The effect of nacre extract on cord blood-derived endothelial progenitor cells: A natural stimulus to promote angiogenesis?

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Abstract

Angiogenesis is a critical parameter to consider for the development of tissue-engineered bone substitutes. The challenge is to promote sufficient vascularization in the bone substitute to prevent cell death and to allow its efficient integration. The capacity of nacre extract to restore the osteogenic activity of osteoarthritis osteoblasts has already been demonstrated. However, their angiogenic potential on endothelial progenitor cells (EPCs) was not yet explored. Therefore, the current study aimed at investigating if nacreous molecules affect EPC behavior.

The gene and protein expression levels of endothelial cell-specific markers were determined in EPCs cultivated in presence of a nacre extract (Ethanol Soluble matrix (ESM) at two concentrations: 100µg/mL and 200µg/mL (respectively abbreviated ESM100 and ESM200)). Cell functionality was explored by proangiogenic factors production and i*n vitro* tube formation assay. ESM200 increased the expression of some endothelial cell-specific genes. The *in vitro* tube formation assay demonstrated that ESM200 stimulated tubulogenesis affecting angiogenic parameters. We demonstrated that a stimulation with 200µg/mL of ESM increased angiogenesis key elements.

This *in vitro* study strongly highlights the proangiogenic effect of ESM. Due to its osteogenic properties, previously demonstrated, ESM could constitute the key element to develop an ideal prevascularized bone substitute.

Keywords: ethanol soluble matrix, nacre, angiogenesis, endothelial progenitor cells, stimulus

Introduction

Bone is a highly vascularized tissue. Vessels play a pivotal role in bone remodeling allowing the maintenance of bone capital. Vascularization provides nutrients, oxygen and growth factors to bone cells. In the case of a bone injury, vascularization will bring inflammatory cells as well as other cells including mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs) and endothelial cells (ECs) which will ultimately allow bone reconstruction at the lesion site [(1)–(3)].

Unfortunately, in the case of critical-sized bone defects, the vascularization will not be able to attract enough cells to replace the affected bone. In these cases, the solution is the replacement with autologous bone graft, which is considered as the "gold standard". However, these surgeries involve important financial costs and present some significant limits such as significant post-operative morbidity, the need for an additional invasive procedure (second site), a limited availability and a resorption that is difficult to anticipate [(4),(5)]. These limitations have induced the development of bone substitutes such as allograft, xenograft and alloplastic materials [(6)–(8)]. However, these techniques produce unsatisfactory results especially regarding the lack of vascularization in the new-formed bone [(9),(10)]. It has opened the road toward the creation of an ideal prevascularized tissue engineered bone substitute.

Establishment of an efficient vascularization in bone substitute is the key element to the success of the treatment of critical-sized bone defects. Angiogenesis must be induced in order to provide sufficient blood supply to the defect site. Indeed, angiogenesis is a crucial physiological phenomenon in bone regeneration, associating complex and diverse cellular interactions, such as membrane and extracellular matrix (ECM) degradation, endothelial cells (ECs) proliferation and migration, tube formation and maturation into functional blood vessels [(11)–(13)]. Moreover, this phenomenon stimulates osteogenesis during bone healing [(14),(15)]. The development of a functional vascular network is essential for bone formation,

osteointegration and subsequently material replacement by a newly formed bone. Then, the use of a stimulus that promotes both angiogenesis and osteogenesis is indispensable to achieve efficient osteointegration of a bone substitute.

The effect of biochemical stimuli such as growth factors on stimulation of angiogenesis is well documented [16],[17]. However, limited research has addressed the effect of natural stimuli on this event.

Nacre is the internal layer of some mollusk shells. This natural and acellular « biomaterial » is known since three decades in the promotion of osteogenesis. Nacre, or mother-of-pearl, is a calcified structure, composed by 97% of aragonite crystals (calcium carbonate) and 3% of organic matrix (proteins, peptides, glycoproteins, chitin, lipids, pigments). It is found in the internal layer of many mollusk shells [18]. The main advantages of this natural osteogenic biomaterial are their properties of biocompatibility, biodegradability, tissue remodeling, cell adhesion, proliferation, differentiation and less toxicity [19]. It has been shown that nacre organic matrix contains osteoinductive compounds [20]–[22]. As reviewed by Zhang *et al.*, it has been reported by means of in vivo experiments that nacre (pieces, powder or extracts) induces a new bone formation without any inflammatory reaction or fibrous formation when it is implanted in bone environment [23]. Studies have suggested that the biodegradation of nacre progresses as induction of biomineralization when implanted in rodents, sheeps or humans [24]–[27]. It has been reported that powdered nacre was totally resorbable [6],[7],[22]. Kim *et al.* demonstrated on ovariectomised-induced osteoporotic mouse model that water-soluble nacreous factors can both stimulate osteoblastic bone formation and inhibit osteoclastic bone resorption [28]. In humans, it was shown that a six-month nacre supplementation of postmenopausal women led to a 5% increase of bone mineral density without any adverse drug reaction [29]. Molecules of the organic matrix can be extracted with aqueous or organic solvents. In our study, we chose to use the organic fraction soluble in ethanol, also called

"Ethanol Soluble Matrix" (ESM). Indeed, this nacre fraction has previously shown its ability to induce mineralization in mouse pre-osteoblastic cell line MC3T3- E1, and also the capacity to restore mineralization defect in human subchondral osteoarthritic (OA) osteoblasts [30],[31]. It is interesting to consider ESM as a source of osteoinductive compounds. Although the enhanced osteogenic potential of ESM has been previously demonstrated [30], its angiogenic potential remains unknown and needs to be explored.

Therefore, we tried to evaluate, in this *in vitro* study, the impact of ESM on the angiogenic capacity of human EPCs. EPCs are classically used as *in vitro* reference cell model to evaluate vessel formation. Indeed, they take place in angiogenesis and endothelial repair due to their abilities to differentiate into endothelial cells and to secrete protective cytokines and growth factors.

Material and methods

Extraction of Ethanol Soluble Matrix (ESM)

ESM was obtained from nacre powder of *Pinctada margaritifera* oyster shell as previously described [30]. Briefly, nacre powder (250 g) was stirred with 500 mL of ethanol absolute anhydrous containing 0.1% HCl for 24 h at 40 °C at 100 rpm by Rotavapor (Heidolph, Hei-Vap). The suspension was then centrifuged (20 min, 3000 rpm, 4°C) and filtered (0.22µm, Millipore) before being evaporated in order to obtain the nacre extract known as "Ethanol Soluble Matrix" (ESM). ESM was solubilized in culture medium and cells were treated with ESM at 100 and 200µg/mL.

Isolation and Culture of Endothelial Progenitor Cells (EPCs)

EPCs were isolated from umbilical cord blood. Consents were obtained from women who donated their cord blood (authorization number: TC/17/M/003). EPCs were extracted using a red blood cell lysis buffer containing ammonium chloride and ethylenediaminetetraacetic acid. Cells were cultured in a humidified atmosphere of 5% CO_2 and 21%O_2 at 37°C in Endothelial growth medium (EGM-2, Lonza) supplemented with 10% of fetal bovine serum (FBS). At passage 2, cells were trypsinized and plated in 6 well plates at the density of $5x10^4$ cells/cm². Three culture conditions of EPCs, for a period of 14 days, were defined: EGM-2 supplemented with 2% of FBS as control (abbreviated ESM0) and EGM-2 supplemented with 2% of FBS with either 100 or 200µg/mL of ESM (respectively abbreviated ESM100 and ESM200). Media were changed every two days.

Real-Time Quantitative Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cultured EPCs using RNeasy plus mini kit (Qiagen) according to the manufacturer's instructions. The purity of extracted RNA was evaluated using a nanodrop spectrophotometer. 500ng of total RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA) using the cDNA synthetis mix (Moloney Murine Leukemia Virus Reverse Transcriptase kit (Gibco)). Real-time PCR was carried out iTaq™ Universal SYBR® Green Supermix (BioRad) and home-designed primers coding for endothelial cell-specific genes [Platelet endothelial cell adhesion molecule-1 (PECAM-1), vascular endothelial-cadherin (VE-cadherin), tyrosine kinase with immunoglobulin and EGF homology domains (Tie2), kinase insert domain receptor (KDR) and von willebrand factor (vWF)]. Primers for the selected genes are shown in Table 1. Reactions were carried out using an StepOne system with StepOne software (Applied Biosystems, CA, USA). Cycling parameters were 15 min at 95°C (initial denaturation); 35 cycles of 10 sec at 95°C

(denaturation), 20 sec at the specific temperature of each primer (hybridization) and 15 sec at 72°C (elongation).

Gene expression levels were obtained from three different patient samples cultured in triplicate and calculated by the comparative deltaCt method ($2^{-\Delta Ct}$ formula) after being normalized to the Ct-value of the GAPDH housekeeping gene (Eurogentec).

Western Blotting

EPCs were harvested in RIPA (radioimmunoprecipitation assay buffer) lysis buffer. 15µg of protein lysates were dropped off a precast Tris-Glycine eXtended Stain Free gels (Biorad) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk in Tris buffered saline (TBS) – Tween 20 solution and then probed with primary antibodies against endothelial cell-specific markers (PECAM-1 (Dako), VE-cadherin and VEGFR-2 (Cell Signaling)) and glyceraldehyde 3-phosphate deshydrogenase (GAPDH (Santa Cruz Biotechnology) as loading control. Then, the membranes were incubated with appropriate horseradish conjugated secondary antibody. Bands were detected by chemiluminescence. A quantitative analysis was performed using Image J software [(32)].

In Vitro Tube Formation Assay

The effect of ESM on tubulogenesis was examined in tube formation assays. They were performed to assess *in vitro* angiogenesis using µ-Slide angiogenesis plates (Ibidi, Germany) and extracellular matrix gel (ECM) gel solution (ScienCell). Briefly, after 2 weeks of ESM treatment, EPCs were cultured at a density of 10^3 cells/well in µslide angiogenesis plates filled with ECM gel solution, according to the manufacturer's recommendations. They were incubated with ESM at 100 or 200µg/ml in triplicate. Capillary-like structures in each well

were captured under a light microscope. Tube formation was followed for 8 hours. Images were taken at 40x magnification. The number of junctions and segments as well as the total tube length formed tubules, were quantitated using Angiogenis Analyzer by Gilles Carpentier macro for ImageJ (NIH) (http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ) [33]. All experiments were repeated three times.

MMP2, MMP9 And PDGF Detection By Enzyme-Linked Immunosorbent Assay (ELISA)

To investigate differences in protein secretion, in presence of or without ESM, we have measured the expression levels of various bioactive proteins such as matrix metalloproteinases (MMP-2 and -9) and growth factor Platelet Derived Growth Factor-BB (PDGF-BB). They were quantified using an ELISA kit (R&D Systems) according to the manufacturer's instructions. Cultured cell supernatants were harvested at the end of the culture and tested for production of PDGF, MMP-2 and MMP-9. Data were obtained from three different experiments in tissue culture and ran in triplicate.

Statistical Analysis

Data are expressed as mean +/- SEM for each condition. Each experiment was repeated independently three times (n=3). Comparisons were performed using one-way ANOVA with the Holm-Sidak method for intergroup comparisons (GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Differences were considered significant for p values lower than 0.05.

Results

Gene expression

We have conducted qRT-PCR to evaluate ESM effects on the expression levels of endothelial cell-specific genes by EPCs (fig. 1). Gene expression levels of PECAM-1, VE-cadherin, vWF, KDR and Tie-2 were measured 14 days after culturing EPCs with ESM at 100 or 200µg/mL. EPCs cultured for 14 days without ESM were used as control.

As shown in Figure 1, gene expression levels of PECAM-1, VE-cadherin and vWF were increased around 0.5 times in presence of ESM at a concentration of 200µg/mL (ESM200) compared to the control. In contrast, there was no significant difference in presence of ESM100. Gene expression levels of KDR and Tie-2 were not modified.

Protein expression

In order to confirm gene expression results, we have investigated the expression of endothelial cell-specific proteins such as PECAM-1, VE-cadherin and VEGFR-2. As shown in figure 2, the protein levels of PECAM-1, VE-cadherin and VEGFR-2 were not significantly different after stimulation with ESM for 14 days. These results were confirmed by the quantitative analysis of band density.

Effect of ESM on EPC function

To understand the influence of ESM on EPCs, *in vitro* tube formation assay and protein secretion were measured.

In vitro tube formation assay

The potential pro-angiogenesis effect of ESM on EPCs was evaluated by a tube formation assay. We have tested whether EPCs would form pseudo-vascular structures on ECM gel by using an *in vitro* tube formation assay. As presented in Figure 3, pseudo-vascular structure

networks were observed within 8 h after seeding the cells on ECM gel. EPCs formed capillary-like structures on ECM gel in presence of basic medium, and after stimulation with ESM 100 with no significant difference. However, the formation of vascular-like networks was remarkably enhanced by ESM200 treatment. The quantitative analysis of segment numbers, segment length and number of junctions has shown that ESM200 improved twice the control results. EPCs were organized into networks exhibiting many ramifications particularly in response to ESM200 treatment consistently with the increase in the number of junctions. Thus ESM200 has a significant stimulatory effect on EPCs in regard to pseudo-vascular structure formation. With ESM100, numbers of segments and junctions were similar to the control condition. The segment length was decreased with this ESM concentration.

ELISA

To study EPC pericyte recruitment capacity, we have quantified PDGF-BB protein in cell culture supernatants. Our results have shown no effect of ESM compared to the control condition, where cells are cultured with EGM-2 medium alone. We also studied the EPC invasion capacity by investigating their secretion in culture supernatant of two metalloproteases MMP-2 and MMP-9. Our studies have revealed no significant difference between our culture conditions (fig. 4).

Discussion

For bone repair, angiogenesis is a critical process that provides the cells with nutrients and renewable autologous cells to heal the defect. Then, angiogenesis constitutes a critical point in bone tissue engineering. Indeed, the survival of the newly formed tissue depends on the vascular supply in this new bone [(8)]. Strategies have been suggested to accelerate angiogenesis [(34),(35)]. Bone tissue regeneration requires a stimulus that could favor both bone formation and

establishment of an angiogenic environment. In order to develop an ideal bone substitute, it is necessary to use a stimulus which promotes angiogenic capacity of cells. Angiogenesis begins with increased permeability of the basement membrane to favor capillary sprouting, followed by the activation, proliferation and migration of EPCs. We have chosen to evaluate angiogenic capacity of cord blood-derived EPCs because these cells are less immunogenic than peripheric blood-derived EPCs, due to their fetal origin [(36),(37)].

It is well documented that nacre can stimulate osteogenesis. For decades, marine compounds have been used in maxillofacial and orthopedic surgery. Nacre from *Pinctada* oyster has shown, since almost 30 years, its capacity to create a newly functional bone [(38)–(40)]. Then different methods to extract active compounds from nacre were investigated. First, extraction using water was tested. This fraction was called WSN (Water-Soluble Nacre). WSN has facilitated wound healing in a deep second-degree burn porcine model by accelerated angiogenesis in skin [(41)] and has induced early mineralization from mouse preosteoblasts MC3T3 [(20)]. Nacre active compounds extracted with ethanol (ESM) have already shown their efficacity on osteogenesis. Previous studies have demonstrated the ability of these extracts to restore mineralization potential of osteoarthritis osteoblasts [(30)].

However, the role of ESM in promotion or inhibition of vessel formation, including its potential effect on EPC behavior, an important parameter for optimizing bone regeneration, was never explored. In our study, we investigated the possible angiogenic effect of ESM on EPCs isolated from umbilical cord blood.

We have also investigated the expression of endothelial cell-related genes, included vWF, PECAM-1, VE-cadherin, Tie-2 and KDR by EPCs stimulated or not by different concentrations of ESM. These genes are classically present in endothelial cells and are considered as EC markers. PECAM-1 is an adhesive glycoprotein, expressed by ECs and

EPCs [42]. It is implicated in EC and EPC adhesion and migration and is essential for vessel formation and stabilization [43]. Figure 1 has highlighted that PECAM-1 expression by EPCs was higher in presence of ESM200 than that with control EGM-2, suggesting an increase in cell migration in presence of ESM200. Tie-2 is known as an angiopoietin receptor, which plays a role in the regulation of vascular network and remodeling. vWF is produced only by functional mature ECs and constitutes a marker of EC differentiation [44]. ESM200 has promoted gene expression of vWF, which could express the stimulation of EPC differentiation into mature ECs in presence of ESM200. Taken together, the expressions of angiogenic genes of ECs were stimulated in presence of ESM200. This latter concentration could provide a more suitable environment promoting angiogenesis by EPCs. We did not observe variations in VE-cadherin, PECAM-1 and VEGFR-2 expression of EPCs. It could be due to the fact that translational process can take some time, leading to differences between the gene and the protein expression [45].

The present study has demonstrated that ESM200 has the capacity to promote tubular network formation by EPCs, indicating their pro-angiogenic effects. Formation of tube-like structures by migrated EPCs is critically important in bone healing and regeneration. The *in vitro* tube formation assay has indicated a higher vessel density in ESM200 group compared to those in ESM0 or ESM100 group. The formation of capillary-like tubes has been reported as a marker of endothelial cell differentiation.

Furthermore, we have demonstrated the capacity of ESM at 200µg/mL to enhance endothelial gene expression and functionality of EPCs. The same stimulatory effect of ESM200 was already depicted in studies related to the improvement of osteogenesis capacity of osteoarthritis osteoblasts. [30].

Proangiogenic and antiangiogenic factors such as matrix metalloproteinases, growth factors and enzymes are essential to blood vessel formation and development [46]. A key step in angiogenesis is the invasion capacity of endothelial cells. This invasion is mediated by proteins which are able to destroy the ECM surrounding ECs (such as MMP2 and MMP9) and to recruit other ECs to form the pericytes keeping the wall vessel integrity (PDGF-BB). Li et al., demonstrated the ability of pearl extract to induce fibroblast migration when added to the medium [47]. Here, we have shown that ESM can stimulate the expression of migration markers (MMP2 and -9) and recruitment agent (PDFG-BB) similarly to control.

These results are promising and demonstrate an angiogenic effect of nacre active compounds from *Pinctada margaritifera* extracted with ethanol. Taking previous results into consideration, it would be interesting to investigate the effect of ESM on a coculture of EPCs and MSCs, capable of differentiation into osteoblasts in order to induce angiogenesis and osteogenesis at the same time. These two types of cells could be seeded in a composite scaffold enriched with nacre. Flausse et al. have demonstrated the efficiency of powdered nacre in stimulation of differenciation and bone matrix production of MSCs seeded in alginate hydrogel [48]. Powdered nacre was still used in an injectable form (mixed with venous blood) to fill bone defects in vertebral and maxillar sites of sheep [25]. Composite scaffolds of nacre and polymeric materials have been developed in bone tissue enginnering. Poly(D, L-lactide)/nacre nanocomposite scaffolds have been reported to repair critical size segmental bone defects in rabbits [49].

It could be interesting to encapsulate ESM in hydrogels. These hydrogels would progressively release the nacre extracts in the bone defect to obtain optimal bone regeneration. Another alternative could be to use ESM to develop three-dimensional printing scaffold [50].

Conclusion

In this study, we have evaluated the potential of ESM to promote angiogenesis. This *in vitro* study has strongly demonstrated that ESM is able to trigger pronounced angiogenic effects, even beyond EGM-2, which is considered to be an optimal culture condition for EPCs. This nacre extract interacts more efficiently with EPCs by promoting angiogenesis. A significant increase of cell differentiation and functional properties of EPC in presence of ESM200 was observed as compared to control medium. Therefore, the valuable effect of ESM on angiogenesis might be a result of an additive or synergistic action with growth factors present in EGM-2. This effect will likely be important in therapeutic applications requiring angiogenesis. Considering the potential of nacre as a source of osteogenic compounds [(30)], we suggest that ESM200 could be an attractive stimulus to develop a prevascularized bone substitute, by promoting simultaneously angiogenesis and osteogenesis, which are two major events required for an efficient bone regeneration.

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Figure legends

Fig. 1: Representative graphs showing expression of EC-specific genes: normalized fold expression against the house keeping gene GAPDH for PECAM-1, VE-Cadherin, vWF, KDR, Tie2 genes in EPC cultured with EGM-2 supplemented with either 100 (ESM100) or 200µg/mL of ESM (ESM200) or not, during 14 days. Data are shown as mean+/-standard error of the mean of three samples. * indicates a statistically significant difference ($p<0.05$) between the represented conditions and the control EGM-2

ESM0: EPCs cultured with EGM-2 medium alone, ESM100/ESM200: EPCs cultured with EGM-2 supplemented with 100 or 200µg/mL of ESM respectively.

Fig. 2: (a) Western blot analysis of endothelial protein expression level in EPCs stimulated with different concentrations of ESM or not for 14 days. (b) Quantification of endothelial protein levels (*$p<0.05$, versus control (EGM), n=3). ESM0: EPCs cultured with EGM-2 medium, ESM100/ESM200: EPCs cultured with EGM2 supplemented with 100 or 200µg/mL of ESM respectively.

Fig. 3: EPC pseudo-vascular structure formation evaluated by tube formation on extracellular matrix (ECM) gel assay. Cells were seeded on µ-Slide angiogenesis plates (Ibidi, Germany) coated with ECM gel solution (ScienCell) and incubated for 8h under the influence of medium supplemented with ESM at 100µg/ml (ESM100), or 200µg/ml (ESM200), compared to EGM-2 (used as positive control). (a) Representative pictures of EPC tube formation. (b) Graphical analysis of pictures (quantification of number of junctions, segments and total tubule length). Values were calculated with Image J software. Data are represented as mean +/-standard error of the mean from three different experiments, ran in triplicate. *$p<0.05$. Scale bar = 50µm

ESM0: EPCs cultured with EGM-2 medium alone, ESM100/ESM200: EPCs cultured with EGM-2 supplemented with 100 or 200µg/mL of ESM respectively.

Fig. 4: MMP-2, MMP-9 and PDGF-BB secretion by EPCs stimulated or not by ESM. PDGF-BB, MMP2 and 9 concentrations were measured by enzyme-linked immunosorbent assay (ELISA).

EGM: EPCs cultured with EGM-2; EGM100: EPCs cultured with EGM-2 and 100µg/mL ESM; EGM200: EPCs cultured with EGM-2 medium and 200µg/mL ESM.