–542.
2. Takahashi, T., Tanaka, M., Brannan, C. I., Jenkins, N. A., Copeland, N. G., Suda, T., and Nagata, S. (1994) *Cell* **76**, 969–976.
3. Heller, R. A., and Kronke, M. (1994) *J. Cell Biol.* **126**, 5–9.
4. Miyazaki, T., Liu, Z.-J., Kawahara, A., Minami, Y., Yamada, K., Tsujimoto, Y., Barsoumian, E. L., Perlmutter, R. M., and Taniguchi, T. (1995) *Cell* **81**, 223–231.
5. Collins, S. J., Gallo, R. C., and Gallagher, R. E. (1977) *Nature* **270**, 347–349.
6. Terui, Y., Furukawa, Y., Sakai, T., Kikuchi, J., Sugahara, H., Kanakura, Y., Kitagawa, S., and Miura, Y. (1996) *J. Immunol.* **156**, 1981–1988.
7. Takeda, K., Iwamoto, S., Sugimoto, H., Takuma, T., Kawatani, N., Noda, M., Masaki, A., Morise, H., Arimura, H., and Konno, K. (1986) *Nature* **323**, 338–340.
8. Shabo, Y., Lotem, J., Rubinstein, M., Revel, M., Clark, S. C., Wolf, S. F., Kamen, R., and Sachs, L. (1988) *Blood* **72**, 2070–73.
9. Lozzio, C. B., and Lozzio, B. B. (1979) *Blood* **45**, 321–322.
10. Baggiolini, M., Walz, A., and Kunkel, S. L. (1989) *J. Clin. Invest.* **84**, 1045–1049.
11. Van Damme, J. (1994) The Cytokine Handbook (Thomson, A., ed.), Academic Press, London, **185** 185–208.
12. Yoshimura, T., Matsushima, K., Tanaka, S., Robinson, E. A., Appella, E., Oppenheim, J. J., and Leonard, E. J. (1987) *Proc. Nalt. Acad. Sci. USA* **84**, 9233–9237.
13. Herbert, C. A., and Baker, J. B. (1993) *Cancer Invest.* **11**, 743–750.
14. Matsushima, K., Larsen, C. G., DuBois, G. C., and Oppenheim, J. J.(1989) *J. Exp. Med.* **169**, 1485–1490.
15. Baggiolini, M., Loetscher, P., and Moser, B. (1995) *Int. J. Immunopharmac.* **17**, 103–108.
16. Sgadari, C., Angiolillo, A. L., Cherney, B. W., Pike, S. E., Farber, J. M., Koniaris, L. G., Vanguri, P., Burd, P. R., Sheikh, N., Gupta, G., Feldstein, J. T., and Tosato, G. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 13791–6.
17. Bosco, M. C., Gusella, G. L., Espinoza-Delgado, I., and Varesio, L. (1994) *Blood* **83**, 537–42.
18. Sham, R. L., Phatak, P. D., Belanger, K. A., and Packman, C. H. (1995) *Leukemia Res.* **19**, 1–6.
19. Yang, K. D., Cheng, S.-N., Wu, N.-C., and Shaio, M.-F. (1993) *Pediatric Res.* **34**, 720–24.
20. Abreu-Martin, M. T., Vidrich, A., Lynch, D. H., and Targan, S. R. (1995) *J. Immunol.* **155**, 4147–54.
21. Horuk, R., Chitnis, C. E., Darbonne, W. C., Colby, T. J., Rybicki, A., Hadly, T. J., and Miller, L. H. (1993) *Science* **261**, 1182–1184.
22. Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clark-lewis, I., Sodoroski, J., and Springer, T. A. (1996) *Nature* **382**, 829–832.
23. Arenzana-Seiadedos, F., Virelizier, J.-L., Rousset, D., Clark-Lewis, I., Sodroski, J., Loetscher, P., Moser, B., and Baggiolini, M. (1996) *Nature* **383**, 400.
24. Premack, B. A., and Schall, T. J. (1996) *Nature Medicine* **2**, 1174–1178.
25. Lee, J., Horuk, R., Rice, G. C., Bennet, G. L., Camerato, T., and Wood, W. I. (1992) *J. Biol. Chem.* **267**, 16283–16287.
26. Charo, I. F. , Myers, S. J., Hermar, A., Franci, C., Connolly, A. J., and Coughlin, S. R. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 2752–2756.
27. Samson, M., Labbe, O., Mollereau, C., Vassart, G., and Parmentier, M. (1996) *Biochemistry* **35**, 3362–3367.