Junctional Adhesion Molecule-C Promotes Metastatic Potential of HT1080 Human Fibrosarcoma*

The junctional adhesion molecule (JAM) family is a key molecule in a process called transendothelial migration or diapedesis. Here, we report implications of JAM-C in cancer metastasis. We first determined the mRNA expression of JAMs in 19 kinds of cancer cell lines. JAM-C was expressed in most of tumors having potent metastatic properties. Especially in murine K-1735 melanoma cell lines, the highly metastatic sublines (M2 and X21) strongly expressed JAM-C when compared with the poorly metastatic ones (C-10 and C23). Next, we investigated the role of JAM-C in cancer metastasis by using human JAM-C (hJAM-C) gene-transfected HT1080 fibrosarcoma cells. In comparison with mock-transfected HT1080 cells, these cells showed a significant increase in the adhesion to various extracellular substrates and the invasion across a Matrigel™-coated membrane. The knockdown of hJAM-C using small interfering RNA resulted in the suppression of both the adhesion and the invasion of HT1080 cells, suggesting that endogenous hJAM-C might be involved in tumor metastasis. Finally, we studied the role of hJAM-C in an in vivo experimental metastatic model. The results showed that the overexpression of hJAM-C in HT1080 cells significantly decreased the life spans of the tumor-bearing mice. In contrast, the knockdown of hJAM-Cin HT1080 cells suppressed the weight gain of the lungs with metastatic colonies. We conclude that the expression of JAM-C promotes metastasis by enhancing both the adhesion of cancer cells to extracellular matrices and the subsequent invasion.

Cancer metastasis involves a series of events that include dissociation of malignant cells from a primary site, polarized proteolysis and migration, intravasation into the circulatory system, and adhesion to the vascular endothelium followed by extravasation, invasion, and growth at distant sites (1). Certain cell surface molecules are known to be involved in these processes. For instance, integrins play central roles in regulating cell adhesion, motility, invasion, and angiogenesis (2–4), and matrix metalloproteinases on tumor cells can degrade the extracellular matrix (ECM) (5). In particular, cell adhesion molecules play key roles in tumor adhesion and invasion, resulting in metastasis (6). When compared with normal tissues, malignant tumors are characterized by disrupted tissue architecture and deranged differentiation. Changes in the expression or function of cell adhesion molecules contribute to tumor progression both by altering the adhesion status of the cell and by affecting cell signaling (7). It has been reported that alteration of cell-cell and/or cell-matrix interactions accounts for the ability of cancerous cells to cross tissue boundaries and to disseminate to distant sites. Many adhesion molecules implicated in tumor metastasis have been identified (6), and certain of them belong to the immunoglobulin superfamily (8). Recently, the junctional adhesion molecule (JAM) family, a member of the immunoglobulin superfamily, has become a focus of study in relation to cell-cell and/or cell-matrix interactions.

The JAM family, belonging to the larger cortical thymocyte Xenopus molecular family (9), is a glycoprotein. It has two immunoglobulin folds (VH and C2 type) and two extra cysteine residues in its extracellular region, a potential N-glycosylation site (s), and a PDZ protein-interacting domain in its intracellular region (10). JAM molecules are selectively expressed in a variety of human organs (11–13). In particular, JAMs are strongly expressed in close proximity to tight junction strands of polarized endothelial and epithelial cells and on circulating leukocytes and platelets (14, 15).

JAMs are known to not only form homophilic binding but also to interact with certain ligands in a heterophilic manner (16–20). It has been reported that JAM-A binds to β2 integrin LFA-1 (21), that JAM-B interacts with α4β1 integrin (22), and that JAM-C is a counter-receptor for the leukocyte integrin Mac-1 (15). Several cytoplasmic partners associated with the PDZ domain of the JAMs have also been reported (23–28). These interactions represent the likely determinants for the diverse roles of JAMs such as junction assembly, platelet activation, and leukocyte transmigration (10). In inflammation, the JAMs play an important role on the passage of leukocytes across interendothelial spaces (29, 30). Certain adhesion molecules on leukocytes and endothelial cells are known to control sequential steps in leukocyte migration (31). The steps of leukocyte migration are partly similar to those of cancer metastasis in several respects; e.g. both leukocytes and tumor cells need to adhere to the vascular bed and to cross the border of endothelial cells.

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1 To whom correspondence should be addressed: Dept. of Medical Biochemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga, Shizuoka 422-8526, Japan. Tel.: 81-54-264-5701; Fax: 81-54-264-5705; E-mail: oku@u-shizuoka-ken.ac.jp.
2 The abbreviations used are: ECM, extracellular matrix; JAM, junctional adhesion molecule; FBS, fetal bovine serum; PBS, phosphate-buffered saline;

GFP, green fluorescent protein; EGFP, enhanced GFP; siRNA, small interfering RNA; Mock, mock-transfected.
In the present study, we investigated certain aspects of the participation of JAMs in cancer metastasis. At first, we determined the expression of JAMs in 14 cell lines and also in five sublines of the K-1735 murine melanoma. Next, we focused on the function of JAM-C in cancer metastasis and used HT1080 human fibrosarcoma cells (HT1080 cells) for metastatic studies. HT1080 cells transfected with human JAM-C (hJAM-C) were used in the experiment of cell adhesion, invasion, and in vivo metastasis for evaluating the functions of hJAM-C. The roles of endogenous hJAM-C were determined by examining the properties of HT1080 cells transfected with small interfering RNA for hJAM-C. This study provides the first evidence that hJAM-C promotes cancer metastasis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Murine melanoma K-1735 cell lines were established by I. J. Fdler (The University of Texas M.D. Anderson Cancer Center, Houston, TX) and kindly provided by Dr. J. Yokota (National Cancer Center Research Institute, Tokyo, Japan). Human HT1080 fibrosarcoma, C8161 melanoma, AZ-P7a gastric carcinoma, Colo205 colorectal adenocarcinoma, SUIT-2 pancreatic carcinoma, K562 erythroleukemia, murine B16BL6 melanoma, and Colon26 NL-17 carcinoma cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS; Sigma-Aldrich), and 100 U/ml penicillin, 100 μg/ml streptomycin (MP Biomedicals, Inc., Irvine, CA), and 100 μg/ml streptomycin (MP Biomedicals). Human JAM-C-transfected HT1080 cells were grown in the same medium supplemented with 400 μg/ml hygromycin B (Wako Pure Chemical Industries, Ltd. Osaka, Japan). Human A431 epidermoid carcinoma, HT-29 colon adenocarcinoma, HeLaS3 cervical cancer, Caco-2 colon carcinoma, murine Lewis lung carcinoma, and K-1735 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Chinese hamster ovary (CHO)-K1 cells were maintained in Ham’s F12 medium containing 10% FBS and the same antibiotics. All cells were cultured at 37 °C in a humidified incubator with 5% CO₂.

**Reverse Transcription and PCR**—Total RNA was isolated by using TRIzol® LS reagent (Invitrogen), and cDNAs were generated from total RNA samples (5 μg) by using the SuperScript first-strand synthesis system (Invitrogen). The PCR conditions were as follows: 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min; and 72 °C for 10 min. The primers for human JAM-A were 5’-CGC GAT GGG GAC AAA GGC GC-3’ (sense) and 5’-ACC AGG AAT GAC GAG GTC-3’ (antisense); for human JAM-B, 5’-TAA AAA TCG AGC TGA GAT GAG-3’ (sense) and 5’-TTA AAT TAT AAA GGA TTT TGT G-3’ (antisense); for human JAM-C, 5’-ACT TCT TCC TGC TGC TGC TT-3’ (sense) and 5’-TCT GAA GTC GCC CTC CTC GT-3’ (antisense); for mouse JAM-A, 5’-ATG GCC ACC GAC GAG GGA AAC GC-3’ (sense) and 5’-TCA CAC CAG GAA CGA CGA GG-3’ (antisense); for mouse JAM-B, 5’-ATG GCC CTG AGC CGG CTG CT-3’ (sense) and 5’-TCA GAT AAC AAA GGA CGA TT-3’ (antisense); for mouse JAM-C, 5’-ATG GCG AGG AGC CCC CAA GG-3’ (sense) and 5’-TTA AAT TAT AAA GGA TTT TG-3’ (antisense); and for β-actin, 5’-TGA GGT CAC CCA CAC TGT GCC CAT CTA-3’ (sense) and 5’-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3’ (antisense). The PCR products were applied onto 1.5% agarose gels and visualized by staining with ethidium bromide under UV light.

**Western Blotting**—Anti-JAM-C antibody (goat polyclonal IgG) was purchased from R&D Systems Inc. (Minneapolis, MN). Peroxidase-conjugated secondary antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

The cells on culture plates were rinsed with phosphate-buffered saline (pH 7.4, PBS). Cell extracts were prepared with lysis buffer composed of 10 mM Tris (pH 7.5), 0.1% SDS, 50 μg/ml aprotinin, 200 μM leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 100 μM pepstatin A. Total protein concentration was measured by using a BCA protein assay reagent kit (Pierce) with bovine serum albumin as a standard, according to the manufacturer’s instructions. The cell extracts were subjected to 10 or 15% SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore, Billerica, MA). After blocking for 1 h at room temperature with 5% skim milk in Tris–HCl buffered saline containing 0.1% Tween 20 (TTBS, pH 7.4), the membranes were incubated with 0.1 μg/ml antibody for 2 h at room temperature. Then, they were incubated with peroxidase-conjugated secondary antibody at a dilution of 1:2000 to 1:10,000 for 1 h at room temperature. Each sample was developed by using chemiluminescent substrate (ECL; Amersham Biosciences).

**cDNA Cloning**—The hJAM-C sequence was obtained from GenBank™ (accession number AF444878). Total RNA was isolated from human umbilical vein endothelial cells (Bio-Whittaker, Walkersville, MD) by using TRIzol LS reagent and reverse-transcribed into single-stranded cDNA by using the SuperScript first-strand synthesis system (Invitrogen). The full-length cDNA of hJAM-C was PCR-amplified by using Ex Taq™ (TaKaRa BIO Inc., Shiga, Japan) with specific primers based on the hJAM-C nucleotide sequence that included BamHI and HindIII restriction sites: 5’-ACG TAA GCT TAT GGC GCT GAG GCG GCC ACC-3’ (sense) and 5’-ACG TGG ATC CTC AGA TCA CAA ACG ATG ACT-3’ (antisense). The PCR product was purified by use of a High Pure PCR product purification kit (Roche Diagnostics, Basel, Switzerland). Restriction enzyme digestions of PCR product and pcDNA3.1/Hygro vector (Invitrogen) were carried out at 37 °C for 1 h. Both digestion products were purified with the purification kit and then ligated at 16 °C for 2 h with T4 DNA ligase (DNA ligation kit, Version 1, TaKaRa). Competent Escherichia coli JM109 cells (TaKaRa) were transformed with ligation mixture, plated on Luria-Bertani (LB) agar plates, and grown overnight at 37 °C. Colonies were screened for the insert by restriction enzyme digestion, and DNA sequencing was performed at Macrogen Inc. (Seoul, Korea).

**Transfection with hJAM-C**—Stable GFP transfectant of HT1080 fibrosarcoma was prepared as described previously (32) and used for transfection with hJAM-C. These cells were transfected with 10 μg of pcDNA3.1/Hygro vector containing either full-length hJAM-C or no insert by using Lipofectamine™ 2000 (Invitrogen). Stable transfectant (hJAM-C/HT1080 or Mock/HT1080) was selected with 400 μg/ml hygromycin B. The expression of hJAM-C was confirmed by Western blotting as described.
above. For cell growth assay, hJAM-C/HT1080 or Mock/HT1080 cells (1.0 × 10^5 cells/dish) were cultured for 24, 48, 72, or 96 h. After staining with 0.5% trypan blue, the numbers of living cells were counted at each time point.

Localization of hJAM-C—Human JAM-C/HT1080 or Mock/HT1080 cells were fixed with 3.7% paraformaldehyde in PBS. The cells were then blocked with 3% bovine serum albumin in PBS, incubated with 0.1 μg/ml anti-JAM-C goat polyclonal antibody for 2 h, and immersed in 1.0 μg/ml Alexa Fluor® 555-labeled anti-goat IgG secondary antibody (Molecular Probes Inc., OR) for 1 h. Localization of hJAM-C was determined by using a LSM510 Meta confocal system (Carl Zeiss Co., Ltd.).

Adhesion Assay—Ninety-six-well flat-bottomed plates were dry-coated with Matrigel™ (3 μg/well), fibronectin (0.75 μg/well), vitronectin (0.25 μg/well), laminin (1.25 μg/well), or type IV collagen (1.5 μg/well) and blocked with 1% bovine serum albumin for 1 h. Human JAM-C/HT1080 or Mock/HT1080 cells (5 × 10^4 cells/well) were allowed to adhere to each substrate-coated well at 37 °C for 1 h. After washing with PBS, the adherent cells were stained with crystal violet for 10 min. The pigment was extracted with 33% acetic acid for 5 min, and the absorbance was measured at 570 nm as an indicator of adherent cells.

Invasion Assay—The invasion was evaluated as the capacity of hJAM-C/HT1080 cells to pass through a Matrigel-coated transwell insert. BD Matrigel matrix (25 μg/well; BD Biosciences) was coated on a fluorescence-blocking micropore membrane of a culture insert (8-μm pore size, 0.28 cm², Falcon HTS FluoroBlok™ inserts, BD Biosciences). The culture inserts were set into wells of a 24-transwell plate containing RPMI 1640 medium supplemented with 10% FBS, which had not been heat-immobilized, as a chemoattractant. After the cell density had been adjusted to 5 × 10^5 cells/ml in serum-free medium, 5 × 10^4 cells in 100 μl were introduced into each culture insert. After incubation for 4–5 h at 37 °C in a CO₂ incubator, the cells that traversed the Matrigel layer and attached to the lower surface of the filter were counted in five randomized fields under a fluorescence microscope.

Transfection with siRNA—The nucleotide sequences of siRNA with a two-nucleotide overhang (underline) for hJAM-C were 5'−CAGGAUGGAGAAGUCAAGAACACGAG-3' (sense) and 5'−GGUUCUUGUAACUUUCUCCAUCCGUAU-3' (antisense); those for luciferase were 5'−UGG-GUG-GUUACCAAGGGUGUAAGAG-3' (sense) and 5'−UAAUUGUACACCUUAGGUAAACCAU-3' (antisense); and those for EGFP were 5'−GGCUCAGUCAGGGCCG-GCC-ACC-3' (sense) and 5'−UGCGCUCCUGGACGUAGCCUU-3' (antisense). The sequences for hJAM-C, luciferase, and EGFP correspond to nucleotide regions 829–853, 612–636, and 118–141, respectively. The siRNAs for luciferase and EGFP were used as a control siRNA. All siRNAs were prepared by Hokkaido System Science Co. (Hokkaido, Japan). Stable GFP transfectant of HT1080 fibrosarcoma (31) was transfected with siRNA by using Lipofectamine 2000 according to the manufacturer's instructions. Forty-eight hours after the addition of these complexes. Western blotting and cell growth, adhesion, and invasion assays were performed by methods similar to those described above.

Experimental Metastasis—The influence of the overexpression and the knockdown of hJAM-C on tumor metastasis were evaluated in an experimental pulmonary metastatic model. Cells were harvested from culture plates by brief exposure to

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**FIGURE 1.** Expression of JAM transcripts in various cancer cell lines. Total mRNA was prepared from each cell line, and reverse transcription-PCR was performed. PCR products were visualized by ethidium bromide staining under UV light. A, human cell lines as follows: HT1080, fibrosarcoma; A431, epidermoid carcinoma; HeLaS3, cervical cancer; Caco-2, colon carcinoma; K562, erythroleukemia; HT-29, colon adenocarcinoma; C8161, melanoma; SUIT-2, pancreatic carcinoma; AZ-P7a, gastric carcinoma; Colo205, colon adenocarcinoma. B, rodent cell lines as follows: LLC, murine Lewis lung carcinoma; B16BL6, murine melanoma; Colon26 NL-17, murine colon carcinoma; CHO-K1, Chinese hamster ovarian cancer.

**FIGURE 2.** Expression of JAMs in K-1735 murine melanoma. A, reverse transcription-PCR for detection of JAM-A, JAM-B, and JAM-C transcript was performed. C-10 and C-23 are poorly metastatic sublines, whereas M2 and X21 are highly metastatic ones. P indicates parent K-1735 melanoma. B, Western blotting analysis was performed to determine the expression of JAM-C protein in each K-1735 cell line.
0.02% EDTA/PBS. After the cells had been centrifuged and resuspended in the medium, their concentration was adjusted to 5 × 10^6 cells/ml. For evaluating the effect of overexpression of hJAM-C on metastatic potential, hJAM-C/HT1080 or Mock/HT1080 cells (1 × 10^6 cells/mouse) were injected into the tail vein of 4-week-old BALB/c nu/nu male mice (Charles River Japan Inc., Kanagawa, Japan). The life spans of tumor-bearing mice were monitored (n = 10 for mock/HT1080, n = 11 for hJAM-C/HT1080). In another three mice in each group, the lungs were dissected, weighed, and photographed for evaluating metastasis at 19 days after implantation.

For evaluating the effect of knockdown of hJAM-C on metastatic potential, hJAM-C siRNA- or EGFP siRNA-treated cells (1 × 10^6 cells/mouse) were injected into the tail vein of 9-week-old BALB/c nu/nu male mice (n = 4). The lungs with tumor metastases were dissected, weighed, and photographed at 38 days after implantation.

**Histochemical Analysis**—The lungs with metastatic colonies were embedded in optimal cutting temperature compound (Sakura Finetechanical Co., Ltd., Tokyo, Japan) and frozen at −80 °C. Six-micrometer lung sections were prepared by using a cryostatic microtome (HM 505E, Microm, Walldorf, Germany), stained with hematoxylin-eosin, and examined histopathologically.

**Statistical Analysis**—Statistical analysis (mean value, S.D., independent t test) was performed by using Stat View® 4.0 (SAS Institute Inc., Cary, NC). Results were expressed as the mean ± S.D.

**RESULTS**

The amounts of JAM transcripts in cancer cell lines were determined by reverse transcription-PCR. In this experiment, we used 19 kinds of cancer cell lines. Among them, human HT1080 fibrosarcoma, HT-29 colon adenocarcinoma, C8161 melanoma,
JAM-C Promotes Cancer Metastasis

A

B

C

D

FIGURE 5. Involvement of endogenous hJAM-C in invasion of HT1080 cells. A, Western blotting analysis of hJAM-C expression in hJAM-C-overexpressing hJAM-C/HT1080 cells (lane 1), HT1080 cells (lane 2), luciferase siRNA-transfected HT1080 cells (lane 3), and hJAM-C siRNA-transfected HT1080 cells (lane 4) was performed. Luciferase siRNA-transfected and hJAM-C siRNA-transfected HT1080 cells were used for each experiment at 48 h after transfection. B, the proliferation of luciferase siRNA-transfected (open bars) and hJAM-C siRNA-transfected (closed bars) HT1080 cells was determined at the indicated times. Cell number was counted at each time point. Data are the mean ± S.D. (n = 4). C, adhesion of EGFP siRNA-transfected (open bars) and hJAM-C siRNA-transfected (closed bars) HT1080 cells to extracellular substrates are shown. The experimental procedures were similar to the case of the hJAM-C overexpression study. Significant differences between EGFP siRNA-transfected and hJAM-C siRNA-transfected HT1080 cells are indicated: *, p < 0.05. Bars indicate mean ± S.D. D, invasion assay using luciferase siRNA-transfected (open bars) and hJAM-C siRNA-transfected (closed bars) HT1080 cells was performed. The number of cells that invaded through the Matrigel-coated membrane was counted under a fluorescence microscope. Significant differences between luciferase siRNA-transfected and hJAM-C siRNA-transfected HT1080 cells are indicated: ***, p < 0.001. Bars indicate mean ± S.D.

SUIT-2 pancreatic carcinoma, AZ-P7a gastric carcinoma, and murine Lewis lung carcinoma, B16BL6 melanoma, Colon26 NL-17 carcinoma, K-1735 M2, and X21 melanoma cell lines were known to have highly metastatic characteristics. The transcripts of JAM-A were similar in amount regardless of the cancer cell lines (Fig. 1). In contrast, the expression levels of JAM-B and JAM-C were different among the various cell lines. The transcripts of JAM-C were detected in highly metastatic cell lines such as HT1080 fibrosarcoma, SUIT-2 pancreatic carcinoma, AZ-P7a gastric carcinoma, Lewis lung carcinoma, B16BL6 melanoma, and Colon26 NL-17 carcinoma (Fig. 1). To assess the relationship between the amounts of JAM transcripts in tumor cells and their metastatic potentials, the expression of JAM members was compared in K-1735 murine melanoma subpopulations having diverse biologic behavior (33). Each clone (C-10, C-23, M2, and X21) was previously established from the parent K-1735 cells. The in vivo metastatic potential of M2 and X21 sublines is very high, as is that of the parent K-1735 cells, whereas that of C-10 and C-23 clones is quite poor. As shown in Fig. 2A, the mRNA level of JAM-C was markedly up-regulated in the parent K-1735 and highly metastatic sublines (M2 and X21) when compared with that in the poorly metastatic sublines (C-10 and C-23). In contrast, the mRNA level of either JAM-A or JAM-B was not much different between the highly metastatic and poorly metastatic cells. Actual expression levels of JAM-C protein in K-1735 sublines determined by Western blotting correlated well with those of JAM-C mRNA (Fig. 2B).

JAM-C functions in cancer metastasis were explored by constructing hJAM-C-overexpressing HT1080 fibrosarcoma cells. HT1080 cells were transfected with hJAM-C-encoded pcDNA3.1/Hygro vector (hJAM-C/HT1080) or empty vector (Mock/HT1080). Overexpression of hJAM-C in hJAM-C/HT1080 cells was confirmed by Western blotting. As shown in Fig. 3A, the amount of hJAM-C protein was very abundant in hJAM-C/HT1080 cells than in Mock/HT1080 cells. Human JAM-C in the transfectant was localized on the extensions of its plasma membrane (Fig. 3B). The forced expression of hJAM-C did not affect the cell growth rate (Fig. 3C).

To evaluate the participation of hJAM-C in the interaction of the cells with ECM, the adhesion of hJAM-C/HT1080 cells to various ECM components was investigated. Human JAM-C expression in HT1080 cells significantly increased their adhesion to Matrigel, fibronectin, and vitronectin and slightly increased their adhesion to type IV collagen and laminin (Fig. 4A). Next, the effect of hJAM-C overexpression on tumor cell invasion was examined. The number of cells that invaded through a Matrigel-coated membrane was compared between hJAM-C/HT1080 and Mock/HT1080 cells. The migration of HT1080 cells was significantly accelerated by the overexpression of hJAM-C, suggesting that hJAM-C expression might be correlated with tumor malignancy (Fig. 4B).

To confirm whether endogenous JAM-C was involved in the invasion, hJAM-C siRNA-transfected HT1080 cells were prepared. The expression of endogenous hJAM-C was observed in both control and luciferase siRNA-transfected HT1080 cells to some extent, and the knockdown of endogenous hJAM-C was
confirmed in hJAM-C siRNA-transfected HT1080 cells (Fig. 5A). Transfection with hJAM-C siRNA did not affect the rate of cell growth (Fig. 5B). In an adhesion assay, the knockdown of hJAM-C inhibited the adhesion of hJAM-C siRNA-transfected HT1080 cells to ECM components (Figs. 4A and 5C). Especially, the knockdown of hJAM-C significantly reduced their adhesion to fibronectin and vitronectin. The invasion potential of hJAM-C siRNA-transfected HT1080 cells was significantly less than that of luciferase siRNA-transfected HT1080 cells, indicating that the knockdown of hJAM-C reduced tumor invasiveness (Fig. 5D).

Finally, the influence of hJAM-C expression on the metastatic potential of HT1080 cells was examined in an experimental metastasis model. The formation of pulmonary metastases was observed in both the Mock/HT1080 cell-injected and the hJAM-C/HT1080 cell-injected group since HT1080 cells are highly metastatic by nature (data not shown). The mean survival times after tumor implantation were 18 ± 2 days for the hJAM-C HT1080 cell-injected group and 30 ± 8 days for the Mock/HT1080 cell-injected group (Fig. 6A). The life spans of the hJAM-C/HT1080 cell-injected group were significantly short when compared with those of the Mock/HT1080 cell-injected group (p < 0.01). Forced expression of hJAM-C caused 40% reduction of the life span of tumor-bearing mice, suggesting that hJAM-C expression promoted the pulmonary metastasis of HT1080 cells. At 19 days after the injection, the hJAM-C/HT1080 cell-injected group showed bloody lungs with metastatic colonies, which might occur due to the increased malignancy of the metastatic cells (Fig. 6B). Most of the mice injected with hJAM-C/HT1080 cells died before showing obvious differences in the lung weight gain between Mock/HT1080 and hJAM-C/HT1080 cell-injected mice. As shown in Fig. 6C, the results of the hematoxylin-eosin-staining showed the overexpression of hJAM-C-induced expansion of the metastatic tumors. Histochemical data also showed that the knockdown of hJAM-C in HT1080 cells suppressed the pulmonary metastasis of those cells (Fig. 6D). In the case of the injection of EGFP siRNA-transfected cells, the leakages of blood corpuscle cells were observed around the metastases. This phenomenon indicated that the progress of tumor metastasis of the cells transfected with hJAM-C siRNA was slower than that of cells transfected with EGFP siRNA. Furthermore, the knockdown of hJAM-C suppressed the weight gain of lungs with metastatic colonies after the injection of the cells in comparison with that of the cells transfected with EGFP siRNA (p = 0.0570, Fig. 6E).

**DISCUSSION**

Invasion of metastatic tumor cells to the secondary colonizing organs is often compared with lymphocyte transendothelial migration since several common cell adhesion molecules, such
as selectins and integrins, are involved in both lymphocyte and tumor cell migration. JAMs are known to participate in the transendothelial migration of lymphocytes (10). Recently, the participation of JAMs in tumor angiogenesis has also been the focus of studies (34, 35). Another recent study demonstrated that JAM-C could mediate tumor cell-endothelial cell interactions and suggested the involvement of JAM-C in tumor cell metastasis (20).

In the present study, to understand the participation of JAM family molecules in tumor metastasis, we first examined the mRNA levels of the JAM family in various cancer cells derived from human and rodent sources. As a result, we observed cell line–dependent differences in the mRNA level of JAM-C. Interestingly, most of the JAM-C-expressing cell lines in this study have often been used for experimental models of metastasis because of their high malignancy. In murine K-1735 melanoma cell lines, the highly metastatic sublines strongly expressed JAM-C when compared with the poorly metastatic ones. In addition, the expression levels of JAM-A and JAM-B on K-1735 cells did not correlate with the metastatic potential of these cells. These data suggested that JAM-C might be implicated in cancer metastasis.

It has been reported that JAM-C shows only 32 and 36% identity to JAM-A and JAM-B, respectively (13). The intracellular region of JAM-C is slightly longer than that of JAM-A and JAM-B (13). The relative tissue distribution of each JAM shows a distinct pattern, resulting from cell type–specific expression of the individual JAM (9). In this study, the unique expression pattern of JAM-C was observed in various cancer cell lines. These observations may explain the distinctive roles of JAM-C in cancer metastasis. The full open reading frame for hJAM-C displays 84% identity with that for murine JAM-C (mJAM-C) at the DNA level and 86% identity at the amino acid level (37). The tissue distribution of hJAM-C does not overlap entirely with that of the murine homologue (13). We also examined mJAM-C function in the invasion assay, with the result that overexpression of mJAM-C also promoted tumor invasion as well as hJAM-C did (data not shown).

In this study, the roles of JAM-C in cancer metastasis were examined by using hJAM-C/HT1080 cells engineered to express hJAM-C abundantly. When compared with Mock/HT1080 cells, these cells showed significantly increased adhesiveness to various ECM components and invasiveness across Matrigel-coated membranes. In addition, the experiments using hJAM-C siRNA showed that endogenous hJAM-C would be involved in tumor adhesion and subsequent invasion. It is not clear whether JAM-C expression enhanced the adhesion of HT1080 cells to various ECM components directly or indirectly. In the case of JAM-A, it has been reported that it regulates β1 integrin expression and cellular adhesion property (36). It is possible that JAM-C may affect the cell adhesiveness by controlling other adhesion molecules. In addition, JAM-C may function cooperatively with certain proteins since the degradation of Matrigel requires proteinase activity.

Finally, we demonstrated that hJAM-C promoted pulmonary metastasis in the experimental metastatic model in vivo. We conclude that the expression of JAM-C promotes cancer metastasis by enhancing the adhesion of and subsequent invasion of cancer cells. The present study is the first report that demonstrates that JAM-C expression promotes tumor malignancy and thus provides a new insight into the mechanisms of cancer metastasis.

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