Sonic hedgehog carried by microparticles corrects endothelial injury through nitric oxide release

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ABSTRACT Microparticles (MPs) are small fragments generated from the plasma membrane after cell stimulation. Among the candidate proteins harbored by MPs, we recently showed that sonic hedgehog (Shh) is present in MPs generated from activated/apoptotic human T lymphocytes [Martínez *et al.*, Blood (2006) vol. 108, 3012–3020]. We show here that Shh carried by MPs induces nitric oxide (NO) release from endothelial cells, triggers changes in the expression and phosphorylation of enzymes related to the NO pathway, and decreases production of reactive oxygen species. When PI3-kinase and ERK signaling were specifically inhibited, the effects of MPs were reversed. *In vivo* injection of MPs in mice was also able to improve endothelial function by increasing NO release, and it reversed endothelial dysfunction after ischemia/reperfusion. Silencing the effects of Shh with cyclopamine, a specific inhibitor of Shh, or siRNA, an inhibitor of the Shh receptor Patched, strongly reduced production of NO elicited by MPs. Taken together, we propose that the biological message carried by MPs harboring Shh may represent a new therapeutic approach against endothelial dysfunction during acute severe endothelial injury.—Agouni, A., Mostefai, H. A., Porro, C., Carusio, N., Favre, J., Richard, V., Henrion, D., Martínez, M. C., Andriantsitohaina, R. Sonic hedgehog carried by microparticles corrects endothelial injury through nitric oxide release. *FASEB J.* 21, 2735–2741 (2007)

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AFTER CELL STIMULATION, A SIGNIFICANT and sustained increase in cytosolic Ca²⁺ leads to the collapse of membrane phospholipid asymmetry by acting on pumps responsible for phospholipid transport. Changes in lipid distribution, mainly phosphatidylserine and cytoskeleton degradation by Ca²⁺-dependent proteases, are followed by microparticle (MPs) release (for a review, see ref. 1). MPs are small in size; their protein composition is not completely elucidated, but it differs in function from the cell origin and stimuli of their generation (2). Recent reports show that circulating MPs or those generated *in vitro* from apoptotic T cells display dele-

terious effects on endothelial and/or vasomotor function (3, 4). Enhanced levels of circulating MPs have been detected in several cardiovascular and hematological pathologies (5–9), strengthening the notion that MPs may play a role in these diseases. Indeed, MPs can be regarded as vectors of biological messages such as induction of endothelial dysfunction, angiogenesis, or differentiation (3, 10, 11).

Among the candidate proteins implicated in the ligand-receptor interactions between target cells and MPs, we have reported that the morphogen sonic hedgehog (Shh) is carried by MPs (11). Indeed, MPs generated from activated/apoptotic T cells or from plasma from diabetic patients harbor Shh and are able to induce differentiation and changes in the cell cycle of human K562 pluripotent erythroleukemic and primary CD34 cells (11). Shh is involved in embryonic and adult development (12), and dysregulation of the Shh pathway can lead to tumors (13) and erectile dysfunction (14). This latter effect is associated with a reduction of nitric oxide (NO) production by the corpora cavernosa. Indeed, NO-synthase (NOS) and vascular endothelial growth factor (VEGF) are downstream targets of exogenous Shh signaling, suggesting that Shh can act as a modulator of the regulation of VEGF and NO production (14). It was recently reported that intracellular elements of the Shh signal involve PI3-kinase and Akt (15), which are also associated with NO production in endothelial cells (16).

Recent evidence shows that MPs are important for communication between cells. Among the actions of MPs, it has been described that they can transfer receptors and organelles between cells and deliver mRNA and proteins into cells (17). We consider here engineering human T lymphocytes undergoing activation/apoptosis in order to generate MPs bearing Shh, as described (11). We have examined the effects of this type of MP on endothelial function and dysfunction

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after an ischemia/reperfusion with respect to production NO and reactive species of oxygen (ROS).

MATERIALS AND METHODS

MPs production

The human lymphoid CEM T cell line (ATCC, Manassas, VA, USA) was used for MP production. Cells were seeded at 10^6 cells/ml and cultured in serum-free X-VIVO 15 medium (Cambrex, Walkersville, MD, USA). MPs were produced as described previously (11). Briefly, CEM cells were treated with phytohemagglutinin (5 μ g/ml; Sigma, St. Louis, MO, USA) for 72 h, then with phorbol-12-myristate-13 (20 ng/ml, Sigma) and actinomycin D (0.5 μ g/ml, Sigma) for 24 h. A supernatant was obtained by centrifugation at 750 *g* for 15 min, then at 1500 *g* for 5 min to remove cells and large debris, respectively. MPs from the supernatant were washed after three centrifugation steps (45 min at 14,000 *g*) and recovered in 400 μ l NaCl (0.9% w/v). Washing medium for the last supernatant was used as control. Determination of the amount of MPs was carried out by measuring MP-associated proteins, using the method of Bradford (18), with BSA (Sigma) as the standard.

Cell culture

The Eahy 926 endothelial cell line was cultured in growth medium (DMEM, Ham's F-12, 1:1) supplemented with 1% L-glutamine, 1% HAT, 1% NEAA, 1% Na-pyruvate, 1% streptomycin/penicillin (Cambrex), and 10% FBS (Invitrogen, Cergy Pontoise, France). Cells were grown for 24 h in the absence or presence of 10 μ g/ml MPs preincubated or not with inhibitors [PI3-kinase inhibitor LY294002, 20 μ M (Calbiochem, London, UK), MEK inhibitor U0126, 2 μ M (Calbiochem), NOS inhibitor nitro-L-arginine (L-NA), 100 μ M (Sigma), or cyclopamine 30 μ M (Sigma)].

NO determination by electron paramagnetic resonance (EPR)

Detection of NO production was performed using a technique with Fe^{2+} diethyldithiocarbamate (DETC, Sigma) as spin trap. Briefly, after 24 h of MP treatment, cells were stimulated with either vehicle or bradykinin (20 μ M, Sigma) for 45 min at 37°C; medium was replaced with 250 μ l of Krebs solution, then treated with 250 μ l of colloid $\text{Fe}(\text{DETC})_2$ and incubated for 45 min at 37°C. NO detection was measured *in situ* by EPR. Values are expressed in units/ μ g/ μ l of endothelial cell proteins.

In another set of experiments, animals received i.p. injections of DETC (400 mg/kg), followed by s.c. injection of ferrous citrate; animals were sacrificed 30 min later. Blood and tissues (lung and aorta) were dissected and immediately frozen in plastic tubes using liquid nitrogen. NO spin trapping and EPR studies were performed as described previously (4). Values are expressed in units/ μ l of blood or in units/mg weight of dried tissue (aorta and lung).

Vascular reactivity

All animal studies were carried out using approved institutional protocols. Male Swiss mice (8 to 10 wk old) were treated *in vivo* by i.v. injection into the tail vein of MPs (10 μ g/ml of blood) or the same volume of vehicle in the absence or presence of cyclopamine (10 mg/kg, i.p.). For studies in

normal arteries, aortic rings were isolated 24 h after MPs were injected and mounted on a wire myograph filled with Krebs solution with the following composition in mM: NaCl 130, NaHCO_3 14.9, KCl 3.7, KH_2PO_4 1.2, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2, $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ 1.6, glucose 11 (37°C, 95% O_2 -5% CO_2). Mechanical activity was recorded isometrically by a force transducer (Danish Myo Technology, Aarhus, Denmark) (4). Concentration-response curves were constructed by cumulative application to acetylcholine (1 nM to 10 μ M, Sigma) to vessels with functional endothelium precontracted with U46619 (30 nM, Sigma). For ischemia/reperfusion studies, mice were anesthetized and mechanically ventilated. A thoracotomy was performed and ischemia was induced by ligating the left anterior descending coronary artery proximal to its origin. After 30 min the suture was removed to permit reperfusion for 1 h. Then the heart was removed and immediately placed in cold oxygenated Krebs solution (37°C, 95% O_2 -5% CO_2). A segment of the coronary artery (1.5 to 2 mm in length) distal to the site of occlusion was carefully dissected and mounted on a myograph. Endothelial function of a distal coronary artery was assessed on the basis of vascular studies performed earlier (19, 20). Concentration-response curves to acetylcholine (10 nM to 30 μ M) were performed in serotonin-precontracted segments (10 μ M, Sigma).

Dihydroethidine staining and flow cytometry

After incubation of endothelial cells with MPs at 10 μ g/ml for 24 h in the presence or absence of the PI3-K inhibitor (LY294002, 20 μ M), the MEK inhibitor (U0126, 2 μ M), or the NOS inhibitor (L-NA, 100 μ M), cells were washed three times with PBS, then incubated with the oxidative fluorescent dye dihydroethidine (DHE, 3 μ M, Sigma). Fluorescent-positive cells were determined by flow cytometry.

Western blot

After treatments, cells were homogenized and lysed. Proteins (20 μ g) were separated on 10% SDS-PAGE. Blots were probed with anti-endothelial NOS (eNOS), caveolin-1 (BD Biosciences, San Jose, CA, USA), phospho-caveolin-1 Tyr 14, phospho-eNOS Ser-1177, phospho-eNOS Thr 495, phospho-P42/44 (Cell Signaling, Beverly, MA, USA), and Patched (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. A polyclonal rabbit anti-human β -actin antibody (Sigma-Aldrich) was used at 1/5,000 dilution to visualize protein gel loading. The membranes were then washed at least three times in Tris buffer solution containing 0.05% Tween and incubated for 1 h at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ, USA). The protein-antibody complexes were detected by ECL plus (Amersham) according to the protocol of the manufacturer.

RNA interference and transient transfection

To silence Patched, the Shh receptor, siRNA duplexes specific for human Patched and control, nonsilencing siRNA were obtained from Santa Cruz Biotechnology. Transient transfection of Eahy 926 endothelial cells was done according to the manufacturer's protocol. Briefly, cells were seeded in 6-well plates, grown for 24 h (60% confluence), then transiently transfected with 100 nM of Patched-specific or control siRNA using the transfection reagent provided, which also served as control without siRNA. Medium was replaced 24 h later by fresh medium and cells were grown for an additional 24 h before either Western blot analysis of Patched expression or functional studies. In another set of experiments, cells

were treated with MPs and NO measurements were performed.

Data analysis

Data are represented as mean $\pm$ SEM; n represents the number of animals or experiments as appropriate. Statistical analyses were performed by 1-way analysis of variance (ANOVA), Student's t test (parametric), Mann-Whitney U tests (nonparametric), or ANOVA for repeated measures with a subsequent Bonferroni *post hoc* test. $P < 0.05$ was considered to be statistically significant.

RESULTS

MPs promote NO release and decrease ROS production

MPs induced an increase in NO production in human endothelial cells. This increase was observed in basal conditions and after stimulation with bradykinin (Fig. 1A). We investigated the pathways involved in MP-induced NO production. MPs induced an

increase in eNOS expression and in its phosphorylation at Ser-1177 and Thr 495 (activator and inhibitor sites, respectively) (Fig. 1B), phosphorylation of the activator site being significantly higher than that of the inhibitor site (Fig. 1C), indicating activation of the NO production pathway. When the PI3-kinase pathway was antagonized specifically with LY294002, the effects of the MPs were reversed, suggesting that this cascade is involved in MP-induced NO production. We also found that MPs both decreased the expression of caveolin-1 and enhanced its phosphorylation; these effects were inhibited by the PI3-kinase inhibitor (Fig. 1D). However, not all of the effects of MPs on protein expression and phosphorylation were modified in the presence of the ERK inhibitor, U0126 (not shown). In addition, incubation with MPs reduced ROS production in endothelial cells. This effect was abolished in the presence of the inhibitors of PI3-kinase or ERK but was not affected by the NOS inhibitor, L-NA (Fig. 1E). Neither LY294002, U0126, nor L-NA alone had a significant effect on ROS as measured by flow cytometry (% positive cells: 52 ± 2 , 45 ± 6 , 46 ± 6 , 56 ± 4 for control, LY294002, U0126, or

Figure 1. MPs promote NO-synthase (NOS) expression and activity and decrease ROS production. *A*) Quantification of the amplitude of the NO-Fe(DETC)₂ complex signal in human Eahy endothelial cells. Cells were incubated in the absence (basal conditions) or presence of bradykinin (BK, 20 μ M) for 45 min. Values are expressed as units/ μ g/ μ l of protein in the samples ($n=5$) (** $P < 0.01$). *B*) Human Eahy endothelial cells were treated for 24 h with either medium (CTL) or 10 μ g/ μ l MPs in the absence or presence of the PI3-kinase inhibitor LY294002 (LY). Western blot revealed eNOS expression and phosphorylation of Ser-1177 (activator site) and of Thr 495 (inhibitory site) ($n=6$). *C*) Quantification of the phosphorylation of the activator and the inhibitory site of eNOS (** $P < 0.01$) (AU, arbitrary units). *D*) Human Eahy endothelial cells were treated for 24 h with either medium (CTL) or 10 μ g/ μ l MPs in the absence or presence of the PI3-kinase inhibitor LY294002 (LY). Western blot revealed caveolin-1 expression and phosphorylation ($n=6$). *E*) Human Eahy endothelial cells were treated for 24 h with either medium (CTL) or 10 μ g/ μ l MPs in the absence or presence of PI3-kinase inhibitor LY, ERK1/2 inhibitor U0126, or NOS inhibitor L-NA, then stained with DHE and analyzed by flow cytometry ($n=5$) (*** $P < 0.001$ vs. control cells; † $P < 0.05$ vs. cells treated with MPs alone).

L-NA, respectively). As a positive control, lipopolysaccharide was used (not shown).

MPs enhance endothelium-dependent relaxation and restore endothelial dysfunction through NO release

To evaluate the *in vivo* physiological significance of MPs in NO production, MPs were injected into mice for 24 h. Under these conditions, MPs enhanced the acetylcholine-evoked endothelial relaxation of precontracted vessels (Fig. 2A). Blood, aorta, and lung from control or MP-treated mice exhibited a NO-Fe(DETC)₂ signal that was greater in samples from mice treated with MPs than in those obtained from control mice (Fig. 2B–D). To determine whether this enhanced production of NO by MPs is also associated with protective effects against endothelial dysfunction, we assessed relaxation responses to acetylcholine in coronary arteries from mice subjected to 30 min ischemia (left coronary occlusion), followed by 60 min reperfusion (Fig. 2E). Ischemia/reperfusion (I/R) markedly reduced the coronary relaxation to acetylcholine; however, the endothelial dysfunction was entirely reversed after administration of MPs 24 h before ischemia, showing that MPs may preserve coronary endothelial integrity and functionality in severe acute endothelial injury.

Silencing Shh signaling neutralizes the effects of MPs

To determine whether Shh accounts for the effects evoked by MPs, we examined the effect of Shh inhibition on MP-induced NO production. Treatment of endothelial cells with either the selective Shh inhibitor, cyclopamine, or siRNA to the Shh receptor, Patched, resulted in a reduction of MPs' effects on NO production (Fig. 3A, B). In addition, pretreatment of mice with cyclopamine reversed the enhancement of endothelium-dependent relaxation evoked by MPs (Fig. 3C). Cyclopamine treatment reduced the amount of NO detected in blood, aorta, and lung from mice treated with MPs (Fig. 3D–F). Together, these findings suggest that the effects of MPs are directly mediated by the Shh cascade, as illustrated by endothelial dysfunction in the presence of the Shh pathway inhibitor.

DISCUSSION

The results of the present study clearly show that engineered MPs presenting membrane-bound Shh induce NO production from endothelial cells and tissues; this effect is mediated directly by the Shh pathway, as illustrated by the reduction of NO production when the Shh pathway was silenced. NO generated after *in vivo*

Figure 2. MPs improve endothelial function. *A*) Acetylcholine-induced relaxation in control (CTL) and MP-injected mouse aorta. Results were expressed as a percentage of relaxation of U46619-induced precontraction. (** $P < 0.01$, *** $P < 0.001$ between each acetylcholine concentration; ††† $P < 0.001$ between the two curves) ($n = 6$). *B–D*) Quantification of the amplitude of the NO-Fe(DETC)₂ complex signal in circulating blood (*B*), aorta (*C*), and lung (*D*) from either control (CTL) or *in vivo* MP-treated mice. Values are expressed in units/μl blood ($n = 6$) or in units/mg weight of dried lung or aorta ($n = 6$) (* $P < 0.05$). *E*) Acetylcholine-induced relaxation in control (CTL) and MP-injected mouse coronary arteries after cardiac ischemia/reperfusion (I/R) ($n = 5$) (* $P < 0.05$, ** $P < 0.01$ I/R vs. control; † $P < 0.05$, †† $P < 0.01$ I/R + MPs vs. I/R).

Figure 3. The Shh pathway accounts for the effects induced by MPs. *A, B*) Quantification of the amplitude of the NO-Fe(DETC)₂ complex signal in either control (CTL), MP-, cyclopamine (cycl, 30 μ M) + MP- (*A*), or siRNA of the Shh receptor Patched (*B*) -treated human Eahy endothelial cells (* P <0.05) (n =5). Insert shows Western blot for Patched in the control (lane 1), siRNA scrambled (lane 2), or Patched siRNA (lane 3) -treated Eahy endothelial cells. *C*) Acetylcholine-induced relaxation in control (CTL), MP-, and MPs + cycl-injected mouse aortic rings (n =6) (*** P <0.001 between each acetylcholine concentration; ††† P <0.001 between curves). *D–F*) Quantification of the amplitude of the NO-Fe(DETC)₂ complex signal in circulating blood, aorta, and lung from *in vivo* cycl + MP-treated mice (n =6). Values are expressed as in Fig. 2 (* P <0.05 *vs.* control; † P <0.05 *vs.* the presence of MPs alone).

MP treatment enhances endothelium-dependent relaxation in normal mice but also restores endothelial dysfunction in coronary arteries subjected to ischemia/reperfusion.

The concentration of MPs used here is in the range of those able to promote differentiation of megakaryocytes (11), suggesting that low concentrations of MPs harboring Shh can induce important effects on the cardiovascular system. In addition, the effects evoked by MPs were dependent on the stimuli used for their generation, as suggested by others (21). Indeed, MPs from apoptotic actinomycin D-treated T cells did not produce NO release from endothelial cells (A. H. Mostefai and R. Andriantsitohaina, unpublished results) probably due to the absence of Shh in these MPs (11).

MPs used in the present study induce both the increase of NO release and the decrease in ROS production, with the latter being independent of the former as shown by the absence of effect of the NOS inhibitor. The concomitant effect of NO and ROS production might result in an enhancement of the bioavailability of generated NO by reducing oxidative stress and the subsequent scavenging of NO. The increase in NO release was associated with an enhancement of eNOS expression and activity, as reflected by the increase in eNOS phosphorylation, and with changes in the expression and phosphorylation of caveolin-1. All of these effects of MPs on pathways involved in NO production, except on caveolin-1 expression, were dependent on the PI3-kinase pathway. By contrast, the reduction in ROS induced by MPs was

dependent on PI3-kinase and ERK pathways, as observed in other models (22). Altogether, these results indicate that MPs are able to modulate eNOS expression and activity and reduce oxidative stress in human endothelial cells, leading to a beneficial potential effect on the cardiovascular system.

The generation of NO induced by MPs was corroborated after *in vivo* injection of MPs into mice. MPs were able to enhance endothelial NO relaxation to acetylcholine, which was accompanied by an increase of NO production in tissues and blood. MPs restore endothelial dysfunction after ischemia/reperfusion. It has been accepted that ischemia/dysfunction evoke deleterious consequences at the level of the myocardium and the coronary artery. The endothelial dysfunction described under these conditions is characterized by impaired endothelium-dependent vasodilatation, an exaggerated endothelium-dependent vasoconstriction, and increased production of endothelin-1 and ROS, leading to increased vasoconstriction and reduction of blood flow; the endothelium then becomes dysfunctional and NO formation decreases (23). In the present study, MPs restore endothelial injury probably through their dual ability to increase NO and reduce ROS.

We recently demonstrated that MPs generated from activated/apoptotic T cells carried Shh, a morphogen essential for embryonic development and other functions in the adult. Indeed, Shh signaling participates in the normal and diseased pancreas, hematopoietic differentiation, or erectile dysfunction (14, 24–27). With regard to the latter, injection in the corpora cavernosa of exogenous Shh protein

induces eNOS and VEGF. These results suggest that Shh may prevent the reduction of NO production in erectile dysfunction and highlight the clinical relevance of the Shh pathway for treatment of impotence (14). The Shh receptor Patched is normally expressed in cardiovascular tissues (25). In the present study, all the effects induced by MP treatment were inhibited when the Shh pathway was silenced by cyclopamine or by siRNA against the Shh receptor, indicating that Shh bound to MPs directly produces NO release from endothelial cells. PI3-kinase and Akt belong to the intracellular pathway for endothelial NO release. Here we show that MPs bearing Shh enhance both eNOS expression and activity *via* mechanisms sensitive to the PI3-kinase inhibitor. In conjunction with these studies, it has been reported that PI3-kinase and Akt are essential for Shh signaling (15, 28). Altogether, one can advance the hypothesis that Shh harboring by MPs cause NO release *via* PI3-kinase and Akt pathways.

To our knowledge, this is the first study to demonstrate that engineered MPs from human activated/apoptotic T lymphocytes carrying Shh improve endothelial function and prevent endothelial dysfunction *via* NO release. It was recently reported that Shh gene therapy may have considerable therapeutic potential by improving cardiac function in either ischemia or infarct models and wound healing in diabetes (29, 30). Here we propose that generation of MPs harboring Shh from T cells and the biological message they carried may represent a new therapeutic approach, independent of gene therapy, with which to correct cardiovascular pathologies linked to endothelial dysfunction. [F]

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