MINIREVIEW

Plasma Membrane Microparticles in Angiogenesis: Role in Ischemic Diseases and in Cancer

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Summary

Microparticles are small fragments of the plasma membrane released by activated and/or apoptotic cells. In theory, all type of cells can shed microparticles representing a physiological process in the cell life. Mainly, microparticles generation has been studied in different cardiovascular pathologies due to the facility to obtain blood samples from individuals. Although microparticles have been considered as simply markers of several diseases, in the last decade, several studies support the hypothesis that they participate in the regulation of the cardiovascular system function by carrying biological messages between cells. Among the effects of microparticles, recent data show that they can be implicated in the modulation of neovascularization, an essential function of cells from cardiovascular system during either ischemic diseases or cancer development. Whereas during pathologies associated with ischemia an increase of neovascularization may have beneficial effects, anti-angiogenic strategies represent new approaches for manipulation of tumor development. Here, we give an overview of the mechanisms and factors involved in neovascularization, and finally, we look at the role and the consequences of the modulation of this process by microparticles in pathological situations.

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Vasculogenesis, angiogenesis and neovascularization

There are two distinct but interconnected processes for the formation of postnatal new blood vessels, vasculogenesis and angiogenesis. The former is referred to the formation of the earliest vascular network *via* the differentiation of endothelial progenitor cells (EPCs) (also called angioblasts) into endothelial cells (ECs) (Asahara *et al.* 1997). By contrast, angiogenesis consists in the formation of new capillaries from the pre-existing vasculature (Carmeliet 2003). Thus, it is largely accepted that vasculogenesis might play a key role in embryogenesis, whereas angiogenesis can take place in both pre- and postnatal life. However, recent evidence suggests that in the adult, also EPCs could be considered as a source of cells that can participate in postnatal vasculogenesis. In addition, a third process, arteriogenesis, which describes the development and growth of pre-existing arterioles into physiological relevant arteries forming collateral vessels (Luque Contreras *et al.* 2006, Heilsch and Schaper 2003), complete the expansion and growth the vascular system in adults. It is obvious that, in the literature, these three mechanisms involved in the development of the cardiovascular system are frequently confounded and are not mutually exclusive. In this review, we have summarized the mechanisms implicated in *in vitro* angiogenesis and in the *in vivo* neovascularization, which takes into account the three processes described above.
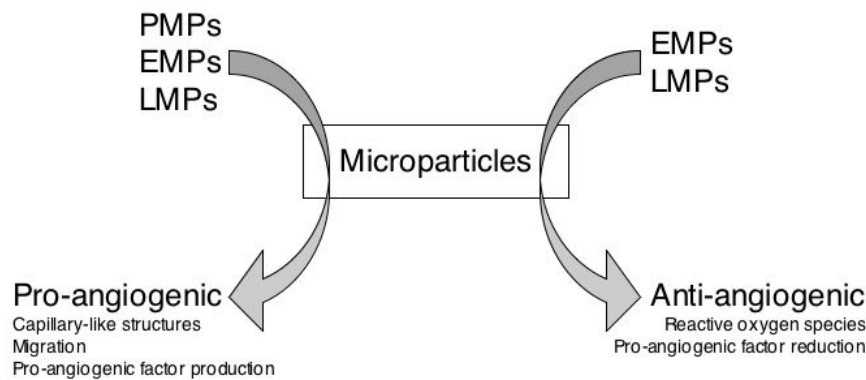
Fig. 1. Differential effects of microparticles on angiogenesis depending on their origin. Microparticles from platelets (PMPs) display pro-angiogenic properties by promoting capillary-like structures and pro-angiogenic factor production. By contrast, both endothelial- and lymphocyte-derived microparticles (EMPs, LMPs) possess pro- or anti-angiogenic properties depending on the stimuli used for their production.

Mechanisms implicated in neovascularization

Neovascularization plays a key role in physiological processes including embryonic development and wound repair as well as in various pathologies such as ischemic diseases, cancer, diabetic retinopathy or chronic inflammation including atherosclerosis (Folkman 1995). The neovascularization process is matched with changes in tissue mass and/or metabolic demands in order to maintain adequate oxygen delivery. This tightly regulated process involves the degradation of extracellular matrix, disruption of cell-cell contacts, migration and proliferation, and capillary tube formation of ECs. More in detail, angiogenic stimuli cause increased EC permeability through dissolution of adherens junctions (Pepper 2001). Briefly, EC proliferation occurs early in neovascularization, and continues as the new capillary sprout elongates. Proteolysis of basement membrane matrix cellular components is necessary to promote endothelial invasion into the surrounding interstitial matrix. Cellular migration is triggered and the sprouting tip of the EC proceeds into the interstitium. Lumen formation occurs as the sprout forms a multi-cellular structure. The new capillary channel forms an anastomosis with a pre-existing capillary, creating a new patent capillary. The final stage requires stabilization of the capillary through the construction of basement membrane, adherent junctions and cessation of ECs activation.

Factors regulating neovascularization

Normal tissues present a balance between pro-angiogenic and anti-angiogenic factor productions, however under pathologic conditions, this balance is dysregulated. Stimuli known to initiate neovascularization include hypoxia, inflammation, and mechanical factors such as shear stress and stretch. These stimuli either directly or indirectly activate ECs, by initiating the autocrine or paracrine production and release of growth factors or cytokines. Here, we briefly describe several factors known by their abilities to modulate angiogenesis.

The most important molecule that controls neovascularization is vascular endothelial growth factor (VEGF). Among the different isoforms of VEGF that have been described, VEGFA is the most potent angiogenic factor *in vivo*. Indeed, VEGFA regulates endothelial proliferation and permeability, as well as chemotaxis and differentiation of EPCs that are the main steps taking place in neo-vascularization (Asahara *et al.* 1999). In addition, inhibition of VEGFA activity is correlated with reduction of growth of tumors, suggesting that approaches considering inhibition of the VEGF activity could represent successful therapies against cancer. Finally, it has been shown that VEGF induces endothelial nitric oxide synthase (eNOS) expression and nitric oxide (NO) release from ECs (Ziche *et al.* 1997), which favors the proangiogenic process of capillary formation (Donnini and Ziche 2002). This is accordingly with studies performed in eNOS knockout mice displaying attenuated VEGF and angiogenesis induced by ischemia (Fukumura *et al.* 2001). Altogether, these data suggest that NO from eNOS as a key mediator of VEGF signaling.

Many other factors regulate the process of neovascularization. Among these, angiopoietins (mainly angiopoietins 1, 2 and 4) activate ECs in a paracrine manner resulting in a pro-angiogenic effect. Also, other

growth factors such as basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) or epidermal growth factor (EGF) display pro-angiogenic properties and induce EC proliferation (Das and Vasudevan 2007). Integrins can affect angiogenesis by mediating the direct interactions between cellular and extracellular matrix proteins (Hsu *et al*. 2007). Several interleukins (IL), in particular IL-6 and IL-8 are described as pro-angiogenic mediators (Motro *et al*. 1990, Park *et al*. 2001).

Anti-angiogenic factors can act directly on the ECs by modifying the regulatory pathways of angiogenesis, or indirectly by clearing pro-angiogenic factors, by blocking the signaling pathways of these factors. Endostatin, an internal fragment of type XVIII collagen (O'Reilly *et al*. 1997), impairs EC adhesion and migration, and interactions between cells and cellular matrix (Dixelius *et al*. 2002). Angiostastin, a cleavage product of plasminogen is able to bind to EC adhesion proteins, such as integrins, and to inhibit EC proliferation and migration (Troyanovsky *et al*. 2001). Another anti-angiogenic factor, trombospondin-1, acts directly inhibiting on EC migration, inducing EC apoptosis or indirectly modulating the effects of various pro-angiogenic factors (Zhang and Lawler 2007).

Cell types involved in neovascularization

In addition to ECs, which are involved in neovascularization, other cells such as EPCs are critical to this process as described above. EPCs may be analogous to the embryonic angioblasts, in a sense that they can circulate, proliferate, and participate in the development of vascular networks by differentiating into mature ECs (Rafii 2000). Many animal studies have now demonstrated that bone marrow-derived cells may play a role in physiological and pathological growth in the adult, both by promoting angiogenesis through the secretion of angiogenic factors and by providing a rich source of progenitor cells that can circulate and differentiate into mature vascular ECs. Recent works implicate EPCs in healing processes of injured tissues including myocardial ischemia and infarction, limb ischemia, wound healing, atherosclerosis, endogenous endothelial repair, and tumor vascularization (for review see Hillen and Griffoioen 2007, Napoli *et al*. 2007). Thus, when these cells are injected into animal models with ischemia, they are rapidly incorporated into sites of neovascularization (Luttun *et al*. 2002).

An inverse correlation between the number of circulating EPCs and risk factors for cardiovascular disease has been reported (Hill *et al*. 2003, Schmidt-Lucke *et al*. 2005). In addition, these authors have found that EPCs from high-risk subjects become senescent more rapidly than those from low-risk subjects. Under these conditions, EPCs may have important vascular clinical applications. Thus, EPCs may provide an opportunity for therapeutic intervention during ischemic complications either through enhancement of the mobilization, migration, or incorporation of endogenous EPCs or through transplantation of exogenous cell populations that have been expanded *ex vivo*.

Under another pathologic context such as tumors, EPCs have been detected at increased frequency in the circulation of cancer patients. Also, tumor production of VEGF was found to correlate with EPC mobilization (for review see Rafii *et al*. 2002). During tumor development, the inhibition of the recruitment of EPCs might provide a novel approach to block tumor angiogenesis.

Taken together, mobilization of EPC might have beneficial effects on pathologies associated with ischemic complications, whereas their effects can be deleterious in cancers.

Plasma membrane microparticles (MP)

All cell types subjected to chemical, physical activation (thrombin, endotoxin or shear stress, respectively) or apoptosis (growth factor deprivation or apoptotic inducers) can virtually release plasma membrane fragments, called microparticles (MP), originally described as inert "cell dust". MP are small (0.1-1 μm) membrane-bound vesicles that circulate in the blood and can mediate inflammation and thrombosis (Martinez *et al*. 2005). The mechanisms of MP formation are complex and not completely elucidated. Briefly, following cell activation or apoptosis, MP formation is dependent on a sustained rise in the cytosolic calcium concentration with the consequent activation of calpain and protein kinases and phosphatase inhibition. These changes result in cytoskeletal reorganization, membrane blebbing and the formation of MP (Wiedmer and Sims 1991, Yano *et al*. 1994, Miyazaki *et al*. 1996). The most abundant MP in the blood are generated from platelets, although MP in the periphery can also arise from leukocytes, erythrocytes, and cells that compose the vessel wall, mainly macrophages, ECs and smooth

muscle cells. MP contain membrane, cytoplasmic and nuclear constituents characteristic of their precursor cells and differ in size and composition from other subcellular structures such as exosomes (Distler *et al.* 2005). Indeed, on the surface, MP bear antigens characteristic of the cell from which they originated allowing their characterization. Thus, functional adhesion complexes such as glycoprotein IIb-IIIa and P-selectin are harbored by MP from platelets (Baj-Krzyworzeka *et al.* 2002). MP of endothelial origin carry CD31 or CD146, whereas CD4, CD3, or CD8 is present at the surface membrane of leukocytic MP (Mallat *et al.* 2000, Martin *et al.* 2004). A large body of data implicates MP in the initiation and amplification of the coagulation cascade, in thrombosis and in the propagation of inflammation, modulation of vascular tone, angiogenesis, stem cell engraftment, tumor metastasis owing to molecules harbored at their surface or within their cytoplasm (for review see Martinez *et al.* 2005) suggesting that MP can be efficient vectors of biological information from one cell type to another and hence, it has been proposed that MP may account for long-range intercellular communication. Most of these processes are associated with alterations of hemostatic parameters and vascular dysfunction including attenuation of endothelium-dependent vasodilatation, alteration of responsiveness of vascular smooth muscle to vasoconstrictor stimuli or both.

MP and *in vitro* angiogenesis

MP can be generated *in vitro* from cultured cells or from human freshly isolated leukocytes or platelets. This type of preparation of MP allows studying the specific role of each type of MP depending on its cellular origin as well as the stimuli used for their generation. Indeed, it has been described that the surface markers on MP depends on the stimulus, and the response they elicit in target cells may vary accordingly (Baj-Krzyworzeka *et al.* 2002, Martinez *et al.* 2006).

Concerning the effects of MP on angiogenesis, contradictory data are reported in the literature (Fig. 1). Endothelial MP have been described as pro-angiogenic through the metalloproteinase (MMP) activity, mainly MMP-2 and MMP-9, that they harbor (Taraboletti *et al.* 2002). Since MMPs are involved in EC invasion and formation of capillaries, MP may promote matrix degradation and favor new vessel formation. By contrast, Mezentsev *et al.* (2005) have reported that endothelial MP decrease formation of capillary-like structures by

increasing the production of reactive oxygen species. Thus, using a superoxide dismutase mimetic in order to neutralize reactive oxygen species, these authors have shown that angiogenesis impaired by endothelial MP was restored. The differences observed between the two studies could be related to the different concentrations of MP used. In particular, low concentrations of endothelial MP could promote angiogenesis, whereas high concentrations could suppress angiogenesis.

Platelet-derived MPs display a pro-angiogenic activity. In particular, this type of MP is able to favor almost all the steps involved in angiogenesis (proliferation, survival, migration and formation of capillary-like structures in ECs) (Kim *et al.* 2004). In addition, platelet MP effects on angiogenesis are mediated by phosphoinositide 3-kinase (PI3-kinase) and extracellular signal-regulated kinase (ERK) pathways. Accordingly, Brill *et al.* (2005) have shown that MP from platelets induced *in vitro* sprouting in aortic rings *via* PI3-kinase and ERK pathways involving growth factors such as VEGF and PDGF, and also displayed *in vivo* effects (see below).

Recently, it has been shown that lymphocyte-derived MP generated *in vitro* after actinomycin D treatment strongly suppressed aortic ring microvessel sprouting. This effect is linked to a down-regulation of the VEGF receptor type 2 and an increase of reactive oxygen species production associated with NADPH oxidase activity (Yang *et al.* 2008). Also, these MP impair vascular survival, proliferation, and migration. Although these authors have not studied the potential role of NO in their study, we have shown that the same type of MP decreased NO production *via* the PI3-kinase pathway (Mostefai *et al.* 2008). Indeed, incubation of ECs with this type of MP decreased NO production that was associated with enhanced phosphorylation of eNOS on its inhibitory site and overexpression of caveolin-1. In addition, we have observed that MP enhanced reactive oxygen species by a mechanism sensitive to xanthine oxidase inhibitor. Thus, lymphocyte-derived MP generated *in vitro* after actinomycin D treatment activate pathways related to NO and reactive oxygen species productions through PI3-kinase, xanthine oxidase and NADPH oxidase (Mostefai *et al.* 2008, Yang *et al.* 2008). Since NO plays a key role in angiogenesis, one can hypothesized that in parallel to reactive oxygen species-mediated MP effects on angiogenesis (Yang *et al.* 2008), the decrease of NO production induced by lymphocyte-derived MPs on ECs (Mostefai *et al.* 2008) may also be

involved in the impairment of angiogenesis. On the other hand, we have observed that when MP are generated from apoptotic/stimulated human lymphocytes, they promote angiogenesis through the increase of pro-angiogenic factor expression, such as VEGF, IL-1β and ICAM-1 (Martinez & Andriantsitohaina, unpublished results). These MP have the particularity to harbor at their surface the morphogen Sonic Hedgehog (Shh) (Martínez et al. 2006), which has been reported to act indirectly on angiogenesis by upregulating two families of angiogenic growth factors, VEGF and angiopoietins (Pola et al., 2001). In addition, we have shown that MP carrying Shh are able to stimulate NO production from ECs by direct activation of the Shh and PI3-kinase pathways (Agouni et al. 2007). Altogether, and depending on the stimuli used for formation of MP derived from lymphocytes, MP could represent potential tools to modulate angiogenesis. Indeed, pro-angiogenic effects of lymphocyte-derived MP are associated with their ability to release NO from ECs, whereas anti-angiogenic effects are linked to oxidative stress and reduced NO release from ECs.

MP and pathologies associated with modifications in angiogenesis

During myocardial infarction, ischemia/reperfusion evoke deleterious consequences on the level of the myocardium, but also on coronary artery mainly at the level of the endothelium. The endothelial dysfunction described under these conditions is characterized by an impaired endothelium-dependent vasodilatation, an exaggerated endothelium-dependent vasoconstriction, increased production of endothelin-1 and reactive oxygen species leading to increase vasoconstriction and reduction of blood flow, then the endothelium becomes dysfunctional and NO formation decreases (Moens et al. 2005). In parallel, it has been described that circulating levels of MP, mainly from platelets and endothelial cells, are increased in patients with myocardial infarction (Bernal-Mizrachi et al. 2003, Zielinska et al. 2005, van der Zee et al. 2006). In addition, MP from patients with myocardial infarction impair endothelial NO transduction pathway, suggesting that MP may account, at least partially, for the endothelial dysfunction observed in these patients (Boulanger et al. 2001). New therapeutic approaches in myocardial infarction try to decrease the deleterious effects of ischemia/reperfusion, to avoid the expansion of the necrotic infarct zone and to favor neovascularization. For the later, multiple studies

performed in animal models corroborate that the stimulation of collateral vessel development may have a clinical potential. Direct infusion of ischemia zone with growth factors or administration of a plasmid vector incorporating VEGF into arteries increases the formation of collateral vessels (Morishita et al. 1999, Isner et al. 1996). Recently, it has been shown that, in rat chronic ischemic heart, injection of MP from platelets into the myocardium increased the number of functioning capillaries (Brill et al. 2005), suggesting that in this pathological state, local application of MP may play an important role in controlling formation of new vessels. With regard to the correction of coronary endothelial dysfunction following cardiac ischemia/reperfusion, we found that i.v. injection of engineered MP from human activated/apoptotic T lymphocytes carrying Shh improved endothelial function and prevented 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 (Kusano et al. 2005, Asai et al. 2006). Our data lead us to advance the hypothesis that generation of MP harboring Shh from T cells (and the biological message they carry) may represent a new therapeutic approach, independent of gene therapy, by which we can correct cardiovascular pathologies linked to endothelial dysfunction, and probably improve neovascularization.

In diabetes, angiogenesis is also altered. Diabetes-associated vascular complications are generally the major clinical problems, which should be treated in diabetic patients, contributing to the significant morbidity and mortality. Thus, diabetic patients have an elevated incidence of macrovascular complications, such as atherosclerosis that increases the risk for myocardial infarction, stroke, and peripheral artery disease (often leading to limb amputation), as well as microvascular complications that consist of retinopathy and nephropathy, causing blindness and renal failure (Wild et al. 2004). Elevated number of total MP, and those from platelets, lymphocytes and ECs, has been detected in diabetic patients (Sabatier et al. 2002). While platelet MP are elevated in rats with streptozotocin-induced diabetic nephropathy (Kobayashi et al. 2008), levels of monocyte-derived MP are increased in patients with diabetic retinopathy (Ogata et al. 2006). Altogether these findings suggest that MP may play a role in the pathogenesis of several symptoms associated with diabetes. Thus, the treatment of peripheral artery disease or retinopathy and
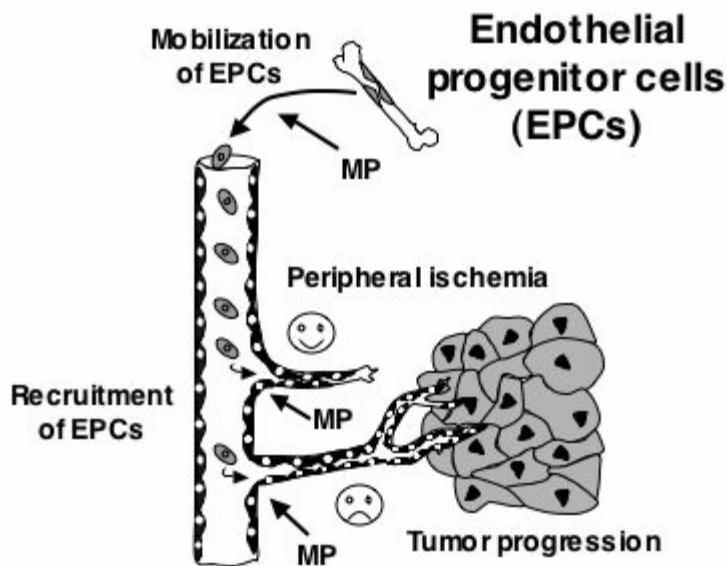
Fig. 2. Endothelial progenitor cells are involved in neovascularization. Mobilization of endothelial progenitor cells from bone marrow is induced under several conditions such as peripheral ischemia or cancer. Whereas in diseases associated with ischemia, endothelial progenitor cells may have a beneficial role and favor new blood vessel formation in order to repair tissues, during tumor development endothelial progenitor cell mobilization and recruitment may have a deleterious role promoting tumor progression. Microparticles (MP) may modulate the mobilization and recruitment of endothelial progenitor cells.

nephropathy may favor or inhibit angiogenesis, respectively. It has been shown that, in a mice model of type 2 diabetes, a plasmid DNA encoded VEGF improved blood flow after femoral artery ligation and increased VEGF protein, suggesting that gene transfer of pro-angiogenic factors are able to promote therapeutic angiogenesis in type 2 diabetes (Li *et al.* 2007). By contrast, anti-angiogenic therapy may be used in therapy for retinopathy and nephropathy in diabetes since recent data have shown that VEGF is, at least partially, responsible for these pathologies (Andreoli and Miller 2007, Zent and Pozzi 2006).

Finally, cancer progression is dependent on abnormal angiogenesis, in particular, exacerbated neovascularization form new vessels that guarantee an adequate supply of nutrients, oxygen, and growth factors to facilitate the growth tumor and metastasis development (Folkman 1995). Tumor cells are able to generated MP both *in vitro* and *in vivo*. Through proteins, such as urokinase, CD147 or sphingomyelin, harbored by MP from tumor cells, MP can modify the adhesive and invasive properties of tumor target cells (Angelucci *et al.* 2000), or the angiogenic activity of ECs (Millimaggi *et al.* 2007). Moreover, it has been shown that platelet MP enhance the *in vitro* invasive potential of breast cancer cell lines, and induce metastasis and angiogenesis in lung cancer (Janowska-Wieczorek *et al.* 2005, 2006). Indeed, injection of platelet MP resulted in metastatic foci in lung mice (Janowska-Wieczorek *et al.* 2005). These data suggest that MP transfer a transcellular signal that could allow tumor progression. Also, MP may represent a sign of vascular complications in patients with lung and gastric cancer (Kanazawa *et al.* 2003, Kim *et al.* 2003). These authors

have reported enhanced circulating monocyte- and platelet-derived MP in patients with lung cancer. In addition, levels of P-selectin associated to platelet MP and tissue factor generated from cancer cells are increased indicating that proteins involved in hemostasis are elevated in patients with cancer (Yu and Rak 2004) and may represent a tool for exacerbated thrombosis.

Future directions

During last years, EPCs have been considered as the key cells implicated in neovascularization. Modulation of their mobilization from bone marrow, or of their recruitment on the ischemic zones during ischemia or in zones contributing to tumor progression may represent novel potential therapeutic strategies by increasing pro-angiogenic EPC properties in ischemia or by decreasing them in cancer (Fig. 2).

Very recently, it has been reported that endothelial MP modulate angiogenic properties of EPCs *in vitro* (Lacroix *et al.* 2007). These authors have demonstrated that endothelial MP expressing urokinase-type plasminogen activator and its receptor at their surface affect tube formation mediated by EPCs. Furthermore, Deregibus *et al.* (2007) have shown that MP derived from EPCs are incorporated in ECs by interacting with integrins. Once this interaction takes place these MP promote the formation of *in vitro* capillary-like structures, which is abolished in the presence of RNase. Analysis of mRNA extract from MP showed that they carried cellular mRNA associated with the PI3-kinase/Akt signaling pathway, which plays an important role in the angiogenic effect of MP (Deregibus *et al.* 2007). These findings

underline the potential role of MP in the modulation of the angiogenic program in ECs *via* the transfer of a biological message through mRNA delivery.

Although all of these studies have been performed *in vitro*, they indicate that modulation of neovascularization through direct action on both ECs or EPCs *via* MP interaction with these cells may represent new therapeutic tool in pathologies with alterations in neovascularization.

Conflict of Interest
There is no conflict of interest.

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