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Original Contribution

Internalization and induction of antioxidant messages by microvesicles contribute to the antiapoptotic effects on human endothelial cells

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ABSTRACT

Microvesicles are plasma membrane-derived fragments released from various cell types during activation and/or apoptosis and posses the ability to deliver biological information between cells. Microvesicles generated from T lymphocytes undergoing activation and apoptosis bear the morphogen Sonic Hedgehog, and exert a beneficial potential effect on the cardiovascular system through their dual capacity to increase nitric oxide and reduce reactive oxygen species production. This study investigated the effect of microvesicles on the apoptosis of human umbilical vein endothelial cells triggered by actinomycin D. Microvesicles prevented apoptosis induced by actinomycin D by modulating reactive oxygen species production: during the early phase of apoptosis, microvesicles might act directly as reactive oxygen species scavengers, owing to their ability to carry active antioxidant enzymes, catalase, and isoforms of the superoxide dismutase. Furthermore, their effects were associated with the ability to increase the expression of manganese-superoxide dismutase in endothelial cells, through the internalization process. Interestingly, microvesicles bearing Sonic Hedgehog induced cytoprotection in endothelial cells through the activation of the Sonic Hedgehog pathway. These findings provide additional evidence that microvesicles from T lymphocytes exert their vasculoprotective effects by promoting internalization and induction of antioxidant messages to the endothelial monolayer.

Introduction

Endothelial cell (EC) apoptosis represents an essential mechanism acting during development and adulthood; it may contribute to blood-vessel regression, remodeling, morphogenesis, and homeostasis. However, alterations in the regulation of endothelial apoptosis lead to loss of endothelial integrity which is linked to various cardiovascular pathologies such as atherosclerosis, thrombosis, and hind-limb ischemia. Moreover, excessive generation of reactive oxygen species (ROS) coupled with their ability to cause oxidative damage in the vascular endothelium can trigger EC apoptosis [1–4]; endothelial-derived nitric oxide (NO) inhibits this process. Thus, EC death can critically disturb the integrity of the endothelial monolayer and may contribute to the initial endothelial injury.

Microvesicles (MVs) constitute nonclassical protein secretion pathways by which cells intercommunicate [5]. Their function is strictly linked to the cargo they carry which is, in turn, dependent on the cell type from which they stem and on the stimulation used for their generation. MVs, small particles (0.1–1 μm) released from plasma membrane blebs on activation and/or apoptosis, are able to take part in different physiological and pathophysiological processes [6,7]. Regarding the effects of MVs on apoptosis, few studies are available. It has been demonstrated

Abbreviations: Act D, actinomycin D; ATP, adenosine triphosphate; BSA, bovine serum albumin; CMH, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine; Cu/Zn-SOD, copper/zinc superoxide dismutase; CUR61414, *N*-((3*S*,5*S*)-1-(benzo[d][1,3]dioxol-5-ylmethyl)-5-(piperazine-1-carbonyl)pyrrolidin-3-yl)–*N*-(3-methoxybenzyl)-3,3-dimethylbutanamide; Cycl, cyclopamine; DETC, diethyldithiocarbamate; EC(s), endothelial cell(s); EC-SOD, extracellular superoxide dismutase; EGF, epidermal growth factor; EPR, electronic paramagnetic resonance; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; FGF-2, fibroblast growth factor 2; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; HUVECs, human umbilical vein endothelial cells; IGF-1, insulin growth factor 1; L-NA, *N*ω-nitro-L-arginine; MAPK, mitogen-activated protein kinase; Mn-SOD, manganese superoxide dismutase; MnTMPyP, manganese(III)tetrakis-(1-methyl-4-pyridyl)-porphyrin pentachloride; MV(s), microvesicle(s); mRNA, messenger RNA; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PI, propidium iodide; PI3-k, phosphatidylinositol 3-kinase; PMA, phorbol-12-myristate-13-acetate; ROS, reactive oxygen species; Shh, Sonic Hedgehog; SOD(s), superoxide dismutase(s); TBS, Tris-buffered saline; TBS-T, Tris-buffered saline containing 1% Tween 20; TCTP, translationally controlled tumor protein; TUNEL, terminal deoxynucleotidyltransferase dUTP nick end labeling; VEGF, vascular endothelium growth factor; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt

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that platelet-derived MVs inhibit apoptosis in human hematopoietic cells [8] and in human ECs [9]; by contrast, they induce apoptosis in human monocytes [10]. A recent report shows that MVs isolated from human mesenchymal stem cells derived from bone marrow confer resistance to apoptosis on tubular epithelial cells by internalizing and horizontal transfer of messenger RNA (mRNA) [11]. Moreover, MVs released from ECs increase endothelial apoptosis by a mechanism implicating oxidative stress [12]. Exosome-like MVs expressing translationally controlled tumor protein (TCTP) act as antiapoptotic vectors in vascular smooth muscle cells [13]. Altogether these data suggest that MVs are components of paracrine apoptotic programs.

On the other hand, data from our laboratory showed that MVs generated from T lymphocytes undergoing both activation and apoptosis that carry the morphogen Sonic Hedgehog (Shh) possess properties that benefit vascular function at least partly because of their ability to correct endothelial injury [14] and favor angiogenesis [15,16]. Additionally, these engineered MVs posses the concomitant ability to increase NȮ release and reduce ROS production [14], providing further consistent evidence for their protective effect on the vascular endothelium.

In the present study, the hypothesis that MVs bearing Shh might also protect ECs against stress-induced apoptosis in response to actinomycin D (Act D), a classical antineoplastic drug known to promote apoptosis, has been tested. The mechanism(s) underlying the action(s) and interaction(s) of MVs were than characterized in detail. We report here a novel observation that MVs protect ECs against apoptosis through dual activation of both the antioxidant defense mechanism and the Shh pathway. One the one hand, MVs counteract the increase of cytoplasmic and mitochondrial ROS depending on the time of exposure; on the other hand, MVs, by carrying antioxidant enzymes, may protect target cells. This evidence may elucidate their capacity to rescue ECs from the induction of apoptosis.

Materials and methods

MV production

The human lymphoid CEM T cell line (ATCC, Manassas, VA) was used for MV production. Cells were seeded at 10^6 cells/ml and cultured in serum-free X-VIVO 15 medium (Lonza, Basel, Switzerland). MVs were produced as described previously [17]. Briefly, CEM cells were treated with phytohemagglutinin (PHA, 5 μg/ml; Sigma Aldrich, St. Louis, MO) for 72 h, and then with phorbol-12-myristate-13-acetate (PMA, 20 ng/ml, Sigma Aldrich) and Act D (0.5 μg/ml, Sigma Aldrich) for 24 h. A supernatant was obtained by centrifugation at 750*g* for 15 min to remove cells, and then at 1500*g* for 5 min to remove large debris. The remaining MV-containing supernatant was subjected to 14,000*g* for 45 min to pellet the MVs. The MV pellet was subjected to two series of centrifugations at 14,000*g* for 45 min. Finally, the MV pellet was added to 400 μl of 0.9% saline solution and stored at + 4 °C until use. Washing medium from the last supernatant was used as control.

In order to remove RNA carried by MVs, MVs were treated as previously described [18,19] with some modifications. Briefly, MVs were treated with 1 U/ml RNase (Sigma Aldrich) for 3 h at 37 °C in the presence of Tween 0.02% and the reaction was stopped by the addition of 10 U/ml RNase inhibitor (Ambion Inc.). Then, MVs were washed by centrifugation and MV pellet was added to 0.9% saline solution and stored at + 4 °C until use. The efficacy of RNase treatment was evaluated by MV-RNA analyses using an Agilent 2100 bioanalyzer (Agilent Tech. Inc., Santa Clara, CA). In addition, RNA extracted from RNase-treated and -untreated MVs was labeled by oligo-dT-driven retrotranscription and analyzed on agarose gel, as described below, to show the complete degradation of Mn-SOD RNA by RNase treatment (Supplemental data, Fig. 1S).

Determination of the amount of MVs was carried out by measuring MV-associated proteins, using the Bradford method and bovine serum albumin (BSA) (Sigma Aldrich) for the standard curve. MVs were used at 1 to 15 μg/ml. The amount of 10 μg/ml of MVs, the most efficient concentration to inhibit apoptosis, to differentiate megakaryocytes [17], to improve endothelial function after ischemia/reperfusion [14], and to favor *in vitro*15 and in vivo angiogenesis [16], was used for experiments.

Cell culture

Pooled primary human umbilical vein endothelial cells (HUVECs) were obtained from ATCC and cultured in EGM-2 endothelial medium BulletKit system (Lonza) consisting of endothelial basal medium supplemented with 5% fetal bovine serum (FBS), vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), insulin growth factor-1 (IGF-1), ascorbic acid, gentamicin sulfate amphotericin, hydrocortisone, and heparin and maintained at 37 °C and 5% CO_2. HUVECs were used between passages 2 and 5. All experiments were carried out when the cells were at 80–90% confluence.

HUVECs were grown for 24 h in the absence or presence of the proapoptotic agent Act D (1 μg/ml), and/or 10 μg protein/ml of MVs. In another set of experiments, ECs were preincubated in the absence or presence of the nonselective caspase inhibitor, z-vad.fmk (50 μM, Enzo Life Sciences International, Plymouth Meeting, PA), phosphatidylinositol 3-kinase (PI3-k) inhibitor, LY294002 (10 μM, Calbiochem, Darmstadt, Germany), extracellular-signal-regulated kinase (ERK) inhibitor, U0126 (10 μM, Calbiochem), nitric oxide synthase (NOS) inhibitor, *N*ω-nitro-L-arginine (L-NA) (100 μM, Sigma Aldrich), Smoothened antagonists, cyclopamine (30 μM, Enzo Life Sciences) and *N*-((3*S*,5*S*)-1-(benzo[d][1,3]dioxol-5-ylmethyl)-5-(piperazine-1-carbonyl)pyrrolidin-3-yl)–*N*-(3-methoxybenzyl)-3,3-dimethylbutanamide (CUR61414, 1–10 μM), xanthine oxidase inhibitor, allopurinol (50 μM, Sigma Aldrich), nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) inhibitor, apocynin (100 μM, Calbiochem), respiratory chain complex I inhibitor, rotenone (5 μM, Sigma Aldrich), the superoxide dismutase (SOD) mimetic, manganese(III)tetrakis-(1-methyl-4-pyridyl)-porphyrin pentachloride (MnTMPyP, 100 μM, Calbiochem), catalase (250 U/ml, Sigma Aldrich), and Shh agonist SAG (0.003–0.3 μM). SAG and CUR61414 were synthesized as described previously [17,20]. All agents were used at concentrations at which no cytotoxicity was observed, as deduced from trypan blue exclusion.

Determination of hypodiploid DNA

After treatments, culture medium was removed, and adherent cells were trypsinized, detached, combined with floating cells from the original culture medium, and centrifuged. Cells were then fixed in 70% ethanol for at least 4 h at 4 °C and washed in 0.01 M phosphate-buffered saline (PBS, NaCl 0.138 M, KCl 0.0027 M, Sigma Aldrich) before resuspension for 10 min in a solution containing type I-A RNase A (0.05 mg/ml, Sigma Aldrich) in PBS at 37 °C. Propidium iodide (PI, Sigma Aldrich) was then added at a final concentration of 12.5 μg/ml. After 15 min in the dark at room temperature, samples were analyzed by the 500 MPL flow cytometer system (Beckman Coulter). Ten thousand events were acquired per sample.