**Characterization and angiogenic potential of xenogeneic bone grafting materials: Role of periodontal ligament cells**

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Adequate revascularization is a prerequisite for successful healing of periodontal bone defects. This study characterized three different xenogeneic bone grafting materials: Gen-Os of equine and porcine origins, and anorganic Bio-Oss. We also investigated their angiogenic potential. All materials were composed of poorly crystalline calcium oxide phosphate, with Bio-Oss exhibiting a carbonated phase and larger particle size and both Gen-Os showing the presence of collagen. Both Gen-Os materials significantly enhanced vascular endothelial growth factor (VEGF) secretion by PDL cells. A significant increase in endothelial cell proliferation was observed in cultures with both Gen-Os conditioned media, but not with that of Bio-Oss. Finally, angiogenesis was stimulated by both Gen-Os conditioned media as demonstrated by an increased formation of capillary-like structures. Taken together, these findings indicate an enhanced angiogenic potential of both Gen-Os bone grafting materials when applied on PDL cells, most likely by increasing VEGF production.

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**Keywords:** Characterization, Angiogenesis, VEGF, Bone substitute, Periodontal ligament cells

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**INTRODUCTION**

Bone grafting materials are frequently applied in post-extraction ridge preservation and intrabony defect regeneration. These materials provide structural and biological support for bone regeneration and allow the migration of bone forming cells into the bone defect\(^1\). Successful bone regeneration is determined by the formation and migration of blood vessels from adjacent vital bone into the bone grafting material. Indeed, failure of rapid revascularization will impair the tissue regeneration process. Not only will there be a lack of oxygen and nutrients supply, but the circulation of cells required for bone tissue formation will be hampered as well\(^2\)–\(^4\).

To stimulate revascularization, researchers have explored the addition of exogenous angiogenic growth factors, like vascular endothelial growth factor (VEGF), into the bone defect site\(^5\)–\(^6\). Although promising, there are several drawbacks with this approach. Due to the complexity of the *in vivo* environment and the short half-lives of growth factors, the natural spatio-temporal release profile of growth factors cannot be easily simulated by exogenous delivery. Often, supra-physiological doses are administered with possible adverse effects\(^5\)–\(^7\). Furthermore, recombinant protein costs represent a significant financial burden for the patient\(^9\).

Interestingly, it has been shown that various bone grafting materials, successfully used in clinic, have a high angiogenic potential. For instance, various *in vitro* studies showed increased VEGF and fibroblast growth factor (FGF-2) secretion by fibroblasts, after direct and indirect contact with 45S5 Bioglass\(^8\) particles, which was associated with increased endothelial cell proliferation and formation of a tubular network\(^9\)–\(^10\). A clinical study demonstrated increased angiogenesis following the application of a nanocrystalline hydroxyapatite paste in post-extractive sockets\(^10\).

This suggests that the use of bone grafting materials with an angiogenic potential for treatment of periodontal bone defects can improve the clinical outcome. This study characterized three commonly used xenogeneic bone grafting materials in implantology and periodontics: Gen-Os cortico-cancellous collagenated bone of equine and porcine origins and Bio-Oss cancellous anorganic bovine bone, and evaluated their angiogenic potential. The rational beyond this study is that, in cases such as alveolar bone resorption after tooth loss and osseous periodontal lesions, the bone grafting material is not only in intimate contact with bone tissue but also with the periodontal ligament (Fig. 1). It is hypothesized that the material may influence periodontal ligament (PDL) cell angiogenic activity, which is a prerequisite for successful bone regeneration. In clinic, some PDL cells are subjected to the bone grafting material directly. However, the major part of PDL cells are subjected to extracts of these materials. This *in vitro* work simulates this clinical situation by subjecting PDL cells to extracts of the three bone grafting materials. This is followed by measurement of VEGF secretion, the most essential growth factor in...
the angiogenic process\textsuperscript{14}. The influence on angiogenesis is further studied by evaluating endothelial cell proliferation and the formation of closed capillary-like structures reflecting the angiogenic capacity in vivo.

**MATERIALS AND METHODS**

Three different xenogeneic bone-grafting materials were assessed (Table 1 and Fig. 2):

- Bio-Oss cancellous anorganic bovine bone grafting material (Geistlich Pharma, Wolhusen, CH).
- Gen-Os cortico-cancellous collagenated bone grafting material of equine origin (OsteoBiol\textsuperscript{a}, Tecnoss\textsuperscript{d}, Giaveno, Italy).
- Gen-Os cortico-cancellous collagenated bone grafting material of porcine origin (OsteoBiol\textsuperscript{a}, Tecnoss\textsuperscript{d}).

**Material characterization**

The three bone grafting materials were characterized by scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), X-ray diffraction (XRD) analyses and Fourier transform infrared (FT-IR) spectroscopy.

Powders sprinkled on carbon tape on an aluminum stub were viewed under the scanning electron microscope (Zeiss MERLIN Field Emission SEM, Carl Zeiss NTS, Oberkochen, Germany) in secondary electron mode at various magnifications. Energy dispersive spectroscopic (EDS) analysis was also performed. Phase analysis was performed by X-ray diffraction. The diffractometer (Bruker D8 Advance, Bruker, Billerica, MA, USA) used Cu Ka radiation at 40 mA and 45 kV and the detector was rotated between 10–60° with a step of 0.02°/0 and a step time of 0.6 degrees/min. The sample holder was spun at 15 rpm. Phase identification was accomplished using search-match software utilizing ICDD database.

**Table 1** Overview of used bone grafting materials (manufacturers’ data)

| Material/Reference | Manufacturer | Origin    | Particle granulometry (µm) |
|--------------------|--------------|----------|---------------------------|
| Bio-Oss/30643.3    | Geistlich    | Bovine   | 250–1,000                 |
| Gen-Os/M1005FE     | Tecnoss      | Equine   | 250–1,000                 |
| Gen-Os/M1005FS     | Tecnoss      | Porcine  | 250–1,000                 |
The dental ligament was minced, and explants were prepared from sound third molars freshly extracted for orthodontics reasons in compliance with French legislation. The extirpated molars were cultured in 100-mm-diameter culture dishes. Confluent cultures were subcultured in minimum essential medium (MEM) supplemented with 1% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Dominique Dutcher, Brumath, France). The endothelial cell organization was observed using a phase-contrast microscope (Carl Zeiss Axiovert200) (Carl Zeiss S.A.S., Marly Le Roi, France) over a three days period. Angiogenic capacity was quantitatively evaluated using ImageJ software by measuring the tubular perimeters.

Statistics

All biological experiments were repeated at least three times, and statistical significance (p<0.05) was determined using the Student t-test to compare the different treatments and their respective controls. Data are expressed as means±standard deviation.

RESULTS

Material characterization

The secondary electron scanning electron micrographs of the three bone grafting materials are seen in Fig. 3. The low power images gave an indication of the particle sizes and shapes of the materials while the higher power images demonstrated the surface morphology. The particle sizes and shapes of the materials varied with the Gen-Os variants having smaller particles sizes and were not as angular when compared to Bio-Oss. The surface textures of the different materials also varied.

All materials were composed of calcium and phosphorus with traces of sodium and magnesium, as calculated from the EDS analyses. Bio-Oss had a lower calcium to phosphate ratio when compared to both porcine and equine Gen-Os (Table 2).

Effect of bone grafting materials on VEGF secretion by PDL cells

Conditioned media were prepared by cultivating PDL cells at 30,000 cells/cm² in 6-well plates, in 1 mL of the extracts. After 24 h or 3 days, the supernatants were collected for VEGF quantification using the DuoSet ELISA kit according to the manufacturer’s instructions (R&D Sytems, Lille, France). Supernatants from cells cultured in the same medium but without any prior contact with the materials were used as control.

Effect of bone grafting materials on HUVEC proliferation and neo-angiogenesis

For the assessment of HUVEC proliferation and angiogenesis, conditioned media were prepared according to the same procedure as described above, in serum-free and growth factor free EBM-2 medium, and for a contact period of 3 days with the PDL cell cultures. For evaluation of HUVEC proliferation, the cells were cultured at a low density (1,000 cells/cm²) in 96-well culture plates. Conditioned media were added onto the cells and proliferation was evaluated after 2, 5 and 7 days using the succinyl dehydrogenase assay (MTT test) as previously described. MTT results are expressed as percentage cellular viability of the cells cultured in the control medium.
Table 2  Calcium to phosphate ratios of test materials

| Material       | Ca/p ratio |
|----------------|------------|
| Gen-Os Porcine | 2.15       |
| Gen-Os Equine  | 2.07       |
| Bio-Oss        | 1.63       |

also gives bands at 3,450 and 1,450 cm\(^{-1}\) from OH stretching vibrations.

**Powder granulometry**

The plots of volume percentage against particle size for the three materials are shown in Fig. 5. The data on particle granulometry as provided by the manufacturer is listed in Table 1. Bio-Oss showed a larger particle size, which verifies the scanning electron microscopy measurements. All the materials exhibited an average particle size distribution between 100–275 µm. The Gen-Os variants had bimodal particle size distribution with particles in the range of 20–80 µm also present.

**Effect of bone grafting materials on VEGF secretion by PDL cells**

All three bone grafting materials significantly increased VEGF secretion by PDL cells after a contact period of 1 and 3 days (Fig. 6). Compared to basal VEGF secretion (755 pg/mL), this increase was about two-fold higher with
Bio-Oss and about three-fold higher with Gen-Os from porcine and equine origins. Moreover, VEGF secretion was significantly higher with Gen-Os from porcine and equine origins as compared to Bio-Oss.

**Effect of bone grafting materials on HUVEC proliferation and neo-angiogenesis**

Conditioned media, obtained after a contact period of 3 days with Gen-Os from porcine and equine origins, significantly enhanced endothelial cell proliferation after 2, 5 and 7 days (Fig. 7). A decline in proliferation was noted after 7 days compared to 2 and 5 days, which can be explained by the cells reaching confluency, decreasing their metabolism and thus the rate of MTT reduction to produce formazan\(^{17}\). Bio-Oss conditioned medium stimulated endothelial cell proliferation significantly after 2 days, but this effect was abolished after 5 and 7 days of contact where cell viability dropped significantly.
Effect of bone grafting materials on neo-angiogenesis in vitro.

When endothelial cells were incubated in conditioned EBM-2 media obtained from three-day PDL cell cultures, they organized into closed structures and formed a capillary-like network. Conditioned medium obtained from PDL cells exposed to serum-free EBM-2 medium for three days was used as control. (a) Representative microscopic images for perimeter quantification in ImageJ software (scale bar=50 µm). (b) Bar graph of the mean tubular perimeters with error bars presenting standard deviation. Asterisk corresponds to a $p<0.05$.

to 66 and 55%, respectively, of the control condition.

After 3 days of culture on Matrigel, the formation of capillary-like closed structures of well-organized endothelial cells was observed with all bone grafting materials and control (Fig. 8). Measurement of the tubular perimeters demonstrated that conditioned media with Gen-Os from porcine and equine origin significantly enhanced the perimeter of the closed structures as compared to the control and Bio-Oss. The tubular perimeters with Bio-Oss were similar to the control.

DISCUSSION

Three bone grafting materials commonly used in the treatment of periodontal bone defects were evaluated in vitro for their angiogenic potential when applied on PDL cells. The rational beyond this work is that some clinical applications of bone grafting materials involve an intimate contact between the material and the periodontal ligament (Fig. 1), and it is hypothesized that this contact may enhance angiogenesis which is essential for the bone regeneration process. Angiogenesis, which is the sprouting of new blood vessels from existing ones, involves endothelial cell stimulation by multiple angiogenic factors such as VEGF, proliferation of endothelial cells and structural rearrangement of these cells to form new well-organized blood vessels.

The materials were characterized using a combination of techniques namely SEM and EDS, which gave an indication of particle sizing and shapes. The EDS analysis revealed the elements present within the materials and their ratios. The XRD analysis showed the material crystallinity and the phases present while FT-IR spectroscopy showed the different chemistry. In this way, the precise material chemistry was determined which contributes to the interpretation of the materials' effects on cellular behavior.

Our results clearly indicate a significant increase in VEGF secretion and a higher angiogenic potential of both Gen-Os materials from porcine and equine origins, compared to Bio-Oss. Indeed, VEGF secretion by PDL cells increased when cultured in Gen-Os conditioned media. This was associated with increased endothelial cell proliferation and formation of capillary-like structures. The formation of these structures reflects angiogenesis in vivo. Indeed, angiogenic capacity can be evaluated in vitro by the Matrigel differentiation assay. With this assay, the formation of capillary-like structures by endothelial cells in response to the biomaterials can be quantitated by counting the number of tubular structures, branching and measurement of tubular perimeters. In this study, tubular perimeters are used as measure of angiogenic potential together with assessment of endothelial cell proliferation.

Bio-Oss conditioned medium, on the other hand, induced lower VEGF secretion and less capillary-like structure formation. FT-IR spectroscopy revealed that Bio-Oss exhibits higher incorporation of carbonate in the apatite. Previous works have shown that hydroxyapatite carbonation was associated with lower VEGF secretion as demonstrated with osteoblast-like MG63 cells exposed to experimental carbonated hydroxyapatite compared to pure hydroxyapatite. Furthermore, a possible toxic effect of Bio-Oss was observed in our study since endothelial cell proliferation was significantly decreased after 5 and 7 days of culture. This result is in line with
the study by Zimmerman and coworkers which reported a toxic effect of Bio-Oss eluates on porcine mesenchymal stem cells likely due to toxic substances eluted from the Bio-Oss material during extract preparation 26.

The clinical effectiveness of Bio-Oss for periodontal intrabony defect filling and post extraction ridge preservation has been demonstrated 27, 28, but it should be noted that various studies utilized Bio-Oss Collagen 25, 26, as opposed to Bio-Oss without collagen used in this study. For both Gen-Os materials, clinical success has been obtained as well 27, 28, and seems to be related to the presence of collagen. The unique manufacturing procedure of Gen-Os materials at low temperature allows the preservation of the natural collagen matrix 29.

The presence of collagen in both Gen-Os materials was confirmed by FT-IR spectroscopy. It has been suggested that the presence of collagen creates a favorable environment for bone regeneration by promoting vascular ingrowth after direct contact between endothelial cells and collagen of the grafting material 30. Indeed, the specific interaction between integrin and the collagenous amino acid sequence 496–507 on the α1(I) chain, allows direct endothelial cell attachment on the material 31.

The results obtained in this work are based on indirect contact between cell cultures and bone grafting materials. Indeed, extracts prepared from the bone grafting materials were utilized to understand the consequences of the application of a bone grafting material on PDL cells in vitro. This work allows illustrating the early molecular and cellular mechanisms underlying the effects observed in clinical studies. As such, this study on the angiogenic potential provides an additional partial explanation for the clinical success observed with Gen-Os materials. Indeed, enhanced VEGF secretion by PDL cells will promote rapid vascularization, which is essential for successful regeneration of the bone tissue in the grafted periodontal defect. Although it remains a short-term in vitro study which cannot fully explain the long-term clinical success of these bone grafting materials, our findings provide an interesting lead to be further explored on the in vivo level.

CONCLUSION

Within the limits of this in vitro study, it was demonstrated that both Gen-Os materials have a higher angiogenic potential compared to Bio-Oss. Both Gen-Os materials have the ability to induce VEGF secretion by PDL cells which is an interesting feature since it mitigates the need for exogenous growth factor delivery. Furthermore, angiogenesis was induced which suggests that the use of Gen-Os materials will favor the bone regeneration process by stimulating early revascularization within the grafted material.

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REFERENCES

1) Liu J, Kerns DG. Mechanisms of guided bone regeneration: a review. Open Dent J 2014; 8: 56-65.
2) Suran U, Gemini Piperni S, Chatterjee S. Role of angiogenesis in bone repair. Arch Biochem Biophys 2014; 561: 109-117.
3) Stegen S, van Gastel N, Carmeliet G. Brining new life to damaged bone: the importance of angiogenesis in bone repair and regeneration. Bone 2015; 70: 19-27.
4) Novosel EC, Kleinhans C, Kluger PJ. Vascularization is the key challenge in tissue engineering. Adv Drug Deliv Rev 2011; 63: 300-311.
5) Gothard D, Smith EL, Kanczler JM, Rashidi H, Qutachi O, Henstock J, Rotheram M, El Haj A, Shakesheff KM, Orefio RO. Tissue engineered bone using select growth factors: A comprehensive review of animal studies and clinical translation studies in man. Eur Cell Mater 2014; 28: 166-207.
6) Du B, Liu W, Deng Y, Li S, Liu X, Gao Y, Zhou L. Angiogenesis and bone regeneration of porous nano-hydroxyapatite/coralline blocks coated with rhVEGF165 in critical-size alveolar bone defects in vivo. Int J Nanomed 2015; 10: 2555-2565.
7) Aryal R, Chen X, Fang C, Hu Y. Bone morphogenetic protein-2 and vascular endothelial growth factor in bone tissue regeneration: new insights and perspectives. Orthop Surg 2014; 6: 171-178.
8) Roux BM, Cheng M-H, Brey EM. Engineering clinically relevant volumes of vascularized bone. J Cell Mol Med 2015; 19: 903-914.
9) Gerhardt L-C, Boccaccini AR. Bioactive glass and glass-ceramic scaffolds for bone tissue engineering. Materials 2010; 3: 3867-3910.
10) Gorustovich AA, Roether JA, Boccaccini AR. Effect of bioactive glasses on angiogenesis: a review of in vitro and in vivo evidences. Tissue Eng Part B Rev 2010; 16: 199-207.
11) Detsch R, Stoor P, Grünwald A, Roether JA, Lindfors NC, Boccaccini AR. Increase in VEGF secretion from human fibroblast cells by bioactive glass S53P4 to stimulate angiogenesis in bone. J Biomed Mater Res A 2014; 102: 4055-4061.
12) Day RM. Bioactive glass stimulates the secretion of angiogenic growth factors and angiogenesis in vitro. Tissue Eng 2005; 11: 768-777.
13) Canuto RA, Pol R, Martinasso G, Muzio G, Gallesio G, Mozzati M. Hydroxyapatite paste Ostim, without elevation of full-thickness flaps, improves alveolar healing stimulating BMP- and VEGF-mediated signal pathways: an experimental study in humans. Clin Oral Implants Res 2013; 24 Suppl A: 42-48.
14) Ferrara N. Vascular endothelial growth factor. Eur J Cancer 1996; 32A: 2413-2422.
15) Mathieu S, Jeannneau C, Sheibath-Othman N, Kalaji N, Fossi H, About I. Usefulness of controlled release of growth factors in investigating the early events of dentin-pulp regeneration. J Endod 2013; 39: 228-235.
16) Schneider CA, Rashband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012; 9: 671-675.
17) Riss TL, Moravec RA, Niles AL, Benink HA, Worzella TJ, Minor L. Cell Viability Assays. Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2013.

18) Folkman J, Shing Y. Angiogenesis. J Biol Chem 1992; 267: 10931-10934.

19) Arnaoutova I, George J, Kleinman HK, Benton G. The endothelial cell tube formation assay on basement membrane turns 20: state of the science and the art. Angiogenesis 2009; 12: 267-274.

20) Tran-Hung L, Mathieu S, About I. Role of human pulp fibroblasts in angiogenesis. J Dent Res 2006; 85: 819-823.

21) Salani D, T这些问题的解码可以纯文本形式表示。

22) Adams BR, Mostafa A, Schwartz Z, Boyan BD. Osteoblast response to nanocrystalline calcium hydroxyapatite depends on carbonate content. J Biomed Mater Res A 2014; 102: 3237-3242.

23) Zimmermann CE, Gierloff M, Hedderich J, Açil Y, Wiltfang J, Terheyden H. Biocompatibility of bone graft substitutes: effects on survival and proliferation of porcine multilineage stem cells in vitro. Folia Morphol (Warsz) 2011; 70: 154-160.

24) Carmagnola D, Adriaens P, Berglundh T. Healing of human extraction sockets filled with Bio-Oss. Clin Oral Implants Res 2003; 14: 137-143.

25) Sweeney SM, DiLullo G, Slater SJ, Martinez J, Iozzo RV, Lauer-Fields JL, Fields GB, San Antonio JD. Angiogenesis in collagen I requires α2β1 ligation of a GFP*GER sequence and possibly p38 MAPK activation and focal adhesion disassembly. J Biol Chem 2003; 278: 30516-30524.