Apoptotic Cells Initiate Endothelial Cell Sprouting via Electrostatic Signaling

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Abstract

Angiogenesis, the development of new blood vessels from preexisting vessels, is crucial to tissue growth, repair, and maintenance. This process begins with the formation of endothelial cell sprouts followed by the proliferation and migration of neighboring endothelial cells along the preformed extensions. The initiating event and mechanism of sprouting is not known. We show that the phenotypic expression of negatively charged membrane surface in apoptotic cells initiates the formation of directional endothelial cell sprouts that extend toward the dying cells by a mechanism that involves endothelial cell membrane hyperpolarization and cytoskeleton reorganization but is independent of diffusible molecules. (Cancer Res 2005; 65(24): 11529-35)

Introduction

Vascular expansion is a multistep process that includes activation of preexisting endothelial cells, formation of sprouts, migration of endothelial cells along the sprouts, formation of tubular structures, and finally, formation of vessels with a distinct blood-transporting lumen (1, 2). Although still unclear, the initiation of angiogenesis has been attributed to changes in the net balance between proangiogenic and antiangiogenic factors in the microenvironment (3, 4) that can be induced by severing the blood supply, by wounding, or by rapid tissue expansion typical of the microenvironment (3, 4) that can be induced by severing the blood supply, by wounding, or by rapid tissue expansion typical of tumor growth. Because oxygen can only diffuse ~ 120 µm from capillaries (5), injury and hypoxia invariably lead to apoptosis (6). Because cell death is an integral component of angiogenesis (7, 8), it is possible that apoptotic cells play a fundamental role in signaling and/or initiating angiogenic endothelial cell responses.

To determine the potential role of apoptotic cells in vascular expansion, we cocultured endothelial cells with autologous apoptotic endothelial cells or xenogeneic apoptotic tumor cells. Surprisingly, nonproliferating endothelial cells extended sprouts exclusively toward apoptotic cells. This process was initiated by the negative charge of apoptotic cell surface and involved endothelial cell membrane hyperpolarization and cytoskeleton reorganization.

Materials and Methods

Cell lines and reagents. Rat kidney endothelial cells were a gift from S. Adler (New York Medical College) and R. Johnson (University of Florida; ref. 9). The K-1735 mouse melanoma cells were obtained from M.L. Kripke (The University of Texas M.D. Anderson Cancer Center; ref. 10). Stable green fluorescent protein (GFP)-expressing K-1735 cell lines were generated as described previously (11). Cationized and anionized ferritin, rabbit anti-ferritin IgG, and cytochrome c from horse heart were purchased from Sigma (St. Louis, MO). Rabbit anti-caspase 3 IgG was purchased from Cell Signaling Technology (Beverly, MA). Monoclonal proliferative cell nuclear antigen (PCNA) antibody was from DAKO (Glostrup, Denmark). Polyclonal antibodies to epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor-2 (VEGFR-2), and phosphorylated EGFR were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antiphosphorylated VEGFR-2 was from Oncogene (Cambridge, MA). The terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) assay kit was obtained from Promega (Madison, WI). Alexa Fluor 594–labeled anti-rabbit IgG, CellTracker Red CMTPX, and membrane potential sensitive fluorescent dye (di-8-ANEPPS) were purchased from Molecular Probes (Eugene, OR). Annexin V apoptosis detection kit was from BD (San Diego, CA), and protein A-Sepharose beads were purchased from Amersham Biosciences (Uppsala, Sweden). AEE788 was obtained from Novartis Pharma (Basel, Switzerland). Compartmental culture dishes with removable separating walls were made of medical-grade silicon Elastomer (Maxxon Scientific, Houston, TX). Ion channel blockers, amiloride for sodium channel, protopine for calcium channels, and glyburide and charybdotoxin for potassium channels were purchased from EMD Biosciences (San Diego, CA).

Cocultures of viable and apoptotic endothelial cells. Endothelial cells were grown to confluency in 60-mm culture dishes in DMEM with 10% fetal bovine serum (FBS) in a 5% CO2 incubator at 37°C. Confluent endothelial cell cultures were overlaid for 2 minutes at 37°C with 0.25% trypsin in a 0.1% EDTA solution. Floating cells were washed off with serum-free DMEM. The remaining endothelial cells were subsequently cultured in serum-free medium. Morphologic changes to the cells in the monolayer margin adjacent to the denuded areas were monitored every 30 minutes by microscopy using an inverted light microscope. Once endothelial cell sprouts developed, the cultures were washed, and the cells were incubated with Annexin V or fixed with paraformaldehyde (15 minutes at 20°C) and subjected to TUNEL assay and PCNA staining following the manufacturer’s protocols.

Microinjection of cytochrome c into endothelial cells. Confluent endothelial cells were partially trypsinized as described above. Detached cells were removed by washing. The remaining cells were then cultured for 5 hours in serum containing DMEM to allow adhesion of any loosely detached cells. The border of a denuded area was marked on the bottom surface of the culture dish and photographed. Microinjection needles with an inner diameter of ~0.1 µm were pulled from glass capillaries with a horizontal electrode puller (Brown Micropipette Puller; Sutter Instrument Co., Novato, CA). Cytochrome c at 10 mg/mL in water containing 0.5 µmol/L CellTracker Red CMTPX (a concentration that does not induce cell death when injected alone) was loaded into the microinjection needles. Cytochrome c was injected into the cytosol at 80 to 100 hps within 0.3 seconds on the stage of an inverted microscope with a Narishige pressure injector (Model IM-5B’ Narishige, Sea Cliff, NY) and micromanipulator (Leitz, Overland Park, KS) as described previously (12). Injection into the cytosol was verified by fluorescence microscopy. Bright-field images were recorded every 5 minutes.

Cell culture manipulation and treatment with the vascular endothelial growth factor receptor/epidermal growth factor receptor inhibitor, AEE788. Microinjection needles were used to produce scratches on the inner bottom surface of the culture dish between a sprouting...
A culture dish was separated into two in a compartmental dish. Particle were selected for continuous monitoring. Denuded endothelial cell cultures. The denuded areas containing a single negatively charged vesicles were carefully added to the medium of anionized or cationized beads and neutral phospholipid vesicles and could substitute for apoptotic cell–dependent endothelial cell sprouting, exclusively. To determine whether negatively charged beads or vesicles produced by drying 1 mg of phosphatidylserine with 1 mg of were then air-dried. Negatively charged multilamellar vesicles were incubated in the presence of ferritin (100 μg/mL). The relative charge of sprouting and apoptotic cells was determined by adding cationized or anionized ferritin following sprout formation. In this case, sprouting cultures were fixed with 4% paraformaldehyde (15 minutes at 20°C) followed by three 10-minute washes with PBS. The cells were then incubated overnight at 4°C with PBS containing 100 μg/mL cationized or anionized ferritin. The cultures were washed and then incubated in blocking buffer (3% bovine serum albumin in PBS for 1 hour at 20°C). Binding was determined immunohistochemically with rabbit anti-ferritin (1:100 dilution for 2 hours at 20°C) followed with fluorescein-conjugated anti-rabbit IgG under the same conditions.

Initiation of sprouting with negatively charged Sepharose beads and phospholipid vesicles. Charged Sepharose beads were produced by antibody-mediated coupling of anionized or cationized ferritin. Briefly, protein A-Sepharose (30 mg) was generated by incubating the beads with ferritin antibody (15 ng in 1 mL PBS) overnight. IgG not bound was removed by washing with five times with PBS. The antibody-conjugated beads were then incubated with positively or negatively charged ferritin (1 mg at 4°C for 3 hours). Excess ferritin was removed by washing. The beads were then air-dried. Negatively charged multilamellar vesicles were produced by drying 1 mg of phosphatidylserine with 1 mg of phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) under nitrogen. The dried vesicles were then hydrated by vortexing in PBS containing 25% sucrose (w/v). Small vesicles were removed by sedimentation at 1 g for 1 hour. Control neutral vesicles were composed of phosphatidylcholine exclusively. To determine whether negatively charged beads or vesicles could substitute for apoptotic cell–dependent endothelial cell sprouting, anionized or cationized beads and neutral phospholipid vesicles and negatively charged vesicles were carefully added to the medium of denuded endothelial cell cultures. The denuded areas containing a single particle were selected for continuous monitoring.

Coculture of endothelial cells and apoptotic K-1735 melanoma cells in a compartmental dish. A culture dish was separated into two compartments by a wall of silicon Elastomer that was 5-mm tall and 1.5-mm wide. Endothelial cells (1 × 10⁶) were plated into one compartment, and GFP-expressing K-1735 cells (1 × 10⁷) were plated into the other. The cultures were then incubated with DMEM containing 10% FBS. Ten hours later, the compartment with K-1735 cells was washed thrice with serum-free medium and reﬁd with serum-free DMEM. Apoptosis of K-1735 cells was induced by incubating cells for an additional 48 hours. The silicon barrier was then removed, and the serum-free DMEM was replaced with DMEM containing 10% FBS. The morphologic changes in the endothelial cells at the boundary were monitored by light and fluorescence microscopy. Once endothelial cell sprouts were visible, the cells were ﬁxed for 5 minutes with 4% paraformaldehyde and stained for caspase-3 using the manufacturers’ protocol.

Dual wavelength imaging membrane potential changes. Di-8-ANEPPS (ﬁnal concentration at 1 μmol/L) was added into the medium of denuded endothelial cell cultures and incubated for 20 minutes in a cell culture incubator. After washing with medium, the cells were monitored with an inverted fluorescent microscope equipped with 450 nm (green emission) and 510 nm (red emission) excitation ﬁlters. Two-second exposures at each excitation wavelength were recorded with a CCD camera. Images from 450 and 510 nm were merged. The morphologies at corresponding time points were also taken with normal light imaging.

Treatment of endothelial cells with ion channel blockers. Before the addition of phosphatidylserine vesicles, denuded endothelial cell cultures were incubated with media containing different ion channel blockers, amiloride (10 μmol/L), protopine (10 μmol/L), glyburide (10 μmol/L), and charybdotoxin (50 nmol/L) for 20 minutes. Images of cells from randomly selected areas ( ﬁve for each sample) were taken before and after the addition of the vesicles. The morphologic changes were monitored continuously for 40 minutes.

Scanning electron microscopy. The surface morphology of normal, sprouting, and apoptotic endothelial cells was visualized by scanning electron microscopy (Model S350, Hitachi Densi, Woodbury, NY).

Results

Nonproliferating endothelial cells sprout toward apoptotic cells. Culturing mildly trypsinized confluent rat glomerular, endothelial cell monolayers resulted in the generation of sprouts that extended from a small number of cells. These sprouts extended from endothelial cells at the margin of the monolayer to rounded and partially detached residual cells that remained in the denuded areas (Fig. 1A and B). To determine whether these “attracting” cells were indeed apoptotic, the cells were assessed for exposure of phosphatidylserine and DNA fragmentation by their ability to bind Annexin V (Fig. 1C-F) and TUNEL staining (Fig. 1G and H), respectively. Fluorescence microscopy showed that the attracting cells were apoptotic. Further examination of the cultures by scanning electron microscopy revealed that in contrast to the rough surface of the nonsprouting endothelial cells, attracting cells had a relatively smooth surface (Fig. 1I). To test whether the correlation between sprouts and apoptotic cells is signiﬁcant, we counted the number of sprouting cells and the number of sprouting cells coupled with apoptotic cells in five randomly selected areas. The average number of sprouting cells was 37, and all had sprouts pointing to apoptotic cells. We did not find any endothelial cells with sprouts that did not point toward apoptotic cells. The distance between the attracting and sprouting cells was typically <200 μm.

To unequivocally determine whether apoptotic cells were indeed the initiating stimulus for sprout formation, single cells along the monolayer margin were triggered into apoptosis by microinjection of cytochrome c into the cytosol. Figure 2A and B shows that within 20 minutes after injection of cytochrome c (red fluorescence), a directional sprout pointing towards the cytochrome c–positive cell began to form from an opposing endothelial cell. Surprisingly, apoptotic cells were also required for the maintenance of existing sprouts. This can be seen from results showing that removal of the sprout-initiating apoptotic cell caused the sprout to retract back into the main cell body of the sprouting endothelial cell (Fig. 2C and D).

Apoptosis was induced in the melanoma side of the plate by serum starvation for 48 hours. No endothelial cell sprouting was observed as long as the two compartments were separate. Removal of the barrier resulted in the formation of endothelial
cell sprouts within 12 hours. Importantly, although some intermixing between cell populations was unavoidable, only apoptotic (caspase positive) melanoma cells attracted sprouts (Fig. 2E and F). We also cultured epithelial origin cells, MCF7 (breast cancer cells), and Du145 (prostate cancer cells) in the presence of apoptotic cells. We did not observe formation of sprouts.

**Initiation of sprouting by electrostatic signaling.** Several distinct mechanisms could be responsible for apoptosis-dependent endothelial cell sprouting. These include a cell expansion proliferation–dependent mechanism, activation of VEGF and/or EGFRs known to participate in angiogenesis, or specific chemotactic factors released by dying cells (13). To determine whether sprouting was dependent on endothelial cell proliferation, mixed viable/apoptotic endothelial cell cultures were stained with PCNA antibody. Figure 3A shows that sprout-producing endothelial cells were nonproliferating (PCNA negative) and closest to but not adjacent to the attracting cells.

To determine whether VEGF or EGF regulate sprouting, trypsinized endothelial cell monolayer was incubated in the presence of AEE788, a potent competitive inhibitor of both EGFR and VEGFR phosphorylation (14). Figure 3 shows that although both EGFR and VEGFR phosphorylation were effectively inhibited (Fig. 3B), sprout formation was not inhibited (Fig. 3C). Surprisingly, AEE788 decreased the fraction of PCNA-positive proliferating endothelial cells (Fig. 3C) and increased the density of sprouts (Fig. 3D) and the fraction of TUNEL-positive apoptotic cells (Fig. 3E and F).

**Figure 1.** Apoptotic cells initiate endothelial cell sprouting. A and B, endothelial cells were cultured to 100% confluency and partially trypsinized to produce denuded areas. The inner periphery contained partially detached cells, where endothelial cells sprouted (arrows) toward cells with typical apoptotic morphology (arrowheads). C-F, sprouts (arrow) extending toward the attracting cell (arrowhead) that was positive for Annexin V binding (white arrows crossing images, green). G and H, TUNEL staining revealed that some attracting cells (arrowhead) are TUNEL positive (green). I, scanning electron microscopy shows that the surfaces of the attracting cells (arrowheads) are smoother than the relatively rough surfaces of the neighbor cells (double-head arrow). Bar, 50 μm.

**Figure 2.** Induction and removal of apoptotic attracting cells. A, cytochrome c microinjection into viable endothelial cells (arrow) together with red fluorescent dye (inset). B, 20 minutes after microinjection of cytochrome c, an endothelial cell formed a sprout toward the injected cell (arrow). C and D, removal of the attracting apoptotic cell (arrowhead) resulted in retraction of the extending sprout within 15 minutes (arrows). E, confluent endothelial cells were cocultured with apoptotic GFP-labeled K-1735 melanoma cells. An endothelial cell sprout (inset, enlarged view of the sprout) directed toward the apoptotic K-1735 melanoma cell (arrowhead, dashed line). F, apoptotic status of the K-1735 cell (yellow) was confirmed by caspase-3 staining with Texas red–conjugated secondary antibody and merged with the green GFP image of K-1735 cells. Bar, 50 μm.
Figure 3. Nonproliferating endothelial cell sprout–independent VEGF and EGF pathways. A, PCNA staining (arrowheads, brown) of control endothelial cells with sprouts showing that the cells that sprout are PCNA negative (arrow, blue stained with hematoxylin) and nearest to but not adjacent to the attracting cells. B, Western blot analysis showing inhibition of VEGFR (pVEGFR) and EGFR (pEGFR) phosphorylation with AEE788. Total VEGFR (tVEGFR) and EGFR (tEGFR) served as controls. C, PCNA staining of endothelial cells treated with AEE788 showing more sprouts (arrowheads) and less proliferating cells as compared with control (A). D, statistical analysis of the number of sprouts in control and AEE788-treated endothelial cells (P < 0.001). E, colocalization of sprouts (arrows) with TUNEL-positive cells (arrowheads, green) in AEE788-treated endothelial cells. Bar, 50 μm.

To test whether concentration gradients of diffusible compounds might be responsible for directional sprouting, the formation concentration gradient was prevented by incubating trypsinized endothelial cells on a horizontal shaker. As shown in Fig. 4A, culturing cells with constant shaking did not prevent the formation of directional sprouting. To rule out that there was no invisible preexisting physical cell-cell contact between the apoptotic cell and sprouting endothelial cells, scratches were made on the surface of the culture dish between attracting apoptotic and sprouting endothelial cells. Scratches did not disturb the extension of the sprout (Fig. 4B and C), and similar to the results shown in Fig. 2, the sprouts retracted only in response to removal of the attracting apoptotic cell (Fig. 4D).

Because apoptosis is associated with an increase in net negative cell surface charge (15, 16), the possibility exists that the initiation of endothelial cell sprouts could be dependent on specific electrostatic charge interactions between the attracting apoptotic cell and the sprouting endothelial cells. To dissipate the polarity differential between the apoptotic and sprouting endothelial cells, trypsinized endothelial cell cultures were treated with cationized or anionized ferritin, respectively. Binding of ferritin to the cells was determined by staining with ferritin antibodies. Figure 4 shows that anionized ferritin bound exclusively to sprouting endothelial cells (Fig. 4E and F). Cationized ferritin, on the other hand, bound only to attracting apoptotic cells (Fig. 4G and H). Interestingly, the addition of cationized (Fig. 4I) but not anionized (Fig. 4J) ferritin to the cultures prevented sprout initiation. In contrast, addition of anionized but not cationized ferritin to endothelial cells bearing established sprouts caused the extending sprout to retract back into the main cell body within 90 to 100 minutes. Ferritin-induced sprout reversal, however, was ineffective once a sprout reached the attracting apoptotic cell (Fig. 4K and L).

To test whether negative surface charge is the primary stimulus by which endothelial cells are triggered to sprout, we attempted to initiate charge-dependent sprouting in an apoptotic cell–free system. For this purpose, Sepharose beads coated with anionized (negative charged) or cationized (positive charged) ferritin and negatively charged phosphatidylserine or neutral phosphatidylcholine vesicles were added to trypsinized endothelial cell cultures. Figure 5A shows that negatively charged but not positively charged (Fig. 5B) Sepharose beads initiated sprouting. Similarly, negatively charged (Fig. 5C) but not neutral (Fig. 5D) vesicles initiated sprouting. Collectively, these results suggest that the negatively charged cell surface expressed in apoptotic cells provides the initiating stimulus that induces the formation of endothelial cell sprouts.

Endothelial cell membrane hyperpolarization and cytoskeleton reorganization triggered by phosphatidylserine phospholipid vesicles. Because endothelial cell membrane is rich in ion channels (20) and is polarized in resting cells (18), we tested whether distant static negative charge can alter the membrane potential of endothelial cells. Dual wavelength imaging analysis (19) of endothelial cells labeled with membrane potential sensitive dye (di-8-ANEPSS) revealed the hyperpolarization of endothelial cell exposed to phosphatidylserine vesicles. This was detected within 15 minutes after exposure to the vesicles (Fig. 6A-D) and preceded the appearance of sprouts (Fig. 6E). No transmembrane potential changes were detected in the control cells (Fig. 6F-I).

Because alteration in membrane potential can trigger cytoskeleton reorganization (20), we tested whether endothelial cell sprouting was associated with reorganization of the cytoskeleton by staining with FITC-conjugated phalloidin. Reorganization of the cytoskeleton occurred during different stages of sprouting (Fig. 7). Nonsprouting endothelial cells exhibited a nondirectional distribution (Fig. 7A), whereas in sprouting endothelial cells, the cytoskeleton polarized (Fig. 7B), elongated in a parallel manner (Fig. 7C), and finally concentrated at the tip of the sprout (Fig. 7D). Because Ca2+ flux changes have been shown to regulate cytoskeleton reorganization (21), we tested whether calcium channel blockers can inhibit sprout formation. Figure 7E and F shows that pretreatment of endothelial cells with protopine completely inhibited phosphatidyserine vesicle–induced sprout formation.

Discussion

Angiogenesis in development, wound healing, and neoplasia is dependent on the initiation of a defined sequence of events that include endothelial cell migration toward the region of (re)vascularization, endothelial cell proliferation, and finally, reorganization into blood carrying tubules. Although the critical
the initiation event for the generation of new blood vessels has been attributed to the production of diffusible growth factors that stimulate endothelial cell migration and proliferation, recent data suggests that endogenous electric fields may also participate in this process (22). Indeed, alterations in electric fields are associated with wounding where they persist until repair is complete (23).

Cells undergoing apoptosis undergo dramatic intracellular and membrane alterations. In particular, the normally asymmetrical transmembrane distribution of membrane phospholipids reorganizes in such a manner that phosphatidylserine, normally localized exclusively in the cell’s inner membrane leaflet, redistributes to the outer membrane leaflet. The expression of anionic phospholipid results in increasingly negative surface charge (24) commonly identified by the ability of the cells to bind Annexin V (25). The data presented here show that endothelial cells produce sprouts in direct response to cell surface electrostatic charge on apoptotic cells. Studies carried out in the presence of the tyrosine kinase inhibitor, AEE788, a dual inhibitor of EGFR and VEGFR phosphorylation, revealed that sprout formation was independent of activation of the VEGFR and EGFR. In addition, continuous agitation to prevent formation of solute concentration gradients failed to affect sprout formation. Taken together, these data indicate that sprout formation towards attracting apoptotic cells is growth factor/growth factor receptor independent.

All the attracting cells were Annexin V positive and bound cationized ferritin that, when added during the early stages of sprout formation, was inhibitory. This suggests that a net negative surface charge is required for the initiation of sprout formation. Additional evidence in support of the concept that cell sprouting can be initiated by electrostatic charge comes from experiments showing that negatively charged beads and phosphatidylserine-containing vesicles (Fig. 5) also initiated sprouting. In contrast to negatively charged apoptotic cells that bound cationized ferritin, sprouting endothelial cells bound anionized ferritin, suggesting that the sprouting cell has a strong
positively charged surface. Although the source and nature of the positive surface charge on sprouting endothelial cells remains unclear, this finding is consistent with reports that endothelial cells derived from angiogenic macrovascular tissues elongate and migrate toward the cathode in a direct current electric field (26, 27).

Using dual-wavelength imaging, we found that endothelial cells respond to distant negative charges by altering membrane potential and becoming hyperpolarized, a phenomenon similar to what occurs in neuronal cells following exposure to electrical fields (19). The nature of endothelial cell membrane hyperpolarization seems unrelated to potassium and sodium channels since glyburide, charybdotoxin (potassium channel blocker), and amiloride (sodium channel blocker) were without effect (data not shown). However, preincubation endothelial cells with calcium channel blocker but no other channel blockers (data not shown) did inhibit sprout formation, indicating that calcium signaling is critical to sprout formation. Ion channel blockers did not reverse preformed sprouts (data not shown).

In conclusion, the data presented here provide evidence that apoptosis is not only important for marking the cell for elimination by phagocytes but also triggers a sequence of events important for angiogenesis and vascular remodeling.
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