Extracellular Membrane Vesicles from Tumor Cells Promote Angiogenesis via Sphingomyelin

Chan Woo Kim, Hwan Myung Lee, Tae Hoon Lee, Chulhun Kang, Hynda K. Kleinman, and Yong Song Gho

ABSTRACT

Actively growing tumor cells shed membrane vesicles into the extracellular milieu both in vivo and in vitro. Extracellular membrane vesicles from tumor cells contain most surface antigens and proteases present on these cells. They facilitate the escape of tumors from immune surveillance and promote tumor cell invasion. Here, we demonstrate that tumor membrane vesicles stimulate an additional important activity for tumor growth and metastasis by promoting endothelial cell migration, invasion, and tube formation, and inducing in vivo neovascularization. Our data show that tumor vesicles are one of the multiple effectors involved in tumor-induced angiogenesis. Heat-treated vesicles and lipid extracts from the vesicles also induce endothelial cell migration and in vivo angiogenesis. We identify sphingomyelin as the active component for vesicle-induced endothelial cell angiogenesis. We identify sphingomyelin as the active component for vesicle-induced endothelial cell migration, tube formation, and neovascularization. Together with previously reported results, our data demonstrate that shed tumor vesicles play multiple roles in tumor growth and metastasis by promoting angiogenesis, tumor invasion, and immune escape.

INTRODUCTION

Activated cells, including endothelial cells, platelets, and monocytes, shed fragments of their plasma membrane into the extracellular milieu under specific physiological conditions (1–3). The process of shedding is elevated in actively growing tumor cells that continually shed membrane vesicles in vitro and in vivo (4–18). Although several hypotheses have been suggested, the exact mechanisms involved in shedding extracellular membrane vesicles are not clear (4, 5). EMVTCs are derived from selected areas of the plasma membrane (6–8) and appear to be enriched in most surface antigens and proteases present on tumor cells (9–18). When compared with the plasma membrane, EMVTCs are composed of a lower content of phospholipids but an increased amount of cholesterol and sphingomyelin, resulting in a more rigid structure (7, 8). Several studies have suggested that EMVTCs are involved in tumor growth and metastasis by playing relevant roles in the escape of tumors from immune surveillance and in promoting tumor cell invasion (9–18). Tumor surface antigens and the immune-suppressing cytokine, transforming growth factor-β, in EMVTCs provide powerful factors that protect tumors from immune attack (9–12). The levels of membrane vesicles in the sera of cancer patients are significantly higher than that of healthy donors (13, 14). Several proteases, including MMPs and plasminogen activator, are enriched in EMVTCs (13–18). These proteolytic enzymes and integrins in EMVTCs are thought to play important roles in tumor cell invasion and metastasis. Both the amounts and the vesicle-associated proteolytic activities of EMVTCs are positively correlated with malignancy in vivo and with the invasiveness of tumor cells in vitro (14, 15).

In addition to immune escape and tumor invasion, angiogenesis, the formation of new capillaries from the preexisting blood vessels, is critical for tumor growth and metastasis (19, 20). Actively growing tumor cells need increased blood vessels to take up nutrients and clean up wastes for their survival. Furthermore, such vessels could provide pathways for metastasis. Here we show that EMVTCs have angiogenic activity and that sphingomyelin may be an active component involved in EMVTC-induced neovascularization.

MATERIALS AND METHODS

Cell Culture. Immortalized HMEC-1 were maintained in RPMI 1640 supplemented with 10% FBS, penicillin/streptomycin, 3 ng/ml human bFGF (R&D Systems, Minneapolis, MN), and 5 units/ml heparin (Sigma-Aldrich, St. Louis, MO). Primary HUVECs were prepared and cultured as described previously (21). HT1080 human fibrosarcoma and DU-145 human prostate carcinoma cells were maintained in RPMI 1640 supplemented with 10% FBS and penicillin-streptomycin. EMVTC Preparation. EMVTCs were prepared as described previously (16). Briefly, confluent HT1080 and DU-145 cells were washed and grown in serum-free RPMI 1640. After a 24-h incubation, conditioned medium was removed and centrifuged at 500 × g for 10 min and then at 800 × g for 15 min. The supernatant was centrifuged at 100,000 × g for 1 h. The resulting pellet EMVTCs were resuspended in PBS, and then the protein concentration was determined (22). EMVTCs were aliquoted and stored at −20°C until use. For heat treatment, EMVTCs were boiled for 10 min.

For further purification of EMVTCs, centrifugation of EMVTCs on a discontinuous sucrose gradient was carried out at 4°C (23). Briefly, 1.5 ml of 1.4 M, 1.0 M, and 0.6 M sucrose in 20 mM HEPES/NaOH (pH 7.2) was added to a Sorvall AH-650 tube. A solution of EMVTCs in PBS (0.5 ml) was overlaid on top of the discontinuous sucrose gradient, and the sample was centrifuged at 150,000 × g for 2 h. Fractions of the gradient (0.8 ml) were diluted in 4.2 ml of PBS and were centrifuged at 100,000 × g for 2 h, and each individual pellet was resuspended in PBS. Then, the protein concentration was determined. The EMVTCs were recovered from the 1.0–1.4-M sucrose interface as reported previously (12).

Migration and Invasion Assay. Endothelial migration and invasion assays were carried out in 48-well microchemotaxis chambers (Neuro Probe Inc., Cabin John, MD) as described previously (21). Polyester membranes with 12-μm pores were coated with a 0.1% gelatin (Sigma-Aldrich) in deionized water and a 0.5 mg/ml Matrigel (Collaborative Biomedical Products, Bedford, MA) in double distilled water for the migration and invasion assays, respectively. Human endothelial cells were resuspended in RPMI 1640 containing 0.1% BSA. The bottom chamber was loaded with 50,000 cells, and the membrane was laid over the cells. The chamber was inverted and incubated for 2 h. Upper wells were then loaded with RPMI 1640 containing 0.1% BSA and test samples. The chamber was then reincubated for 2 h and 4 h for migration and invasion assays, respectively. The membrane filters were fixed and stained using Diff-Quick (Baxter Healthcare Corp., McGraw Park, IL). The number of cells that migrated and invaded through the filter was determined by counting two regions of each well under a microscope. All of the samples used in the experiments with lips contained 0.1% DMSO, and the presence of DMSO did not affect endothelial cell migration. Protease inhibitor cocktail, comprising the mixture of Complete, Mini (Roche Molecular Biochemicals, Mann-
EMVTCs Stimulate Endothelial Cell Migration and Invasion.

Because endothelial cell migration and invasion through extracellular matrix are essential for neovascularization, we first investigated the effects of EMVTCs on endothelial cell migration and invasion. One million HT1080 cells shed 5 μg/ml EMVTCs per day in the absence and presence of serum, respectively. In all of our experiments, we used EMVTCs prepared from serum-free conditions. EMVTCs significantly induced up to a 2.7-fold increase in the migration of HMEC-1s in a dose-dependent manner over migration in the presence of medium alone (Fig. 1A). Endothelial cell migration, stimulated by 5 μg/ml EMVTCs, was similar to levels observed for the positive control bFGF (5 ng/ml). EMVTCs also stimulated the migration of HUVECs with a response comparable with that observed with HMEC-1 (data not shown). EMVTCs, purified by sucrose gradient sedimentation, also induced endothelial cell migration, and the migratory activity was similar to that of EMVTCs prepared by ultracentrifugation. In this experiment, based on comparisons with appropriate control samples tested at the same time.

Statistical Analysis. All of the values are expressed as mean ± SD. P values were calculated from Student’s t test, based on comparisons with appropriate control samples tested at the same time.

RESULTS

EMVTCs Stimulate Endothelial Cell Migration and Invasion.

We next investigated the effect of EMVTCs on the invasion of endothelial cells. EMVTCs significantly induced endothelial cell invasion about 2-fold in a dose-dependent manner (Fig. 2A). Protease inhibitor cocktail, comprising the mixture of Complete, Mini and MMP-2/MMP-9 inhibitor II, blocked EMVTC-induced endothelial cell invasion (Fig. 2B), but it did not affect EMVTC-induced endothelial cell migration (data not shown). Complete, Mini inhibits a broad spectrum of serine and cysteine proteases as well as calpains. Because EMVTCs are enriched with plasminogen activator, MMP-2, and MMP-9, which are essential for cell invasion through extracellular matrix (13–18), we investigated the involvement of these proteases in EMVTC-induced endothelial cell invasion. Aprotinin (3 μg/ml) and MMP-2/MMP-9 inhibitor II (20 ng/ml) blocked EMVTC-induced endothelial cell invasion (Fig. 2C; P < 0.0001). A mixture of these inhibitors further blocked EMVTC-induced endothelial cell invasion, and the inhibitory activity was stronger than that of aprotinin or MMP-2/MMP-9 inhibitor alone (P < 0.001). The presence of the tissue inhibitor of metalloproteinase-1 (1 μg/ml), the tissue inhibitor of metalloproteinase-2 (2 μg/ml), the plasminogen activator inhibitor-1 (10 μg/ml), or the α2-antiplasmin (10 μg/ml) efficiently reduced EMVTC-induced endothelial cell invasion (Fig. 2D; P < 0.0001). All of the protease inhibitors also blocked the basal invasion observed in the absence of EMVTCs, which indicated the expected role of endogenous proteases. These results indicate that both the MMPs and the plasminogen activator present in EMVTCs showed significantly reduced migratory activity compared with that of conditioned medium itself (P < 0.0001). These results suggest that both EMVTCs and factors not associated with EMVTCs in tumor-conditioned medium are involved in stimulating endothelial cell migration.

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ANGIOGENIC ACTIVITY OF EMVTCs

EMVTCs stimulate endothelial cell invasion. In A, EMVTCs significantly induced invasion of endothelial cells over invasion in the presence of medium alone. Positive control, bFGF, showed a 1.5-fold increase in invasion. In B, protease inhibitor cocktail blocked EMVTC-induced endothelial cell invasion. However, this treatment did not affect EMVTC-induced endothelial cell migration (not shown). In C, aprotinin and MMP-2/MMP-9 inhibitor II blocked EMVTC-induced and basal invasion of endothelial cells (\(\beta\)). In D, TIMP, tissue inhibitor of metalloproteinase-1 and -2, plasminogen activator inhibitor-1 and -2, tissue inhibitor of metalloproteinase; TIMP-1, tissue inhibitor of metalloproteinase; PAI, plasminogen activator inhibitor; Antiplasmin, \(\alpha_2\)-antiplasmin. In E, heat-treated EMVTCs, compared with untreated EMVTCs \(\square\), showed a decrease in the ability to stimulate endothelial cell invasion.

EMVTCs Have Angiogenic Activity. We next examined the ability of EMVTCs to promote the formation of capillary-like structures of HMEC-1 cells on Matrigel. This assay measures activities involved in some of the steps in neovascularization, including migration and the differentiation of endothelial cells \(\square\). The presence of EMVTCs at 10 \(\mu\)g/ml, 20 \(\mu\)g/ml, and 40 \(\mu\)g/ml showed a significant 1.4-, 1.8-, and 2.0-fold increase, respectively, in the tube area over control containing medium alone (Fig. 3A; \(P < 0.0001\)). Tube formation stimulated by 20 \(\mu\)g/ml EMVTCs was similar to levels observed for the positive control bFGF (5 ng/ml; 1.8-fold increase). Heat-treated EMVTCs promoted the formation of capillary-like structures at a level similar to that of untreated EMVTCs \(\square\). EMVTCs did not act as a mitogen for endothelial cells (data not shown).

We next examined whether EMVTCs stimulated in vivo angiogenesis using the chick CAM assay. EMVTCs significantly induced neovascularization from preexisting blood vessels (Fig. 3, B and C). The presence of 1 and 3 \(\mu\)g of EMVTCs per egg caused 2.9-fold and 3.6-fold increases, respectively, in the number of newly formed blood vessels compared with that of PBS alone \(P < 0.00001\). Heat-treated EMVTCs also stimulated blood vessel formation in the CAM assay with angiogenic activity comparable with that of the untreated EMVTCs \(\square\). These results clearly indicate that EMVTCs has angiogenic activity and that a component(s) resistant to heat treatment may be involved in its activity.

Sphingomyelin in EMVTCs Stimulates Endothelial Cell Migration and Has Angiogenic Activity. Because the migratory and angiogenic activities of EMVTCs were heat resistant, we examined the angiogenic activities of lipids extracted from EMVTCs. The lipids in the organic phase (after the extraction of EMVTCs by the Folch method) induced endothelial cell migration in a dose-dependent manner \(P < 0.00001\) with migratory activity similar to that of EMVTCs (Fig. 4A; \(P > 0.5\)). TLC analysis showed that both EMVTCs and EMVTCs that were purified by sucrose gradient sedimentation contained several abundant lipids, including sphingomyelin and other phospholipids, and that their lipid composition was similar to each other (data not shown). We examined the angiogenic activities of the lipids present in EMVTCs. Sphingomyelin from bovine brain stimulated the migration of endothelial cells (Fig. 4B; \(P < 0.0001\)), with maximal migratory activity at 0.5 \(\mu\)g/ml (a 4.1-fold increase). Heat-treated sphingomyelin also stimulated the migration of endothelial cells. The purity of the commercially obtained bovine sphingomyelin used in this study is \(\sim 99\%\). Sphingomyelin, further purified by TLC, also stimulated endothelial cell migration, and the activity was comparable with that of the unpurified material (data not shown). The
other major phospholipids, such as phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylserine, that were present in EMVTCs showed little effect on endothelial migration (data not shown). Sphingomyelin stimulated the invasion of endothelial cells, and gelatin zymography showed that the presence of sphingomyelin at concentrations of up to 1 μg/ml did not up-regulate MMP activity in HUVECs (data not shown). Sphingomyelin also promoted capillary tube-like formation of endothelial cells on Matrigel (Fig. 4C). In D, sphingomyelinase almost completely abolished their migratory activity (Fig. 4D). The other lipids in crude lipid extracts and purified sphingomyelin from EMVTCs with sphingomyelinase treatment almost completely abolished their migratory activity (Fig. 4E). In F, sphingomyelinase-treated bovine sphingomyelin (0.5 μg/ml) and crude lipids from EMVTCs lost their migratory activity when compared with untreated material (Fig. 4F). However, sphingomyelinase treatment did not affect the migratory activity of S1P (0.4 μg/ml). The EMVTC-derived lipid concentrations used here are unknown but equivalent to 25 μg/ml EMVTCs used for lipid extraction.

We next purified the sphingomyelin present in EMVTCs by TLC and examined its angiogenic activity. Sphingomyelin, purified from EMVTCs, stimulated the migration of HUVECs (P < 0.001), and this activity was comparable with that of untreated EMVTCs (data not shown). TLC analysis showed that treatment of crude lipid extracts and purified sphingomyelin from EMVTCs with sphingomyelinase caused a disappearance of the spot with an Rf value identical to that of sphingomyelin (Fig. 4E). The other lipids in EMVTCs were not affected by this enzyme. Furthermore, treatment of bovine sphingomyelin and crude lipid extracts from EMVTCs with sphingomyelinase almost completely abolished their migratory activity (Fig. 4F; P < 0.01). To investigate the possibility that S1P, the most potent endothelial cell chemotactic and angiogenic lipid, might be involved in EMVTC-mediated endothelial cell migration, we examined the effect of sphingomyelinase treatment on the migratory activity of S1P. Sphingomyelinase treatment did not deplete S1P as observed by TLC analysis (Fig. 4E), nor did it affect the migratory activity of S1P (Fig. 4F; P > 0.5), which suggested that S1P may not be the active component involved in EMVTC-mediated endothelial cell migration. We further confirmed that the active component of EMVTCs was sphingomyelin by the CAM assay (Table 1). Lipid extracts and purified sphingomyelin from 3 μg of EMVTCs caused 5.2-fold and 5.3-fold increases, respectively, in the number of newly formed blood vessels when using PBS alone (P < 0.00001). These levels of activities were similar to that of EMVTCs (P > 0.1). TLC analysis showed that 3 μg of EMVTCs contained about 0.3 μg of sphingomyelin. Bovine sphingomyelin (0.3 μg) also induced a 5.8-fold increase in neovascularization over that induced by PBS control (P < 0.00001), and this activity is almost identical to that of EMVTCs itself, lipid extracts, and purified sphingomyelin from EMVTCs (P > 0.1). These results suggest that sphingomyelin may be an active component involved in EMVTC-induced angiogenic activity.

We used EMVTCs that were shed by human fibrosarcoma cells in all of the assays described above. We prepared EMVTCs from human prostate adenocarcinoma cells (DU-145) to demonstrate that the angiogenic activity is present in the EMVTCs that come from other tumor cell types. These EMVTCs also induced endothelial cell migration, and heat-treated EMVTCs showed similar migratory activity compared with that of untreated EMVTCs (data not shown). TLC analysis of extracted lipids showed that sphingomyelin and other phospholipids were present in EMVTCs from DU-145 cells (data not shown). These data suggest that the EMVTCs from different tumor cell types have a similar capacity for angiogenesis.

Table 1. Angiogenic activity of lipid extracts, purified sphingomyelin from EMVTC, and bovine sphingomyelin in the chick CAM assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vessel number</th>
<th>Fold increase</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>9.9</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>EMVTCs (3 μg)</td>
<td>61</td>
<td>6.2</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Lipids from 3 μg of EMVTCs</td>
<td>51</td>
<td>5.2</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Sphingomyelin from 3 μg of EMVTCs</td>
<td>52</td>
<td>5.3</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Bovine sphingomyelin (0.3 μg)</td>
<td>57</td>
<td>5.8</td>
<td>&lt;0.00001</td>
</tr>
</tbody>
</table>
DISCUSSION

The major findings of this report are that EMVTCs have angiogenic activity and that sphingomyelin may be the active component involved in this activity. EMVTCs prepared from human fibrosarcoma cells significantly induced endothelial cell migration, morphogenesis, and in vitro neovascularization, and heat treatment did not affect these activities. EMVTCs did not act as a mitogen for endothelial cells. In addition to factors not associated with membrane vesicles, EMVTCs are also involved in tumor-induced angiogenesis. EMVTCs stimulate endothelial cell invasion. Proteases, including MMPs and plasminogen activator that are enriched in membrane vesicles, are involved in EMVTC-induced endothelial cell migration. Extracted lipids from EMVTCs also induced endothelial cell migration and in vivo angiogenesis. Furthermore, sphingomyelin, one of the major lipid components in EMVTCs, showed almost identical migratory and angiogenic activities as EMVTCs. Heat treatment did not affect the migratory activity of sphingomyelin. Sphingomyelinase-treated sphingomyelin and crude lipid extracts from EMVTCs lost their migration-promoting activity, whereas the migratory activity of S1P was not affected by sphingomyelinase. Our data demonstrate that EMVTCs from another human cancer cell line, DU-145, also stimulated endothelial cell migration and contained sphingomyelin.

It is surprising that lipid components, such as sphingomyelin, are involved in EMVTC-mediated neovascularization. Sphingomyelins are one of the major membrane phospholipids, and most are localized on the outer leaflet of the mammalian plasma cell membrane (26). The sphingomyelin levels of highly metastatic cancer cells are significantly higher than that of less metastatic variants (27). EMVTCs are composed of increased amounts of sphingomyelin relative to that of the plasma membrane (7, 8). Sphingolipids play diverse roles in cell proliferation, differentiation, apoptosis, and migration (28–31). Sphingolipids, including S1P, sphingosylphosphorylcholin, sphingosine, and gangliosides, are also involved in angiogenesis via their intrinsic angiogenic activities or promotion of the angiogenic response of microvessels stimulated by angiogenic factors (32–36). In our studies, phosphorylated cholamine, phosphorylatedinositol, and phosphatidylinositol, which are present in EMVTCs, showed little effect on endothelial cell migration. It has been reported that sphingomyelin is less potent for cell migration than S1P is (32). However, our results clearly indicate that sphingomyelin itself, as well as the sphingomyelin present in EMVTCs, has angiogenic activity. Furthermore, sphingomyelinase treatment did not either degrade S1P nor affect its migratory activity, whereas sphingomyelinase-treated sphingomyelin and sphingomyelinase-treated crude lipid extracts from EMVTCs lost almost all of their migration-promoting activity with this treatment. These results suggest that S1P may not be the active component involved in the EMVTC-mediated angiogenesis. Although we could not completely exclude the possibility that other lipid components are involved in EMVTC-induced angiogenesis, our results indicate that sphingomyelin may be a major active component.

It is important to note that the serum level of shed vesicles in cancer patients (80 µg/ml) relates closely to the amounts used here in our assays (6–50 µg/ml) and that have been shown to be active for angiogenesis. The level of extracellular membrane vesicles observed in cancer patients is approximately five times that observed in normal patients, as expected because normal cells shed much less material than do cancer cells (14, 37). Recently, it was demonstrated that membrane vesicles that were shed by activated endothelial cells were enriched with MMPs and promoted endothelial cell invasion and tube formation on Matrigel (37). Although it has not been tested, one would expect that the shed vesicles from normal cells may also have angiogenic activity based on the lipid composition.

What might be the significance of the angiogenic activities of EMVTCs in tumor growth and metastasis? Several studies have suggested that EMVTCs are involved in both immune escape and tumor invasion via EMVTC-associated tumor cell antigens, immune-suppressing cytokines, integrins, and proteases (9–18). Actively growing tumor cells shed EMVTCs via a vital process, and the rate of shedding is increased in malignant tumors. Evidence suggests that the serum amounts of EMVTCs in cancer patients are significantly elevated, and that both the amounts and the vesicle-associated proteolytic activities of EMVTCs are positively correlated with malignancy and the invasiveness of tumor cells in vitro (13–15). In addition to tumor cell invasion and the escape of tumors from immune surveillance, angiogenesis is essential for tumor growth and metastasis. It is worthwhile to note that even a single molecule, such as bFGF, and several soluble cell-adhesion molecules, including E-selectin, vascular cell adhesion molecule, and intercellular adhesion molecule-1, perform dual functions: angiogenesis and escape from immune surveillance (21, 38–40). EMVTCs, composed of multiple proteins and lipids, may play an additional role in angiogenesis as well as in immune escape and tumor invasion. Together with previously reported results, our data suggest that EMVTCs may play a major role in tumor cell survival in the host. EMVTCs are fully equipped with the tumor antigens, proteases, and angiogenic molecules needed for immune escape, tumor invasion, and neovascularization and, thus, play a triple role in tumor growth and metastasis. Although additional work on the physiological significance of EMVTC-induced angiogenesis in tumor growth and metastasis is required, our findings suggest that blocking the shedding of EMVTCs could be a worthwhile approach for cancer therapy.

REFERENCES

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