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Role of Osteoprotegerin in vasculogenesis.

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Osteoprotegerin, a new actor in vasculogenesis, stimulates Endothelial Colony-Forming Cells properties

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Abstract

Background: Osteoprotegerin (OPG), a soluble receptor of the tumour necrosis factor family, and its ligand, the receptor activator of nuclear factor-κB ligand (RANKL), are emerging as important regulators of vascular pathophysiology.

Objectives: We evaluated their effects on vasculogenesis induced by endothelial colony-forming cells (ECFC) and on neovessel formation *in vivo*.

Methods: Effects of OPG and RANKL on *in vitro* angiogenesis were evaluated after ECFC incubation with OPG or RANKL (0-50ng/mL). Effects on microvessel formation were evaluated with an *in vivo* murin Matrigel plug assay. Vascularization was evaluated by measuring plug haemoglobin and VEGF-R2 content 14 days after implantation.

Results: We found that ECFC expressed OPG and RANK but not RANKL mRNA. Treatment of ECFC with VEGF or SDF-1 upregulated OPG mRNA expression. OPG stimulated ECFC migration ($p<0.05$), chemotaxis ($p<0.05$) and vascular cord formation on Matrigel® ($p<0.01$). These effects were correlated with SDF-1 mRNA overexpression, which was 30-fold higher after 4 hours of OPG stimulation ($p<0.01$). OPG-mediated angiogenesis involved the MAPK signalling pathway as well as Akt or mTOR cascades. RANKL also showed pro-vasculogenic effects *in vitro*. OPG combined with FGF-2 promoted neovessel formation *in vivo*, whereas RANKL had no effect.

Conclusions: OPG induces ECFC activation and is a positive regulator of microvessel formation *in vivo*. Our results suggest that the OPG/RANK/RANKL axis may be involved in vasculogenesis and strongly support a modulatory role in tissue revascularization.

Keywords: Endothelial colony-forming cells, Osteoprotegerin, RANKL, Vasculogenesis.

Introduction

Angiogenesis and vasculogenesis are central to revascularization of ischemic tissue, as well as tumour formation and progression. Molecular regulation of these processes is the subject of many studies [1-2]. Recent publications underscore the possible contribution of osteoprotegerin (OPG), a soluble receptor of the tumour necrosis factor (TNF) family, traditionally implicated in the regulation of bone remodelling [3-5]. OPG is primarily known to prevent RANKL, a TNF-family member, from binding to its receptor RANK on osteoclast precursors, and thereby to inhibit osteoclast maturation [7,8]. By neutralizing RANKL, OPG protects the skeleton from excessive bone resorption [7-9]. Treatment with exogenous recombinant OPG inhibits osteolysis *in vivo*, reducing bone loss resulting from osteoporosis and breast cancer bone metastasis [9-12]. It reduces the skeletal tumour burden in an *in vivo* murine model of skeletal metastasis [13-16].

There is growing evidence that OPG underlies a possible link between the osseous and vascular systems. Recent experimental, clinical and epidemiological data have linked high OPG serum levels to the severity of cardiovascular diseases such as atherosclerosis and myocardial ischemia [17-18]. Ueland *et al.* showed that OPG levels were markedly higher in the acute phase than in the chronic phase (>1 month after myocardial infarction) of human heart failure [19], further supporting the influence of ischemia on OPG secretion. These authors also found enhanced OPG/RANKL/RANK expression in ischemic versus non ischemic areas of myocardium. Interestingly, elevated serum OPG has been linked to high RANKL levels through a negative feedback loop. Coexistence of severe osteoporosis and vascular calcification has been observed in OPG knockout mice [20-22]. According to Corallini *et al.,* OPG acts as an autocrine/paracrine growth factor for vascular smooth muscle cells and might contribute to the progression of atherosclerotic lesions [23].

OPG is expressed in a variety of tissues, including heart, arteries and veins, and is constitutively secreted by endothelial cells [20,24]. By comparison, RANKL and RANK are expressed in calcified arteries of OPG-deficient mice but not in normal vessels. OPG is physically associated with von Willebrand factor, both within Weibel-Palade bodies and following secretion from endothelial cells [25-27]. It can thus be released rapidly from Weibel-Palade bodies in endothelial cells in response to inflammatory stimuli following an ischemic event, raising the intriguing possibility that it may play a modulatory role in vascular injury and angiogenesis. Recent data show that OPG protects microvascular endothelial cells from detachment and apoptosis [3-5,28,29] and induce the formation of cord-like capillary structures by HUVEC cultured on Matrigel [5].

Endothelial Progenitor Cells (EPC) are marrow-derived circulating cells involved in postnatal vasculogenesis. These cells are recruited from bone marrow and are incorporated into sites of active revascularization, attracted by proangiogenic factors produced by the local inflammatory response [30]. They contribute to neovascularization after ischemic injury and are involved in regeneration of injured endothelium [30]. Different cell populations have been isolated which play a role in angiogenesis, but only one population, called “endothelial colony-forming cells” (ECFC) have been shown to possess all the characteristics of a true endothelial progenitor and to form neovessel *in vivo* [31]. We thus investigated the effect of OPG and RANKL on angiogenic properties of ECFC *in vitro* and *in vivo*. We found that both proteins activate the functional activity of ECFC *in vitro* and induces new blood vessel formation, at least for OPG, in a mouse model of extracellular matrix revascularization *in vivo*.

Materials and Methods

Materials

Recombinant human OPG and RANKL were from R&D Systems (France). Basic fibroblast growth factor (FGF-2) and Vascular endothelial growth factor (VEGF-A) and Stromal cell-derived factor-1 (SDF-1) were from Abcys (France). Growth factor-reduced Matrigel was from Becton Dickinson (France). Culture cell products were from Invitrogen (France), Cell culture medium from Lonza (Belgique) and other biochemical reagents from Sigma-Aldrich (France).

Cell characterization, culture and pretreatment

Umbilical cord bloods were collected from consenting mothers (n=20). The study was approved by local ethics committee of "Hôpital des Instructions et des Armées de Begin (France)(201008043234797) and protocol conformed to ethical guidelines of Declaration of Helsinki. ECFC were isolated from human umbilical cord blood, expanded and characterized as previously described [32].

The endothelial cell phenotype was shown by double positivity for DiI-AcLDL uptake and BS-1 lectin binding. Further endothelial characterization was obtained by FACS analysis (FACSCalibur, Becton Dickinson) of combined expression of cell-surface antigens of the endothelial lineage, namely CD31,KDR,Tie-2,CD144,CD34 and Flt-1. One day before all experiments, cells were growth-arrested for 18 hours in EBM2, 2% FCS and released from growth arrest by adding EBM2, 5% FCS, with or without 6.25 to 50ng/ml OPG or RANKL, for various incubation times at 37°C, then washed, detached with versene/0.01% collagenase (1/1) and washed twice with buffered Hank's, 0.5%BSA before use in *in vitro* angiogenesis assays. All assays were performed in triplicate. All the following experiments were performed during the first 30days of culture.

Proliferation assay

The effect of various concentrations of OPG and RANKL on ECFC proliferation was determined by measuring acid phosphatase activity (pNPP, Sigma) at 405nM (Fluostar optima; BMG labtech) as previously described [32].

Chemotaxis assay

Chemotaxis was examined in 24-well Boyden microchemotaxis chambers (Costar) with 8-µm pore-size polyvinylpyrrolidone-free polycarbonate Nucleopore filters. The chamber was inserted in a 24-well culture dish containing EBM2, 5% FCS (negative control), EBM2, 5% FCS with 20ng/ml VEGF (positive control) or OPG or RANKL concentrations ranging from 6.25 to 50ng/ml. Then $1.5x10^4$ control and pretreated-cells in suspension in EBM2, 5% FCS were placed in the upper chambers. Migration was allowed to proceed for 6 hours at 37°C, 5%CO2. Cells remaining on the upper surface of the filters were mechanically removed, and the filters were then fixed with 1.1% formaldehyde and stained with Giemsa. Number of migrated cells was determined by counting under a high-power microscope.

***In vitro* tube formation assay.**

Untreated ECFC and ECFC treated for 24 h with OPG or RANKL were seeded on Matrigel in growth factor-depleted basal medium, cultured for 18 h at 37°C, 5%CO2. Cells were then fixed with 1.1%glutaraldehyde for 15min and stained with Giemsa. Total length of tube structures was quantified on the entire surface of each well by using Videomet software (Microvision Instruments, France).

Flow cytometry

Expression of cell-surface antigens on ECFC was analysed by measuring immunofluorescence with a FACSCan flow cytometer. Labelling with mouse anti-human monoclonal KDR, Flt-1(Sigma), and VE-cadherin(CD144,Tebu-Bio) was visualised by using

RPE-conjugated goat anti-mouse F(ab')$_2$(Beckman Coulter). Anti-human Tie-2(Beckon Dickinson) and CD144(Beckman Coulter) were used directly PE-conjugated. CD34(Beckman Coulter) was used directly FITC-conjugated. In each immunofluorescence experiment isotype-matched mouse IgG antibody was used as a control and the fluorescence intensity of stained cells was gated according to established methods. Data were analysed with CellQuest™ software.

Real-time (RT) polymerase chain reaction

Total RNA was extracted either directly with the RNABle® technique (Eurobio GEXEXTOO-OW), or following pre-treatment with VEGF (20ng/ml), SDF-1 (100ng/ml), OPG (25ng/ml) or RANKL (25ng/ml) for 4 and 24 hours. Total RNA (250ng/µl) was reverse-transcribed. Transcripts of the TBP gene, an endogenous RNA control coding for the TATA box-binding protein, were quantified and target gene expression was normalized on the basis of its TBP content. PCR was performed with the SYBR® Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems), in duplicate for each data point. Primers for TBP and the target genes (sequences available on request) were chosen with the assistance of Oligo 5.0software (National Biosciences, USA).

Western blot

Cells treated with 10 to 100ng/ml OPG for 10 and/or 30min were analyzed in RIPA buffer. After electrophoretic transfer, an Immobilon-P membrane (Millipore, USA) was blotted with antibodies against p-mTOR (Ser 2448), p-AKT (Ser 473) and ERK 1/2 (Thr202/Tyr204), or total forms of protein quoted above (Cell Signaling, USA). The membrane was probed with a secondary antibody coupled to horseradish peroxidase. Antibody binding was then visualized with the ECL kit (Roche Molecular Biomedicals).

Matrigel plug assay

Animal care conformed to French guidelines (Services Vétérinaires de la Santé et de la Production Animale, Ministère de l'Agriculture, Paris, France), and experiments were performed in keeping with the guidelines of the Paris-Descartes University Institutional Committee on Animal Care and Use (C75.06.02). Ice-cold Matrigel (BD) was mixed with PBS, FGF-2, OPG alone or supplemented with FGF-2, and RANKL alone or supplemented with FGF-2, then kept on ice. Male C57BL/6J mice (8 weeks old) were obtained from Janvier breeders. They were anesthetized with ketamine/xylasine (80/16 mg/kg), and 500 µL of Matrigel was injected subcutaneously at the dorsal posterior. The mice were sacrificed on day 14 and the Matrigel plugs were recovered in RIPA lysis buffer 50 mM Tris, 150 mM NaCl, 1% NP40, 0.50% sodium deoxycholate, 0.10% SDS, 10 mM EGTA, 1 mM PMSF, 10 mM EDTA, 1 mM orthovanadate Na, 10 mM NaF, a protease inhibitor cocktail (2 mM AEBSF, 1 mM EDTA, 130 µM bestatin, 14 µM E-64, 1 µM leupeptin and 0.3 µM aprotinine from CIP SIGMA) at pH 7.2 and 4°C. The haemoglobin concentration was measured in the supernatants with Drabkin's reagent (Sigma). For VEGFR-2 ELISA, tubes were centrifuged for 30 min at 1500*g* and the supernatants were analyzed with the R&D kit for mouse VEGFR-2.

Statistical analysis

Data are expressed as means ± SEM of at least three independent experiments. Significant differences were identified by ANOVA followed by Fisher's protected least significant difference test. The Statview software package (SAS) was used for all analyses. Differences with probability values below 0.05 were considered significant.

Results

We explored whether OPG and RANKL were able to modulate angiogenic process. We focused on ECFC because this cell type is currently proposed as the cell type at the origin of newly formed vessels. These cells were isolated from human umbilical cord blood on the basis of CD34 expression. The presence of Weibel-Palade bodies and combined expression of endothelial markers (CD31, Tie-2, KDR, Flt-1, CD144) as shown in Table 1 unequivocally confirmed the endothelial phenotype of the ECFC thus obtained. Furthermore ECFC do not express leuko-monocytic markers such as CD14 and CD45 [33].

Human ECFC express OPG and RANK mRNA but not RANKL mRNA.

Expression of RANKL and its two receptors OPG and RANK was evaluated in the isolated ECFC by qRT-PCR. We found, for the first time, that ECFC express OPG and RANK mRNA in steady-state culture. In contrast, no RANKL mRNA was detected. We then used qRT-PCR to examine whether OPG and RANK mRNA synthesis was regulated by the proangiogenic factors VEGF-A and SDF-1. VEGF is one of the most potent angiogenic factors, stimulating proliferation and regulating vessel permeability and endothelial cell differentiation. SDF-1 is known for its chemoattractant and proangiogenic properties [34]. In our experimental conditions, VEGF-A and SDF-1 markedly increased the expression of OPG mRNA ($p<0.01$ and $p<0.05$ respectively). OPG mRNA expression increased 9-fold and 2.5-fold after respectively 4 and 24 hours of stimulation with VEGF-A and 6-fold and 3-fold, respectively, after stimulation with SDF-1 (Figure 1A). In contrast, no significant increase in RANK mRNA expression was observed (Figure 1B).

OPG and RANKL differently modulate ECFC proliferation.

Proliferation was examined by measuring cell phosphatase activity based on the release of pNPP measured at OD 405nm after 24, 48, 72 and 96 h of incubation in basal medium

(EBM2, 5% FCS) containing 6.25 to 50ng/ml of recombinant OPG or RANKL. RANKL enhanced ECFC proliferation in a concentration-dependent manner, starting at 6ng/ml after 96 h of incubation (Figure 2A), whereas no significant difference in cell numbers was observed between untreated ECFC and ECFC treated with OPG (Figure 2B), even at 100ng/ml (data not shown).

OPG and RANKL stimulate ECFC migration.

We then investigated the effect of OPG and RANKL on ECFC chemotaxis and migration, to see whether they might be involved in ECFC recruitment to sites of neovascularization. As shown in Figures 3A-B, OPG and RANKL stimulated the chemotactic motility of ECFC, with an optimal and statistically significant effect up to 25ng/ml ($p<0.05$ and $p<0.01$ respectively). ECFC were maximally attracted by 25ng/ml RANKL ($p<0.001$), more strongly than by 20ng/ml VEGF (figure 3B). Furthermore, treatment with 25ng/ml OPG stimulated ECFC migration to a level comparable to that of VEGF 20ng/ml (Figure 3C), reported to be an optimal concentration for VEGF-stimulated endothelial cell migration [35]. At the same concentration (25ng/ml), RANKL-treatment had no effect on ECFC motility.

OPG and RANKL induce tubular morphogenesis by ECFC in Matrigel

Angiogenesis may require changes in the functional properties of blood vessels, and not only stimulation of ECFC growth or migration. We incubated ECFC overnight in starvation medium then stimulated them for 24 h with different concentrations of OPG and RANKL in basal medium supplemented with 5% FCS, before seeding on Matrigel for 18 h. As shown in Figure 4A, without OPG or RANKL pretreatment, ECFC did not form tubular structures. In contrast, pretreatment with OPG or RANKL promoted ECFC organization into branched structures and pseudotubes with enclosed areas (Figures 4B-C). The tubular network was respectively 2.5 and 2.1 times more extensive with 25ng/ml OPG ($p<0.01$, Figure 4E) and

25ng/ml RANKL ($p<0.01$, Figure 4F) compared to control, reaching the same order of magnitude as with ECFC culture medium (EGM2) containing proangiogenic growth factors (Figure 4D). Effect of OPG was confirmed by using neutralizing polyclonal anti-human OPG antibody associated with OPG treatment. We found that anti-human OPG antibody totally abolished ECFC tubulogenesis induced by OPG (data not shown).

OPG enhances FGF-2-induced neoangiogenesis *in vivo*.

To confirm these *in vitro* results and to evaluate the effects of OPG and RANKL on neo-vessel formation, we used an *in vivo* Matrigel plug assay. In this model, host endothelial cells and EPC migrate into the implanted plug attracted by local injected growth factors and, 14 days later, form a capillary networks. We first injected 0.5ml of Matrigel, containing PBS (control), 500ng/ml of OPG or RANKL, or 350ng/ml FGF-2 (positive control), into the flank region of C57BL/6 mice. As shown Figure 5A, PBS control plugs were pale, indicating little or no vessel formation. Plugs containing OPG or RANKL showed few blood vessel formations, indicating the very beginning of a vasculature (Figures 5C,E). But either OPG or RANKL has a modest non stimulatory effect on angiogenesis comparing to FGF-2 (Figures 5B,G,H).

To investigate the possible synergy of OPG/FGF-2 and RANKL/FGF-2, we used the same model, injecting each mouse with 0.5ml of Matrigel containing 350ng/ml FGF-2 alone or combined with 500ng/ml OPG or RANKL. Analysis of the haemoglobin and VEGF-R2 contents showed enhanced neoangiogenesis in OPG/FGF-2-containing plugs ($p<0.001$ Figures 5D,G,H) as compared to FGF-2-containing plugs (Figures 5B,D,G,H). The RANKL/FGF-2 combination was as effective as FGF-2 alone (Figures 5B,F,G,H).

Proangiogenic activity of OPG correlates with SDF-1 mRNA overexpression and angiogenic signalling pathway activation.

We then evaluated the effect of OPG and RANKL on VEGF-A and SDF-1 mRNA expression by ECFC *in vitro*, with qRT-PCR in order to determine whether the observed pro-angiogenic effects of OPG and RANKL was mediated by enhanced synthesis of these two factors. OPG and RANKL induced a slight but non significant increase in VEGF-A mRNA after 24 hours, but not after 4 hours (data not shown). In contrast, OPG and RANKL both markedly increased the expression of SDF-1 mRNA (Figures 6A,B). The increase in SDF-1 mRNA was 30-fold after 4 hours of OPG stimulation ($p<0.01$), and 5-fold after 24 hours (Figure 6A), compared to respectively 31-fold ($p<0.001$) and 6-fold with RANKL (Figure 6B).

We then evaluated the capacity of OPG to activate the angiogenic signalling pathways MEK/ERK and PI3K/Akt/mTOR. OPG strongly activated both the MEK/ERK pathway and Akt, which is known to be essential for angiogenesis (Figure 6C). Increased phosphorylation of ERK1/2, Akt and mTOR was detected in the absence of any measurable change in the total amount of these proteins. These effects were time- and concentration-dependent.

Discussion

We examined whether recombinant human OPG and RANKL were able to activate ECFC and to induce new blood vessel formation *in vivo*. These bone marrow-derived progenitor cells are recruited to sites of vascular injury and also contribute to tumour angiogenesis [1,36]. They participate directly in collateral vessel formation and display specific functional properties of mature vessel-wall endothelial cells. ECFC extracted from human cord blood exhibit a stronger angiogenic capacity than mature endothelial cells (HUVEC), proliferating more strongly and producing significantly more tubular structures than HUVEC in the presence of the proangiogenic growth factor FGF-2 [32].

We first confirmed that human ECFC express both OPG and RANK mRNA but not RANKL mRNA. This is not surprising, as OPG is expressed by a variety of cells, and especially mature vascular endothelial cells [4,20,23]. Its production is modulated by SDF-1 and VEGF, as we found that its mRNA expression was enhanced by both proangiogenic cytokines after 4h and 24 h of stimulation. Cytokines, such as TNFα and IL-1, have been shown to enhance OPG expression in mature endothelial cells [4], but until now neither SDF-1 nor VEGF had been shown to have such an effect. Constitutive expression of RANK on ECFC allows them to respond to RANKL and might prevent their apoptosis, as described with mature endothelial cells [37,38]. The presence of RANKL on mature endothelial cells [3,37] but not on immature endothelial cells (ECFC) may provide a clue to the role of RANKL in ECFC recruitment and adhesion to activated endothelium. RANKL is also expressed by vascular smooth muscle cells and might interact with ECFC in a paracrine manner.

We then found that OPG is able to affect proangiogenic capacity of CD34+ endothelial cell progenitors. Human ECFC treatment with recombinant OPG induced a proangiogenic phenotype, involving both an early angiogenic event (migration) and a late event (differentiation into vascular cords). Indeed, OPG attracted ECFC in the Boyden chamber assay, and promoted their motility. These results are similar to those obtained with microvascular endothelial cells however using 500 ng/ml OPG, a concentration 20 times higher than the one used in this study [35]. Furthermore, OPG-pretreated ECFC showed enhanced migration. OPG drastically enhanced the formation of vascular cord-like structures in Matrigel, in a concentration-dependent manner starting at 12,5ng/ml, as potently as growth factors contained in EGM2. These results are similar to those obtained with human dermal microvascular endothelial cells at 100ng/ml, a concentration 8 times higher than the one tested [5]. Interestingly EGM-2-induced tube formation was partly inhibited in the presence of the anti-human OPG antibody as observed by a significant 25% decrease in tube length

($p<0.01$ – data not shown) suggesting the existence of an OPG autocrine loop. Our findings therefore suggest that OPG is more effective on $CD34^+$ immature cells than on mature endothelial cells.

Several studies have shown that recombinant OPG can promote the survival/proliferation of mature vascular endothelial cells when cultured in basal medium [3,5,28,29,39]. The precise mechanism for this remains to be identified but is unlikely to involve protection from growth factor deficiency [5]. However in the same experimental conditions, we observed no direct effect of OPG on ECFC proliferation, even at concentrations up to 100 ng/ml. ECFC might be more resistant than mature endothelial cells to cytotoxic effects induced by serum depletion [40].

In the same experimental conditions, RANKL promoted ECFC angiogenesis *in vitro*, as potently as OPG. These *in vitro* findings are relevant to the *in vivo* situation, at least for OPG. Indeed, we found that OPG potentiated the effect of FGF-2 and is able to recruit endothelial cells to form vascular structures within Matrigel plugs implanted in wild-type mice. It should be emphasized that in our experimental conditions, RANKL does not exhibit any proangiogenic activity *in vivo*. Nevertheless, Kim YM *et al.* have observed new blood vessel formation for RANKL concentrations 6 times greater than the one tested (3 µg/ml) well above physiological ones [41]. Our findings therefore point to a role of OPG in new blood vessels formation.

These proangiogenic activities correlated with activation of both the extracellular signal-regulated kinase (ERK)1/2 and the PI3K/Akt/mTOR signalling pathway, both of which are linked to cell survival and angiogenesis. Experiments underway in our laboratory indicate that neither OPG nor RANKL affects SDF-1 (CXCR4) or VEGF (KDR) receptor expression whereas they induce β3 subunit and αvβ3 overexpression by ECFC (data not shown). In association with αv, β3 promotes cell adhesion and subsequent migration to the underlying

extracellular matrix. According to Kobayashi-Sakamoto *et al*, integrin αv activity correlates with ERK1/2 activation in mature vascular endothelial cells [35]. This integrin profile could potentially allow circulating ECFC to migrate more efficiently to ischemic sites, where they can proliferate and form vascular tubes. Our results suggest that OPG interacts directly with the cell membrane [42] and acts by enhancing SDF-1 secretion by ECFC. Enhanced SDF-1 expression and production is essential for the recruitment of stem cells and for their maintenance *in situ* [32]. OPG may induce ECFC differentiation and migration by interacting with glycosaminoglycans present on the cell membrane that can transduce the intracellular signals required to induce an angiogenic phenotype [42].

Several studies have investigated the therapeutic potential of recombinant OPG in osteolysis associated with cancer [6,8,10,12-14,16]. Studies of patients with bone disease related to breast carcinoma or multiple myeloma indicate that a single dose of recombinant OPG is at least as effective in reducing bone resorption marker levels as an acceptable treatment for these diseases [11]. Lamoureux *et al.* propose the use of chemotherapy combined with RANKL inhibition by OPG administration to suppress tumour-induced osteolysis and to inhibit the growth of skeletal tumours [43]. However, our findings suggest that OPG injection could promote tumour vascularisation and growth.

How does OPG influence neovessel formation? More and more studies are highlighting the crucial role of this protein in vascular disorders [3-5,7,15,17-20,22]. Our findings, together with recently published data, throw light on the OPG mechanism of action in angiogenesis. To reach the ischemic tissue, ECFC must adhere to and extravasate through the activated endothelium, attracted by SDF-1 and VEGF. Close contact with vascular endothelial cells would directly expose ECFC to OPG, RANKL and cytokines released by activated endothelium. This would promote ECFC activation and trigger signalling events linked to cell survival and angiogenesis [3-5,20,28,36,39,44,45] inducing their migration and their

development into new blood vessels. It should be emphasized that OPG can modulate SDF-1 production and inflammatory responses by interfering with RANKL and other members of the tumour necrosis factor (TNF) family signalling pathway, such as the TNF-related apoptosis-inducing ligand (TRAIL), a death-inducing ligand sharing 35% homology with RANKL [4,15,42,46,47]. A resulting elevation of OPG levels within the bone marrow microenvironment or around a tumour might thus contribute to new blood vessel formation.

In conclusion, our findings partly clarify how OPG intervenes in vasculogenesis. There is strong evidence of OPG release in ischemic tissues. OPG induces SDF-1 overexpression by ECFC and is thereby able to participate in the recruitment of active stem cells involved in blood vessel formation. Thus, OPG-based strategies designed to limit bone resorption must take into account the risk of unwanted tumorigenic effects [48, 49].

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Author contributions

Conceived and designed the experiments: ZBA, DH, CBV

Performed the experiments: ZBA, RB, IB, BD, AL, IL, CBV

Analyzed the data: ZBA, RB, BD, IB, AMF, DH, CBV

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Contributed reagents/materials/analysis tools: IGF, DS

Wrote the paper: ZBA, DH, CBV.

Conflict of Interest Statement: none declared

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Table1: Means fluorescence intensity of ECFC phenotypic markers analysed by flow cytometry (MFI ± S.D., n = 5)

Markers	*ECFC D30 (MFI ±SD)*
CD34	4,0 ± 2,0
Tie2	6,1 ± 0,5
KDR	8,8 ± 0,4
CD144	21,8 ± 0,3
Flt-1	7 ± 0,1
CD31	36,3 ± 0,3

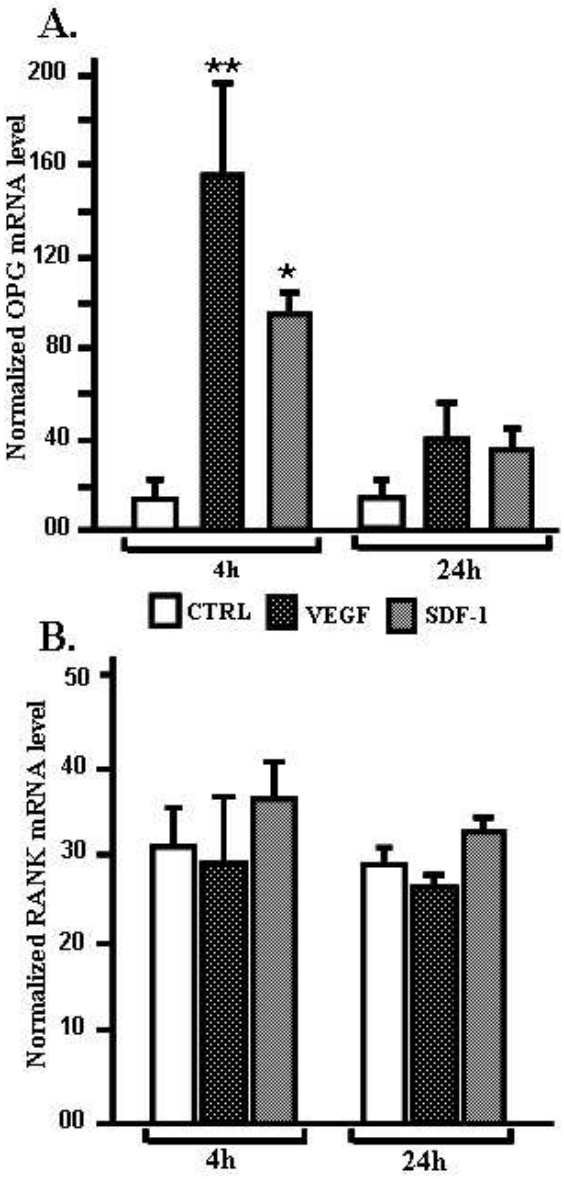

Figure 1: ECFC express OPG and RANK mRNA in steady-state culture. Proangiogenic cytokines VEGF and SDF-
1 modulate OPG mRNA expression (A) but not RANK mRNA expression (B) in human ECFC. Quantitative Real Time-PCR using total RNA isolated from human primary ECFC incubated for 4h and 24h in the presence or absence of 25 ng/ml VEGF and 100 ng/ml SDF-1 (A) as described under methods. Means±SEM, n=3. *:p<0.05, **:p<0.01.
82x173mm (96 x 96 DPI)

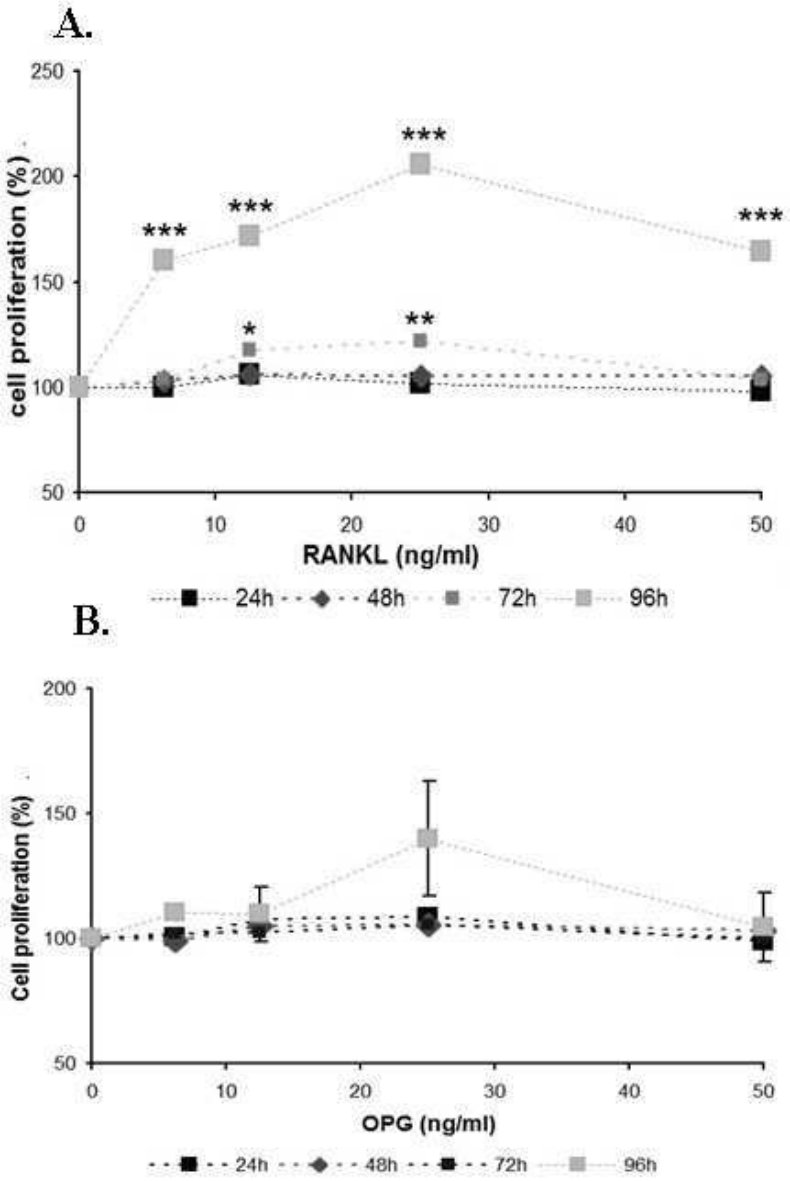

Figure 2: RANKL induces ECFC proliferation in a concentration-dependent manner 72h after incubation (A),
but no significant difference in cell numbers was observed between untreated ECFC and ECFC treated with OPG (B). Cell numbers were determined by pNPP colorimetric assay. Results are normalized to untreated ECFC, n=3. *:p ≤ 0.05, **:p ≤ 0.01, ***:p ≤ 0.001.
114x173mm (96 x 96 DPI)

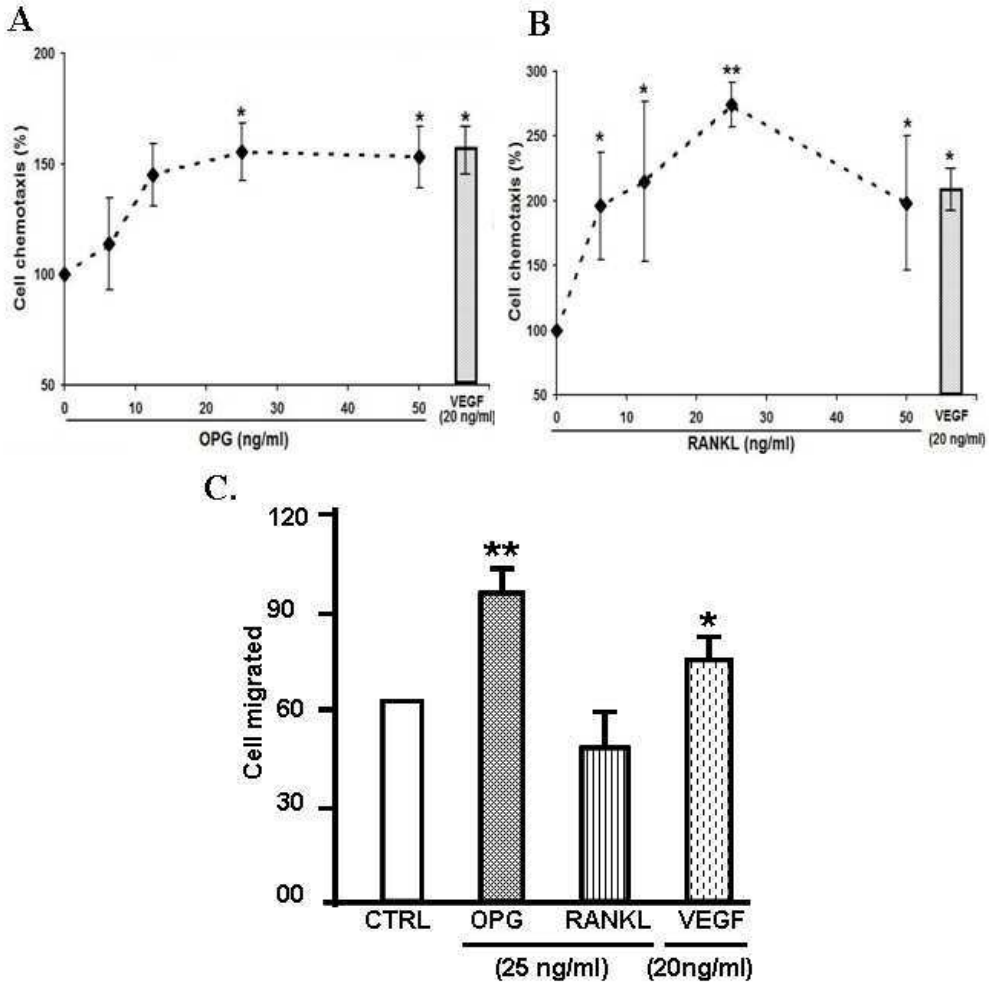

Figure 3: OPG and RANKL stimulated ECFC motility and migration. A-B: ECFC were seeded in the upper
chamber and let to migrate toward various concentrations of (A) OPG and (B) RANKL. C: Before the migration assay, ECFC were incubated with or without OPG and RANKL (25 ng/ml) for 24h. The migration assay was performed in chemotaxis chamber without any chemoattractants. Data are presented as percentages, 100% corresponding to untreated cells. Means±SEM, n=4. *:p ≤ 0.05, **:p ≤ 0.01. Positive control VEGF 20 ng/ml.
173x173mm (96 x 96 DPI)

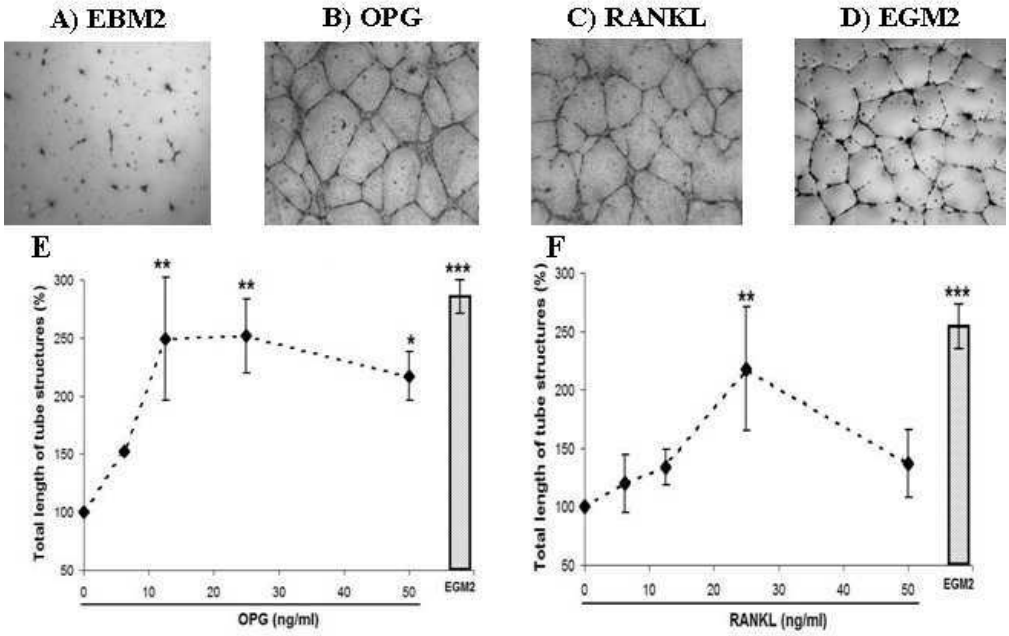

Figure 4: OPG and RANKL induce tubular morphogenesis by ECFC in Matrigel. After 24h of culture in basal
medium, in the presence or absence of various concentrations of OPG or RANKL, ECFC were immediately seeded on Matrigel in growth factor-depleted basal medium. After 18h of culture the cells were fixed and stained with Giemsa (Phase-contrast micrograph, original x20). A-D: Light micrographs showing the typical appearance of tubules formed by control and pretreated ECFC in Matrigel (G x4). A: untreated ECFC (EBM2, 5% FCS, negative control); B: OPG-treated-ECFC (25 ng/ml OPG); C: RANKL-treated-ECFC (25 ng/ml RANKL); D: ECFC in EGM2 (positive control contains proangiogenic growth factors). E-F: Comparison of the mean (± SEM, n=3) total length of tubules (% control) formed in each assay, with OPG- (E) and RANKL-pretreated ECFC (F).
*:p<0.05, **:p<0.01, ***:p<0.001.
173x113mm (96 x 96 DPI)

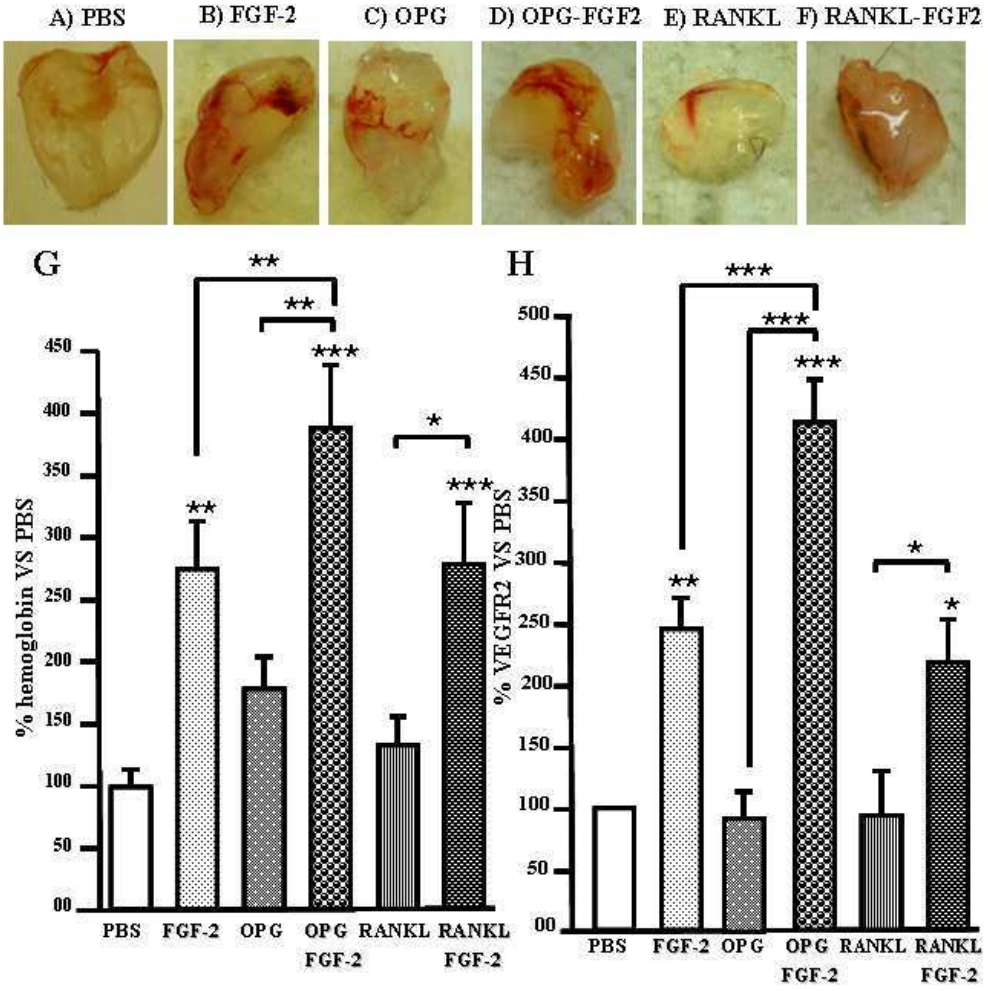

Figure 5:OPG (500 ng/ml) stimulates FGF-2-induced angiogenesis in Matrigel injected subcutaneously in
C57BL/6 mice (n=10 in each group) (G x4) (A-F). Representative photos of plugs excised at day 14 and processed for haemoglobin (G) or VEGF-R2 (H) content. (A) Plugs containing PBS (negative control); (B) Plugs containing FGF-2 (350 ng/ml, positive control); (C) Plugs containing OPG; (D) Plugs containing OPG/FGF-2; (E) Plugs containing RANKL; (F) Plugs containing RANKL/FGF-2. Means±SEM, n=3. *:p<0.05, **:p<0.01, ***:p<0.001 versus PBS.
173x173mm (96 x 96 DPI)

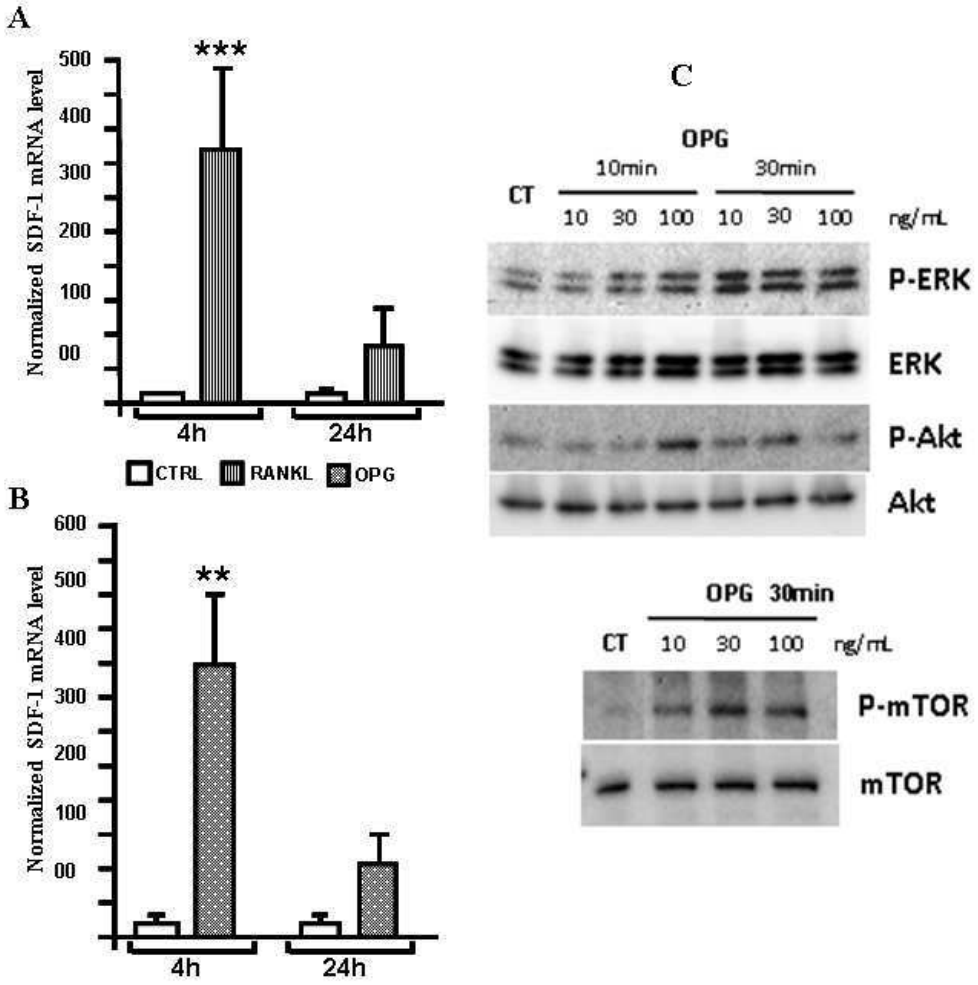

Figure 6: As determined by quantitative real time PCR, OPG (A) and RANKL (B) induce SDF-1 mRNA overexpression in ECFC. OPG activates the MEK/ERK pathway and AKT signalling pathways in a time- and dose-dependent manner as revealed by western blot analysis (C). All experiments were repeated four times and a representative blot is shown.
172x173mm (96 x 96 DPI)