Overexpression of LAMP3/TSC403/DC-LAMP Promotes Metastasis in Uterine Cervical Cancer

Hiroyuki Kanao,¹ Takayuki Enomoto,¹ Toshihiro Kimura,¹ Masami Fujita,¹ Ryuichi Nakashima,¹ Yutaka Ueda,¹ Yuko Ueno,¹ Takashi Miyatake,¹ Tatsuo Yoshizaki,¹ Gregory S. Buzard,² Akira Tanigami,³ Kiyoshi Yoshino,⁴ and Yuji Murata¹

¹Department of Obstetrics and Gynecology, Osaka University Faculty of Medicine, Osaka, Japan; ²Basic Research Program, Science Applications International Corporation-Frederick, Frederick, Maryland; and ³Fujii Memorial Research Institute, Osaka Pharmaceutical, Co., Ltd, Shiga, Japan

Abstract

LAMP3 (DC-LAMP, TSC403, CD208) was originally isolated as a gene specifically expressed in lung tissues. LAMP3 is located on a chromosome 3q segment that is frequently amplified in some human cancers, including uterine cervical cancer. Because two other members of the LAMP family of lysosomal membrane glycoproteins, LAMP1 and LAMP2, were previously implicated in potentially modulating the interaction of vascular endothelial and cancer cells, we hypothesized that LAMP3 might also play an important part in metastasis. To clarify the metastatic potential of LAMP3 in cervical cancers, we transfected a LAMP3 expression vector into a human uterine cervical cancer cell line, TCS. In an in vitro invasion assay, the migration of LAMP3-overexpressing TCS cells was significantly higher than in control TCS cells. In an in vivo metastasis assay, distant metastasis was detected in 9 of 11 LAMP3-overexpressing TCS cell–injected mice and in only 1 of 11 control mice. Histologic study showed that LAMP3-overexpressing cells readily invaded into the lymph-vascular space. In clinical samples, quantitative real-time reverse transcription-PCR (RT-PCR) analyses showed that LAMP3 mRNA was significantly up-regulated in 47 of 47 (100%) cervical cancers and in 2 of 15 (13%) cervical intraepithelial neoplasias, compared with a low level of LAMP3 mRNA expressed in normal uterine cervixes. Interestingly, high LAMP3 expression was significantly correlated with the overall survival of patients with stage I/II cervical cancers. These findings indicate that LAMP3 overexpression is associated with an enhanced metastatic potential and may be a prognostic factor for cervical cancer. (Cancer Res 2005; 65(19): 8640-5)

Introduction

LAMP3 was first cloned as a gene specifically expressed in lung tissues, which was called TSC403 that was subsequently found to be overexpressed in primary cancers of the esophagus, colon, fallopian tube, ovary, breast, and liver, although its expression was barely detectable in the corresponding normal tissues (1). TSC403 is now better known as the cell surface marker gene DC-LAMP (2) or CD208, but it codes for a 416-amino-acid protein that is the third member of the lysosome-associated membrane glycoprotein (LAMP) family, thus, the HUGO Gene Nomenclature Committee has designated this gene and its protein LAMP3/LAMP3, respectively. We will use the LAMP3 nomenclature for the protein and LAMP3 for the gene throughout, although DC-LAMP has been used more frequently in the literature. LAMP3 is a member of the LAMP family. LAMP1 and LAMP2 are located primarily in the lysosomal membrane and are rarely present on the surface of normal cells (3). They are the major carriers for poly-N-acetyllactosamines, including those with sialyl-Leα termini (4, 5), which are critical ligands for the E-selectin present on endothelial cells and platelets (6–9). Cancer cells show increased levels of sialyl-Leα determinants and adhere to vascular endothelial cells through their interaction with E-selectin (10, 11). LAMP1 and LAMP2 are constitutively expressed on the surface of some colon cancer cell lines, being much more associated with highly metastatic than less metastatic colon carcinoma cell lines (12). These observations suggest that cancer cells can modulate the display of E-selectin ligands on their cell surface in part by regulating expression of LAMP1 and LAMP2, and that up-regulation of surface-localized LAMP1 and LAMP2 may contribute significantly to the metastatic process. Although a definitive function of LAMP3 has not been clarified yet, LAMP3 may promote metastasis in the same way as LAMP1 and LAMP2.

LAMP3 is located on chromosome 3q27, a region that is often amplified in several types of cancer, especially squamous cell carcinomas, including uterine cervical cancers (13–15). Heselmeyer et al. (16) found a gain of 3q in 9 of 10 cases of invasive cervical carcinomas, but in only 1 of 13 cases of severe dysplasia. They suggested that a functionally important gene for cervical carcinogenesis might exist at 3q24-27. LAMP3 is a suitable candidate gene and its altered expression may play a role in cervical carcinogenesis, especially during late events such as tumor cell migration into lymph vessels.

To date, several candidates have already been suggested as being the potentially critical, frequently amplified, 3q oncogene. For example, PIK3CA at 3q26 (17), which encodes the p110α catalytic subunit of phosphatidylinositol 3-kinase, is an oncogene in ovarian and uterine cervical cancers (18, 19). Another possible 3q gene is hTR, which encodes the RNA component of human telomerase (20). Telomerase plays an essential role in stabilizing telomere length and, consequently, contributes to the processes of cellular immortality and tumorigenesis. Differential expression of telomerase activity has been reported during cervical cancer development (21). Other possible 3q locus genes are p63 at 3q28 (22) and eIF-5A2 at 3q26 (23).
LAMP3 Promotes Metastasis

In this article, we propose that LAMP3 should now also be considered as one of the relevant oncogene candidates at 3q.

Materials and Methods

Overexpression of LAMP3 in TCS cells. The complete open reading frame of the LAMP3 gene was cloned into a pCMV-script vector (Stratagene, La Jolla, CA). The human uterine cervical cancer cell line TCS was purchased from the Riken Gene Bank (Tokyo, Japan). Stable transfection of the construct into TCS cells was done using LipofectAMINE-Plus Reagent (Invitrogen Corp., Carlsbad, CA). LAMP3-overexpressing clones were selected with G418 (600 μg/mL) and were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37°C in a 5% CO2 incubator.

In vitro invasion assay. An in vitro invasion assay was done using a 24-well transwell unit with Chemotaxicell (8 μm pores on polycarbonate filters; Kurabo, Osaka, Japan). The upper side of the filter was coated with serum-free medium containing 0.1% bovine serum albumin. Cells (1 × 10⁶/200 μL) were placed in the upper part of the transwell plate, incubated for 24 hours, fixed with 10% buffered formamide, and stained with trypan blue. The invasive phenotype was determined by counting the cells that migrated to the lower side of the filter, with microscopy at 250-fold magnification. Assay was done on four isolated clones of LAMP3-overexpressing cells. Ten high power fields were counted per 100 invasive cells.

Quantitative reverse transcription-PCR. For cDNA synthesis, 1 μg of total RNA was reverse-transcribed in a 20 μL reaction using 0.5 μL of the TaqMan Universal PCR Master Mix (Applied Biosystems), 0.5 μL of each primer (forward and reverse), 5′-GCTCTGTCTCACTACGACATG-3′ (forward) and 5′-CGGTCTCTGAGTCGACACGATGTT-3′ (reverse) for GAPDH, and 5′-GAM-CGCAGCTGTCGTCGGAACACATCACCAC-TAMRA-3′ (probe). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was quantified using the TaqMan GAPDH Control Regent for normalization of the LAMP3 gene expression. Templates were loaded into a 96-well Reaction Plate (Applied Biosystems, Foster City, CA). In brief, 5 μL of cDNA template was placed in microwells for each tissue sample, followed by the addition of 25 μL of the TaqMan Universal PCR Master Mix (Applied Biosystems), 0.5 μmol/L of each primer (forward and reverse), 0.2 μmol/L of each probe, 5 μL of H2O to a total volume of 50 μL. The GeneAmp 7700 Sequence Detection System (Perkin-Elmer Life Analytical Sciences, Inc., Boston, MA) was used for amplification using a 5-second denaturation at 95°C, followed by 50 cycles of 1-second denaturation at 92°C, 1-second annealing at 56°C, and 20-second extension at 74°C. The fluorescence was measured automatically after each cycle. The data were analyzed using the Sequence Detection System software (Perkin-Elmer Life Analytical Sciences, Inc., Boston, MA). The mRNA levels were normalized to the mRNA levels of GAPDH.
were similar between the control mice and the TCS/LAMP3-transfected mice. The number of gross metastases per organ was significantly higher in the TCS/LAMP3-injected mice than in control mice (Fig. 1B). The number of invading cells of TCS/LAMP3 and TCS were not different, indicating that transfection with the empty vector did not affect cell migration. The cell counts for all of four isolated TCS/LAMP3 clones were 3.5, and 11.5, respectively. The RT-PCR analysis was done in triplicate and showed similar results. Western blotting analysis showed a single band of 70 kDa in size. An increase of LAMP3 protein in the TCS/LAMP3-transfected cells was apparent and is consistent with mRNA (Fig. 1D). Therefore, a higher ΔCt indicates a higher copy number of LAMP3 mRNA. Results were calibrated against normal brain tissue cDNA.

**Western blot analysis.** TCS/LAMP3 and TCS cells were harvested and boiled in SDS buffer for 5 minutes. Equal aliquots (20 μg) of total protein from the whole cell extracts were fractionated on a 10% denaturing SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Non-specific interactions were blocked with 5% non-fat milk/0.05% Tween 20. Proteins were identified using mouse monoclonal antibodies against DC-LAMP/LAMP3 (1:50; Immunotech, Marseilles, France), with a 1-hour incubation at room temperature followed by incubation with peroxidase-conjugated secondary antibodies (goat anti-mouse) for 30 minutes. Membrane-bound antibodies were detected using ECL Western Blotting Detection System (Amersham Biosciences Corp., Piscataway, NJ). Immunoblots were exposed on X-OMAT-AR film (Eastman Kodak, Rochester, NY) under standard conditions. Equivalent loading of proteins was checked using β-actin antibody (1:1,000).

**Immunohistochemistry.** Sectioned tissues were dewaxed, blocked with methanol, and incubated with a 1:10 dilution of the anti–DC-LAMP/LAMP3 antibody (Immunotech). The mouse anti-human recombinant E02B02 monoclonal antibody was originally produced by de Saint-Vis et al. (2). Signals were detected by use of a VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA).

**Statistical analyses.** Univariate analyses were done by the Mann-Whitney U test and the Student’s t test. Survival rates were calculated by the Kaplan-Meier method. For all tests, the level of significance was set at \( P < 0.05 \).

**Results**

**Overexpression of LAMP3 in TCS cells.** To establish a cell line overexpressing LAMP3, an expression vector for LAMP3 was stably transfected into the human uterine cervical cancer cell line TCS. Quantitative real-time reverse transcription-PCR (RT-PCR) showed that the TCS/LAMP3 cells expressed ~250-fold more LAMP3 mRNA than the TCS cells (Fig. 1A). ΔCt of TCS/pCMV and TCS/LAMP3 were 3.5, and 11.5, respectively. The RT-PCR analysis was done in triplicate and showed similar results. Western blotting analysis showed a single band of 70 kDa in size. An increase of LAMP3 protein in the TCS/LAMP3 cells is apparent and is consistent with mRNA (Fig. 1B).

**TCS/LAMP3 cells display increased cell motility.** Using an in vitro invasion assay, we observed higher motility in TCS/LAMP3 cells than in the parental TCS cells. Figure 1C shows the cells migrating to the lower side of the filter, indicating invasive phenotype. The average number ± SD of invaded TCS cells in 10 high power fields of each filter was 4.0 ± 1.5; for TCS/pCMV, it was 5.8 ± 2.1; for TCS/LAMP3 (clone 1), it was 89.4 ± 6.1; for TCS/LAMP3 (clone 2), it was 71.2 ± 10.0; for TCS/LAMP3 (clone 3), it was 73.2 ± 12.9; for TCS/LAMP3 (clone 4), it was 73.0 ± 9.0; and for TCS/pCMV without Matrigel, the number was 16.6 ± 4.5 (Fig. 1D).

Cell counts for all of four isolated TCS/LAMP3 clones were significantly higher than for vector control (TCS/pCMV). The number of invading cells of TCS/pCMV and TCS were not different, indicating that transfection with the empty vector did not affect cell migration. The cell counts of TCS/pCMV without Matrigel were higher than TCS/pCMV, showing that 25 μL of Matrigel was
sufficient for the invasion assay. These findings indicate that overexpression of LAMP3 enhances cell invasion.

**TCS/LAMP3 transfectants possess increased metastatic potential in vivo.** S.c. injection of TCS/LAMP3 or TCS/pCMV (vector control) cells into SCID mice produced xenografts in the back of the mice and subsequent metastases. Metastatic tumors were detected more frequently in TCS/LAMP3-injected mice than in control mice (Fig. 2A): 9 of 11 (83%) TCS/LAMP3-injected mice developed metastases and only 1 of 11 (9%) of the control mice did. The metastatic target sites were the liver, lung, and para-aortic and mesenteric lymph nodes. The number of gross metastases was significantly higher in TCS/LAMP3-injected mice (18.8 ± 12.2 versus 0.009 ± 0.3) as shown in Fig. 2B. On the other hand, the growth curves of the primary s.c. tumors were similar between the control mice and the TCS/LAMP3-injected mice (Fig. 2C), suggesting that overexpression of LAMP3 do not affect primary tumor growth. H&E-stained sections of the metastatic tumors of a TCS/LAMP3-injected mouse are shown in Fig. 3. Aggressive invasion into the lymph-vascular space was observed in the TCS/LAMP3-injected mice, but not in the control mice. In the immunohistochemistry for LAMP3, tumor cells that were invading into the surrounding tissues were more strongly positive than other areas. The cytoplasm of the tumor cells was stained, suggesting that LAMP3 was located in or near the lysosomal membrane as are other LAMP family members, LAMP1 and LAMP2. These findings indicate that overexpression of LAMP3 is actively involved in tumor invasion through increased migration into lymph-vascular spaces.

**Expression of the LAMP3 gene in primary uterine cervical cancers.** In clinical samples, quantitative real-time RT-PCR showed that cervical cancers expressed significantly higher levels of LAMP3 mRNA than others. The expression of LAMP3 mRNA was presented as ΔCt (GAPDH Ct - LAMP3 Ct). Therefore, higher ΔCt indicates higher copy number of LAMP3 mRNA. In 47 cervical cancers tested, 47 samples (100%) revealed overexpression of LAMP3 mRNA. In 15 CINs, two samples did (a single case of CIN2 and a single case of CIN3). Overexpression was not detected in five normal cervixes. The averages ± SD of ΔCt in normal cervix, CIN1, CIN2, CIN3, and cervical cancer were 0.37 ± 0.26, 0.13 ± 0.48, 0.78 ± 1.42, 0.74 ± 1.05, and 7.34 ± 2.1, respectively.

Immunohistochemistry. In an immunohistochemical study using clinical samples, we found that LAMP3 protein was highly expressed in primary cervical cancers (Fig. 5A). Metastatic tumors in lymph nodes and tumor cells in lymph-vascular spaces also
showed a high expression of LAMP3 as well (Fig. 5B-D). Squamous epithelium of the normal uterine cervix had negligible LAMP3 staining (data not shown).

High LAMP3 expression was significantly correlated with poor prognosis in patients with stage I or II cervical cancers. For the 47 cervical carcinomas we examined, 24 patients were evaluated for their prognosis. All of them were Fédération Internationale des Gynaecologistes et Obstétristes stage I or II (24). Radical hysterectomy, with or without postoperative whole pelvic radiation, was applied to all the patients. To clarify the significance of the LAMP3 gene expression in prognosis, we divided these 24 patients into groups A and B by the degree of expression of LAMP3 mRNA of the tumor. Group A included nine cases with a ΔCt of >7; group B included 15 cases with a ΔCt of <7. The correlation between expression of LAMP3 mRNA and overall patient survival was analyzed by the Kaplan-Meier method (Fig. 6). The prognosis for group A was significantly worse than that of group B by the log-rank test (P < 0.05). This suggests that high levels of LAMP3 are associated with uncontrollable distant metastasis and a higher mortality.

Discussion

Surgery and radiation therapy are relatively effective for controlling uterine cervical cancer at its primary site. Mortality from this tumor is usually caused by its metastasis to lymph nodes and distant organs. Our data indicate that the presence of overexpressed LAMP3 is significantly associated with the promotion of a metastatic potential both in vitro and in vivo. Furthermore, in clinical samples, we found, by quantitative real-time RT-PCR, overexpression of LAMP3 mRNA in cervical cancer compared with normal uterine cervix and CINs. Interestingly, we also observed that higher LAMP3 expression was significantly correlated with poorer prognosis of patients with stage I/II cervical cancer.

Metastasis consists of several sequential steps (i.e., growth at the primary site, invasion into the stroma, migration into blood vessels or lymphatics, arrest at a distant site, extravasation, and proliferation). Recent studies have shown that vascular endothelial growth factors (VEGF)-C and VEGF-D play pivotal roles in lymphangiogenesis and in lymph node metastasis in human cancers. Skobe et al. (25) have shown that overexpression of VEGF-C in human breast cancer cell line increases both lymphangiogenesis and lymphatic metastases in mice. VEGF-D also promotes the metastatic spread of tumor cells via the lymphatic systems (26). In their study, VEGF-D promotes not only lymphatic metastasis but also tumor growth and angiogenesis. These effects were inhibited by anti-VEGF-D antibodies. Association between overexpression of VEGF-C and/or VEGF-D and lymph node metastasis has been reported in various human cancers as reviewed by Pepper et al. (27). Thus, the molecular mechanisms of lymphangiogenesis in tumor have begun to be elucidated.

Certain mechanisms of metastasis have been found to be similar to those of inflammation in that many of the mediators involved are similar in both processes. For example, the chemokine receptors CXCR4 and CCR7, which were originally identified in leukocytes, are highly expressed in human breast cancer cells and metastases. Their respective ligands, CXCL12/SDF-1α and CCL21/6Ckine, exhibit the highest levels of expression in organs representing the first destinations of breast cancer metastasis (28). Correlations between overexpression of CCR7 in tumor cell and lymph node metastasis have been reported in gastric cancers (29), murine melanoma (30), non–small cell lung cancer (31), and head and neck cancer (32). These similarities between metastasis and inflammation have suggested that further study of the
crosstalk between inflammation and the metastatic process may be worthy of consideration.

Migration of cancer cells into lymph vessels is of keen interest, but its mechanism is largely unknown. On the other hand, the mechanisms of cellular traffic of peripheral dendritic cell in lymphatics have been studied extensively, as reviewed by von Andrian et al. (33). After exposure to inflammatory stimuli, peripheral dendritic cell undergoes maturation that allows them to enter lymph vessels and to access the T-cell area in draining lymph nodes. In this process, CCR7 participates in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes (34), during which the dendritic cell marker LAMP3 (DC-LAMP) is up-regulated transiently (2). Thus, LAMP3 is transiently expressed in dendritic cell upon maturation and may be a key molecule for their migration from the periphery into lymph vessels, although such a definitive function for LAMP3 has not yet been proven. Taken together, by mimicking dendritic cell, tumor cells expressing LAMP3 may migrate into surrounding adjacent lymph vessels and reach the draining lymph nodes.

Additionally, LAMP3 is on chromosome 3q, where many previous studies have implicated the existence of an oncogene(s). Therefore, we propose that LAMP3, which promotes lymph node metastasis, should be given serious consideration as being one of the candidates for this implied oncogene.

In a support of our idea, using a comparative genetic hybridization technique, Allen et al. (33) have reported that a high occurrence of a 3q gain was more frequent at the primary site of uterine cervical cancers with lymph node metastases than in those without metastases (64% versus 28%), although not statistically significant. In their 37 cases of stage Ib uterine cervical cancers, the patient mortality was linked to a higher presence of 3q amplification compared with survivors (80% versus 37%), indicating the importance of 3q amplification in lymph node metastasis and prognosis.

Our data give additional new evidence for similarities between cancer metastasis and inflammation at the level of LAMP3 expression. Because this similarity is common in human cancers, overexpression of LAMP3 may also occur in other types of cancer as well.

In conclusion, our data suggest that LAMP3 promotes metastasis and can thus be a prognostic factor and serve as a novel target antigen for the therapy of patients with cervical cancers that are refractory to standard treatment modalities.

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