

SCAFFOLDS COMBINED WITH STEM CELLS AND GROWTH FACTORS IN RECONSTRUCTION OF LARGE BONE DEFECTS

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Abstract

Tissue engineering is recognized as the possibility to reconstruct *in vitro* a specialized tissue ready for transplantation. This application is very appealing for all the surgeons who foresee an off the shelf engineered tissue to substitute sick or deficient tissues instead of organ or tissue transplantation harvesting tissue from living or cadaver donors. Orthopaedic applications are very interesting for the tissue, bone, can be created by combining vital cells from mesenchymal progenitors or from osteoblastic lineage with a solid three-dimensional scaffold with specific mechanical and biochemical properties. Therefore a valid technique of orthopaedic tissue engineering should deal with selection of osteogenic lineage cells, culture and replication of these cells with the use of growth factors that promote differentiation of osteoblasts and creation of a scaffold with distinct shape, physical construction, chemical surface properties that allows seeding of the cells. This engineered craftwork may be developed in several ways, of different complexity. The tissue may be formed by simply adding all the elements in a favourable environment, like a defect surrounded by bone and form the final construct as a normal healing procedure, or can be arranged completely *in vitro* and then implanted as an already living tissue.

Introduction

Reconstructions of large lesions or defects often requires a bone graft or a bone substitute to promote healing. In common practice the reconstruction of a bone defect is dependent on the size and the site of the lesion: in long bones intercalary defects may be managed with Ilizarov technique of bone transport and distraction osteogenesis or the use of a free or pedicled vascularized bone graft, like vascularized fibula. For cavitory defects the available surgical options include auto, allo or xenograft or the use of other materials to promote bone regeneration on a synthetic scaffold. Autologous cancellous bone is recognized as the most biologically active graft material because it is a tridimensional scaffold, possesses bone growth factor in its non mineralized matrix and usually preserves a relative percentage (from 10% to 90%) of vital osteoblastic cells. However, autologous bone harvest is associated with significant morbidity for the patient, it is time consuming in the theatre and not always available. Biomaterials or allografts do not encounter these limitations, but have no osteogenic and limited osteoinductive potential. Moreover the normal process of bone healing needs the presence of mesenchymal stem cells that have the potential to differentiate in osteoblastic precursors. The triangle resulting by merging scaffold, cells and growth factors has been recently revised by Giannoudis in a diamond figure where, looking at non-union and post-traumatic defects, the fourth side is the mechanical stability. Reviewing the most recent data on tissue engineering the authors think that a pentagon should be a more appropriated project, where the last side is the environment needed to obtain bone regeneration. This fifth side could be an *in vitro* bioreactor or an *in vivo* bioreactor from the simplest, a cavity surrounded by bleeding bone, to the most complex, a muscular pouch to obtain a vascularised graft.

Literature review

Checking the literature we can separate four major varieties of cell-based tissue engineering: 1) the stimulation of connective tissue progenitors in the site of the defect; 2) transplantation of autogenous connective progenitors to improve the present cell number; 3) transplantation of cultured cells or genetically modified cells; 4) transplantation of a fully formed tissue. The first method is to stimulate tissue formation by stimulating the activation, migration, proliferation, and/or differentiation of local connective tissue progenitors. Implant of an allograft or a synthetic scaffold, but also demineralized bone matrix or bone morphogenetic proteins can simply explain this strategy. Biophysical stimulation, such as mechanical loading, electromagnetic stimulation or ultrasound can also be taken into account when dealing with this approach. In the second way the addition of osteoblastic cells at the defect is thought to stimulate bone formation. Autologous bone graft is one of the most prevalent and relatively effective example of cell transplantation. Also injection of only stem cells of bone marrow fraction have been used to obtain a similar results and the success rate is dependent on the number of stem cells [2]. In the third method the cells are harvested and expanded in culture medium and then reimplanted alone, with growth factors associated or seeded on a scaffold. This method seems very promising and some data on animal and clinical studies have been reported. On the other side culture expansion adds substantial cost and some risks, such as contamination with bacteria or viruses or depletion of the proliferative capacity of the stem cells which seems to arrive at a plateau also *in vitro*. Along with *in vitro* expansion stem cells can also be genetically modified to express different factors (BMPs the most popular). The complexity of the procedure will probably confine its clinical use in the setting of inherited genetic defects (e.g., osteogenesis imperfecta). The fourth but by sure not the least method to obtain bone regeneration is the creation of a fully organized and vital tissue by *ex vivo* method followed by transplantation and integration. Quarto et al. [3] trying to create a bone model in a clinical setting chose the option of replicate the biologic environment in a extracorporeal way. To obtain implantable three-dimensional (3D) living constructs, cells isolated from the patients' bone marrow stroma were expanded in culture and seeded onto porous hydroxyapatite (HA) ceramic scaffolds designed to match the bone deficit in terms of size and shape. Patients where studied at a long-term follow up and data reported by Marcacci et al. [4]. In one patient, an angiographic evaluation was performed at 6.5 years follow-up. No major complications occurred in the early or late postoperative periods, nor were major complaints reported by the patients. No signs of pain, swelling, or infection were observed at the implantation site. Complete fusion between the implant

and the host bone occurred 5 to 7 months after surgery. In all patients at the last follow-up (6 to 7 years postsurgery in patients 1 to 3), a good integration of the implants was maintained. This study and its long-term follow up is perhaps the first description of a clinically proven bone engineering. The idea of using a living animal as a bioreactor was applied by Terheyden and co-workers [5,6] who designed a pilot study in minipigs to obtain a prefabricated vascularised bone graft to perform mandibular reconstruction. In nine minipigs 600 µg rhOP-1 were used with 8 ml xenogenic bone mineral as a carrier and inserted into a pouch prepared in the M. latissimus dorsi. After 6, 12, and 24 weeks the grafts were harvested. A high yield of newly formed bone was obtained on the osteoconductive scaffold of the xenogenic bone. It was possible to create a vascularized osseous graft in the given shape of the BioOss blocks. The reconstructive result was significantly superior in the prefabrication technique, assessed by histology and computerized tomography (CT).

Also Heliotis et al. [7] described the generation in a patient of a vascularized pedicled-bone flap useful for reconstruction of a hemi-mandible; the flap was obtained after intramuscular implantation of a HA/rhBMP-7 composite without any addition of harvested bone, bone marrow, or stem cells.

The experiment on the minipigs was repeated by the same group of Terheyden [8] to repair an extended mandibular discontinuity defect by growth of a custom bone transplant inside the latissimus dorsi muscle of an adult male patient. The subject had received an ablative tumor surgery 8 years previous to the reconstruction; three-dimensional computed tomography (CT) scanning and computer-aided design techniques were used to produce an ideal virtual replacement for the mandibular defect. These data were used to create a titanium mesh cage that was filled with bone mineral blocks and infiltrated with 7 mg recombinant human bone morphogenetic protein 7 and 20 mL of the patient's bone marrow. The patient served as his own bioreactor as the scaffold was implanted into his latissimus dorsi muscle to allow for growth of heterotopic bone and ingrowth of vessels from the thoracodorsal artery. After 7 weeks the mandible replacement was transplanted, along with the adjacent vessel pedicle, into the mandibular defect. The vessel pedicle was anastomosed onto the external carotid artery and the cephalic vein by microsurgery. During the first 6 months the patient reported a continuous improvement both in the quality of life and in self-confidence. In-vivo skeletal scintigraphy showed bone remodelling and mineralisation inside the mandibular transplant both before and after transplantation. CT provided radiological evidence of new bone formation. Even if with a short follow up the experiment showed the possibility to apply in large bone defect a technique of bio-reactor which is relative simple, does not need ex-vivo cell expansion and creates a large amount of bone which is viable and, if considered necessary, can be transplanted with its own vascularisation.

Vogelin and co-workers [9], following the idea of a previous study where a vascularized bone graft was prefabricated in a heterotopic site in a rat model, and four factors, blood supply, osteoprogenitor cells in a periosteal flap, a biocompatible matrix, and recombinant human bone morphogenetic protein-2 (rhBMP-2), were found to be the optimal combination for increased bone formation, repeated the study in a rat model in a critical defect. A carrier matrix D,D-L,L-poly-lactic and hyaluronan acid (OPLA-HY) with or without rhBMP-2 was implanted in a 1-cm-long femoral defect and secured with a plate and screws. In some groups, a vascularized periosteal flap was harvested from the medial surface of the tibia. In group 1, the femoral defects in the animals were filled with the OPLA-HY matrix alone; in group 2, the OPLA-HY matrix was covered by the vascularized periosteal flap; in group 3, 20 µg of rhBMP-2 was added to the OPLA-HY matrix; and in group 4, the femoral defect containing the OPLA-HY matrix and 20 µg of rhBMP-2 was wrapped circumferentially by the vascularized periosteal flap. The presence and density of new bone formation in the femoral defect were evaluated radiographically, histologically, and with histomorphometry at four and eight weeks postoperatively. In groups 1 and 2, which were not treated with rhBMP-2, showed no radiographic or histologic evidence of mature bone formation at four or eight weeks. Both groups 3 and 4, which were treated with rhBMP-2, demonstrated excellent bone formation. However, with the periosteal flap, group 4 demonstrated more bone formation on histomorphometric analysis at eight weeks (43.1%) than did group 3 (28.3%) ($p < 0.01$). Additionally, heterotopic bone formed outside the boundaries of the defect in eight of the fifteen animals in group 3, which had no periosteal flap. This animal study shows some very promising data. One of the resulting conclusion is that a very powerful growth factor like a

recombinant protein can stimulate bone healing in a critical defect on a synthetic scaffold. When scaffold is not osteoinductive, like in this study, the presence of vascularisation is not sufficient to stimulate differentiation of cells along the osteoblastic lineage. When all components are present (scaffold, cells, vascularity, growth factors and stability) the bone engineering goes fast and in a controlled way. The deficient environment (absence of a vascular flap in this model) can provide for bone formation but without control. To reinforce these findings on the importance of vascularisation are the similar results reported by studies of Terheyden and co-workers on minipigs.

Dallari and co-authors [10] tried to study the synergistic effect of cells, growth factor and scaffold in a clinical setting of high tibial osteotomies, without performing the tissue but using the defect as a bioreactor. A prospective, randomized, controlled study was performed, and a standardized clinical model was applied. Thirty-three patients undergoing high tibial osteotomy to treat genu varum were enrolled and assigned to three groups. During the osteotomy, lyophilized bone chips with platelet gel were implanted into eleven patients (Group A), lyophilized bone chips with platelet gel and bone marrow stromal cells were implanted in twelve patients (Group B), and lyophilized bone chips without gel were placed in ten patients as controls (Group C). Six weeks after surgery, computed tomography-guided biopsies of the grafted areas were performed and the specimens were analyzed by histomorphometry. Clinical and radiographic evaluation was performed at six weeks, twelve weeks, six months, and one year after surgery. Histomorphometry at six weeks showed significantly increased osteoblasts and osteoid areas in both Group A and Group B in comparison with controls, as well as increased bone apposition on the chips, which was greater in Group B than in Group A. Group B showed significantly higher revascularization than the controls ($p = 0.004$). Radiographs revealed a significantly higher rate of osseointegration in Groups A and B than in the controls at six weeks. At the final evaluation at one year, the osseointegration was still better in Groups A and B than in Group C; however, all patients had complete clinical and functional evidence of osseointegration. The only limitation of this study is the small number of patient in each group, which underpowered statistical analysis. In addition the chosen clinical setting, i.e. high tibial osteotomy, is not a critical one and this may invalidate some of the results. However the defect, filled with only scaffold, scaffold and growth factors and scaffold, growth factors and stem cells, did show different rate of osteointegration and healing, proving the importance of the different agents in order to arrive to a normal tissue.

Personal experience

Using a concept similar to that later utilized by Dallari and co-workers, since 2000 till February 2006 we have treated 82 cases of large bone defects with a combination of homologous bone, growth factors (platelet derived growth factors, PDGF) and fresh bone marrow. From these series of patients we retrospectively selected and reviewed those with cavitory defects and those with orthopaedic problem, mainly pseudoarthrosis, to assess the safeness, the results and complications of the procedure [11]. Thirty-seven patients required healing of large defects: 20 males and 17 females. The age of the patients was 19 on average (6-54). The lesions were located in 22 cases in the femur, 11 cases in the humerus, 4 cases in the tibia, 2 cases in the scapula and 2 in the calcaneus and in one patient at the fibula. The original diagnosis for surgery was an aneurismal bone cyst in 18 cases, unicameral bone cyst in 11 cases, fibrous dysplasia in 6 cases, a giant cell tumour in 4 cases, chondroblastoma in 2 cases and one case of benign fibrous hystiocitoma. In 14 cases (9 patients) of the above mentioned series the lesion was treated percutaneously. The 26 patients operated with open technique healed at 121 days on average (58-279). The medium follow up is 25 months on average (6-39). We had two patients with recurrent disease: one patient with an aneurismal bone cyst of the proximal humerus and one patient with a giant cell tumor of the elbow at 10 months and 26 months from the original surgery. All the remaining 24 patients experienced a successful result both clinically and radiologically. We did not experience any surgical site infection or wound complication in this series. No additional recovery time was needed for the procedure, and the absence of autologous bone graft harvesting surgical procedure obviously was associated with the total lack of additional complications such as pain and blood loss. The surgical time was prolonged by one hour on average, which was the time required for the iliac crest bone marrow harvest and for operating field changing, for during bone marrow processing and bone graft

preparation surgery proceeded without differences. Our study was not designed to evaluate osteointegration of the composite graft or to show differences in the procedures, for the same protocol was applied. Our first end point was to demonstrate the safeness of the procedure and a secondary end point was to assess the healing capacity. During the study period we applied the procedure to patient with larger defects that in our experience would have requested several weeks to heal and we obtained very good clinical results. Ethically we decided not to apply the technique in smaller defects where the healing could be historically obtained within 8 weeks with conventional technique, i.e. allograft bone. The technique of harvesting platelet rich plasma by an aphaeresis the day before surgery is no longer applied, while we collect platelet rich fibrin matrix (PRFM) from peripheral blood during surgery using a commercially available the Cascade System (Cascade Medical Enterprise, LLC, Plymouth 60-PL2, Devon Uk) developed in collaboration with Musculoskeletal Trasplant Foundation (MTF) (fig. 1). We are going to promote a controlled study to evaluate the effect of the PRFM and the concentration of colony forming unit in the cells preparation on the final result.



Fig. 1: CASCADE preparation of platelet rich fibrin matrix, resulting in a dense gel preparation, easily handled, ready to be mixed to bone chips. Two doses have been prepared from 18 cc of peripheral blood.

In the non union cases we have treated 67 cases of established non unions from 2001 till December 2007. Thirteen cases of non unions affected the upper limb (11 humeri, 2 ulnae) and 54 cases were localized at the lower limb: 28 cases in the femur, 25 cases in the tibia and 1 case of the hindfoot. Precognizing the “diamond concept” we changed the hardware in 11 cases out of 13 in the upper limb and in 50 cases out of 54 in the lower limb. In the upper limb we have always used a new plate with angular stability, while in the lower limb we have always used a reamed nail both removing a plate and changing a previous nail. In 16 cases we have treated the non union with the use of fresh bone marrow, platelet rich plasma and allograft bone. These patients healed in 87,5% of the cases, with a delay from treatment to definite healing of 5 months on average. In the remaining 51 cases we utilized Osigraft alone or in combination of bone graft. These patients showed a 89.4% rate of healing with a time for union of 4 months on average. The use of Osigraft raised through the years and has completely replaced other growth factors, due to the relatively high percentage of success and the simple modality of surgical procedure. The conclusion of our retrospective study in the non union cases is that BMP-7 is a simple and effective method to treat also difficult non unions, provided that mechanical stability is obtained; the cost of the protein will probably lower, but some studies have already proved the economic value of this kind of approach and satisfaction of the patients is definitely superior to other conventional approaches.



Fig 2 a: Presentation of a non union in the femur with osteosynthesis failure



Fig 2 b: Healing obtained after revision of osteosynthesis and application of Osigraft.

Discussion

Bone healing process has been extensively studied, yet not thoroughly comprehended. Recent studies about stem cells and growth factors as way of implementation of bone grafts introduced new perspectives, new expectations and moreover new doubts of efficacy and safety. A very small number of studies, to our knowledge, has been published about the clinical use of growth factors and stem cell with bone graft in clinical setting. With the limitation of the relatively small number of patients our retrospective study proved that a composite graft is easy to obtain and manipulate in clinical setting; it offers good results, superior to historic controls, presents no disadvantages in respect to safety issues or additional contraindications. The routine use of tissue engineering is not advisable in all clinical settings, but has proven to be effective to treat large bone defect in order to reduce the time of healing and functional impairment for the patients. The option of using as bioreactor the patient itself, or by creating a local chamber with vascularised flap, or by inserting the product in a muscular pouch and then performing a vascularised transplant is probably a tissue engineering solution at reach by economic and ethic consideration and should be taken into account in difficult cases.

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