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Resorption pattern of a porcine-derived bone substitute

Keywords bone substitutes; confocal microscopy; porcine bone; osteoclasts; resorption.

ABSTRACT

Background Resorption of a graft material could be a relevant issue to drive the choice of a surgeon for the selection of the best clinical option to fulfil the needs of a given clinical situation. The aim of the present study was to evaluate the in vitro formation, adhesion and morphology of human osteoclasts (OCLs) generated on a porcine-derived bone substitute (OsteoBiol Apatos Sp-Block, Tecnoss, Coazze (TO), Italy).

Materials and Methods Peripheral blood mononuclear cells from healthy volunteers were used to generate OCLs in vitro in the presence of macrophage colony stimulating factor (M-CSF) and receptor activator of NF-kB ligand (RANKL) on bovine bone (positive control) and porcine bone slices. Morphological and biochemical methods were used to assess OCLs formation and activity.

Results Cells generated after 21 days of culture on the porcine bone substitute showed morphologic features resembling those on the positive control and displayed typical OCL markers. These findings indicate that the porcine-derived biomaterial supported OCL formation. With regard to the resorption, on porcine bone OCLs formed smaller discontinuous lacunae.

Conclusions This study demonstrates that the porcine-derived bone substitute undergoes a cell-mediated resorption process.

INTRODUCTION

Resorption of a graft material could be a relevant issue to drive the choice of a surgeon for the selection of the best clinical option to fulfil the needs of a given clinical situation. However, while graft material characteristics relevant for bone formation have been extensively investigated, little is known about bioresorption (1-5).

The bone resorption cascade involves a series of steps, which lead to the removal of both the mineral and organic phases of bone matrix by osteoclasts (OCLs), aided by osteoblasts (OBLs). The role of the OCL as a major resorbing cell, and its structure and biochemical properties have been well characterized (6). The first stage of the bone resorption cascade involves the recruitment of OCL progenitors to bone. The OCL precursors originate from haemopoietic cells (7) related to the monocyte-macrophage lineage (8). The progenitor cells are recruited from the haematopoietic tissues such as bone marrow and splenic tissues to bone via the circulating blood stream. They proliferate and differentiate into OCLs through a mechanism based on cell-to-cell interac-

tion with OBL (9). The next step involves the removal of the unmineralized osteoid layer by the lining OBLs, and thus the production of a variety of proteolytic enzymes such as metalloproteinases, collagenases and gelatinases (10). This process promotes the adhesion of the OCL to the underlying mineralized bone, which occurs via the integrin superfamily of adhesion receptors; specifically, an important functional role in bone resorption is carried out by the $\alpha_v\beta_3$ subunit of vitronectin receptor (11,12). The latter process involves the formation of ruffled borders and clear zones, and finally, OCL activation at the surface of the mineralized bone.

The physiologic bone resorption cascade is very closely mimicked by bone substitute materials which undergo a cell-mediated resorption process (13). A variety of bone grafts are currently used for reconstruction of bone defects (1,14-17). The guidance provided by biomaterials may facilitate restoration of structure and function of damaged or dysfunctional tissues, both in cell-based therapies, such as those where carriers deliver transplanted cells or matrices induce morphogenesis in bioengineered tissues constructed ex vivo, and in acellular therapies, such as those where materials induce ingrowth and differentiation of cells from healthy residual tissues in situ. Such materials should provide a provisional three-dimensional (3-D) mechanic support to interact biomolecularly with cells to control their function, guiding the spatially and temporally complex multicellular processes of tissue formation and regeneration (18). Virtually, these bone substitute materials are intended to degrade slowly after implantation in the patient and to be replaced by new bone tissue (3,19).

Among biomaterials, xenografts are very popular (20, 21), especially for sinus elevation procedures. They are highly attractive as these natural materials show great osteoconductive properties due to their chemical-physical characteristics similar to those of human bone (22, 23). The most common source of xenograft for bone replacement is bovine bone; as an alternative, materials of porcine origin have been used (24, 25). To our best knowledge, there are very few studies on in vitro bioresorption of xenografts (4.) It is only known that xenogenic materials are very slowly resorbed, because they are found in the augmented sites many years after implantation (23, 26). The baseline hypothesis connected to the usage of xenografts is the assumption of temporary structural support, integration in the surrounding bone, bioresorption and replacement with vital bone. However, while the graft material's characteristics relevant for osteoconduction and bone formation have been extensively examined, their bioresorption process has not been much investigated (1-5). A better understanding of the degradation of a bone substitute material caused by OCLs could help to clarify the cells-substrate interaction and the process of bioremodelling due to the coupling of osteoclasts and osteoblasts activity (2, 27).

The aim of the present study was to evaluate OCL formation, adhesion, morphology and resorptive activity on a new xenogenous bone grafting material of porcine origin.

MATERIALS AND METHODS

Bovine bone and porcine bone preparation

Bovine bone and porcine bone blocks (12x12x21 mm) (OsteoBiol Apatos Sp-Block, Tecnoss, Coazze (TO), Italy) were cut into slices at a thickness of approximately 200µm using a Buehler low speed saw

equipped with a diamond wafer blade (Buehler Isomet, Lake Bluff, IL, USA). The slices were cut to small squares, processed through washes in distilled water (d-H_2O), sonicated, then further processed through washes in d-H_2O and acetone prior to sterilization in 70% ethanol. Substrates were dried, labelled and then re-sterilized using ethanol.

Generation of human OCLs from peripheral blood mononuclear cells (PBMCs)

Human peripheral blood was obtained from healthy adult volunteers in syringes containing 1000 U/ml of preservative-free heparin. Blood was diluted 1:1 in phosphate buffered saline (PBS) (Oxoid Ltd., Basingstoke, UK) containing 2mM glutamine (G), 100 IU/ml benzyl penicillin and 100mg/ml streptomycin (P/S) (Invitrogen, Gibco, Paisley, Scotland), gently layered over Ficoll-Plaque (Sigma-Aldrich, Poole, UK) and centrifuged. The lymphocyte fractions of mononuclear cells were collected into tubes containing PBS, centrifuged again and resuspended in Minimal Essential Medium Eagle (MEME) (Sigma-Aldrich, Poole, UK), supplemented with 15% heat-inactivated fetal calf serum (Sera Lab, Crawley, UK), G and P/S (concentrations as above). Cells were then seeded at a concentration of 3 x 10^5 cells on bovine bone and porcine bone slices in 96 multi-well plates, incubated for 4 h in 5.0% CO_2, 95% air at 37°C. Unattached cells were removed by washing the substrates in PBS and then transferring them to a new culture dish where the cells attached were then cultured in MEME supplemented as above in the presence of 25ng/ml of human macrophage colony stimulating factor (M-CSF) (Genetic Institute, Cambridge, MA) for three days in 5.0% CO_2, 95% air at 37°C. Cultures were fed every 3-4 days by replacing half of the medium with fresh medium containing M-CSF and 30ng/100µl of receptor activator of NF-KB ligand (sRANKL) (Amigen, CA, USA), whilst collecting supernatants for ELISA analysis.

At day 21, cells were fixed and processed for confocal, light and reflection microscopy analysis. All experiments were performed in quintuplicate and repeated four times using cells from different volunteers. Bovine bone was used as positive control, and both substrates, cultured under the same conditions without cells, were used as negative controls to monitor the occurrence of non-cell mediated hydrolytic degradation.

Immunofluorescence for confocal laser scanning microscopy

Cells on the substrates were fixed in a 40:60 mixture of MEME with fixation buffer (3.5% paraformaldehyde and 2% sucrose in PBS), rinsed in PBS and then placed in ice cold permeabilizing buffer (20mM HEPES, 300mM sucrose, 50mM NaCl, 3mM MgCl2, 0.5% Triton X-100 and 0.5% sodium azide in PBS), before being rinsed again in PBS. Substrates were incubated with monoclonal antibodies to human $\alpha_v\beta_3$ subunit of the vitronectin receptor (VNR) (supplied by Prof. Michael Horton, UCL, UK). The antibodies were recognized by applying either rabbit anti-mouse conjugated to fluorescein isothiocyanate (FITC) (Dako, Glostrup, Denmark) secondary antibody. Finally, they were incubated with rhodamine-phalloidin (Molecular Probes Inc., Eugene, OR) to stain for actin and then examined by scanning laser confocal microscopy on a Leica TCS NT System (Heidelberg, Germany). Fluorescent images were collected in sequential 1µm steps through the OCLs for FITC and TRITC fluorochromes at 500 and 575 emission wavelengths. OCLs were defined as multin-

ucleated cells expressing VNR ($\alpha_v\beta_3$) and F-actin rings or patches. Images were displayed in XY orientation plane.

Staining for light and reflection microscopy

OCL formation was evaluated by the presence of tartrate-resistant acid phosphatase (TRAP)-positive cells. At day 21 the substrates were washed in d-H_2O, fixed in 10% neutral buffered formalin, further washed in d-H_2O before being histochemically stained for TRAP, according to the manufacturer's protocol (Sigma-Aldrich, Poole, UK; KIT 387-A). TRAP positive cells, containing three or more nuclei, were examined and imaged by light microscopy. Preparations were then counterstained using 0.1% toluidine blue to assess the presence of resorption pits using reflective microscopy.

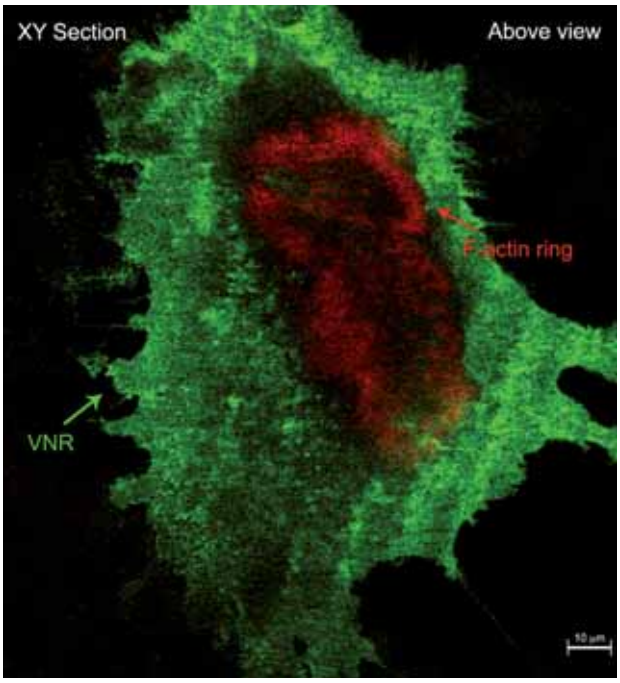

Fig. 1 High power confocal microscopy xy view of an osteoclast. Green shows positive staining for monoclonal antibody 23C6 to human $\alpha_v\beta_3$ complex of vitronectin receptor. Red shows F-actin enriched patches and rings found in activated osteoclasts. Mgnification x630. Bar = 10µm.

RESULTS

Cell generated were large and showed microvilli and filopodia (Fig. 1). Their size and appearance did not differ particularly between test and control samples, although features of actively-resorbing osteoclasts tend to be slightly different. A lower yield of TRAP-positive cell was produced on porcinee bone samples (Fig. 2); therefore, resorptive activity appears to be decreased in the presence of porcine bone as compared to the control group. Cells generated on control samples produced typical lobulated zig-zag track-like resorption lacunae (Fig. 3a), while cells generated on porcine bone formed small discontinuous island-like resorption lacunae (Fig. 3b).

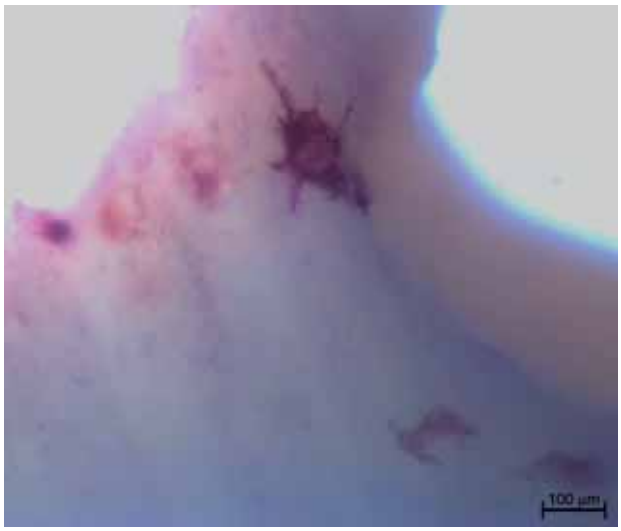

Fig. 2 Light microscopy image of osteoclasts generated from peripheral blood mononuclear cells on porcine bone substitute at day 21 of culture. Red indicates a TRAP+ cell. Magnification x200. Bar = 100µm.

DISCUSSION

Investigating the degradation of xenografts is one way of determining a desirable

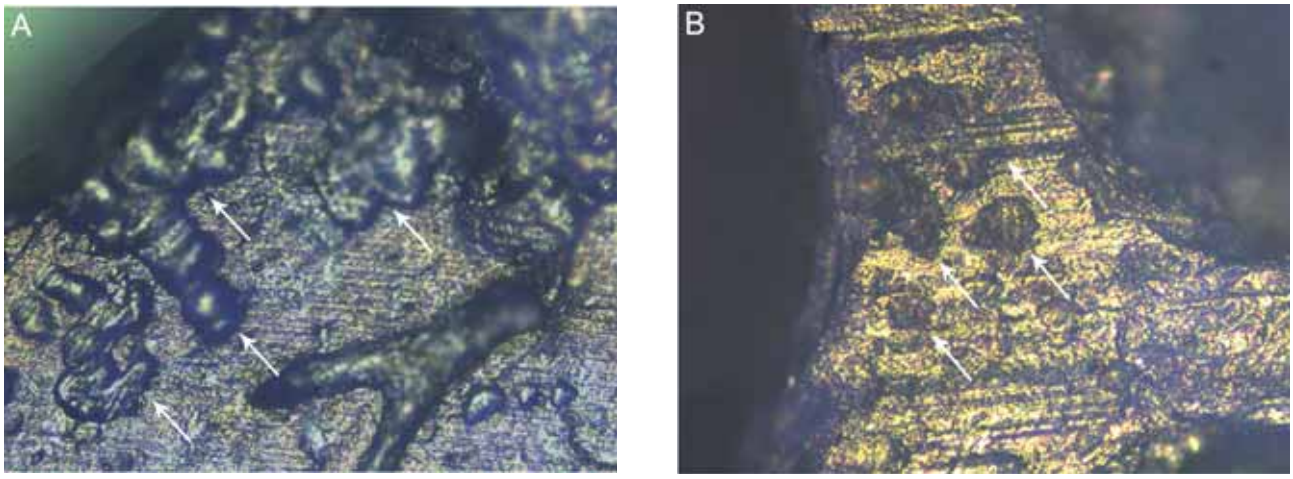

Fig. 3 Reflection microscopy images of osteoclasts generated from peripheral blood mononuclear cells on control (a) and porcine bone substitute (b) at day 21 of culture. Resorption pits (white arrows) are present on both substrates, though on porcine discontinuous resorption is evident, while on control track-like lacunae are present. Toluidine blue staining. Magnification x200.

material with appropriate characteristics to fulfil clinical needs. However, the properties of xenografts tend to vary depending on the preparation. Consequently, interpretation of data obtained in different studies is often contradictory. In an effort to prevent this confusion and provide a clear comparison of osteclast resorption activity, xenografts have to be individually analyzed.

The present study clearly shows that OCLs resorbed porcine bone. However, osteoclastic resorption did not always become as extensive as on positive control. This has already been shown in studies on in vitro OCL resorption of bone substitute (4,13). Taylor et al. (4) examined the resorptive behaviour of neonatal rabbit OCLs grown on bovine cortical bone as compared to samples of commercially available biomaterials (bone-derived, synthetic hydroxyapatite (HA), synthetic non-HA). They concluded that bone-derived materials demonstrated a greater OCL attachment, spreading and resorptive activity than was evident on synthetic hydroxyapatite (HA), synthetic non-HA. However, deproteinated and demineralized samples groups exhibited cells in lower numbers and of less typical morphology than on control groups. Perrotti et al. (13) investigated the in vitro resorption potential of human OCLs on a xenogenous bone mineral (XBM). By using morphologic and biochemical methods, they observed that OCL formation occurred on XBM and these cells were positive for the major OCL markers. Regarding OCL activity, resorption pits were detected on XBM by reflection and confocal microscopy. However, biochemical analysis revealed that collagen degradation was significantly lower in XBM supernatants when compared to control, suggesting that XBM underwent a much slower resorption over time.

In our conditions, osteoclasts produced typical lobulated track-like resorption lacunae resembling those ordinarily formed on bone, while on the porcine substrate the resorption pattern was characterized by discontinuous resorption lacunae, easily distinguishable from the non-resorbed surface, but still different from the natural ones. This resorption pattern has been observed on other biomaterials of ceramic

or animal origin, such as ,-TCP (2), synthetic HA (4) and XBM (13). Many factors have been proposed as regulators of osteoclastic activity either directly or via osteoblasts. Particularly important is the microenvironment of the OCL-substratum interface, since the OCL cell membrane must be sealed to the substrate by means of cell surface receptors and proteins of the integrin family (28). This mechanism is not impaired on the porcine substrate investigated in the present study since expression of vitronectin receptor, one member of the integrin superfamily with a major role in mediating OCL attachment to bone matrix, was detected. Therefore, the discontinuity of OCL resorptive activity on such substrate could be due to its complex three-dimensional structure characterized by voids and peaks, which could represent an obstacle for a continual resorption.

In conclusion, this study represents the first in vitro evidence of generation of human OCLs on this bone substitute of porcine origin. From our data, differentiation of human OCLs and resorption of the material occurred, though to a lower extent than for native bone.

Such findings can be useful for clinicians to tailor the usage of this porcine bone substitute materials to clinical situations where a material with such characteristics is needed.

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