NH₂-Terminal Pentapeptide of Endothelial Interleukin 8 Is Responsible for the Induction of Apoptosis in Leukemic Cells and Has an Antitumor Effect in Vivo

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Abstract
We have reported that endothelial interleukin 8 (IL-8) induces apoptosis in leukemic cells in vitro and in vivo, and that interaction between endothelial cells and leukemic cells causes induction of apoptosis through the release of endothelial IL-8. It has been reported that the pentapeptide Ala-Val-Leu-Pro-Arg (AVLPR) significantly inhibited growth of i.p. and s.c. tumor masses of K562 cells and induced apoptosis in these cells in vivo. The active site of endothelial IL-8 is the NH₂-terminal pentapeptide AVLPR, and this may serve as a new therapy for hematological malignancies.

Introduction
Leukemic cells are known to undergo apoptosis under several conditions, and the basic strategy of leukemia therapy is the induction of apoptosis (1). We have purified an apoptosis-inducing factor to homogeneity from a medium conditioned by phorbol-12,13-dibutyrate-treated HL-60 cells (2). NH₂-terminal sequence analysis revealed that apoptosis-inducing factor is identical to endothelial IL-8 (3). Moreover, we also demonstrated the antitumor effect of endothelial IL-8, which has an additional five amino acids at the NH₂ terminus as compared with monocyte-derived IL-8. Endothelial IL-8, which has an additional five amino acids at the NH₂ terminus as compared with monocyte-derived IL-8, is active in the induction of apoptosis. We showed that interaction between endothelial cells and leukemic cells induces apoptosis in leukemic cells through the release of endothelial IL-8 (3). Moreover, we also demonstrated the antitumor effect of endothelial cells in vitro and the antitumor effect of endothelial IL-8 in vivo. It has been reported that the pentapeptide Glu-Glu-Asp-Cys-Lys (EEDCK) has inhibitory effects on normal hematopoietic cells and malignant cells, especially leukemic cells, because of a redox-mediated mechanism for demand-induced hematopoietic regulation (4, 5). This pentapeptide EEDCK was also found to protect stem cells from cytotoxic agents both in vitro and in vivo, suggesting that this pentapeptide may be useful for the purging of tumor cells from stem cell grafts (6). No peptide is known to induce apoptosis in malignant cells or normal cells. In this study, we first examined whether the pentapeptide Ala-Val-Leu-Pro-Arg (AVLPR), which corresponds to the NH₂-terminal amino acids residues of endothelial IL-8, has the ability to induce apoptosis in leukemic cells in vitro and in vivo.

Materials and Methods
Reagents. Recombinant human TNF-α was purchased from R & D Systems, Inc. (Minneapolis, MN). Recombinant human endothelial IL-8 was purchased from Genzyme (Cambridge, MA). All peptides were synthesized by TaKaRa Co. (Shiga, Japan).

Cell Lines and Cell Culture. A human chronic myelogenous leukemia cell line, K562 (7), was obtained from American Type Culture Collection and was maintained in GIT medium (Wako, Tokyo, Japan; Ref. 8). Human myelogenous leukemia cell line HL-60, human monocytic leukemia cell line U937, human T-cell leukemia cell line Jurkat, and human myeloma cell line Daundy were also obtained from American Type Culture Collection.

TUNEL Assay. Residual cells were incubated with a digoxigenin-dUTP terminal deoxynucleotidyl transferase mixture and subsequently stained with peroxidase-conjugated antibody to digoxigenin (Apop Tag PLUS; Oncor, MD; Ref. 9), counterstained with 1% methyl green in sodium acetate (pH 4.0), and mounted. Specimens were examined and photographed under a microscope. The percentage of apoptotic cells was determined by counting >200 cells. Statistical analysis was performed by 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 10⁶ K562 cells injected into the peritoneal space. A preparation of the NH₂-terminal or COOH-terminal pentapeptide of endothelial IL-8 was injected into the i.p. tumor masses of K562 cells daily for 2 days. As controls, saline and endothelial IL-8 were injected. i.p. cells were collected daily and washed with 5 ml of PBS. Cell counting, Wright-Giemsa staining, and the TUNEL assay were also performed. Mice were inoculated with 5 × 10⁵ viable K562 cells by s.c. injection in the midline ventral position in a total volume of 0.1 ml of PBS. Test mice bearing s.c. established K562 tumors (confirmed 4 days after inoculation) were treated daily (for 11 days) with the NH₂-terminal or COOH-terminal pentapeptide of endothelial IL-8 in a total volume of 0.1 ml saline, injected into the tumor. As controls, saline and TNF-α were injected. Tumor size was calculated using the formula described by Kyriazis et al. (10): tumor volume = width² × length × 0.4.

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3 The abbreviations used are: IL, interleukin; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; VP-16, etoposide.
Results

Induction of Apoptosis in K562 Cells by the NH$_2$-Terminal Pentapeptide AVLPR of Endothelial IL-8. Endothelial IL-8 induces apoptosis on leukemic cells, but monocyte-derived IL-8 does not. Because we noticed a difference in the NH$_2$ terminus of these two forms of IL-8, the NH$_2$-terminal pentapeptide Ala-Val-Leu-Pro-Arg (AVLPR) and the COOH-terminal pentapeptide Arg-Glu-Ala-Asn-Ser (REANS) were synthesized, and we examined their ability to induce apoptosis in K562 cells. When K562 cells were cultured with the NH$_2$-terminal pentapeptide AVLPR, the percentage of apoptotic cells increased to 15.0 ± 1.5% from 1.0 ± 0.5% (Fig. 1A). On the other hand, when K562 cells were cultured with the COOH-terminal pentapeptide REANS, there was no increase in the percentage of apoptotic cells compared with the control (saline only). This indicates that the NH$_2$-terminal pentapeptide AVLPR of endothelial IL-8 is effective to induce apoptosis in K562 cells.

We also investigated the dose dependency of the effect of NH$_2$-terminal pentapeptide AVLPR in terms of induction of apoptosis (Fig. 1B). Apoptotic cells significantly increased to 5.0 ± 1.0% from 1.0 ± 0.5% of K562 cells in the presence of 10 $\mu$M AVLPR, and the percentage of apoptotic cells became 15.0 ± 1.0% maximally in the presence of 1000 $\mu$M AVLPR. This indicates that the induction of apoptosis in K562 cells by the NH$_2$-terminal pentapeptide AVLPR occurs in a dose-dependent manner.

We also examined the susceptibility of K562 cells in each cell cycle phase to apoptosis induced by AVLPR using the counterflow centrifugal elutriation as described in the previous study (8). K562 cells could be separated into the fractions enriched for G$_0$-G$_1$ phase (100% of cells), S-phase (75.5%), and G$_2$-M phase cells (87.1%), and then the cells from each elutriated fraction were seeded at $1 \times 10^5$ cells/ml and cultured in the absence or presence of AVLPR. After 24 h, the percentage of apoptotic cells was determined microscopically by the TUNEL method. When the cells in G$_0$-G$_1$, S, and G$_2$-M phases were cultured without AVLPR, the proportion of apoptotic cells after 24 h was 4.9 ± 1.5%, 6.8 ± 1.0%, and 5.8 ± 0.5%, respectively. When the cells in G$_0$-G$_1$, S, and G$_2$-M phases were cultured with AVLPR for 24 h, the proportion of apoptotic cells was significantly increased and reached 34.4 ± 4.7%, 9.3 ± 0.9%, and 8.5 ± 1.2%, respectively ($P < 0.01$ between G$_0$-G$_1$ and S or G$_2$-M).

We also examined the effect of the pentapeptide AVLPR on fresh leukemic cells. Fresh leukemic cells prepared from 12 cases with acute leukemia (7 cases of acute myelogenous leukemia and 5 cases of acute lymphocytic leukemia) were cultured with or without AVLPR for 48 h, and then TUNEL assay was performed. In ten cases (6 of acute myelogenous leukemia and 4 of acute lymphocytic leukemia), the percentage of apoptotic cells in fresh leukemic cells was significantly increased to 43.9 ± 8.7% by AVLPR as compared with controls (10.1 ± 2.9%; $P < 0.01$). Moreover, we investigated the effect of combination of AVLPR and VP-16. When fresh leukemic
Deletion Analysis of the Active Site of NH$_2$-Terminal Pentapeptide AVLPR. To investigate the active site of the NH$_2$-terminal pentapeptide AVLPR, various derivatives of this peptide were synthesized. When K562 cells were cultured with the peptide AVLPR, AVL, or VLP, the percentage of apoptotic cells increased significantly to 17.0 ± 1.2%, 13.0 ± 1.1%, and 16.0 ± 1.4%, respectively (Fig. 1D). On the other hand, the peptides AVL, VLP, and LPR failed to induce apoptosis in K562 cells (Fig. 1D). These findings indicate that AV or PR is an essential residue in the NH$_2$-terminal pentapeptide AVLPR for induction of apoptosis in K562 cells.

To confirm that this phenomenon is not limited to K562 cells, we examined whether the NH$_2$-terminal pentapeptide AVLPR could induce apoptosis in other leukemia cell lines, i.e., HL-60, U937, Jurkat, and Daudi. This peptide induced apoptosis in 18.6% of the HL-60, 11.2% of the U937, 10.8% of the Jurkat, and 19.1% of the Daudi cells, respectively. These results demonstrate that the NH$_2$-terminal pentapeptide AVLPR can induce apoptosis in various leukemic cell lines.

NH$_2$-Terminal Pentapeptide AVLPR Suppresses Tumor Growth and Induces Apoptosis of K562 Cells in Vivo. We also investigated whether the NH$_2$-terminal pentapeptide AVLPR can induce apoptosis or suppress the growth of leukemic cells in vivo. The peptide was injected daily for 2 days into i.p. tumor masses of K562 cells in nude mice (Fig. 2). Apoptosis and suppression of cell growth were observed after 2 days (Fig. 2), and the apoptotic cells were phagocytosed by macrophages. After 2 days, the percentage of apoptotic cells increased to 12.0 ± 4.0%. Moreover, the number of K562 cells decreased to 26.7% of the control level. The COOH-terminal pentapeptide REANS did not significantly suppress cell growth or induce apoptosis in i.p. K562 cells.

In addition, we investigated whether AVLPR could suppress cell growth or induce apoptosis in the case of s.c. implanted K562 cells (Fig. 3). The peptide was injected daily from day 4 to day 11 into s.c. K562 cell tumors established in nude mice, and its antitumor and apoptosis-inducing effects were examined. The mice showed an obvious response to intratumor administration of AVLPR, and apoptosis was evident (data not shown). The tumor size in the case of AVLPR treatment declined to 50.1% of the size observed in the case of the control (saline; Fig. 3), and TNF-α treatment decreased the tumor size to 41.3% of the size observed in the case of the control (Fig. 3). On the other hand, the COOH-terminal pentapeptide REANS did not induce apoptosis or suppress the growth of s.c. implanted K562 cells (Fig. 3).

Antitumor Effect of NH$_2$-Terminal Pentapeptide AVLPR Is Attributable to Induction of Apoptosis. To investigate whether the antitumor effect of the NH$_2$-terminal pentapeptide AVLPR is attributable to induction of apoptosis, pathological examination was performed by H&E staining and TUNEL staining (Fig. 4). Histologically, s.c. K562 tumors that responded to AVLPR (10 mice/group were examined) generally displayed homogenous central necrosis with intratumor bleeding (Fig. 4E). Within the viable tumor tissue, many tumor cells became smaller than control cells and showed either condensation or fragmentation of nuclei (Fig. 4E). Neutrophil and lymphocyte infiltration was unremarkable in both groups. Control K562 tumors (saline- and REANS-treated groups) displayed little or no tumor necrosis and showed no change in cell size or nuclei (Fig. 4, A and C). Tumor sections were stained by the TUNEL method, which is specific for apoptotic cells. The TUNEL assay showed that apoptosis was induced in 45.2% of the K562 tumor cells in mice treated with AVLPR (Fig. 4F). However, control tumor cells showed little apoptosis (Fig. 4, B and D).

Discussion

In a previous study, we purified an apoptosis-inducing factor derived from differentiated HL-60 cells (2). This apoptosis-inducing factor was found to be identical to endothelial IL-8. Human recombinant endothelial IL-8 is effective to induce apoptosis in most leukemic cell lines, whereas monocyte-derived IL-8 is not. IL-8 was originally isolated from culture supernatants of stimulated human monocytes and was identified as a protein consisting of 72 amino acid residues (11, 12). The open reading frame of IL-8 cDNA encodes a polypeptide consisting of 99 amino acid residues (13), and the mature form is processed further at the NH$_2$ terminus, yielding several biologically active truncation analogues (14–16). Of the two major

![Fig. 2. Inhibition of cell growth and induction of apoptosis by treatment with endothelial IL-8 in nude mice. The peritoneal space of nude mice (five examined) was inoculated with K562 cells, and saline, endothelial IL-8 [IL-8 E type], the NH$_2$-terminal pentapeptide AVLPR, or the COOH-terminal pentapeptide REANS was injected daily as described in “Materials and Methods.” A, inhibition of the growth of K562 cells by the NH$_2$-terminal pentapeptide AVLPR. Horizontal bars, means. B, the percentage of apoptotic cells was determined microscopically by counting ≥200 cells on slides after in situ staining. Columns, means of three independent experiments; bars, SD. Statistical analysis was performed by Student’s t test. *, P < 0.01.](Image 335x519 to 533x741)
forms, the 72-amino acid form (monocyte-derived IL-8; AVLPRSAKELRC. . . ) is predominant in cultures of monocytes and fibroblasts (18). Endothelial IL-8 has five extra NH2-terminal amino acids lacking in monocyte-derived IL-8, and it is converted to a form identical to monocyte-derived IL-8 by serine proteases such as thrombin (19). The difference of five extra NH2-terminal amino acids may decide the effectiveness in induction of chemotaxis or apoptosis. In this study, we observed that the NH2-terminal pentapeptide AVLPR derived from endothelial IL-8 is effective to induce apoptosis in leukemic cells such as K562, HL-60, U937, Jurkat, and Daudi cells. Moreover, this pentapeptide displays the ability to inhibit the growth of K562 tumor cells and was subsequently injected with saline (A and B). The difference of five extra NH2-terminal amino acids may decide the effectiveness in induction of chemotaxis or apoptosis. In this study, we observed that the NH2-terminal pentapeptide AVLPR derived from endothelial IL-8 is effective to induce apoptosis in leukemic cells such as K562, HL-60, U937, Jurkat, and Daudi cells. Moreover, this pentapeptide displays the ability to inhibit the growth of K562 tumor cells and to induce apoptosis in vivo. It is evident that the NH2-terminal region of endothelial IL-8 corresponding to the peptide AVLPR plays an important role in the induction of apoptosis by endothelial IL-8.

Various short peptide fragments derived from the prohormones proopiomelanocortin and proenkephalin A have been studied and described (20). Methionine enkephalin is a pentapeptide with a variety of functions; it displays an antinecrotic effect, and it is effective in activation of the immune system (21). Previous studies have shown that the pentapeptide Glu-Glu-Asp-Cys-Lys (EEDCK) and the peptide acetyl-Ser-Asp-Lys-Pro (acetyl-SDKP) function as negative regulators in normal hematopoietic cells and malignant cells, especially leukemic cells (22, 23). In our preliminary experiments, the pentapeptide AVLPR did not inhibit colony formation by hematopoietic stem cells, although the pentapeptide EEDCK and the peptide acetyl-SDKP showed an inhibitory effect on colony formation by stem cells. These findings suggest that the pentapeptide AVLPR may provide us with a new therapy for hematological malignancies.

Recently, it has been demonstrated that vascular cells are important participants in antitumor host defense. The mechanism of the antitumor host defense system associated with vascular cells has not been clarified, although vascular cells expressing nitric oxide synthase in response to stimulation by IFN-γ and TNF-α can kill leukemic cells (24). Many researchers have discussed the role of angiogenesis in tumor vascularization and tumor metastasis (25). Tumor cells secrete a metalloproteinase that acts to destroy matrix proteins and to damage endothelial cells, and then they invade extravascular spaces (26). In our previous study, it was demonstrated that endothelial IL-8 can protect against tumor invasion (3). This is a novel function of endothelial cells involving tumor cell eradication in the body, mainly via endothelial IL-8. In this system, the NH2-terminal pentapeptide AVLPR may be released or produced from endothelial IL-8 and then induce apoptosis in tumor cells. This peptide may have an important role in induction of apoptosis in tumor cells in the bloodstream when they become anchored to endothelial cells.

Surprisingly, in our in vivo experiments, the NH2-terminal pentapeptide AVLPR inhibited the growth of K562 cell tumors in the same manner as TNF-α. We demonstrated that TNF-α does not directly suppress cell growth or induce apoptosis in K562 cells, but rather it allows endothelial cells to secrete IL-8, and it can indirectly kill leukemic cells (3). TNF-α modulates the expression of various biological molecules in endothelial cells (27). The in vivo effect of TNF-α on killing tumor cells may be explained in terms of both a direct death signal mediated through its receptor and in terms of 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 the secretion of endothelial IL-8 from intratumor endothelial cells, and then the NH2-terminal pentapeptide AVLPR may be released from endothelial IL-8. The peptide AVLPR was found to inhibit the growth of K562 cell tumors more strongly than endothelial IL-8. However, angiogenesis for tumor vascularization would aid tumor progression, and tumor cells produce and secrete some endogenous regula-
tors such as endostatin and angiostatin (28, 29). In this study, injected TNF-α acted on endothelial cells growing in tumors, and endothelial IL-8 might then be released from endothelial cells. We noticed that the difference of the quantities of endothelial IL-8 versus pentapeptide administered in the in vivo experiments was a log order of magnitude. This result suggests two possibilities of differences in stability of molecules and affinity to cells. The shorter peptides may not have enough stability to act biologically because of ubiquitous peptidases such as aminopeptidase N (CD13). The pentapeptide AVLPR lost the biological activity to induce apoptosis when only two amino acids of NH₂ or COOH terminus were deleted from AVLPR. We need to modify the pentapeptide to stabilize it. There may be some differences in binding characteristics. IL-8 has two major receptors, IL-8RA and IL-8RB, and binds to receptor by its internal sequence ELR (13), which is the COOH-terminal site of AVLPR. In contrast, binding characteristics of AVLPR have not been clarified yet. AVLPR production from endothelial IL-8 may occur in the cells after internalization of IL-8/IL-8 receptor complex. Therefore, a higher concentration of the pentapeptide AVLPR is needed to induce apoptosis than that of endothelial IL-8.

If we understand how to stimulate the release or production of the NH₂-terminal pentapeptide of endothelial IL-8, we may be able to develop a new approach to treatment. In 10 of 12 clinical cases, we have observed that apoptosis was significantly induced in fresh leukemic cells by the NH₂-terminal pentapeptide of endothelial IL-8 in vitro. Endothelial IL-8, with five extra NH₂-terminal amino acid residues, can induce apoptosis, whereas monocyte-derived IL-8, which lacks them, cannot. Because most IL-8 in blood plasma exists in the form of monocyte-derived IL-8 (30), which is not effective to induce apoptosis in leukemic cells (2), this phenomenon may be important in relation to leukemia therapy. In cases of monoblastic leukemia with poor prognosis, a large amount of monocyte-derived IL-8 is released, and this may act as a competitor of endothelial IL-8. 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 μM VP-16 and 1.0 mM AVLPR for 48 h, the percentage of apoptotic cells increased as compared with treatment with only 0.1 μM VP-16. This result suggests that the NH₂-terminal pentapeptide of endothelial IL-8 can enhance the effect of VP-16 in induction of apoptosis, and it may therefore be clinically promising in combination with VP-16.

Our observations concerning the NH₂-terminal pentapeptide AVLPR are consistent with data on endothelial cells as follows; about 15% of the cells. In conclusion, endothelial cells involved in a novel apoptotic mechanism similar to that of endothelial IL-8.

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References

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