Possible Role of Placental Leucine Aminopeptidase in the Antiproliferative Effect of Oxytocin in Human Endometrial Adenocarcinoma

Yuka Suzuki, Kiyosumi Shibata,1 Fumitaka Kikkawa, Hiroaki Kajiyama, Kazuhiko Ino, Seiji Nomura, Masafumi Tsujimoto, and Shigeziko Mizutani

Department of Obstetrics and Gynecology, Nagoya Graduate University School of Medicine, Nagoya 466-8850, Japan, and Riken (Laboratory of Cellular Biochemistry, The Institute of Physical and Chemical Research), Wako-shi, Saitama, Japan

ABSTRACT

Purpose: Oxytocin (OT) was reported to inhibit the proliferation of various neoplastic tissues and cells, however, the regulation system remains unclear. This study examined the role of OT and its regulatory ability in endometrial adenocarcinoma.

Experimental Design: To investigate the possible function of placental leucine aminopeptidase (P-LAP) in endometrial adenocarcinoma, we transfeced P-LAP cDNA into A-MEC cells, showing the lowest enzyme activity of P-LAP. Also we examined P-LAP protein expression in human endometrial adenocarcinoma.

Results: We demonstrated the presence of P-LAP, which is identical to cysteine aminopeptidase as oxytocinase, in human endometrial adenocarcinoma tissues and found that the expression of P-LAP increase with advances in the grade. Exposure of endometrial adenocarcinoma cell lines to OT caused dose- and time-dependent inhibition of growth. Treatment with 10−7 M OT for 72 h reduced cell growth by 62, 25, and 30% in A-MEC, HEC1A, and Ishikawa cells, respectively. P-LAP-transfected cells not only partially recovered from OT-induced growth inhibition but also showed a higher growth rate than parental cells under condition without OT. An OT receptor antagonist and a protein kinase A inhibitor blocked OT-induced growth inhibition in A-MEC and A-MEC-pc cells but not in A-MEC-LAP cells.

Conclusions: These findings suggested that P-LAP might be functionally positive on carcinoma cell growth by degrading suppressive peptides such as OT.

INTRODUCTION

OT,2 a nonapeptide hormone, is secreted from the neurohypophysis and acts on target cells. Previous studies showed that the biological effects of OT are involved in various reproductive functions through OTR in an autocrine and paracrine manner (1–3). OTR is also present in human endometrium and localized in predominantly in the epithelial cells and glands, with little or none detected in the stroma (4). The expression of OTR in endometrium may be regulated by the sex steroids, and OT may play an important role of implantation (4–6). Cassoni et al. (7) demonstrated that endometrial adenocarcinoma cells also expressed OTR using immunohistochemical staining and reverse transcriptase-PCR. OT was reported to act as a negative regulator of cell proliferation in various carcinoma cell lines, including human breast carcinoma, osteosarcoma, and endometrial adenocarcinoma, all of which express OTR (7–9). P-LAP, a cell surface aminopeptidase, is identified with oxytocinase (10). P-LAP is also referred to as insulin-regulated membrane aminopeptidase associated with the glucose transporter 4-containing vesicle (11). We have cloned P-LAP cDNA and found the wide-spread tissue distribution of P-LAP by immunohistochemical staining and Northern blot analysis (10, 12, 13). Because P-LAP can degrade several small peptide hormones such as OT, arginine vasopressin, and angiotensin III (14), this enzyme would be involved in many cellular functions in carcinoma cells as well as normal cells.

There are several reports of cellular functions of OT and OTR, however, little is known about the possible role of P-LAP in carcinoma cells. In this study, we demonstrated that P-LAP expressed in epithelial cells of normal endometrium and endometrial endometrioid adenocarcinoma tissues and the expression level increased with advancement in the grade of differentiation. Furthermore, we present experimental evidence that P-LAP may play an important role in regulating the carcinoma cell growth by transfection of P-LAP cDNA to endometrial adenocarcinoma cells.

MATERIALS AND METHODS

Tissues. Human endometrial neoplasm tissues were obtained from 35 patients consisting of atypical endometrial hyperplasia (n = 5) and endometrial endometrioid adenocarcinomas (n = 30) at Nagoya University Hospital between 1994 and 1999. Normal endometrial tissue samples were also obtained from 10 women with uterine myoma. Informed consent was obtained from each patient for sample use. All tissue samples

1 To whom requests for reprints should be addressed, at Department Obstetrics and Gynecology, Nagoya Graduate University School of Medicine, Tsurumai-cho 65, Showa-ku, Nagoya 466-8550, Japan. Phone: 81-52-744-2263; Fax: 81-52-744-2268; E-mail: shiba@med.nagoya-u.ac.jp.

2 The abbreviation used are: OT, oxytocin; OTR, OT receptor; P-LAP, placental leucine aminopeptidase; SCO, spiroindenylpiperidine camphorsulfonamide oxytocin; PKA, protein kinase A.
were fixed in 10% formalin, embedded in paraffin, and routinely stained with H&E for histological examination. The differentiation of endometrial adenocarcinomas were graded according to the criteria of the WHO and classified as grade 1 (well differentiated, n = 10), grade 2 (moderately differentiated, n = 10), and grade 3 (poorly differentiated, n = 10).

**Immunohistochemistry.** Antihuman monoclonal antibody against OTR was kindly gifted from Dr. Tadashi Kimura (Osaka University). Antihuman P-LAP polyclonal antibody was prepared in our laboratory. 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 0.01M citrate buffer were treated three times for 5 min at 90°C at 750 W with a H2500 microwave oven. Sections were incubated with 0.3% hydrogen peroxide for 20 min and then additionally incubated with 10% normal goat serum. Antihuman OTR monoclonal antibody or antihuman P-LAP polyclonal antibody was added to the tissue sections and incubated for 1 h. The binding of the antibodies was followed by biotinylated goat antirabbit IgG and horseradish peroxidase-conjugated streptavidin (Histofine SAB-PO kit; Nichirei). Chromogenic development was performed by immersion of the sections in 3-amino-9-ethylcarbazole (Nichirei). The slides were counterstained with Mayer’s hematoxylin. Immunostaining intensity was scored semiquantitatively on a three-tiered scale (negative = −, weakly positive = +, and strongly positive = +++) relative to the known positive and negative controls.

**Cell Lines and Culture Condition.** We used three human endometrial endometrioid adenocarcinoma cell lines (A-MEC, HEC1A, and Ishikawa). A-MEC was kindly gifted from Aich Medical University, Ishikawa from Dr. Masato Nishida (Kasanigaura Hospital, Ibaragi, Japan), and HEC1A from Dr. Hiroyuki 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.

**Reverse Transciptase-PCR.** Total RNA was isolated from the A-MEC, HEC1A, and Ishikawa cells using Trizol regent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacture’s protocol. Oligonucleotide primers used for the amplification of OTR cDNA were 5’-CCTTTCATCGTG-GCTGACG-3’ (forward) and 5’-CTAGGGACAGGCAC-TATG-3’ (reverse). Primers for P-LAP were 5’-TGGTGGCCAC-TACGCTATGAACTCA-3’ (forward) and 5’-ACTGCTGAC-ATAAAGGTCTACCTTG-3’ (reverse). PCR consisted of 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The reverse transcription-PCR amplified samples were visualized on 1.5% agarose gels using ethidium bromide.

**Enzyme Activity.** P-LAP activity was measured spectrophotometrically as reported previously with minor modification (15). Briefly, subconfluent cultures in 96-well plates were washed twice with PBS and incubated at 37°C for 1 h in 50 μM Tris-HCl (pH 7.4) containing 1.6 mM L-leucine-p-nitroanilide and 20 mM L-methionine. Absorbance was measured with a spectrophotometer (Multiskan Bichromatic; Labsystems, Helsinki, Finland) at 405 nm. The enzyme activities were expressed in pmol/min/10⁵ cells.

**Plasmid Construction and Transfection.** The eukaryotic expression vector pcDNA3.1(-) (Invitrogen Japan K.K., Tokyo, Japan) was used to drive the expression of inserted P-LAP cDNA. Transfections were carried out using Lipofectamine according to the manufacturer’s instructions (Invitrogen Japan K.K.). A-MEC cells were transfected with pcDNA3.1(-) (A-MEC-pc) or pcDNA3.1(-) inserted with P-LAP cDNA (A-MEC-LAP). Stable transfectants were selected by growth in medium supplemented with 400 mg/ml G418 (Sigma Chemical Co.). 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.

**Immunoblotting.** Tissues were homogenized for 10 min on ice in lysis buffer composed of PBS, 1% Triton X. Tissue extracts and cell lysates were cleared by centrifugation at 14,000 × g for 10 min at 4°C and stored at −80°C. Samples were electrophoresed on 7.5% 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 P-LAP. The membrane was washed with PBS, 0.05% Tween 20 for 15 min three times 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 ECL-Western blotting detecting reagent (Amersham Biosciences K.K., Tokyo, Japan).

**Drug Treatment and Cell Growth Analysis.** OT was purchased from Peptide Institute, Inc. (Osaka, Japan). To evaluate the effect of OT on cell proliferation, cells were seeded in triplicate in 96-well plates at a density of 5000 cells in a volume of 200 μl. Twenty-four h after plating, OT was added to the culture medium at concentrations ranging from 10⁻¹¹ to 10⁻⁶ M. An OTR antagonist, SCO (Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan), and a PKA inhibitor, 14–22 amide (Calbiochem, La Jolla, CA), were further added to the medium. The medium was changed every 24 h. At 24, 48, and 72 h of culture, cell viability was assayed using a modified tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay by Cell Titer 96 Aqueous One Solution Cell Proliferation Assay kit (Promega Corp., Tokyo, Japan) according to the manufacturer’s instructions. Absorbance was measured at 490 nm by a microplate reader (Multiskan Bichromatic; Labsystems).

**Measurement of Intracellular cAMP.** Cells (1 × 10⁵) were plated in 96-well plates in serum-free medium. After 6 h, cells were treated with or without OT in serum-free medium. After 30 min, intracellular cAMP was measured using a EIA kit (Amersham Pharmacia Biotec, Little Chalfont, United Kingdom).

**Statistical Analysis.** The nonparametric Kruskal-Wallis test and Mann-Whitney t test with Bonferroni correction. Student’s t test and ANOVA with Bonferroni correction were applied to compare the number of cells and concentration of cAMP among cell lines. P < 0.05 was considered significant.
Fig. 1 Immunochemical localization of P-LAP in human normal endometrium and endometrioid adenocarcinoma tissues (Magnification, ×200). A, P-LAP in normal endometrium; B, P-LAP in endometrial hyperplasia; C, P-LAP in grade 1 endometrioid adenocarcinoma; D, P-LAP in grade 2 endometrioid adenocarcinoma; E, P-LAP in grade 3 endometrioid adenocarcinoma; F, Western blotting analysis of P-LAP. Lanes as follows: Lane 1, normal endometrium tissue; Lane 2, grade 1 endometrioid adenocarcinoma tissue; Lane 3, grade 2 endometrioid adenocarcinoma tissue; and Lane 4, grade 3 endometrioid adenocarcinoma tissue. G, the intensity of immunoreactivity was scored as negative (−), weakly positive (+), and strongly positive (++). A significant difference is observed by Kruskal-Wallis test (+, P < 0.05; ++, P < 0.01).
RESULTS

Detection of P-LAP and OTR. Fig. 1 shows immunohistochemical staining of P-LAP. Epithelial cells of normal endometrium were P-LAP positive in 4 of 10 tissue samples (Fig. 1A). Four of 5 endometrial hyperplasia tissues were immunostained with P-LAP (Fig. 1B), and one case was strongly stained. In low-grade (grade 1) endometrioid adenocarcinomas, only 3 of 10 were weekly immunostained with P-LAP (Fig. 1C). On the contrary, enhanced P-LAP expression was observed in high-grade adenocarcinomas. Tumor cells showed a diffuse and intense cytoplasmic P-LAP distribution in the strongly positive cases (Fig. 1, D and E). The intensity of immunohistochemical staining was scored and plotted in Fig. 1G, showing the significant tendency of increased immunoreactivity with advancement of grade ($P < 0.05$). In 24 of 30 adenocarcinomas, OTR was positive, but the no differences were observed among tumor grade (data not shown).

Expression of P-LAP protein was examined in the endometrial adenocarcinoma tissues and cell lines by immunoblotting. We detected an immunoreactivity of P-LAP with molecular mass of 165 kDa. The levels of immunoreactivity were in accordance with the results of immunohistochemistry in the tissue samples (Fig. 1F). In three cell lines immunoreactivity of P-LAP was detected, and the level was lowest in A-MEC cells (data not shown). The presence of P-LAP and OTR was also confirmed in three cell lines by reverse transcriptase-PCR (Fig. 2A).

Growth Inhibitory Effects of OT. Cells were exposed to $10^{-7}$ M OT and then counted at daily intervals during a 72-h period of culture. Exposure to OT for 72 h reduced cell number to 38, 75, and 70% of untreated cells in A-MEC, HEC1A, and Ishikawa cells, respectively (Fig. 2B). Lower concentrations up to $10^{-11}$ M still resulted in a significant inhibition of cell proliferation in A-MEC cells.

Involvement of P-LAP in OT-induced Growth Inhibition. To investigate the possible effect of the P-LAP in endometrial adenocarcinoma cells, we transfected P-LAP cDNA into A-MEC cells (A-MEC-LAP), which had the lowest P-LAP expression among three cell lines. Although both parental A-MEC cells and vector-transfected A-MEC-pc cells expressed little P-LAP, A-MEC-LAP cells expressed a remarkably high level of P-LAP on immunoblotting (Fig. 3A). The enzyme activity of P-LAP was in accordance with the results of immunoblotting (Fig. 3B). As shown in Fig. 3C, A-MEC-LAP cells exhibited clear resistance to the growth inhibitory effect of OT. After 72 h of treatment with $10^{-7}$ M OT, cell viability (percentage of untreated cells) were 72% in A-MEC-LAP cells, which is higher than that in A-MEC or A-MEC-pc cells (39% in A-MEC cells and 40% in A-MEC-pc cells). Interestingly, A-MEC-LAP cells increased cell growth compared with A-MEC or A-MEC-pc cells in 10% FCS medium without OT. After 72 h, A-MEC-LAP cell viability increased ~1.3-fold compared that of A-MEC or A-MEC-pc cells (Fig. 3D).

Inhibitory Effect of OT on Tumor Cell Growth through the OTR-cAMP-PKA Pathway. SCO, an OTR inhibitor, at a concentration of $10^{-5}$ M did not affect the proliferation of A-MEC, A-MEC-pc, and A-MEC-LAP cells under basal growth conditions. However, even at the concentration of $10^{-6}$ M, SCO inhibited the most of the antiproliferative effect of $10^{-7}$ M OT in both A-MEC and A-MEC-pc cells but not in A-MEC-LAP cells. Also, 14–22 amide, a PKA inhibitor, did not affect the cell proliferation under basal growth conditions at a concentration of 1 ng/ml. At the same concentration, 14–22 amide partially inhibited the antiproliferative effect of $10^{-7}$ M OT in both A-MEC and A-MEC-pc cells but not in A-MEC-LAP cells (Fig. 4).

Because OT was reported to stimulate cAMP production in carcinoma cells (8), we examined cAMP levels after OT stimulation in A-MEC and its transfectant cells. Fig. 5 shows that OT increased significantly intracellular cAMP level at $10^{-7}$ M in A-MEC and A-MEC-pc cells but not in A-MEC-LAP cells ($P < 0.01$).

DISCUSSION

In the current study, we provide the first demonstration of the expression of P-LAP in human carcinoma tissues and cell lines (Fig. 1 and 2). More importantly, we showed the increased immunoreactivity of P-LAP with advances in the tumor grade in human endometrial endometrioid adenocarcinomas (Fig. 1G).
The involvement of several aminopeptidases such as neutral endopeptidase (CD10), dipeptidyl peptidase IV (CD26), and aminopeptidase A in carcinoma proliferation was reported in several types of carcinomas (16–18). In contrast to these aminopeptidases, P-LAP is positively correlated with advances in tumor grade or progression. However, we could not find any difference with OTR among tumor grades. In contrast, a recent study has demonstrated the more increased OTR in less-differentiated tumors than the better-differentiated ones (8). Because it is reported that OT acts as a negative regulator of cell proliferation in various carcinoma cell lines, the more increased OTR in less-differentiated tumors might be contradictory. It is difficult to explain the discrepancy between the biologically inhibitory effect of OT on cell proliferation and the extensive distribution of OTR in poorly differentiated carcinomas compared with the focal expression in well-differentiated ones.

Fig. 3  The effect of P-LAP transfection. A, Western blotting analysis of P-LAP expression in cell lysates. Lanes as follows: Lane 1, A-MEC cells; Lane 2, A-MEC-pc cells; and Lane 3, A-MEC-LAP cells. B, the enzyme activity of P-LAP. C, effect of oxytocin on cell proliferation in A-MEC, A-MEC-pc, and A-MEC-LAP cells. Treatment with oxytocin inhibited cell proliferation at concentration of 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-7} M. A-MEC-LAP cells showed partially ablation of the inhibitory effect of oxytocin compared with A-MEC and A-MEC-pc cells. D, cell proliferation of A-MEC, A-MEC-pc, and A-MEC-LAP cells in medium containing FCS. A-MEC-LAP cells grew faster than A-MEC or A-MEC-pc cells under ordinary conditions.
Because P-LAP actively degrades OT, the increment of P-LAP with advancing tumor grade might be reasonable in relation to the tumor growth in view of the inhibition of carcinoma cell growth by OT.

This data showed that OT inhibited the growth of endometrial adenocarcinoma cell lines dose dependently and time dependently (Fig. 2B). Our data confirmed that OT acts as a negative regulator of cell proliferation in various carcinoma cell lines, including human breast carcinoma, osteosarcoma, and also endometrial adenocarcinoma, all of which express OTR (7–9). In addition, the most dominant inhibitory effect of OT was demonstrated in A-MEC cells, which contained the least P-LAP protein among the three cell lines examined. Because the overexpression of P-LAP remarkably neutralized the OT-mediated growth inhibition in A-MEC cells (Fig. 3C), it is probable that P-LAP degrades OT and neutralizes the OT-mediated growth inhibition. Furthermore, PLAP-transfected cells showed higher potential of cell growth than parental and vector-transfected cells without OT (Fig. 3D). The reason why P-LAP itself stimulates the growth of tumor cells is not clear at present. However, it is provable that the serum contains certain suppressive peptides on carcinoma cell growth, which are degraded by P-LAP. Thus, it is quite reasonable that the expression level of P-LAP increases with advances in tumor grade. The potential of using P-LAP inhibitor to treat endometrial adenocarcinoma seems promising, although extensive work is required before using this method on humans.

This data showed that OT significantly increased the intracellular cAMP level in A-MEC but not in A-MEC-LAP cells (Fig. 5). This data also confirmed the findings by Cassoni et al. (7), that the treatment of breast carcinoma cells with OT caused a significant increase in cAMP. OTR is a G protein-coupled receptor located on the plasma membrane, and the binding of
TO its receptor leads to various functions in reproductive organs and cells (2, 3, 5), and the signal pathways of OT have been extensively studied (19, 20). OT increases intracellular Ca\(^{2+}\), inositol 1,4,5-triphosphate, and 1,2-diacylglycerol, resulting in activation of protein kinase C. However, there were a few reports about the signal pathways of OT in tumor cells (7, 8). Cassoni et al. (7) reported that treatment of breast carcinoma cells with OT did not modify either the inositol phosphate and intracellular Ca\(^{2+}\) level. Thus, the functions and signal pathways of OT may differ between normal and tumor cells or from cell type to type. Several studies have previously discussed the possible role of OT as a factor-regulating cell proliferation, but those studies were controversial. In endometrial, breast, and nervous tumor cells, OT inhibited cell proliferation, and this inhibitory effect was mediated via the cAMP-PKA pathway (7, 8). We showed that the inhibition of cell proliferation by OT was completely blocked by SCO, an OTR antagonist, and partially blocked by 14–22 amide, PKA inhibitor (Fig. 4). These results suggest that OT works through OTR, and there are other signal pathways for the effects of antiproliferation by OT besides CAMP-PKA pathway. Further study on the signal pathway of the effects of antiproliferation by OT will be required for the clarification of the mechanisms.

In conclusion, our present data demonstrated that P-LAP is present in both human endometrial adenocarcinoma tissues and cells and that it works as a regulator of the inhibition of carcinoma cell growth by OT, which works through OTR and via signal pathways, including CAMP-PKA pathway. We also showed that immunostaining of P-LAP might provide the useful information in patients with endometrial adenocarcinoma. A more extensive study of P-LAP in endometrial adenocarcinoma will be needed to clarify the mechanism of carcinoma cell growth in relation to OT and to develop new therapeutic strategies against endometrial adenocarcinoma.

REFERENCES

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