Adipocyte-Derived Leucine Aminopeptidase Suppresses Angiogenesis in Human Endometrial Carcinoma via Renin-Angiotensin System

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ABSTRACT

Purpose: Angiotensin (Ang) II was reported to induce vascular endothelial growth factor (VEGF) expression in various cells. Adipocyte-derived leucine aminopeptidase (A-LAP) is a novel member of the M1 family of zinc metallopeptidases. Enzymatic characterization demonstrated that A-LAP hydrolyzes Ang II. This study examined the role of A-LAP in angiogenesis of human endometrial carcinoma.

Experimental Design: We investigated whether Ang II induces VEGF expression in human endometrial carcinoma cells. To investigate the possible function of A-LAP in angiogenesis of endometrial carcinoma, we transfected A-LAP cDNA into HEC-1A cells, showing the lowest expression of A-LAP.

Results: In the present study, we showed that Ang II enhanced VEGF expression in a dose-dependent manner in endometrial carcinoma cells (HEC-1A cells). Overexpression of A-LAP attenuated Ang II-induced VEGF expression in HEC-1A cells. In addition, Human umbilical vascular endothelial cell migration was increased in conditioned media from Ang II-treated wild-type cells, but not in conditioned media from Ang II-treated A-LAP-overexpressing cells (HEC-1A-A-LAP cells). In an in vivo study, we showed that A-LAP overexpression in endometrial carcinoma cells results in a reduction of VEGF immunoreactivity and the number of blood vessels within tumors.

Conclusions: Our study demonstrates that it is feasible to overexpress Ang II-degrading enzymes in cultured cells and that this overexpression attenuated some effects of exogenous and endogenous Ang II. These experiments are a first step toward the development of novel strategies to selectively antagonize locally generated Ang II and suppress VEGF-induced angiogenesis in endometrial carcinoma.

INTRODUCTION

It is now widely recognized that the growth of a solid tumor depends on angiogenesis. Angiogenesis is an important physiological process associated with neovascularization, growth, and metastasis of many different tumors (1–5). Vascular endothelial growth factor (VEGF) is the most important stimulator of angiogenesis with a specific mitogenic action on endothelial cells as well as a potent capacity for increasing vessel permeability. Previous studies showed that angiotensin (Ang) II stimulated VEGF expression in human mesangial cells (6) and human vascular smooth muscle cells (7). Recently, it was suggested that Ang II is not only a vasoconstrictive agent but also a growth factor and similar to cytokines. To date, many scientists have focused on Ang II synthesis and its signal transduction via Ang II receptors on this important peptide; however, there are few studies on the degradation of Ang II. We have studied Ang II-degrading protease in human placenta since 1976 and cloned human adipocyte-derived leucine aminopeptidase (A-LAP), which degrades Ang II (8). However, there are few reports on the relation between Ang II and VEGF, and there is no report on the possible role of angiotensinase in carcinoma.

We detected Ang II receptor, Ang II, and its degrading protease, A-LAP, in human endometrial carcinoma. In addition, we investigated whether Ang II stimulates VEGF secretion from endometrial carcinoma cells and whether A-LAP functions as a regulator of the local concentration of Ang II in endometrial carcinoma.

MATERIALS AND METHODS

Reagents. Ang II was purchased from the Peptide Institute, Inc. (Osaka, Japan). A selective antagonist of AT1, candesartan (CV11974), was purchased from Takeda Chemical Industries Ltd.

Cell Culture and Tissue Specimens. We used three human endometrial adenocarcinoma cell lines (A-MEC, HEC-1A, and Ishikawa). A-MEC was generously donated by Aichi Medical University (Aichi, Japan), Ishikawa was donated by Dr. M. Nishida (Kasumigaura Hospital, Ibaragi, Japan), and HEC-1A was donated by Prof. H. Kuramoto (Kitazato University, Kanagawa, Japan). Cells were maintained in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FCS and penicillin-streptomycin. These cells were incubated at 37°C in a humidified atmosphere of 5% CO2. Human umbilical vascular endothelial cells (HUVECs) were established in primary cell cultures in Humedia-EG 2 (Kurabo Industries Ltd., Osaka, Japan). Surgical specimens of human endometrial adenocarcinoma tissues were used after obtaining informed consent from the patients. All tissue samples were fixed in 10% forma-
lin, embedded in paraffin, and routinely stained with H&E for histological examination.

**Immunohistochemistry.** Immunohistochemical staining was performed using the avidin-biotin immunoperoxidase technique (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). Sections were cut at a thickness of 4 μm and immunostained by the streptavidin/biotin/peroxidase method. Deparaffinized sections in 0.1 M citrate buffer were treated three times for 5 min each at 90°C and 750 W using an H2500 microwave oven. Sections were incubated in 0.3% hydrogen peroxide for 20 min and then further incubated with 10% normal goat serum in NaCl/Pi for 10 min to block the endogenous peroxidase activity and nonspecific immunoglobulin binding, respectively. Rabbit polyclonal antibody (sc-1173; Santa Cruz Biototechnology, Inc., Santa Cruz, CA) was used at a 1:200 dilution for AT1, rabbit polyclonal antibody (Santa Cruz Biotecnoology, Inc.) was used at a 1:200 dilution for VEGF, rabbit polyclonal antibody was used at 10 μg/ml for A-LAP, and rabbit anti-Ang II serum (IHC 7002; Peninsula Laboratories Inc., a division of Bachem) was used at 1×10^5 g/ml for A-LAP, and rabbit anti-Ang II serum (IHC 7002; Peninsula Laboratories Inc., a division of Bachem) was used at 1:500 dilution for Ang II. Antibodies were added to the tissue sections and incubated for 1 h in a moist chamber at room temperature. Binding of the antibodies was followed by biotinylated goat antirabbit IgG and horseradish peroxidase-conjugated streptavidin (Histofine SAB-PO; Nichirei). Chromogenic development was performed by immersion of the sections in 3-amino-9-ethylcarbazole (Nichirei). The slides were counterstained with Mayer’s hematoxylin.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR).** Total RNA was isolated from A-MEC, HEC-1A, and Ishikawa cells using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s protocol. Oligonucleotide primers were designed to amplify a 330-bp fragment of human AT1 cDNA (forward primer, 5’-GGAAA-CAGCTTGGTGTTGAT-3’; reverse primer, 5’-GCAGC-CAAAATGATGATGAGCAG-3’). PCR consisted of 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The RT-PCR-amplified samples were visualized on 1.5% agarose gels using ethidium bromide.

**Plasmid Construction and Transfection.** Full-length cDNA for A-LAP was isolated from a human adipose tissue cDNA library (9). The eukaryotic expression vector pcDNA3.1(−) (Invitrogen Japan K.K., Tokyo, Japan) was used to drive the expression of inserted A-LAP. Transfections were carried out using LipofectAMINE according to the manufacturer’s instructions (Life Technologies, Inc., San Diego, CA). HEC-1A cells were transfected with pcDNA3.1(−) (HEC-1A-pcDNA) or pcDNA3.1(−) inserted with A-LAP cDNA (HEC-1A-A-LAP). Stable transfectants were selected by growth in medium supplemented with 400 μg/ml G418 (Sigma). Several hundred clones resistant to G418 were obtained, and polyclonal cells from these transfectants were used in the following experiments to eliminate any effects that could be attributed to clonal variation.

**Western Blotting.** Samples were electrophoresed on 10% SDS polyacrylamide gel under reducing conditions. After electrophoresis, the proteins were transferred electrophoretically to Immobilon membrane (Millipore, Bedford, MA). After blocking, the membrane was incubated for 1 h with rabbit polyclonal antibody against human A-LAP at a 1:1000 dilution. Anti-VEGF antibody was used at a 1:500 dilution. Anti-human β-actin antibody (Abcam Ltd.) was used as a control. The membrane was washed three times (15 min each time) with TBS-T and then incubated with peroxidase-conjugated goat antirabbit IgG for 1 h. After washing with PBS, 0.05% Tween 20, the membrane was subjected to enhanced chemiluminescence Western blotting detecting reagent (Amersham Biosciences K.K.).

**Cell Treatments and Measurement of VEGF Protein by Enzyme-Linked Immunosorbent Assay (ELISA).** A-MEC, HEC-1A, and Ishikawa cells (1×10^5) were plated onto 24-well plates. After resting for 12 h, stimulation was performed in 1 ml of serum-free medium with Ang II concentrations ranging from 10^-10 to 10^-7 M in the presence or absence of CV11974. After 24, 48, and 72 h, media were centrifuged at 5000 × g for 10 min, and then supernatant was used as conditioned media (CM). VEGF protein in CM was measured by a VEGF ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. VEGF protein concentrations were corrected using total cellular proteins.

**Migration Assay.** Endothelial cell migration was assayed in 24-well Transwell cell culture chambers (Costar Corp., Cambridge, MA). HUVECs (10^4 cells) were suspended in the upper chamber of the Transwell plate in 0.1 ml of CM or those neutralized with anti-VEGF polyclonal antibody. The lower chamber was filled with 0.6 ml of RPMI 1640 with 0.1% BSA. After 8 h of incubation, the remaining tumor cells on the upper surface of the filter were removed by wiping with cotton swabs, and cells that had migrated onto the lower surface were stained with May-Grünwald-Giemsa staining. The number of cells on the lower surface of the filters was counted under a microscope at ×200 magnification. Cells were counted in 5 fields/filter in triplicate.

**In Vivo Studies.** Female nude mice (BALB/c) at 6 weeks were obtained from Chubu Kagaku (Nagoya, Japan). HEC-1A-pcDNA and HEC-1A-A-LAP cells (1×10^5 cells/150 μl of medium/mouse) were injected into the dorsal surface of the mice. Four weeks after carcinoma cell injection, the mice were sacrificed, and tumors were collected. Consecutive 4-μm sections of paraffin-embedded tissues were immunostained for VEGF using a rabbit polyclonal antihuman VEGF antibody (Santa Cruz Biotecnoology, Inc.) and for CD34 using a rabbit monoclonal antimouse CD34 antibody (HyCult Biotechnology b.v., Uden, the Netherlands).

**Statistical Analysis.** Student’s t test and analysis of variance with Bonferroni correction were used to compare the VEGF levels and migration cell number, and P < 0.05 was considered significant.

**RESULTS**

**Expression of AT1, Ang II, and A-LAP in Endometrial Carcinoma Tissues and Cells.** We first examined the expression of A-LAP, Ang II, AT1, and VEGF in human endometrial carcinoma tissues by immunochemistry. In 25 of 30 endometrial adenocarcinomas, tumor cells were A-LAP positive. In all positive tumor cells, A-LAP staining was intense and localized at the periphery of the cytoplasm, close to the cell membrane (Fig. 1A). In 26 of 30 cases, tumor cells were Ang II
positive. Ang II was stained at both the cell membrane and cytoplasm (Fig. 1B). In 23 of 30 endometrial adenocarcinomas, tumor cells were AT1 positive. The staining level of tumor cells was almost the same as that of vascular endothelial cells (Fig. 1C). VEGF protein was expressed in 26 of 30 cases and localized in the cytoplasm of the tumor cells (data not shown). A-MEC, HEC-1A, and Ishikawa cells were all positive for AT1 and A-LAP on Western blotting (Fig. 2, A and B). Ang II was stained in both the cell membrane and cytoplasm on the three cell lines (data not shown). The 330-bp PCR products corresponding to AT1 mRNA were also observed in all cell lines (Fig. 2C).

**Induction of VEGF Expression by Ang II.** Using human VEGF ELISA kits, we measured the effect of Ang II on the secretion of VEGF in HEC-1A cells. As shown in Fig. 3A, Ang II enhanced VEGF secretion in a dose-dependent manner after 48 h of incubation, and the greatest effect (1.9-fold increase) was obtained at a dose of 10^{-8} M. These results were confirmed by Western blotting of cell lysates, demonstrating that Ang II treatment led to synthesis of VEGF protein in HEC-1A cells (Fig. 3B). Similar data were obtained from A-MEC and Ishikawa cells, whereas the induction was greatest in HEC-1A cells among the three cell lines. To determine the subtype of Ang II receptor involved in Ang II-induced up-regulation of VEGF expression in HEC-1A cells, cells were preincubated with CV11974, a selective antagonist of AT1 receptor. Supplementation of culture medium with CV11974 (10 μM) alone had no effect on VEGF secretion from HEC-1A cells. However, increased VEGF secretion from HEC-1A cells induced by Ang II (10^{-8} M) was abolished by pretreatment with 10 μM CV11974 (Fig. 3C).

**Involvement of A-LAP in Ang II-Induced VEGF Secrecion of Endometrial Cancer Cells.** To investigate the effect of A-LAP in endometrial carcinoma cells, we transfected A-LAP into HEC-1A cells (HEC-1A-A-LAP cells). Both parental HEC-1A cells and vector-transfected HEC-1A-pcDNA cells expressed little A-LAP, whereas HEC-1A-A-LAP cells expressed a remarkably high level of A-LAP on Western blotting.
HEC-1A-A-LAP cells also secreted A-LAP in culture medium, whereas HEC-1A-pcDNA cells did not secrete A-LAP (Fig. 4B). As shown in Fig. 4C, a single dose of 10^{-9} to 10^{-7} m Ang II for 48 h led to a significant increase in VEGF secretion in HEC-1A-pcDNA cells (P < 0.05). In contrast, the same concentration of Ang II failed to stimulate VEGF secretion in HEC-1A-A-LAP cells.

Increased Endothelial Cell Migration by Ang II-Induced VEGF. Because Ang II increased VEGF secretion from HEC-1A cells, we sought to determine whether VEGF up-regulated by Ang II might influence endothelial cell migration, which is an indicator of angiogenic response. CM were collected from HEC-1A cells and HEC-1A-A-LAP cells treated with or without Ang II and used to stimulate HUVEC migration. Incubation of HUVECs with CM from Ang II-treated HEC-1A cells increased the number of migrated HUVECs by 2-fold compared with that from untreated cells (Fig. 5, 1 and 2). The addition of VEGF antibody (50 pg/ml) inhibited the migration of HUVECs in CM from Ang II-treated HEC-1A cells, whereas nonspecific IgG had no effect (Fig. 5, 3–5). HUVEC migration after incubation with CM from HEC-1A-A-LAP cells treated with or without Ang II was almost the same as that of untreated HEC-1A cells (Fig. 5, 6 and 7). VEGF (10^{-8} m) increased HUVEC migration by 2-fold compared with control medium, but Ang II (10^{-8} m) had no effect (Fig. 5, 8 and 9).

Effect of A-LAP on VEGF Expression and Blood Vessel Density in Vivo. We also investigated whether A-LAP suppressed VEGF expression and the number of blood vessels in endometrial carcinoma using nude mice. Decreased VEGF expression was observed in the tumors of HEC-1A-A-LAP cells (Fig. 6B) compared with HEC-1A-pcDNA cells (Fig. 6A). Tumors formed by HEC-1A-pcDNA cells (Fig. 6C) were highly vascularized, whereas tumors formed by HEC-1A-A-LAP cells were observed to have a remarkably decreased vascular density and central necrosis (Fig. 6D).

DISCUSSION
The present study showed for the first time the localization of AT1 receptor, Ang II, and A-LAP in human endometrial carcinoma tissues (Fig. 1) and their cell lines (Fig. 2). We also confirmed the localization of VEGF in these cells, as reported previously by Guidi et al. (10) and Giatromanolaki et al. (11). VEGF is well known to act as a potent angiogenic factor as well as a survival factor in tumor endothelial cells. Previous studies showed that Ang II stimulates VEGF expression (6, 7). VEGF expression is also regulated by many growth factors and cytokines, such as insulin-like growth factor (12), interleukin 6 (13), transforming growth factor β (14), and platelet-derived growth factor (15).
factor (15). Recent studies have shown that Ang II activates nuclear factor-

\( \text{NF}-\kappa \text{B} \) in vascular smooth muscle cells through Ang type 1 receptors (AT1; Ref. 16). In 1999, we cloned A-LAP, which is a protein highly homologous to placental leucine aminopeptidase/insulin-responsive aminopeptidase and showed that A-LAP cleaves stepwise the NH\(_2\)-terminal amino acid of Ang II. We showed that A-LAP cleaves Ang II and Ang III and converts kallidin to bradykinin. Therefore, it is suggested that the possible interaction among the effects of Ang II via AT1, A-LAP, and VEGF might influence the growth via angiogenesis in human endometrial carcinoma.

The present data showed that Ang II enhanced VEGF secretion from HEC-1A cells in a dose-dependent manner (Fig. 3, A and B). Peak response was observed at 10 nM, whereas higher concentrations had less stimulatory effect on VEGF expression. Our findings are similar to those in some previously reported experiments (15, 17). Similar data were obtained from A-MEC and Ishikawa cells. It was interesting that the induction of VEGF was greatest in HEC-1A among the three cell lines, whereas A-LAP protein was the lowest in HEC-1A among the three cell lines (data not shown). Our data also showed that the induction of VEGF by Ang II occurs via AT1 (Fig. 3C).

The present data also showed that Ang II affects endothelial cell migration, which is an indicator of angiogenic response, and migration by Ang II is due to VEGF (Fig. 5). Interaction of VEGF and its receptor is thought to play a major role in angiogenesis in human tumors. It is reported that vascular invasion in endometrial adenocarcinoma is related to VEGF expression (10) and thus to poor prognosis of endometrial carcinoma patients (11).

Ang II is the main effector peptide of the renin-Ang system, and it exerts a variety of actions on the cardiovascular and renal systems. Although the roles of Ang II and its receptors have been studied extensively in the cardiovascular field, there are few reports in relation to tumors (18–21). However, a retrospective cohort study of many patients receiving Ang-converting enzyme inhibitor has already suggested the possible importance of Ang II in carcinoma (22). Recently, Yoshiji et al. (23) reported that several types of Ang-converting enzyme inhibitors and AT1 antagonists inhibited tumor development as well as angiogenesis in a murine hepatocellular carcinoma experimental model, suggesting an interaction between VEGF and Ang-converting enzyme activity in the tumor. It is suggested that the amount of Ang II relates not only to cardiovascular disease but also to carcinoma. Needless to say, the amount of Ang II depends on both the production and degradation of Ang II.

In this study, we showed that overexpression of A-LAP in HEC-1A cells failed to stimulate VEGF secretion by Ang II (Fig. 4) and also failed to stimulate the migration of endothelial cells by VEGF possibly stimulated by Ang II (Fig. 5). Furthermore, we showed that A-LAP overexpression in endometrial carcinoma cells results in a reduction in the number of blood vessels within tumors in vivo (Fig. 6). Because our immunohistochemical study showed that A-LAP localizes mainly in the
cytoplasm in endometrial carcinoma cells (Fig. 1), Ang II might also be catalyzed in the cytoplasm of carcinoma cells by A-LAP. Several studies showed that Ang II is internalized into the cells via an AT1-mediated process (24, 25), and intracellular Ang II stimulates the cell growth of vascular smooth muscle cells. These effects were inhibited by AT1 antagonist (losartan; Ref. 26). In 1981, we demonstrated the existence of aminopeptidase A in human placenta (27), and this enzyme cleaves the NH$_2$-terminal amino acid of Ang II, aspartic acid, converting it to Ang III. Recently, we have shown the existence of aminopeptidase A in human endometrium and its possible roles in endometrial function (28). Whereas aminopeptidase A is localized mainly in the stromal region, A-LAP is localized in the cytoplasm in tumor cells of endometrial adenocarcinoma. Therefore, the present data suggest that A-LAP is another angiotensinase in human endometrium, which inactivates Ang II before its binding to AT1 receptor. A-LAP in endometrial carcinoma cells might regulate the local concentration of Ang II, which plays an important role in the regulation of angiogenesis via VEGF. However, expression and involvement of aminopeptidase A were not previously investigated in endometrial carcinoma; therefore, further study is required.

In a recent study, Miyashita et al. (29) showed that mouse A-LAP was induced during mouse endothelial cell differentiation from mouse embryonic stem cells by VEGF and involved in angiogenesis. We showed that A-LAP catalyzes Ang II and suppresses VEGF-induced angiogenesis in endometrial carcinoma. The potential for using angiotensinase A-LAP in treating cancer by regulating the concentration of Ang II might be promising, although extensive work is required.

In conclusion, this is the first report on the existence of AT1 receptor, Ang II, and A-LAP in endometrial carcinoma. Although Ang II stimulates VEGF secretion from carcinoma cells, A-LAP functions as a regulator of the Ang II concentration by promoting its degradation. Therefore, A-LAP in carcinoma cells could play an important role in tumor growth via inhibition of angiogenesis and endothelial cell migration by regulating the Ang II concentration. However, in the present study, Ang II-enhanced VEGF expression was not very strong (the greatest effect showed a 1.9-fold increase). Ang II is one of several factors promoting VEGF expression, and

**Fig. 6** Immunohistochemical analysis for expression vascular endothelial growth factor (VEGF) and CD34 in HEC-1A-pcDNA cells and HEC-1A-A-LAP cells (magnification, ×200). Four weeks after carcinoma cell injection, mice were sacrificed, and tumors were collected. Sections were immunostained by VEGF and CD34 and antibody. A, VEGF expression in the HEC-1A-pcDNA tumor cells. B, VEGF expression in HEC-1A-A-LAP tumor cells. C, CD31 expression in HEC-1A-pcDNA tumor cells. D, CD31 expression in HEC-1A-A-LAP tumor cells.
the manner in which its degrading aminopeptidase, A-LAP, might be involved in angiogenesis in endometrial carcinoma should be further elucidated. Further study is required to clarify the biological action of A-LAP in endometrial carcinoma.

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