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Tissue engineering of bone: search for a better scaffold

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Structured Abstract

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Background – Large bone defects still represent a major problem in orthopedics. Traditional bone-repair treatments can be divided into two groups: the bone transport (Ilizarov technology) and the graft transplant (autologous or allogeneic bone grafts). Thus far, none of these strategies have proven to be always resolving. As an alternative, a tissue engineering approach has been proposed where osteogenic cells, bioceramic scaffolds, growth factors and physical forces concur to the bone defect repair. Different sources of osteoprogenitor cells have been suggested, bone marrow stromal cells (BMSC) being in most cases the first choice.

Methods and Results – In association with mineral tridimensional scaffolds, BMSC form a primary bone tissue which is highly vascularized and colonized by host hemopoietic marrow. The chemical composition of the scaffold is crucial for the osteoconductive properties and the resorbability of the material. In addition, scaffolds should have an internal structure permissive for vascular invasion. Porous bioceramics [hydroxyapatite (HA) and tricalcium phosphate] are osteoconductive and are particularly advantageous for bone tissue engineering application as they induce neither an immune nor an inflammatory response in the implanted host. Earlier, we first reported a cell-based tissue engineering procedure to treat three patients with long bone segmental defects. Cells were loaded on a 100% HA porous ceramic. These scaffolds proved to have good osteoconductive properties resulting in a good functional recovery, but they have not been resorbed after more than 5 years from the implant. In addition, due to the high density of the mineral and the relatively low porosity (50–60%), it was very difficult to monitor the patient recovery during the post-surgery time using X-rays.

Conclusions – We report here some pre-clinical testing of new scaffolds. To compare these second generation ceramic scaffolds more suitable for a tissue engineering approach we had to first establish animal models and analysis procedures

including the use of X-ray-computed microtomography associated with X-rays synchrotron radiation.

Key words: animal model; bioceramic scaffolds; bonemarrow stromal cells; engineered bone tissue; X-ray-computed microtomography

Introduction

Bone damage, either due to pathologies or traumas, is a very common occurrence and represents a major problem in orthopedics. Osteotomy followed by bone distraction (the Ilizarov technique) and auto- or allografting are the most currently used therapeutic approaches (1–6).

The Ilizarov technique, is based on the bone regeneration potential. The relatively high rate of success obtained by this technique is counterbalanced by the high inconvenience, the long recovery time and the high number of complications for the patient.

Autologous bone implants can be either non-vascularized or vascularized grafts and are the most successful, but complications, such as infections, non-unions, etc. are very frequent especially in large shaft reconstructions. Furthermore, large reconstructions by autologous bone requires a second operation, not always possible, for the harvest of healthy tissue resulting in an important donor site morbidity and increased surgery costs.

Allografts involve the risk of blood-borne diseases and as they are strictly law-regulated can be used only in countries in which fresh frozen grafts are stored and correctly donor–receiver matched; moreover, many grafts are produced from cadavers by procedures such as heat and dehydration which are expensive and time-consuming.

The use of bioceramics as synthetic osteoconductive materials has been proposed (7–17). Ceramic scaffolds provide advantageous features when compared to other materials. Generally speaking, these biomaterials were used either as inert or bioactive artificial substitutes and have proven successful only in the case of relatively small bone defects.

Possibly the best solution for large bone-defect repair is a tissue engineering approach, i.e. the use of a combination of a suitable scaffold with osteogenic cells (18–23).

Porous bioceramics made of hydroxyapatite (HA) and/or tricalcium phosphate (TCP) and with an internal structure reminiscent of the structure of natural cancellous bone are widely used for bone repair. A relatively higher concentration of TCP in the bioceramic usually results in a higher scaffold resorption. The presence of a mineralized scaffold micro-environment is a required factor for the onset of osteogenesis by committed human cells (24). The increased surface due to the internal porosity favors bone deposition by the osteogenic cells and may also facilitate scaffold resorption.

Bone marrow stromal cells (BMSC), also named mesenchymal stem cells (MSC), are an easily accessible source of cells that are able to differentiate osteo-, chondro and adipogenic lineages. In standard culture conditions, these cells do not behave as true stem cells and lose their proliferation rate and differentiation potential. The use of selected animal sera or the addition of FGF-2 to the culture medium maintains the cells in an undifferentiated state and greatly improves BMSC differentiation potential. Indeed, porous ceramic scaffolds in combination with BMSC have been used for the repair of bone lesions by several authors (20,25–29).

We first reported a clinical application of this cell-based tissue engineering procedure to treat patients with long bone segmental (4 to 7 cm) defects (29). BMSC from three patients were expanded *ex vivo* and loaded on porous ceramic scaffolds (50–60% porosity; 100% hydroxyapatite). All three patients recovered limb function between 6 and 12 months. During the more than 5 years follow-up of these patients, no major problems were reported.

Nevertheless, we consider results so far obtained in bone repair by this tissue engineering approach very encouraging, but not optimal. Before tissue engineering could become a routine procedure, additional experimental and clinical work is necessary with regard to cells (Are BMSC the optimal choice? Can we control

immunoresponse and use allogeneic cells?), scaffolds (testing scaffold with different chemical composition, architecture and resorption rate), implant preparation (optimizing cell culture condition, including the use of serum-free media; development and use of bioreactors), surgical procedures and patient follow-up (need to standardize and validate clinical protocols).

Particularly, with regard to the scaffold, the 100% HA porous ceramic used in our initial pre-clinical and clinical studies proved to have good osteoconductive properties resulting in a good functional recovery, but was not resorbed and was essentially unchanged after more than 5 years from the implant. In addition, due to the high density of the mineral and the relatively low porosity (50–60%), it was very difficult to follow by X-rays the new bone formation and therefore, to monitor the patient recovery during the post-surgery time.

We therefore decided to search for second generation ceramic scaffolds more suitable for a tissue engineering approach to bone repair. To this purpose we had to establish first procedures and animal models to compare these scaffolds.

Materials and methods

Porous ceramics

Bioceramic scaffolds analyzed were: 1) 100% hydroxyapatite with a Ca/P ratio of 1.66 ± 0.5 from FinCeramica (Faenza, Italy). Two different types of scaffolds with the same chemistry but a different geometry were used. The first was made from a sponge structure with a $60 \pm 5\%$ porosity. The second, made through a foam-based technology, had a $80 \pm 3\%$ porosity; 2) 100% synthetic calcium-phosphate multiphase biomaterial containing 67% silicon-stabilized TCP (Si-TCP) and 33% hydroxyapatite/beta TCP (HA/beta-TCP). This scaffold had a 60% porosity and was produced by Millenium Biologix Inc. (Kings-ton, ON, Canada).

Cell culture and histology

Marrow aspirates were obtained from iliac crest of sheep as part of a protocol approved by the competent ethical authority. Detailed protocol for BMSC expansion *in vitro*, as well as histology protocols have been described elsewhere (28,30).

Porous ceramic/bone marrow stromal cells constructs implanted in the immunodeficient mouse

The immunodeficient mouse model has been already described (31). Briefly, porous bioceramic cubes of about $4 \times 4 \times 4$ mm were used. Marrow aspirates were harvested in heparin (about 200 units/ml final) from adult sheep iliac crest under total anesthesia, as described elsewhere (32). BMSC were isolated from marrow aspirates and expanded in culture. Bioceramic scaffolds loaded with *in vitro* expanded BMSC were subcutaneously implanted in immunodeficient mice (CD-1 nu/nu, Charles River, Calco, Italy). Scaffolds not loaded with cells were also implanted as control. After 8–16 weeks, implants were harvested, fixed in paraformaldehyde 4% in PBS for 2 h at 4°C and kept in PBS for future micro-CT analysis.

X-ray-computed microtomography

Micro-CT experiments were performed at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) on beamline ID19 with the following operating conditions: monochromatic beam with an energy of 27 keV; sample-to-detector distance 15 mm; detection system: Gadox scintillator (Proxitronic, Bensheim, Germany) associated to FReLoN CCDcamera (ESRF Development) (34). The acquisition setup was based on 3D parallel tomography which is described in detail elsewhere (33–35).

Typical scans included 1100 projections of the sample over 180 degrees. Images were recorded on a 2048×2048 CCD detector, with the pixel size set to $4.91 \mu\text{m}$, yielding a field of view of 10 mm. 3D images of implants were reconstructed from the series of 2D projections using a 3D filtered back projection algorithm implemented at ESRF (34,35). A volume of interest, of about $(950)^3$ voxels was reconstructed for each sample. Each voxel of the reconstructed image was cubic with a $4.91 \mu\text{m}$ size in the three directions of space.

Different image analysis techniques may then be used for quantitative analysis. A first approach consisted in analyzing the thickness of the newly formed bone in a direction roughly perpendicular to the boundary between the scaffold and the bone itself. Thickness was evaluated by using the Image J program (Public Domain Image Processing Program, NIH, Bethesda, MD, USA) with plug in radial sums

measurements. The measurements were made for each pore of each section from the series of 2D projections obtained (more details are given in Ref. (36)). By a different approach, the thickness of newly formed bone was quantified directly in 3D by measuring the diameter of maximal balls filling the 3D structure (37).

Large animal model

Two-year-old ewes were involved in this study upon proper approval of competent ethical committees and legal authorities. Implant preparation and surgical procedure were performed essentially as previously described (28). Briefly, BMSC ($0.5\text{--}1.0 \times 10^8$) were resuspended in 2–3 ml fibrinogen and adsorbed by gently rolling to porous ceramic cylinders. Hundred NIH Units of thrombin were applied to achieve fibrin polymerization. At the time of surgery, 5 cm from the central third of the left tibia were surgically resected and replaced with the porous bioceramic cylinder. Mechanical stability was obtained either by an internal plate or by external fixation. After surgery and every month during the experimentation period, the animals were monitored by X-rays. At killing, tibial diaphysis specimens including the implanted scaffold were explanted and evaluated by gross examination, computed tomography (CT), microradiography and histology.

Results and discussion

Our past pre-clinical experiments and pilot clinical studies were performed taking advantage of a 100% hydroxyapatite (HA) scaffold manufactured from a sponge structure with an approximate 60% porosity.

We have published that marrow-derived osteogenic progenitor cells combined with this porous HA scaffold promote segmental healing of critical size bone defects in sheep (28). The same cell therapy approach was used to treat three patients who presented large tibial, ulnar and humeral diaphyseal gaps ranging in size from 3.0 to 28.3 cm³ (29). External fixation was used to stabilize the grafts. An initial integration at the bone/implant interface was already evident 1 month after surgery. Patients have been followed-up to 6 years.

Although a good functional recovery was observed in treated patients within 6–7 months after surgery, the used scaffold presented some disadvantages. In fact its

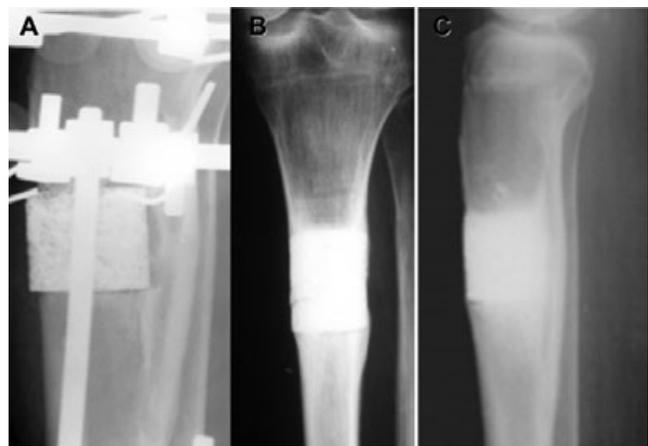


Fig. 1. Tibia repair by a tissue engineering approach in a human subject. Radiographs obtained immediately post (panel A) and 18 months (panel B) and 5.5 years (panel C) after surgery).

relatively high density was making it difficult to monitor the new bone formation within the scaffold itself and therefore to decide on the patient handling during the surgery follow-up. In particular it was difficult to establish the right time for the fixator removal. In addition the scaffold was essentially unresorbable and remained unchanged after more than 5 years from surgery (Fig. 1). Our goal was instead to obtain a complete regeneration of the patient bone without any remaining interference of the scaffold used for cells delivery.

A search for more suitable bioceramic scaffolds was therefore initiated. To test scaffold performances, pre-clinical studies taking advantage of both a small animal model (ectopic bone formation in an immunodeficient mouse) and a large animal model (critical size gap in sheep tibia) were performed.

Quantitative analysis of formed bone in the immunodeficient mouse model

Bone formation by autologous/syngeneic BMSC was first reported in small animals for the repair of small experimentally induced osseous defects in mice and rats (18,26). Ectopic bone formation by xenogeneic (including human) BMSC were instead assessed by implanting small porous ceramic cubes loaded with cells in immunodeficient mice (20,31,38).

These subcutaneous implants form a highly vascularized primary bone tissue. Bone forming efficiency of *in vivo* transplanted BMSCs is strongly dependent on the conditions of their *in vitro* expansion. We have reported that when BMSC are cultured in the presence of FGF-2, their osteogenic potential is maintained longer (30).

Transplanted osteoprogenitor cells into ceramic scaffolds were also used to create vascularized bone flaps (39,40).

While qualitative analysis of the new bone formed in these implants can be easily performed on tissue sections by conventional histology, 3D structure information and quantitative analysis are difficult to obtain. We have described a computer-based method for the quantification of bone in the histological sections of decalcified samples (41). In principle, a 3D structure of the newly formed bone could be obtained by analyzing serial sections of the implant but this approach is not practical and since samples are decalcified before sectioning, the information on the residual scaffold in the implant is lost.

Direct 3D quantitative analyses of the newly formed bone and of the scaffold remaining is very difficult. Previous attempts with commercially available micro-CT failed because the two phases had similar contrasts in the images.

Good results were obtained at the ESRF by the use of X-ray-computed microtomography associated with X-ray synchrotron radiation. Unlike laboratory and commercially available X-ray sources, synchrotron radiation offers the possibility to keeping selected X-rays with a small energy bandwidth from the wide and continuous energy spectrum, maintaining at the same time the photon flux rate high enough for efficient imaging. This allows high spatial resolution images to be generated (from 10 μm to 1 μm) with high signal-to-noise ratio (34,35,42). Variations of the sample's refractive index due to inhomogeneities are registered for each rotation of the sample on a micrometer scale. Recorded images called projections form a set, which is then converted, using algorithms from conventional tomography to obtain three-dimensional (3D) images of the sample (33).

Fig. 2 shows a 3D-image of an HA scaffold loaded with sheep BMSC and implanted in an immunodeficient mouse. The scaffold, the newly formed bone and the organic phase other than bone are distinguishable because of the different colors. The newly formed bone was not found when a HA scaffold without cells was implanted in the mouse. This was in agreement with our previous observation made by histology that bone is formed in the ectopically implanted ceramic scaffold only when the scaffold is pre-loaded with osteogenic cells (31).

By image processing, it is possible to 'cancel' one or more phases to allow a more detailed observation of

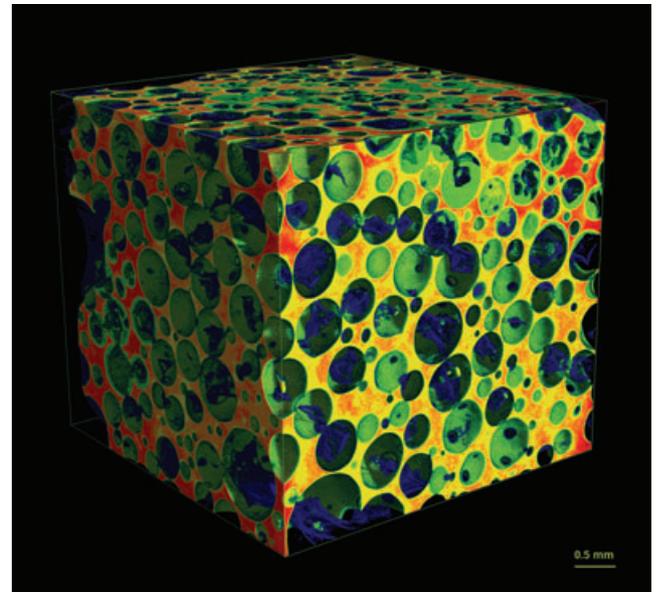


Fig. 2. Three dimensional image of an HA scaffold loaded with sheep BMSC and implanted in an immunodeficient mouse, showing newly formed bone (green) onto the inner surface of the scaffold (yellow and red); the organic phase is blue.

the 3D organization of each phase. Examples of this data treatment are in Ref. (36).

By micro-CT, it was also possible to measure the 3D thickness of the newly formed bone into each pore. A preliminary statistical analysis of the distribution of the newly formed bone thicknesses into pores of several diameters revealed a progressive increase of bone thickness with increased time of implant (data in progress).

Comparing scaffolds with the same chemistry and a different porosity and structure

Two different hydroxyapatite scaffolds with identical chemical composition (100% HA), but with different architecture and different total porosity (A: $60 \pm 5\%$; B: $80 \pm 3\%$), pore size distribution and interconnection pathway were compared. The SR micro-CT setup developed on beam line ID19 at the ESRF was used to image the scaffolds loaded with BMSC before and after 8–16 weeks of the implant in immunodeficient mice. The voxel size was set to 4.91 μm .

The tomographic images allowed the characterization of the 3D structure of the scaffolds. The contrast in the images enabled the visualization of the newly formed bone after implantation. Three-dimensional analysis was performed to extract quantitative parameters on scaffolds and newly formed bone. The 3D

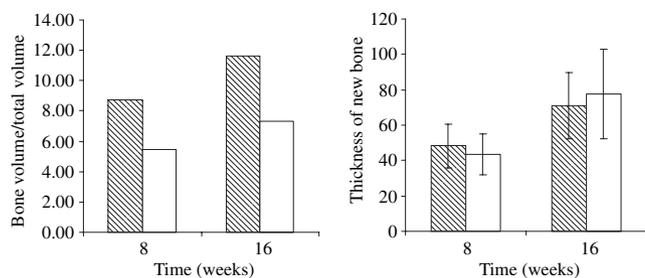


Fig. 3. New bone volume and thickness from segmented images of different scaffolds loaded with BMSCs and implanted in an immunodeficient mouse. Implants (100% HA) were recovered after 8 and 16 weeks. Empty columns, sponge structure $60 \pm 5\%$ porosity; striped columns, foam structure $80 \pm 3\%$ porosity.

porosity, mean 3D wall thickness and mean 3D pore thickness were first derived from the images of the scaffold alone before the implant. Then, after the implant of the same scaffold, the newly formed bone was segmented and its volume as well as its mean thickness was estimated.

Measured quantitative parameters of the scaffolds were in agreement with those given by the manufacturer.

In Fig. 3, we report the results of an experiment where we analyzed the scaffolds at 8 and 16 weeks after the implant. We observed a significant increase in the time of the newly formed bone. However, more bone was formed on the scaffold with higher porosity, higher bone surface to bone volume, higher isotropy, and thinner walls. Interestingly, in agreement with the fact that the scaffold chemistry was the same, the thickness of the newly formed bone did not vary in the two scaffolds. This first experiment demonstrates the feasibility of this analytical approach and suggests that the scaffold characterized by an engineered structure with a higher total porosity and a higher degree of interconnections among pores is a better material for bone reconstruction.

Investigating highly resorbable scaffolds in large animal models

Few large animal models have been developed to evaluate the use of bioceramics in the repair of very extensive, experimentally induced bone defects in weight bearing long bones.

Dog autologous BMSC loaded onto porous ceramic cylinders were implanted in critical-sized segmental defects in the femora of adult animals. After 4 months, both woven and lamellar bone had filled the pores of the implants. On the contrary, an atrophic non-union was observed in the femora of control dogs (25,43).

In our laboratory, we implanted ceramic cylinders with and without the addition of autologous *ex vivo* expanded BMSC at sites of surgical removal of significant portions of the tibial shaft in the sheep (28,44). In ectopic immunodeficient mouse model, bone is formed only when the scaffold is loaded with osteogenic cells. On the contrary, we have shown that in the tibia gap model the orthotopic bone formation was observed also in the implant not loaded with cells. The BMSC supplement resulted in a more rapid and efficient callus formation and bone repair decreasing the time of recovery.

Similar results were also obtained by Petite et al. (27) by implanting a combination of a coral scaffold with *in vitro*-expanded marrow stromal cells in a large segmental defect in sheep tibiae.

Resorbability of a 100% synthetic calcium phosphate multiphase biomaterial containing 67% Si-TCP and 33% HA/beta-TCP was tested in a sheep critical size tibia gap model.

Diaphyseal segments of 5 cm were resected from the tibiae of adult sheep and substituted with ceramic implants of the same size and shape. Fixation was performed with suitable metallic plate. Sheep were killed at different time intervals from 3 months to 2 years from surgery. Plates were removed after 5–6 months and animals were able to walk with no functional impairment. Good integration between the ceramic implants and the bone stumps was observed radiographically and confirmed by histology and micro-X-ray of the implants and adjacent tissues resected at the time of animal sacrifice. Interestingly, while a progressive increase in the new bone formation was observed, a contemporary progressive resorption of the ceramic scaffold was observed. At 1 year from surgery the amount of remaining scaffold was calculated to be approximately 10–20% of the scaffold initially implanted, while after 2 years it was essentially completely resorbed (Fig. 4).

Conclusions

Bone tissue engineering is a very promising alternative approach to repair bone defects. A wide range of synthetic scaffolds have been proposed, each one with different mechanical, chemical and biological properties.

Initial pre-clinical experimental surgery performed taking advantage of large animal (sheep or dogs) models and a pilot small clinical study demonstrated



Fig. 4. Radiographic time course of bone regeneration in sheep. Panels (A and B) after surgery; panel (C and D) after 1 year implant and panel (E) after 2 year implant.

the possibility of repairing a critical size lesion in a long bone by a tissue engineering approach. In those cases, 100% hydroxyapatite (HA) scaffolds with an approximate 60% porosity were used. Results so far obtained in bone repair by a tissue engineering approach and utilizing this ceramic scaffold are very encouraging but susceptible of major improvements. In particular, testing of new ceramic scaffolds with different chemical composition and geometry is mandatory.

The ideal scaffold should provide an initial support for osteoprogenitor cells to deposit mineralized bone matrix; then it should be slowly resorbed at the same time the newly formed bone tissue grows inside the scaffold. A high porosity and a high degree of interconnection among the pores are an absolute requirement for the vascularization of the implant and the new bone formation. Chemical composition plays a major role in the resorbability of the biomaterial.

Since more than one decade an ectopic bone formation model has been proposed. New bone formation is observed by implanting small porous ceramic cubes loaded with xenogeneic (including human) cells in immunodeficient mice (20,31,38). Nevertheless, new methods of analysis should be developed if one wants to get information about the rate of bone deposition and the percentage of the scaffold remaining at different time from the implant. Recently we have demon-

strated (36) the possibility of further non-destructive, quantitative analysis of tissue engineering constructs to determine the volume distribution of the newly formed bone into implant by using the micro-CT technique associated with X-ray synchrotron radiation. This methodology offers major advantages including the possibility of investigating the influence of scaffold parameters such as pore size and spatial distribution with regard to the growth of bone within the implant. This should allow to determine the efficiency of bone formation in scaffolds with different microstructural parameters or under different circumstances such as: variable sources of osteogenic cells, increased or decreased mechanical loading, etc. Eventually, new scaffolds should be tested in large animal models before their clinical application.

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