

Mesenchymal stem cells-derived vascular smooth muscle cells release abundant levels of osteoprotegerin

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Although several studies have shown that the serum levels of osteoprotegerin (OPG) are significantly elevated in patients affected with atherosclerotic lesions in coronary and peripheral arteries, the cellular source and the role of OPG in the pathophysiology of atherosclerosis are not completely defined. Therefore, we aimed to investigate the potential contribution of mesenchymal stem cells in the production/release of OPG. OPG was detectable by immunohistochemistry in aortic and coronary atherosclerotic plaques, within or in proximity of intimal vascular smooth muscle cells (SMC). In addition, bone marrow mesenchymal stem cell (MSC)-derived vascular SMC as well as primary aortic SMC released in the culture supernatant significantly higher levels of OPG with respect to MSC-derived endothelial cells (EC) or primary aortic EC. On the other hand, *in vitro* exposure to full-length human recombinant OPG significantly increased the proliferation rate of aortic SMC cultures, as monitored by bromodeoxyuridine incorporation. Taken together, these data suggest that OPG acts as an autocrine/paracrine growth factor for vascular SMC, which might contribute to the progression of atherosclerotic lesions.

Key words: osteoprotegerin; mesenchymal stem cells; smooth muscle cells; atherosclerosis.

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Atherosclerosis is a form of chronic low-grade inflammation resulting from interaction between modified lipoproteins, monocyte-derived macrophages and vascular smooth muscle cells (SMC) (Libby, 2002). Although the prevalent view is that intimal vascular SMC found in atherosclerotic plaques derive from cells migrating from the tunica media of the same artery (Libby, 2002), accumulating data indicate that also bone marrow (BM) mesenchymal stem cells (MSC), also known as multipotent stromal cells, have the potential to migrate in sites of vascular injury or inflammation and to differentiate into vascular SMC (Hillebrands *et al.*, 2001, Li *et al.*, 2001).

Several studies have clearly demonstrated that the serum levels of the soluble member of the TNF-receptor super-family osteoprotegerin (OPG) are elevated in patients with coronary or carotid artery disease, especially those with clinically unstable atherosclerotic plaques (Jono *et al.*, 2002, Schoppet *et al.*, 2003, Secchiero *et al.*, 2006a, Shin *et al.*, 2006, Abedin *et al.*, 2007, Avignon *et al.*, 2007, Gulbiken *et al.*, 2007, Kadoglu *et al.*, 2008a, Omland *et al.*, 2008). Despite the fact that neither the cellular source nor the physiological and pathological effects of elevated serum levels of OPG are well understood, a possible pathogenic link between elevated levels of OPG and inflammation has been suggested by recent *in vitro* studies of our and others research groups (Zauli *et al.*, 2007, Mangan *et al.* 2007).

Therefore, in order to assess the potential contribution of MSC in the pathogenesis of atherosclerosis, we have evaluated the release of OPG in the culture supernatants of BM-derived MSC differentiating along the vascular SMC or endothelial cell (EC) lineages. In addition, we have investigated the effect of recombinant human OPG on aortic SMC proliferation.

Materials and Methods

Immunohistochemical detection of OPG

Samples of human ascending aortas and coronary vessels with atherosclerotic plaques were obtained from the Thoracic Surgery Department of University of Udine, as approved by the local research ethics committee. All specimens were fixed with 4% formaldehyde, paraffin-embedded and cut in serial sections of 5 μ m. Tissue morphology was evaluated by hematoxylin and eosin staining. Immunohistochemical detection of OPG was performed by using anti-human OPG Ab (clone 69127; R&D Systems, Minneapolis, MN) and the Super Sensitive TmPolymer HRP IHC detection system (Bio Genex, San Ramon, CA), according to the manufacturer's instructions. 3,3'-diaminobenzidine (DAB) was used as a chromogen, with subsequent nuclear counterstaining with hematoxylin. Staining evaluation was essentially qualitative by visual inspection of the samples by microscope.

Cell cultures

BM MSC, obtained from BM samples as previously described (Beltrami *et al.*, 2007), were cultured on fibronectin-coated dishes in expansion medium containing 10% FBS. In order to induce MSC-differentiation into SMC and EC, MSC were exposed to standardized differentiation-inducing conditions, as previously described (Beltrami *et al.*, 2007). Expression of lineage-specific markers was monitored by immunofluorescence analysis with anti-von Willebrand Factor and anti- α -smooth muscle actin (SMA; Dako, Copenhagen, Denmark) Abs. Primary human aortic vascular EC and SMC were obtained by Lonza (Walkersville, MD) and used between passages 4 and 8. OPG released in culture supernatants was measured by using a sandwich-type enzyme-linked immunosorbent assay (ELISA) kit, purchased from Alexis Biochemicals (Lausen, Switzerland). The sensitivity of the kit used was 2.8 pg/mL and the intra- and inter-assay coefficients of variation were 9% and <10%, respectively. For cell cycle analysis, SMC were made in a quiescent state by using serum-reduced (0.1% FBS) medium, treated with recombinant OPG (R&D Systems), and then incubated with 5-bromodeoxyuridine (BrdU; Sigma, Saint Louis, MO). Next, anti-BrdU Ab bounded to BrdU incorporated into newly synthesized DNA was detected by fluorescein isothiocyanate-conjugated secondary

Ab, as described (Secchiero *et al.*, 2006b). Cells were stained with propidium iodide (PI) and analyzed by flow cytometry; live cells were gated tightly using forward and side scatter, as described (Vitale *et al.*, 1997).

Statistical analysis

Comparison of group means was performed by Bonferroni and Student's T test methods. Statistical significance was defined as $p < 0.05$.

Results

OPG is expressed in vascular SMC of atherosclerotic aortic and coronary plaques

Immunohistochemical analysis demonstrated very low levels of OPG immunostaining in normal vessels (Figure 1 A). On the other hand, a striking OPG staining was observed in sections of aortic and coronary plaques, mainly confined to the fibrous cap (Figure 1 A). A strong cellular immunoreactivity in the atherosclerotic lesions was accompanied by a faint OPG expression in the interstitial space, according to the fact that OPG is a secreted protein (Zauli and Secchiero, 2006). A parallel immunohistochemical analysis performed on serial histological sections with α -smooth muscle actin (SMA) Ab indicated a preponderance of vascular SMC in the atherosclerotic fibrous cap (Figure 1 B). In particular, a good correspondence was observed between the elongated cells expressing OPG and cells identified as vascular SMC (Figure 1 B).

Bone marrow MSC-derived SMC and primary aortic SMC release high levels of OPG

Since it has been previously established that BM MSC can differentiate into either vascular EC or SMC (Hegner *et al.*, 2005, Kashiwakura *et al.*, 2003), we next investigated the ability of MSC-derived SMC and EC to produce and release OPG in culture (Figure 2). Undifferentiated MSC (day 0) released detectable amounts of OPG in their culture supernatants. While induction of differentiation along the endothelial lineage did not substantially modulate the amount of OPG released, the production/release of OPG showed a progressive and sustained increase paralleling the degree of SMC differentiation (Figure 2). Consistently with these findings, proliferating human aortic SMC released in culture supernatant significantly

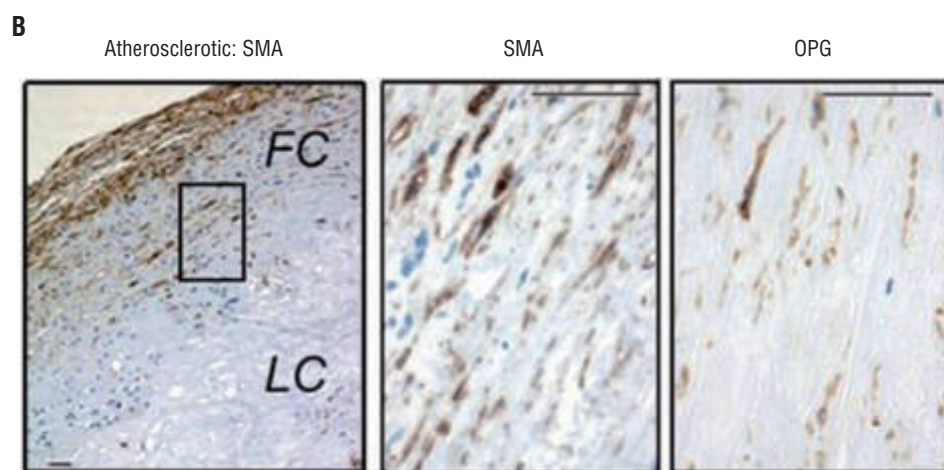
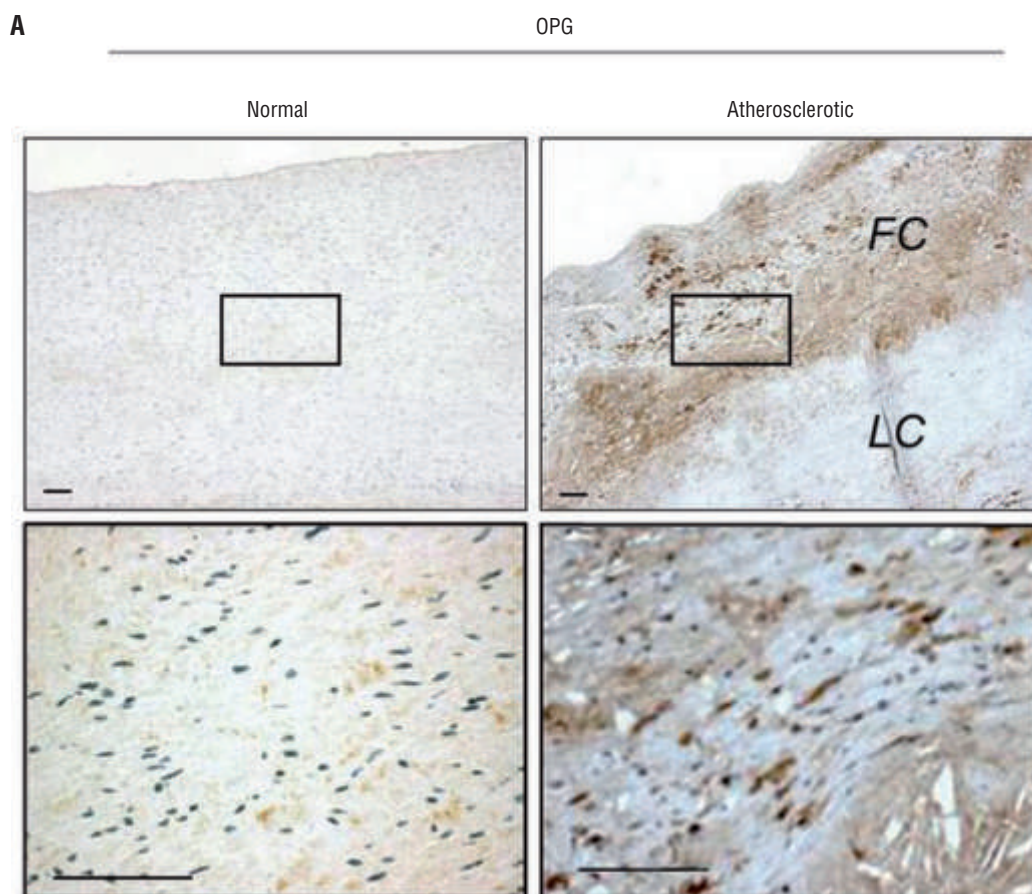


Figure 1. OPG expression in SMC of human atherosclerotic plaques. (A) immunohistochemical detection of OPG in samples of human ascending aortas with or without (normal) atherosclerotic plaque. FC, Fibrous cap; LC, Lipidic core. **(B)** comparison of immunohistochemical staining of a plaque performed with α -smooth muscle actin (SMA) and OPG Abs indicates a good correspondence between the elongated cells expressing OPG and the SMC of atherosclerotic fibrous cap (FC). Scale bar: 100 μ m. Representative pictures are shown.

($p < 0.01$) greater amounts of OPG with respect to aortic EC (Figure 2). Moreover, in agreement with recent findings demonstrating that recombinant OPG stimulates the proliferation of human pulmonary SMC (Lawrie *et al.*, 2008), we could confirm that recombinant OPG also stimulates the

proliferation of aortic SMC (*data not shown*). Thus, vascular SMC represent the major cell type involved in the production of OPG in atherosclerotic lesions and therefore OPG could act as a paracrine/autocrine growth factor for vascular SMC.

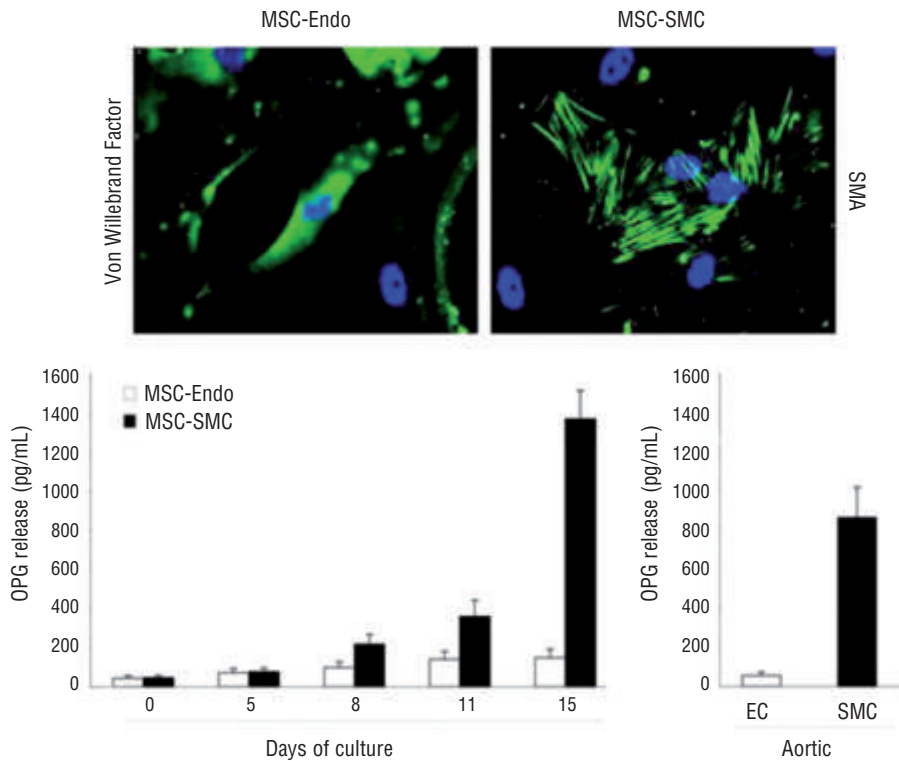


Figure 2. High levels of OPG are released by BM MSC-derived SMC and primary aortic SMC. BM MSC were induced to differentiate into endothelial (MSC-Endo) and SMC (MSC-SMC). Culture differentiation was monitored by immunofluorescence analysis of lineage-specific markers: von Willebrand Factor for endothelial cells and α -SMA for myocytes (both in green). DAPI was used in all fluorescent images to label nuclei in blue. Original magnification 40X. Release of OPG in culture supernatants was measured by ELISA at different time-points of culture. OPG released by aortic EC and SMC cultures is also shown. Measurements were done in duplicate and normalized for cell number. Data is expressed as means \pm SD of results from three independent experiments.

Discussion

Previous studies have shown that the expression and release of OPG by vascular cells is markedly up-regulated in response to inflammatory cytokines, such as TNF- α and platelet-derived growth factor (Zhang *et al.*, 2002, Olesen *et al.*, 2005). Conversely, OPG production is inhibited by PPAR- γ ligands (Fu *et al.*, 2002, Kadoglou *et al.*, 2008b), agents associated with anti-inflammatory and anti-atherogenic effects *in vitro* and *in vivo*. In this context, our current demonstration that BM MSC spontaneously and progressively increase the production of OPG when differentiating along the SMC lineage is of particular interest. Moreover, the observation that OPG was predominantly detected in vascular SMC within the atherosclerotic plaques, suggests that vascular SMC play a major role in releasing OPG also *in vivo*. Our assumption is nevertheless indirect because a simultaneous expression for α -actin and OPG was not assessed at the same time in our experiments. The potential ability of recombinant OPG to significantly increase the proliferation rate of aortic SMC suggests that OPG could act as an autocrine/paracrine growth factor for vascu-

lar SMC. In the context of our study, it is noteworthy that BM-derived MSC are able to engraft all layers of atherosclerotic plaques but not normal segments and could play double roles in the progress of atherosclerosis (Caplice *et al.*, 2003). Differentiating into EC, they contribute to regenerate the damaged endothelial layer, whereas differentiating into SMC, they contribute to worsen the atherosclerotic lesion (Caplice *et al.*, 2003). Therefore one mechanism by which MSC of bone marrow origin may contribute to atherosclerotic progression is through the release of OPG, which could act as a paracrine/autocrine growth factor for vascular SMC. OPG could also contribute to worsen the degree of atherosclerosis by inhibiting the TNF family member TRAIL (Vitovski *et al.*, 2007), which displays anti-inflammatory activity *in vitro* (Zauli *et al.*, 2003) and anti-atherosclerotic activity *in vivo*, as documented by studies in the apo-E null mice model (Secchiero *et al.*, 2006b).

In conclusion, our data suggests that OPG produced by MSC-derived vascular SMC can significantly influence the elevated OPG serum levels and may induce the progression of atherosclerosis by promoting proliferation of vascular SMC.

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