Human T-Cell Lymphotropic Virus Type I-Infected Cells Extravasate through the Endothelial Barrier by a Local Angiogenesis-Like Mechanism

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ABSTRACT
Extravasation of tumor cells through the endothelial barrier is a critical step in cancer metastasis. Human T-cell lymphotropic virus type I (HTLV-I)-associated adult T-cell leukemia/lymphoma (ATL) is an aggressive disease characterized by visceral invasion. We show that ATL and HTLV-I-associated myelopathy patients exhibit high plasma levels of functional vascular endothelial growth factor and basic fibroblast growth factor. The viral oncoprotein Tax transactivates the promoter of the gap-junction protein connexin-43 and enhances gap-junction-mediated heterocellular communication with endothelial cells. The interaction of HTLV-I-transformed cells with endothelial cells induces the gelatinase activity of matrix metalloproteinase (MMP)-2 and MMP-9 in endothelial cells and down-regulates the tissue inhibitor of MMP. This leads to subendothelial basement membrane degradation followed by endothelial cell retraction, allowing neoplastic lymphocyte extravasation. We propose a model that offers a mechanistic explanation for extravasation of HTLV-I-infected cells: after specific adhesion to endothelia of target organs, tumor cells induce a local and transient angiogenesis-like mechanism through paracrine stimulation and direct cell–cell communication with endothelial cells. This culminates in a breach of the endothelial barrier function, allowing cancer cell invasion. This local and transient angiogenesis-like sequence that may facilitate visceral invasion in ATL represents a potential target for ATL therapy.

INTRODUCTION
Metastasis is the colonization of selective, secondary organ sites by dissemination of tumor cells from their primary location (reviewed in Refs. 1–3). Early steps in the metastatic cascade include the shedding of cancer cells from the primary neoplasm, the invasion of extracellular matrices, and the intravasation of tumor cells into lymph and/or blood vessels (reviewed in Refs. 4–6). On circulation, many cancer cells are eradicated by physical forces exerted on them during passage through the microvasculature of secondary organs (reviewed in Ref. 7) and by host defense mechanisms. The few surviving tumor cells are thought to colonize selective organ sites by recognizing organ-specific adhesion molecules expressed on the surfaces of vascular endothelial cells (1–3, 8–10). This step is usually followed by cancer cell extravasation and penetration into the secondary organ. However, little is known about the mechanism by which cancer cells traverse this endothelial barrier.

Angiogenesis is critical for the growth of solid tumors (reviewed in Refs. 11–14) or hematological malignancies (15–17) and is induced by angiogenic factors such as vascular endothelium growth factor (VEGF) and basic fibroblast growth factor (bFGF; Refs. 18, 19) secreted by the tumor and/or stromal cells. Angiogenesis involves activation of endothelial matrix metalloproteinases (MMPs) involved in the degradation of subendothelial basement membrane, which is a prerequisite for directed migration and proliferation of endothelial cells (reviewed in Ref. 20).

Human T-cell lymphotropic virus type I (HTLV-I) is the causative agent of adult T-cell leukemia/lymphoma (ATL; Ref. 21) and tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP/HAM; Ref. 22). ATL is an aggressive malignancy of mature activated T-cells characterized by frequent visceral invasion by tumor cells (reviewed in Ref. 23). On the other hand, TSP/HAM is characterized by central nervous system invasion by HTLV-I-infected lymphocytes (24). The invasive nature of HTLV-I-associated diseases strongly suggests a possible interaction between HTLV-I-infected cells and endothelial cells. In that sense, we have recently shown that HTLV-I-positive cells communicate with endothelial cells through both paracrine stimulation and direct gap-junction-mediated heterocellular communication (25). Indeed, ATL-derived cells specifically secrete high levels of the angiogenic factors VEGF and bFGF, induce endothelial tube formation in vitro, and establish functional gap-junction-mediated communication with endothelial cells. These gap junctions are clusters of transmembrane channels composed of structurally related proteins known as connexins (26), which play an important role in tissue organization and cellular differentiation (27–29).

In this report we demonstrate that plasma from ATL and TSP/HAM patients contains very high levels of VEGF or bFGF, which suffice to induce angiogenesis. We also show that the HTLV-I oncoprotein Tax transactivates the connexin-43 promoter and enhances the formation of functional gap junctions with endothelial cells. Interaction of HTLV-I-transformed cells with endothelial cells induces the production of functional MMPs by endothelial cells, resulting in the degradation of subendothelial basement membrane and allowing extravasation of transformed lymphocytes between endothelial cells. Using ATL as a model, we propose that after adhesion of tumor cells to endothelia of the target organ, tumor cells induce an angiogenesis-like mechanism that leads to a breach in the endothelial barrier and allows cancer cells to invade.

MATERIALS AND METHODS
Cells and Antibodies. HuT-102 and MT-2 cells are HTLV-I-transformed CD4+ T-cell lines that constitutively express HTLV-I virus. The HTLV-I-negative CD4+ T-cell lines CEM, Molt-4, and Jurkat were used as control cells. Cells were grown in RPMI 1640 containing 10% heat-inactivated FCS (Life Technologies, Inc., Paisley, United Kingdom) and antibiotics. A cell concentration of 2×10^6 cells/ml was chosen for seeding for all experiments unless otherwise stated. The human cell lines ECV-304, HeLa, and MCF-7 were grown in the same medium. Human aortic endothelial cells (HAECs;
Clonetics, San Diego, CA) were grown in medium provided by the supplier at an initial concentration of $4 \times 10^4$ cells/ml.

Rabbit polyclonal antibodies to MMP tissue inhibitor-1 were obtained from Serotech Ltd. (Oxford, United Kingdom). Mouse monoclonal anti-Tax (168-A51) was obtained from the NIH AIDS Research and Reagent Program. Rabbit antiactin (Santa Cruz Biotechnology, San Jose, CA) antibodies were used to assess protein loading.

**RNA Isolation and Reverse Transcription-PCR.** cDNA was synthesized from 500 ng of total cellular RNA and isolated by use of Trizol reagent (Life Technologies, Inc.) with reverse transcriptase (Super script II; Life Technologies, Inc.). The cDNA was then amplified by PCR using Taq DNA polymerase (Life Technologies, Inc.). We used PCR primers that recognize all VEGF isoforms, MMP-2, MMP-9, and β-actin-specific primers. Amplification of the housekeeping gene β-actin was used to verify RNA quality and reverse transcription-PCR techniques. The PCR conditions used to amplify VEGF, MMP-2, MMP-9, and β-actin consisted of a precycle of 95°C for 3 min followed by 35 cycles consisting of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. A final extension at 72°C for 7 min was then performed. The primers used were as follows: β-actin forward primer, 5'-CGC CTG CTC GTG TGC GAC A-3'; β-actin reverse primer, 5'-GTC ACG CAC CTA TTT CCC GCT-3'; VEGF forward primer, 5'-TGC GGC CTC CGG AAA CCA TGA-3'; VEGF reverse primer, 5'-CCT GGT GAG AGA CTG TGT CCA CCC CTC AGA GC-3'; MMP-2 forward primer, 5'-GTC ACG CTC GTG TGC GAC A-3'; MMP-2 reverse primer, 5'-TTG CCA TCC TTC TCA CAA GGT TAG G-3'; MMP-9 forward primer, 5'-CAG TGT CCA CCC CTC AGA GC-3'; MMP-9 reverse primer, 5'-GCC ACT TGT CGG CGA TAA GG-3'; MMP-14 forward primer, 5'-CGC TAC GCC ATC CAG GGT CTC AAA-3'; and MMP-14 reverse primer, 5'-CGG TCA TCG GCC AGC ACA AAA-3'. PCR products were electrophoresed in 1.5% agarose gels and visualized with ethidium bromide staining.

**Western Blot Analysis.** Approximately 10° cells were solubilized at 4°C in lysis buffer consisting of 0.125 M Tris-Cl (pH 6.8), 2% SDS, 2.5% β-mercaptoethanol, and 10% glycerol. Samples were loaded onto a 12% SDS–polyacrylamide gel, subjected to electrophoresis, and transferred to nitrocellulose membranes. After blocking of the membrane in 5% skim milk in Tris-buffered saline containing 0.05% Tween 20, the blots were incubated with specific antibodies. The blots were washed, and protein bands were visualized by chemiluminescence (Amersham, Buckinghamshire, United Kingdom). The cDNA was then amplified by PCR using Taq DNA polymerase (Life Technologies, Inc.) with reverse transcriptase (Super script II; Life Technologies, Inc.) according to the manufacturer’s recommendations. Luciferase activity was quantified 24 h later by the Luciferase Assay System (Promega, Madison, WI). Values were normalized with the Renilla activity.

**Matrix-induced Capillary Tube Formation.** Endothelial cells (4 × 10°) were seeded in 12-well plates precoated with 200 µg/well growth factor-reduced Matrigel (Becton Dickinson, San Jose, CA) and cultured for 18 h. Induction of endothelial cell tube formation was assessed after incubation with test plasma at 37°C for 48 h. Function-blocking anti-VEGF antibodies (10 ng/ml; Santa Cruz Biotechnology) or MMP inhibitor (5 µM, 1,10-phenanthroline; Sigma, St. Louis, MO) were added concomitantly to the plasma where indicated. Cells were then stained with 0.5 µg/ml Hoechst (Molecular Probes, Eugene, OR) for 10 min and observed by fluorescence microscopy.

**Functional Assay of Adhesion and Communication.** HeLa cells were transiently transfected with pSG5-Tax or empty vector using Lipofectamine plus (Life Technologies, Inc.) according to the manufacturer’s recommendations. Transfected HeLa cells or HTLV-I-positive cells were labeled with the membrane-permeable dye calcein-AM (Molecular Probes) as described previously (25). Confluent monolayers of endothelial cells grown in 35-mm Petri dishes were cocultured for 1 h at 37°C with calcein-labeled cells. Unbound cells were removed by a single wash. Endothelial cells and adhered HuT-102 or HeLa cells were then released from the growth surface with trypsin/EDTA in HBSS, washed once with HBSS, fixed in 4% formaldehyde in PBS, and

**Fig. 1. High plasma levels of functional angiogenic factors in patients with adult T-cell leukemia/lymphoma (ATL) and tropical spastic paraparesis/human T-cell lymphotropic virus type I associated myelopathy (TSP/HAM).** A. ELISA measurement of plasma vascular endothelium growth factor (VEGF) and basic fibroblast growth factor (bFGF) in 17 ATL patients (mean VEGF, 234 ± 241 pg/ml; mean bFGF, 22 ± 16 pg/ml), 1 patient with tropical spastic paraparesis (mean VEGF, 248 ± 299 pg/ml; mean bFGF, 14 ± 12 pg/ml), and 9 HTLV-I-seronegative healthy control individuals. Plasma was prepared by centrifugation of blood collected in citrate-containing tubes and stored at −80°C. VEGF and bFGF protein levels in patient plasmas or in the cell-free supernatants were determined by ELISA (R&D Systems, Minneapolis, MN) as recommended by the manufacturer. Luciferase Assays. Connexin-43-Luc construct in pGL-2 (provided by Dr. Stephen Lye, McGill University, Montreal, Canada) was cotransfected into HeLa cells with the internal control Renilla luciferase and either pSG5-Tax or empty vector using Lipofectamine plus (Life Technologies, Inc.) according to the manufacturer’s recommendations. Luciferase activity was quantified 24 h later by the Luciferase Assay System (Promega, Madison, WI). Values were normalized with the Renilla activity.
analyzed by flow cytometry (FACSscan; Becton Dickinson). Alternatively, confluent monolayers of endothelial cells grown in coverslip-fitted 35-mm Petri dishes were cocultured with calcine-labeled HuT-102 cells and observed under fluorescence microscopy at different time intervals.

**Gelatin Zymography.** Endothelial cells, cocultured with HTLV-I-positive or-negative cells or with their cell-free supernatant for 72 h, were washed three times in PBS before their protein extracts were analyzed by zymography. The gap-junction inhibitor 18-α-G (Sigma; 50 μM) was added to the cell coculture when indicated. Proteins (20 μg) from cell extracts in 2× sample buffer (0.25 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 0.01% bromophenol blue) were electrophoresed on 10% polyacrylamide gel copolymerized with 1 mg/ml gelatin (Sigma), washed twice for 30 min with 2.5% Triton X-100 (Sigma), and then incubated overnight in substrate buffer [150 mM NaCl, 5 mM CaCl₂, 50 mM Tris-Cl (pH 8.0)] at 37°C with gentle shaking. The gels were then stained with 0.25% Coomassie Brilliant Blue R-250 in 45% methanol–10% acetic acid–45% water for 4 h at 37°C with gentle shaking and destained in doubly distilled water.

**Invasion Assay.** Six-well tissue culture plates were fitted with inserts (8 μm pore size) coated with growth factor-reduced Engelbreth Holm-Swarm (Matrigel, 125 ng/cm²). Endothelial cells were grown in the inserts to confluency and then seeded with calcine-labeled tumor cells. After 24 h of coculture, inserts were removed, and the endothelial cell layer was gently removed with a cotton swab. The membrane of the insert was then removed, mounted on a microscopic slide, and examined by fluorescence microscopy. Fluorescent tumor cells that successfully invaded through the endothelial layer were photographed and counted. At least four fields at ×20 magnification were counted per experiment (n = 3), averaged, and reported as the mean ± SD. Statistical comparison was performed with a t test with unequal variances.

**RESULTS**

**High Plasma Levels of Functional Angiogenic Factors in HTLV-I-Associated Diseases.** We evaluated plasma levels of VEGF and bFGF in 17 ATL patients, 29 TSP/HAM patients, and 9 HTLV-I seronegative healthy controls. Patients with HTLV-I-associated diseases exhibited significantly elevated levels of VEGF (mean ± SD, 243 ± 277 pg/ml) and bFGF (17 ± 14 pg/ml) compared with HTLV-I seronegative healthy controls (mean VEGF, 29 ± 3 pg/ml; bFGF below assay detection limit in 8 donors, and 10 pg/ml in 1 donor; P = 0.025 and 0.001, respectively; Fig. IA). We observed no significant difference in mean plasma VEGF levels between ATL (234 ± 241 pg/ml) and TSP/HAM patients (248 ± 299 pg/ml; P = 0.871) or in mean plasma bFGF levels (ATL patients, 22 ± 16 pg/ml; TSP/HAM patients, 14 ± 12 pg/ml; P = 0.074), although ATL patients had slightly, but nonsignificantly higher bFGF plasma levels compared with TSP/HAM patients.

The biological activity of elevated plasma levels of angiogenic factors in patients with HTLV-I-associated diseases was evaluated on endothelial cells in vitro. In the presence of eight different ATL plasma samples and six different TSP/HAM plasma samples, HAEC and ECV-304 cells formed a network of capillary-like tubules with multicentric junctions (Fig. 1B and not shown). These experiments demonstrate that the circulating angiogenic factors in the plasma of patients with HTLV-I-associated diseases are sufficient to induce biological function. Minimal or no effect was observed with plasma.

![Fig. 2. Human T-cell lymphotropic virus type I (HTLV-I)-positive cells establish functional gap junctions with endothelial cells: adhesion and dye transfer from HTLV-I-transformed cells to endothelial cells. A, flow cytometric analyses of cocultures of unlabeled human aortic endothelial cells (HAEC; a) with calcine-labeled HTLV-I-positive cells (HuT-102*; b). The relative height of the peak for HuT-102 cells compared with that of HAECs is a measure of cell adhesion. The increase in the mean fluorescent intensity (MFI) of endothelial cells reflects dye transfer through gap junctions from the leukemic cells. Endothelial cells were cocultured for 1 h with HuT-102 cells at ratios of 1:1 (c); 1:2 (d); or 1:2 (e) in the presence of the gap-junction inhibitor heptanol (1 mM). Histogram analysis of mean fluorescent intensity of HAECs under these different conditions is presented. B, phase-contrast (a-d) and fluorescence microscopy (e-h) of cocultures of unlabeled endothelial cells (ECV-304) with the calcine-labeled HTLV-I-positive cells (HuT-102) at initiation of coculture (a and e), 30 min of coculture (b and f), 1 h of coculture (c and g), and 1 h of coculture with the gap-junction inhibitor 18-α-G (d and h). Panel d represents an overlay of phase-contrast and fluorescence microscopy for a better identification of the two cell types. In panels f-h, intensely labeled cells represent calcine-labeled tumor cells, whereas endothelial cells can be seen in the background. Fluorescent endothelial cells (panels g and h) on coculture with tumor cells indicate progressive dye transfer through gap junctions.
from HTLV-I seronegative healthy controls (Fig. 1B). Addition of function blocking anti-VEGF antibody attenuated (two patients) or abolished (four patients) the angiogenic activity of plasma from four tested ATL and two tested TSP/HAM patients (Fig. 1B and not shown), implicating VEGF in the induction of angiogenesis by plasma from HTLV-I-infected patients.

**Tax Expression Increases Adhesion and Gap-Junction Communication with Endothelial Cells.** Coculture experiments between leukemic cells and the endothelium revealed that HTLV-I-transformed cells adhere and communicate with endothelial cells, in agreement with our previous report (25). Coculture of HAECs with calcine-labeled HuT-102 cells produced a progressive transfer of fluorescence from the leukemic cells to the HAECs (Fig. 2A). Indeed, cocultures at HuT-102:HAEC ratios 1:1 and 2:1 for 1 h produced an increase in mean fluorescence intensity in the HAECs to 37 and 92, respectively (Fig. 2A, graphs c and d). The addition of the gap-junction inhibitor heptanol sharply reduced endothelial cell fluorescence from 92 to 44 (compare graphs d and e in Fig. 2A), confirming the specificity of the assay. Similar results were observed in coculture experiments between calcine-labeled HuT-102 cells and ECV-304 cells, in which a progressive transfer of fluorescence from the leukemic cells to the endothelial cells was observed (Fig. 2B, compare panels f and g to e). This transfer was significantly inhibited by the addition of 50 μM of the gap-junction inhibitor 18-α-G (Fig. 2B, compare panels h and g).

We investigated the role of HTLV-I viral genes in the expression of functional gap junctions by ATL-derived cells. Coculture for 1 h at a cellular ratio of 1:1 of endothelial cells with HeLa cells transfected with the viral transactivator Tax produced a significant up-regulation of both cell–cell adhesion and gap-junction-mediated heterocellular communication between the two cell types (Fig. 3A). The endothelial cell mean fluorescence intensity significantly increased on coculture with HeLa-Tax cells compared with coculture with HeLa cells, indicating that Tax expression in Tax-transfected cells (Fig. 3B) leads to enhancement in the functional gap-junction activity. These results directly implicate viral gene expression in the formation of functional gap junctions with endothelial cells. Because HTLV-I-transformed cells were shown to express the gap-junction protein connexin-43 (25), we investigated the role of Tax expression on the transcriptional activity of the connexin-43 gene. HeLa cells were cotransfected with Tax, and a reporter plasmid in which the promoter region of the

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** Tax increases adhesion and gap-junction communication with endothelial cells and transactivates the connexin-43 promoter. **A.** Adhesion and dye transfer from Tax-transfected HeLa cells to endothelial cells (ECV-304). Flow cytometry analyses of cocultures of unlabelled endothelial cells (EC) with calcine-labeled (*) HeLa cells transfected with Tax (HeLa Tax) or empty vector (HeLa φ). The relative height of the peak for HeLa cells compared with that for the endothelial cells is a measure of cell adhesion. The increase in the mean fluorescence intensity (MFI) of endothelial cells reflects dye transfer through gap junctions from HeLa cells. Endothelial cells were cocultured for 1 h with calcine-labeled HeLa cells at a ratio of 1:1. The bottom histogram is an overlay to demonstrate the shift in endothelial cell MFI on coculture with calcine-labeled HeLa-Tax. The graph represents the mean and SD (bars) MFI from two independent experiments with endothelial cells cocultured with HeLa-Tax* or HeLa-φ*. B. Western blot analysis using anti-Tax antibodies. C. Connexin 43-Luc (Cx43-Luc) construct in pGL-2 corresponding to the luciferase gene under the control of the connexin-43 promoter was cotransfected into HeLa cells with the internal control Renilla luciferase and either pSGTax or empty vector. Luciferase activity was quantified 24 h later and normalized with the Renilla luciferase activity. D. Molt-4 cells transfected with Tax (Molt-4 Tax) or empty vector were analyzed for vascular endothelium growth factor (VEGF) mRNA expression by reverse transcription-PCR using 100 ng of total RNA. E. HeLa cells were transfected with pCMV-Tax or empty vector. Levels of vascular endothelium growth factor (VEGF) in the cell supernatant were measured with a commercial ELISA.
gap-junction protein connexin-43 controls the luciferase cDNA. Tax expression induced a 6-fold increase in connexin-43 promoter activity as measured by the luciferase assay (Fig. 3C). Furthermore, Tax expression led to a significant increase in the number of VEGF transcripts in Tax-transfected Molt-4 cells (Fig. 3D) and a significant increase in VEGF secretion in Tax-transfected HeLa cells (Fig. 3E), confirming our previous findings that HTLV-I-positive cells synthesize and secrete VEGF, presumably as a result of Tax-induced transcriptional activation of the VEGF gene (25).

**HTLV-I-Transformed Cells Induce MMP Activity by Endothelial Cells.** HTLV-I-transformed cells interact with endothelial cells through both paracrine stimulation and direct gap-junction-mediated communication (25). We tested the effect of this dual interaction on endothelial cells function by assessing the production of MMP transcripts and gelatinase activity by endothelial cells after exposure to HTLV-I-transformed cells or their cell-free supernatants. Confluent ECV-304 cells in culture produce low amounts of MMP-2 and MMP-9 transcripts (Fig. 4A and not shown).

**Paracrine Stimulation.** Endothelial MMP-9 transcript levels were significantly increased after incubation with 72-h cell-free supernatant from HTLV-I-positive HuT-102 cells but not from HTLV-I-negative CEM cells (Fig. 4A). In contrast, transcript levels of endothelial MMP-2 were unaffected (not shown). However, endothelial MMP-14 transcript levels (Fig. 4A) were up-regulated after incubation with 72-h cell-free supernatant from HuT-102 cells and significantly down-regulated after incubation with 72-h cell-free supernatant from HTLV-I-negative CEM cells. MMP-14 has been reported to convert proMMP-2 to active MMP-2 (30). However, proMMP-2 was undetectable by zymography in unstimulated confluent ECV-304 cells, suggesting that cell-free supernatant from ATL-derived cells stimulates translation of preexisting MMP-2 mRNA. Furthermore, production of MMP tissue inhibitor-1 by endothelial cells was significantly down-regulated after incubation with 72-h supernatant from HuT-102 cells and CEM cells (Fig. 4C). Importantly, zymogram analysis (Fig. 4B) of endothelial extracts clearly indicated that incubation of endothelial cells with 72-h cell-free supernatant from HTLV-I-transformed cells (HuT-102 and MT2) significantly up-regulated MMP-2 activity and, to a lesser extent, MMP-9 activity, whereas incubation with 72-h cell-free supernatant from HTLV-I-negative cells (CEM and Jurkatt cells) led to minimal induction of MMP-2 and MMP-9 activity. These results indicate that HTLV-I-transformed cells stimulate the production and/or activation of functional MMPs by endothelial cells through a paracrine mechanism.

**Gap-Junction-Mediated Communication.** Similarly, coculture of endothelial cells with supernatant-free HTLV-I-transformed cells at a 1:2 cellular ratio also up-regulated MMP-2 and MMP-9 activity through both paracrine stimulation and direct gap-junction-mediated communication (25). We tested the effect of this dual interaction on endothelial cells function by assessing the production of MMP transcripts and gelatinase activity by endothelial cells after exposure to HTLV-I-transformed cells or their cell-free supernatants. Confluent ECV-304 cells in culture produce low amounts of MMP-2 and MMP-9 transcripts (Fig. 4A and not shown).

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The gap-junction inhibitor 18-α-G (50 μM) impaired gap-junction-mediated communication in the induction of MMP by endothelial cells (Fig. 4D). Importantly, this gap-junction inhibitor 18-α-G (50 μM) had no detectable toxicity on endothelial cells (not shown) and did not affect VEGF transcript level in HuT-102 cells (Fig. 4E).

Finally, the Matrigel assay clearly demonstrated that in the presence of a >1:1 cellular ratio of supernatant-free HTLV-I-transformed cells or after incubation with 72-h cell-free supernatant, ECV-304 and HAEC endothelial cells formed a network of capillary-like tubules with multicentric junctions, whereas cell-free supernatant from CEM cells did not affect (Fig. 4, F and G). This in vitro angiogenesis was completely inhibited when an MMP inhibitor, 1,10-phenanthroline (5 μM) was added (Fig. 4H). Altogether, our results indicate that interaction of HTLV-I-transformed cells with endothelial cells induces an angiogenesis-like mechanism characterized by the production of functional MMPs by endothelial cells, leading to the degradation of subendothelial basement membrane.

**HTLV-I-Transformed Cells Invade Endothelial Cells by an Angiogenesis-Like Mechanism.** We then set out to document, using time-lapse photography and video microscopy, extravasation process of HTLV-I-transformed cells in vitro. Confluent monolayers of HAEC or ECV-304 cells were seeded with HuT-102 cells and observed by light microscopy over a period of 8 h. Within 15–60 min of coculture, HuT-102 cells adhered to and established pseudopod junctions with the luminal aspects of endothelial cells (Fig. 5A, top panel (ECV-304) and bottom panel (HAEC); 60 min, arrow). Most observed HuT-102 cells eventually settled between two adjacent endothelial cells, and the effect of their interaction became noticeable. ATL-derived cells sent cytoplasmic processes in the area where a cleft between two adjacent endothelial cells appeared (Fig. 5A, 120 min, top panel, arrow) indicative of possible extravasation of HTLV-I-transformed cells. Finally, the morphology of endothelial cells became significantly altered [Fig. 5A, top panel (ECV-304) and bottom panel (HAEC); 240 min, arrow]. In contrast, CEM cells exhibited very low overall activity, remained loosely attached to endothelial cells and resulted in no dramatic changes in endothelial cell morphology (Fig. 5A). The inclusion of 50 μM 18-α-G throughout the coculture time used for video microscopy impeded most of the morphological alterations observed when HuT-102 cells were cocultured with endothelial cells (not shown). These observations were repeated with the breast cancer-derived cell line MCF-7, which secretes high levels of VEGF. Video microscopy revealed that within 60 min of coculture, MCF-7 cells adhered to and established pseudopod junctions with the luminal aspect of endothelial cells (Fig. 5A, 60 min, arrow). Similar to HuT-102 cells, MCF-7 cells induced endothelial retraction with clear cytoskeletal rearrangement and the creation of a local cleft between two neighboring cells (Fig. 5A, 240 min, white arrow). This “retrac-

![Fig. 5. Interaction between human T-cell lymphotropic virus type I-transformed cells and endothelial cells.](image)

**A.** Confluent monolayers of ECV-304 or HAEC cells were seeded with HuT-102 cells and observed by light microscopy over a period of 8 h. Time sequence images are displayed. 30 min, tumor cells (black arrow) adhere to the luminal aspect of endothelial cells (white arrow); 60 min, HuT-102 and MCF-7 cells establish pseudopods junctions with endothelial cells (arrows); 120 min, HuT-102 and MCF-7 cells start sending cytoplasmic processes in the area where a cleft between two adjacent cells appeared (arrow); 240 min, the morphology of endothelial cells change (black arrows), and a cleft between two endothelial cells become visible (white arrow in MCF-7). **B.** Invasion assay of tumor cells (MCF-7 and HuT-102) through endothelial cell layer. Invasion of HuT-102 cells is attenuated by the gap-junction inhibitor 18-α-G (18αG).
tion” was not observed between endothelial cells that had no contact with cancer cells.

We assessed the ability of tumor cells to traverse the endothelial cell barrier by use of an invasion assay with a two-compartment model. This overcomes the limitation of video microscopy of cells plated directly on plastic dishes, where the tumor cells do not have a physical space to invade. Both HuT-102 and MCF-7 cells efficiently invaded the endothelial monolayer as judged by the number of fluorescent tumor cells in the abluminal surface of the 35-mm culture filter insert (mean ± SD, 37 ± 18 and 60 ± 20, respectively, after 24 h). Addition of 18-α-G (100 μM) led to a significant reduction in the number of HuT-102 cells (8 ± 3; P < 0.001) and MCF-7 (21 ± 5; P < 0.05) that invaded the insert. These data highlight the critical role of gap-junction-mediated communication in the invasive potential of ATL-derived and MCF-7 cells.

**DISCUSSION**

In this report we show that patients with HTLV-I-associated diseases have high plasma levels of functional angiogenic factors. The origin of VEGF and bFGF in the plasma of these patients remains to be determined. However, because HTLV-I-transformed cells specifically secrete high levels of these two angiogenic factors (25), it is most likely that they are produced by the HTLV-I-infected cells, presumably as the result of Tax-dependent transcriptional activation of the VEGF gene (25). Most HTLV-I-infected leukemic cells express fms-like tyrosine kinase 1, one of the receptors for VEGF (31). Hence, elevated plasma VEGF levels may contribute to the proliferation of HTLV-I-infected cells through autocrine stimulation of growth and may represent a good target for anti-VEGF therapy.

ATL is characterized by a particularly high frequency of cutaneous and visceral invasion by leukemic cells. A link between high plasma VEGF levels and extranodal involvement in ATL has been suggested (32). This invasive potential of ATL cells dictates an interaction between leukemic cells and the endothelium. Here we show that ATL-derived cells communicate with endothelial cells through both angiogenic factor-mediated paracrine stimulation and direct gap-junction-mediated heterocellular communication. A role for Tax in this interaction is demonstrated by transcriptional induction of VEGF promoter (25) and connexin-43 promoter and by increased functional heterocellular communication on Tax transfection. This dual interaction between ATL-derived cells and endothelial cells induces the production of functional MMPs by endothelial cells. The gelatinase activity of MMPs leads to the degradation of subendothelial basement membrane and retraction of endothelial cells, allowing extravasation of ATL-derived cells. In TSP/HAM, a similar angiogenesis-like mechanism, triggered by HTLV-I-infected cells, may be responsible for passage of HTLV-I-infected cells through the blood–brain barrier into the central nervous system. Moreover, using the breast cancer-derived cell line MCF-7, which produces high levels of functional VEGF, we showed that these malignant cells also induce morphological modifications of endothelial cells (Fig. 5). Hence, tumor-cell-induced angiogenesis-like events may represent a general mechanism of tumor cell extravasation.

Several reports have implicated high plasma levels of angiogenic factors (32), signaling through adhesion molecules, and production of MMPs by tumor cells (33, 34) in extranodal organ invasion by tumor cells. However, this model does not explain how tumor cells extravasate through the endothelial barrier. Indeed, a generalized response of the endothelium to high plasma levels of VEGF and bFGF may produce detrimental consequences for many physiological functions and does not explain the preference of organ invasion in cancer (35, 36). In addition, MMPs produced by tumor cells on the endoluminal side of the endothelial cells will have no access to the subendothelial basement membrane and hence have little relevance in the context of extravasation. Their importance should be emphasized in tissue invasion once a tumor cell has extravasated. Finally, on the specific binding of a tumor cell to the endothelium, three mechanisms potentially influence endothelial cells: local reciprocal paracrine interaction, signaling through adhesion molecules, and the direct exchange of regulatory molecules through gap junctions (37). Although many
studies have been dedicated to elucidate the partners and players of adhesion events between endothelial cells and tumor cells, little is known about the other two mechanisms. On the basis of our data and the fact that most tested malignant cells produce angiogenic factors, we propose a novel role for these angiogenic factors, at the site of cancer cell extravasation, in cancer metastasis (Fig. 6): (a) Adhesion of tumor cells to endothelial cells leads to signaling through adhesion molecules and/or through gap-junction-mediated direct cytoplasmic exchange of messages that influence endothelial barrier function. (b) After this interaction, tumor cells and endothelial cells will be at such proximity as to allow for paracrine reciprocal interaction. The binding of angiogenic factors, such as VEGF produced by tumor cells, to their receptors on the endothelial cell surfaces sets a mechanism for transient and localized angiogenesis into motion. (c) This mechanism includes activation of endothelial MMPs involved in the degradation of subendothelial basement membrane. (d) The result of this activation is translated into cytoskeletal rearrangement and de-adhesion of adjacent endothelial cells (38). This is perceived by the adhered tumor cells as a breach of endothelial cell barrier. (e) Tumor cells will then send processes that will adhere to the subendothelial matrix, and extravasation ensues. The directed migration out of the vessel lumen (extravasation) of tumor cells is driven by chemokines and other degradation products of the extracellular matrix, which are known chemoattractant factors for many cancer cells, that result from the degradation of subendothelial basement membrane. This model, which offers a mechanistic explanation for tumor cell extravasation based on local and transient angiogenic reactions, supports a novel role for angiogenic factors and cell–cell communication in cancer metastasis and could explain the effectiveness of antiangiogenic treatment in preventing tumor cell dissemination and organ invasion (39).

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