Microparticles harbouring sonic hedgehog
Role in angiogenesis regulation

Raffaella Soleti and María Carmen Martínez*

CNRS UMR 6214-INSERM 771; Faculté de Médecine; Université d’Angers; Angers, France

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Sonic Hedgehog (Shh) is a morphogen involved in embryonic development of nervous system. Also, it has been shown that recombinant Shh can modulate angiogenesis under ischemic conditions. However, angiogenic effects of endogenous Shh have not been completely elucidated. Using small membrane-derived vesicles expressing Shh (MPsShh+), we have shown that, although MPsShh+ decrease endothelial cell proliferation and migration, they are able to favour angiogenesis through the increase of endothelial cell adhesion and expression of pro-angiogenic factors. Activation of proteins implicated in cell adhesion, such as Rho A, as well as upregulation of pro-angiogenic factors were sensitive to inhibition of Shh pathway. Although whole composition of MPsShh+ needs to be characterized to understand potential effects of MPsShh+, these results highlight a new role of MPsShh+ in vascular pathophysiology and may have significant implication for therapy in pathologies associated with altered angiogenesis in order to re-address angiogenic switch.

Despite being previously believed inert dust devoid of precise function, several studies have revealed the unequivocal capacity of microparticles (MPs) to transfer biological information between cells, which confers them an active role in the development and regulation of pathophysiological processes. MPs are plasma membrane-derived vesicles (0.05–1 μm diameter) released from various cell types during activation by agonists, physical or chemical stress, including apoptosis. At their surface, MPs bear not only membrane antigens characteristic of the cell from which they are generated, but also cytoplasmic constituents (proteins, mRNA, microRNA,…). Global composition of MP proteins can be related to stimulus at their origin. In this respect, composition of MPs is variable and may explain, at least in part, the beneficial or deleterious effects elicited by MPs in physiological or pathological conditions. Moreover, several mechanisms have been proposed to explain how MPs may mediate intercellular communication (reviewed in ref. 6). In fact, MPs can bear combination of ligands that would engage, simultaneously, different cell-surface receptors. Thus, MPs may provide interaction between cells without direct cell contact. Also, MPs can bind to target cell membrane, which would, then, bear new surface antigens and acquire new biological properties and activities. Alternatively, MPs can also be internalized into target cells and transfer bioactive molecules. Furthermore, MPs have been detected in biological fluids of healthy subjects and, in general, MP levels are increased in several pathologies associated with pro-inflammatory states, for instance cancer, metabolic syndrome or sepsis. Because release of MPs can occur virtually in any cell types during activation or apoptosis, MP generation can be in vitro engineered and thus, MPs can be considered as vectors or cargo, in order to modify the expression of specific antigenic profiles capable of trigger “desired” responses. Recently, we have shown that engineering human T lymphocytes undergoing activation and apoptosis generate MPs bearing the morphogen Sonic Hedgehog (Shh).

Shh morphogen exerts developmentally relevant activities in many biological processes via an intricate signalling cascade. A great number of studies describe a key role for Shh during embryonic development especially with regard to nervous system. More interestingly, it is largely accepted that Shh signalling remains active in adult physiology and pathology. In fact, during post-natal life, Shh pathway orchestrates diverse processes, such as cell proliferation, differentiation and angiogenesis (reviewed in ref. 11). It has been described that activation of Shh cascade using recombinant Shh protein induces formation of in vitro capillary-like structures and...
in vivo new vessel generation. Moreover, the effects promoted by Shh affect modulation of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) activities. These findings represent a source to develop novel therapeutic strategies acting on Shh pathway, in order to treat diseases strictly connected to defects in angiogenesis. In this context, Shh gene therapy promotes neovascularization in ischemia models and wound healing. Moreover, it has been shown that administration of recombinant Shh protein induces angiogenesis in ischemic limbs and cornea of mice. However, the role of endogenous Shh in pathologies associated with ischemia is not completely elucidated. Taking advantage of MPs harboring Shh (MPsShh+), an exclusive study to test the involvement of the endogenous Shh in vascular function, we have analyzed the effects of MP-associated Shh on angiogenesis. It is interesting to highlight that Hedgehog (Hh) family members are the only examples of signalling proteins known to be covalently modified by cholesterol. This modification is essential for the activity of Hh signalling. Furthermore, since Hh proteins are anchored on the cell membrane attributing to lipidic modification, it has been suggested that MPsShh+ might be considered as cargo mediating long-range Shh signal, even if Shh concentration into MPs is low. Indeed, MPsShh+ are functionally active and induce intracellular responses in target cells, probably due to ligand/receptor interaction. For these reasons, in relation with recombinant Shh protein, it may be possible that MPs provide lipid and protein environment necessary to better support Shh activity.

We have shown that, in a model of mice coronary arteries subjected to ischemia/reperfusion, MPsShh+ restore endothelial dysfunction, probably through their dual ability to increase nitric oxide (NO) and reduce reactive oxygen species (ROS) production. Accordingly, modulation exerted by MPsShh+ on different pathways leads to beneficial potential effects on the cardiovascular system. The question raised is whether MPsShh+ are able to modify angiogenic profile of endothelial cells (ECs). Although the implication of MPs in angiogenesis modulation is reported in the literature, the effects described are controversial, because of responses they generated depend on stimulation, activation status of origin cell and their concentration (reviewed in ref. 5) and besides, the mechanisms involved in their formation are not completely known. We have demonstrated that MPsShh+ are able to regulate multiple steps related to in vitro angiogenesis. However, not all the events implicated on angiogenesis and triggered by MPsShh+ are modified in presence of Shh pathway inhibitor, cyclosporine, indicating that other molecules carried by MPs play a role in this phenomenon.

Although in our study MPsShh+ treatment induces an inhibition of EC migration and proliferation, independently from Shh cascade, other steps of angiogenic process are activated. Indeed, the formation of capillary-like structures is an angiogenic stage strongly promoted by MPsShh+. In addition, the mechanism favouring this effect is directly mediated by Shh pathway as demonstrated by the inhibition of formation of capillary-like structures using cyclosporine. Moreover, EC adhesion is increased by MPsShh+ treatment. Cell adhesion is an extremely important aspect of angiogenesis, which allows expansion, maturation, branching and remodelling of new vessels and in which is required a sequence of precisely spatially- and temporally-coordinated adhesive interactions between vascular cells. It is known that various cell adhesion molecules including integrins, members of the immunoglobulin superfamily, selectins and cadherins participate in cell adhesion. In our study, augmented EC adhesion is independent to Shh pathway but sensitive to Rho-associated coiled-containing protein kinase (ROCK) inhibitor, Y27632. Interestingly, expression of Rho A and phosphorylation of focal adhesion kinase, two proteins implicated in EC adhesion, are enhanced in response to MPsShh+. Indeed, it has been reported that intracellular Shh signalling is, at least in part, mediated by the activation of Rho A and its downstream effector, ROCK. Beside, MPsShh+ can modulate angiogenesis both at post-transcriptional and at transcriptional levels modifying expression profiles of key factors that belong to the complex control system under which angiogenesis is submitted. In fact, the analysis of transcripts of a large number of genes involved in angiogenesis revealed that MPsShh+ strongly influence its switch, as shown by upregulation of intracellular adhesion molecule-1, hepatocyte growth factor, VEGF A, VEGF receptor 1, interleukin-1β and matrix metalloproteinase-1 and downregulation of transforming growth factor β2 transcripts.

Collectively, our data demonstrate that MPsShh+ act at different phases of vessel formation (Fig. 1). As consequence, our study suggests new insights into the role of MPsShh+ in vascular pathophysiology and may have significant implications for therapy in diseases associated with an impairment of angiogenesis, in order to re-address angiogenic switch. Promoting angiogenesis is desirable in situations in which vascularisation needs to be re-established or extended, such as post tissue or organ transplantation, or to stimulate collateral circulation in ischemic conditions, including cardiovascular diseases associated with peripheral ischemia.

Therapy of angiogenesis with MPsShh+ is attractive and promising for several reasons: (1) MPsShh+ are able to create a natural bypass around vessels, (2) increase NO and (3) reduce ROS production. MPsShh+ may represent an autologous therapy, may decrease risk of systemic toxicity, and may allow the modulation of excessive or improved angiogenic response. Elucidation of whole MPsShh+ composition, as well as, the underlying mechanisms involved in their effects will help us to develop additional interventional strategies for prevention and treatment of diseases associated with alterations of angiogenesis.

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Figure 1. Schematic representation of effects evoked by microparticles harbouring Sonic Hedgehog (MPs Shh+) on endothelial cells (ECs) in different steps of angiogenesis. MPs Shh+ decrease mRNA expression of transforming growth factor β2 (TGFβ2), which is an anti-angiogenic factor, whereas they enhance mRNA levels of pro-angiogenic mediators such as vascular endothelial growth factor (VEGF) and its receptor FLT1, hepatocyte growth factor (HGF), and interleukin-1β (IL-1β). Also, MPs Shh+ increase mRNA expression of matrix metalloproteinase-1 (MMP-1), which is implicated in extracellular matrix degradation. Moreover, they regulate EC adhesion by augmenting expression of the intracellular adhesion molecule-1 (ICAM-1), Rho A and activity of focal adhesion kinase (FAK). Although both EC proliferation and migration are inhibited, all other effects of MPs Shh+ contribute to generation of capillary-like structures.

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