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Tissue engineering of bone: an ectopic rat model

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TABLE OF CONTENTS

1. ABSTRACT

Tissue engineering is attempting to recreate the complexity of living tissues. In order to test a variety of scaffolds or cells that are constantly being developed, we describe here a model where tissue engineering of bone in a non-osseous environment at subcutaneous thoracic site of DA rats generates. In this model, cell - matix interactions can mimic the normal cascade of bone development into a well organized ossicle like structure including newly formed bone marrow, during 3-4 weeks. Histogenesis of cartilage, bone and bone marrow is closely related to changes in molecular expression of essential early transcriptional regulators of osteoblast differentiation. We tested different organic, anorganic and polymeric scaffolds and their interaction with mesenchymal stem cells present in fresh bone marrow. In another series of experiments we tested mesenchymal populations separated from cultures of calvaria and periosteum for their ability to form bone in the same rat model. It is concluded that this *in vivo* model is very potent in studying cell-scaffold interactions affecting the temporal and spatial tissue engineering of bone.

2. INTRODUCTION

On the onset of mankind genesis, tissue engineering of a human being is described as a scafolding procedure of a human rib bone covered with flesh (cells) (Genesis, Chapter2, sentence 21) (1); being the first report that generation of tissues and organs depends very much on cell-matrix interactions. Today, it is well accepted that bone matrix and its cellular environment is the best known niche of adult stem cells both for hematopoiesis and for most of mesenchymal tissues.Yet, it is still the ultimate goal of tissue engineering (TE) to recreate some of these processes in order to replace and regenerate structural and functional deficits in tissues. For the restoration of structural and functional deficits of human body tissues, beyond their natural healing capacity, the external regenerative resources include cells, scaffolds, and growth/trophic factors (GF), provided either in combination or as single constituents (2).

The general strategy is usually to seed cells within a scaffold that is a structural device who defines the geometry of the replacement tissue, providing

environmental cues to promote tissue regeneration (2,3). In addition to providing cell-directing elements, ECM is itself highly responsive to the actions of cells. Tissue-engineering scaffolds can be designed to interact with cells by emulating key molecular features of the ECM. ECM contains many macromolecules such as proteoglycans, collagens, laminins, fibronectin and sequestered growth factors, and it is primarily this molecular information that confers its bioactivity. Similarly, an emerging philosophy in tissue engineering is that rather than attempting to recreate the complexity of living tissues *ex vivo*, we should aim to develop synthetic materials that establish key interactions with cells in ways that unlock the body's innate powers of organization and self-repair (3). At best, the cell-scaffold interaction should mimic the interplay between cell-surface receptors and ECM molecules, which is crucial in regulating cellular functions, including survival, adhesion, proliferation, migration, differentiation, and matrix deposition. As well as requiring information from each other, cells derive a vast wealth of information from their environments, including the material that surrounds and separates them within tissues, the extracellular matrix (ECM) (4).

As the multidisciplinary nature of the field implies, understanding the specific nature of the cell surface receptor-ECM interactions at the nanoscale level will undoubtedly provide a foundation for the development of functional biomaterials designed to promote tissue regeneration (5). Underlining the role of this biophysical interplay, an interesting study demonstrated that stem cell differentiation can be directed to different lineages solely by adhering to matrices of different elastic moduli (6). Evidently, the characteristic requirements of the scaffolds are tissue-specific and depend on the type of material with its very chemical structure and its architectural design .

Clues for how to construct bioactive artificial scaffolds come from natural bioactive scaffolds. More than 40 years ago, implantation of demineralized bone matrix (DBM), bone from which mineral and cells have been removed, leaving mainly collagenous matrix proteinaceous material with variable amounts of bone morphogenetic proteins (BMPs), was able to induce the formation of bone in the surrounding muscle tissue (7,8). Osteoinductivity refers to the ability of a graft to transform non-differentiated mesenchymal cells or local fibroblasts to differentiate into cartilage and bone cells. While, osteoconductivity refers to the situation in which the scaffold supports the environment for osteoprogenitor cells to form bone by providing an interconnected structure through which new cells can migrate and new vessels can form and migrate. Although, demineralized freeze-dried bone allograft (DFDBA), which is prepared in advance before surgery, usually from human or animal donors, is considered to be osteoinductive (9-11), in most instances is only osteoconductive (12). The hydroxyapatite bone mineral (HA) and its calcium phosphate derivatives that lack the organic components of bone, are used in bone grafting procedures to restore or fill bone defects, is also considered to be osteoconducive. It is well established that ceramic HA has the capability to support osteogenic differentiation when combined with mesenchymal stem cells (13-16). In contrast, SiO2 incorporated in HA via the sol–gel processing technique incorporates bioactive properties, which by absorbing serum components bind actively cells from their near environment, allowing their osteogenic expression directly on its surface (17). Although, various scaffold constructs are available in development of tissue-engineered bone, it should be noted that an active blood vessel network is an essential pre-requisite for these to survive and integrate with existing host tissue (5).

Development and formation of the skeleton (ossification) occurs by two distinct processes: intramembraneous and endochondral ossification. Both may occur in close proximity during woven bone development. Intramembraneous ossification is characterised by invasion of capillaries into the mesenchymal zone, and the emergence and differentiation of mesenchymal cells into mature osteoblasts. In order to test a variety of scaffolds or cells that already are employed in the clinic or those which are constantly being developed for tissue engineering of bone, there should be an animal model where cell matix interaction can mimic the normal cascade of bone development in a non-osseous environment. Several different animal models are available to test cells combined with scaffolds in order to optimize procedures for tissue engineering of bone. Most of these use surgically produced bone defects or large gaps between bone fragments. Although those models normally simmulate conditions where tissue engineering of bone procedures are needed, their intimacy with the "old" bone does not permit to isolate and define the variety of molecular changes which initiate generation of bone. The most accepted model for tissue engineering of bone in a non-osseous environment mostly uses calcium phosphate minerals, combined with mesenchymal stem cells (14-16). In general, it permits to evaluate the efficiency and probability of scaffolds or cells to generate bone, however, the bone is formed in this system in a chaotic non organised fashion.

Here, we describe an animal model where tissue engineering of bone in a non-osseous environment at subcutaneous thoracic site of DA rats generates (18-21). The DA rat carry alleles that cause stronger skeleatal structure and strength (22). In this model, bone is developing into a well organised ossicle like structure including newly formed bone marrow, during 3-4 weeks. Here, histogenesis of cartilage, bone and bone marrow is closely related to changes in molecular expression (23).

It is therefore competent to compare the effectiveness and bioactivity of different scaffolds for tissue engineering of bone, as well as evaluating cell-scaffold interactions. We tested different scaffolds and their interaction with mesenchymal stem cells present in fresh bone marrow (24,25). In another series of experiments we tested mesenchymal populations separated from cultures of calvaria and periosteum for their ability to interact with demineralized bone matrix (DBM) to form bone.

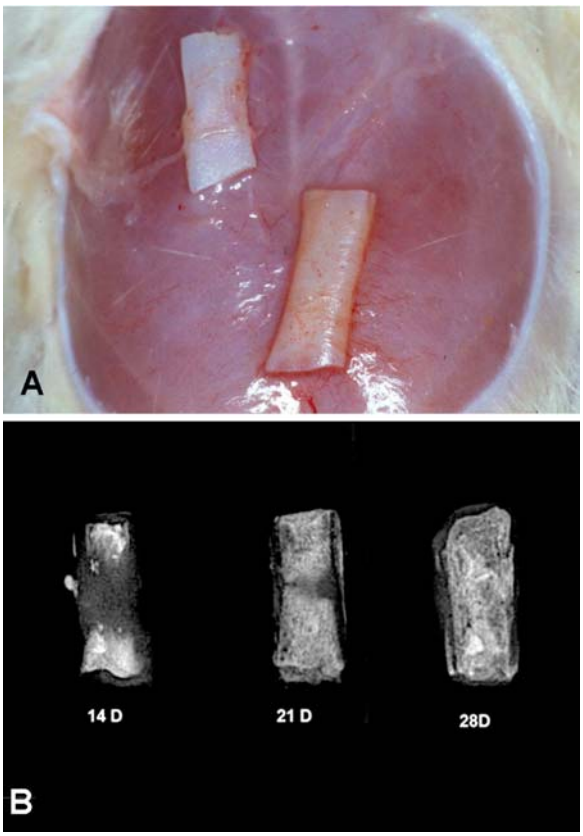

Figure 1. A: DBM cylinders at time of their excision from their subcutaneous site. See the rich vasculture around the DBM cylinders. Also, no fibrous tissue is seen. B : Microradigraphy of DBM's removed at designated times after implantation. The ingrowth of bone inside the DBM's from 14 days untill they merge after 28 days.

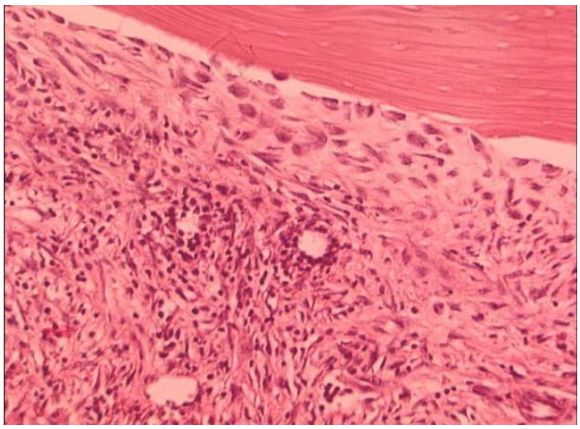

Figure 2. Histology of DBM cylinder 3-5 days after subcutaneous implantation. Sections through the longitudinal axis of the cylinders are prepared and stained with H&E. A typical cellular interface with DBM inner surface is seen at this stage of development. Several layers of osteoprogenitors (OP) are lining the DBM inner surface. Many undifferentiated small cells (F) and blood vessels (Arrow) are present toward the center of the DBM.

3. ANIMAL MODEL FOR TESTING TISSUE ENGINEERING COMPONENTS

Current procedures to restore bone defects by providing structural and mechanical support, include the use of variety of scaffolds and cell tissue engineering procedures. Many of these alternatives use a variety of materials, including natural and synthetic polymers, ceramics, and composites, whereas others have incorporated factor- and cell-based strategies that are used either alone or in combination with other materials.

We propose here a rat model where bone is generated inside an open chamber, in a thoracic subcutaneous non-osseous environment (19-21). In this model the chamber consists of demineralized diaphysis cylinder (DBM) of rat femur in which fresh marrow is placed. In brief, demineralized bone cylinders (DBM) are prepared from diaphyses of femurs derived from Dark Agouti (DA) rats. The femurs are excised from 3-4 month old DA rats after they were sacrificed. The ephiphyseal ends are discarded and the trabecular medullary bone and marrow is reammed away and cleaned with sterile water. The clean cortical diaphyseal bone cylinders are demineralized by immersing them in 0.6N of HCl during 24 hours. The DBM cylinders are rinsed with PBS and kept in 70% alcohol. Fresh marrow which is removed from a femur of another 6-8 weeks old DA rat is placed inside the lumen of the DBM cylinder. Two DBM cylinders including the fresh marrow are then surgically implanted subcutaneously at thoracic sites of 2-month-old DA rats (Figure 1a). The DBM cylinders are open on their both sides, being exposed to the subcutaneous cellular and extracellular environment.

The process of bone engineering depends on a normal cascade of wound healing. It requires a complex interplay between programs for cellular growth and differentiation; signals that rely on various cytokines and hormones. In this rat model, the newly formed bone is first seen on microradiographs inside both orifices of each DBM cylinder, 10-14 days after implantation. The bone growth is advancing along the ingrowth of blood vessels untill both sides merge, at 3-4 weeks (Figure 1b). 4-5 days after fresh marrow within DBM cylinders were implanted subcutaneously at thoracic site of DA rats, few layers of osteoprogenitor cells are developing next to inner DBM surface, mostly close to the orifices (Figure 2). Next to layers of progenitors, blood vessels surrounded by young fibroblast like cells are seen (Figure 2). 3-5 days later typical chondroblasts surrounded by calcified rim of matrix are seen apposed to the inner surface of DBM (Figure 3a). In the near proximity small blood vessels surrounded by cuboidal osteoblast cells and new osteoid are seen (Figure 3b). The development of microvasculature and microcirculation is critical for the homeostasis and regeneration of living bone, without which, the tissue would simply degenerate and die (26). In Figure 3b, the close proximity between the angiogenic and osteogenic cells is highlighting the cell-cell and cell-matrix interactions during the multi step process of bone development and repair. The generation of bone is

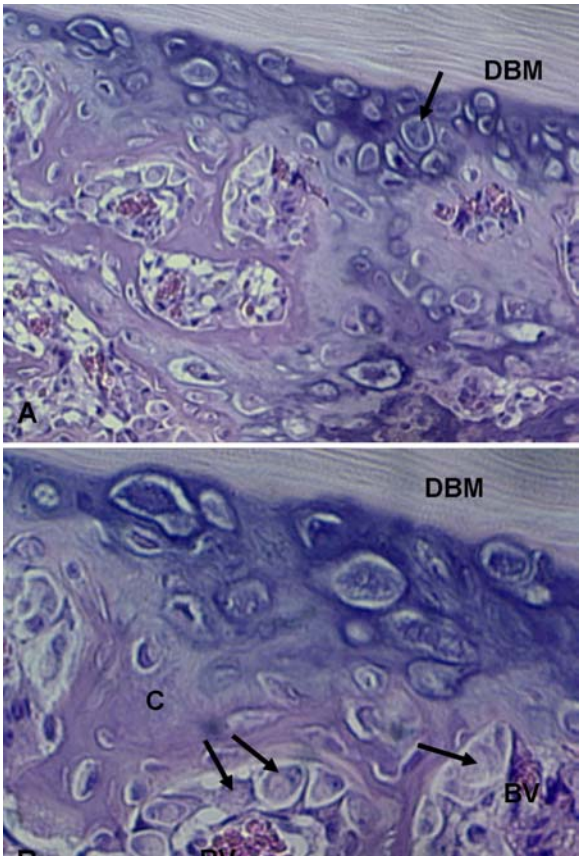

Figure 3. (A) Histology of DBM cylinders 7 days after subcutaneous implantation. Cartilage and bone cells surrounded by their calcified matrix are seen on the inner surface of the DBM(arrow). (B) The intimacy between blood vessels (BV) and differentiated osteoblasts (arrows) is seen. On their opposite surface the osteoblasts are apposed to cartilage matrix (C).

following the ingrowth of blood vessels from the host, inard the cylinder untill they merge and form an ossicle (Figure 1b). It is worthwile to note, that while chondroblasts are mostly apposed to the inner surface of DBM, osteoblasts are seen mostly next to blood vessels.The final architecture is very similar to a true ossicle which consists of outer thin cortical bone surrounding a network of trabeculli and a cell rich newly formed marrow with many blood vessels (23). We reported (21) that as early as 3 days after subcutaneous implantation of the DBM cylinder with marrow the genes expression of osteogenic phenotype is significantly expressed. Overall, the *in vivo* results showed up-regulation of essential early transcriptional regulators of osteoblast differentiation and down-regulation of muscle differentiation factors as the bone regeneration process advances (23). The FGFR3 mRNA expression was not detected in the fresh marrow prior to transplantation and was immediately elevated (day 3) and increased to its highest level of expression, 4.5-fold, at day 7 (21,23). This receptor is known to play an important role in angiogenesis, mesenchymal cell proliferation, and chondrocyte differentiation during endochondral bone development. Also, NOV (CCN3), a member of the CCN family of matricellular proteins, has recently emerged as a biological regulator of skeletal development, wound repair, and angiogenesis (28) was low in the fresh marrow prior to transplantation. It was considerably up-regulated at the early stage at day 3 (21). The transplanted cells expressed significantly high levels (11.4-fold, $p<0.05$) at day 7 and remained significantly high throughout the regeneration process (23). Also, greater level of osteogenic markers as osteocalcin and BSP indicating osteoblastic function of matrix mineralization (23).

The initial stages after surgical implantation of the DBM cylinders is reminiscent of the wound healing process, being a unique biological process involving a cascade of events that begins with an inflammatory response. This reaction is followed by the recruitment, proliferation and differentiation of mesenchymal stem cells, synthesis of extracellular matrix proteins, and angiogenesis. The newly formed bone is woven bone, formed by both endochondral and intramembranous ossification pathways, which is ultimately replaced by lamellar bone through a process of bone remodeling. The outcome is the reconstitution of tissue continuity and thereby tissue regeneration. The remarkable capacity of skeletal tissues to develop may depend on the molecular signaling pathways regulating skeletogenesis which are shared during fetal development and adult wound healing.

We have recently reported that DBM sitmulated marrow-directed endochondral ossification while mineralized bone matrix (MBM) stimulated osteogenesis without prior cartilage formation (21). However, bone mineral (HA) inhibited osteogenesis, therefore, suggesting that organic matrix is necessary for cell-matrix interactions thus, supporting generation of bone (21).

In another set of experiments (24), biocompatibility of non-degrading porous polymer composite, based on bulk-copolymerisation of 1-vinyl-2-pyrrolidinone (NVP) and n-butyl methacrylate (BMA), was evaluated in the DBM model. Various combinations of NVP and BMA materials, differing with respect to composition and, hence, hydrophilicity, were introduced into the centre of DBMs.The ends were closed with rat bone marrow, and ectopic bone formation was monitored after 4, 6, and 8 weeks, both through X-ray microradiography and histology. The 50:50 scaffold particles were found to readily accommodate formation of bone tissue within their pores, whereas this was much less the case for the more hydrophilic 70:30 counterpart scaffolds (24). New healthy bone tissue was encountered inside the pores of the 50:50 scaffold material, not only at the periphery of the constructs but also in the center. Also, active osteoblast cells were found at the bone-biomaterial interfaces. It indicated that the hydrophobicity of the biomaterial is, most likely, an important design criterion for polymeric scaffolds which should promote the healing of bone defects.

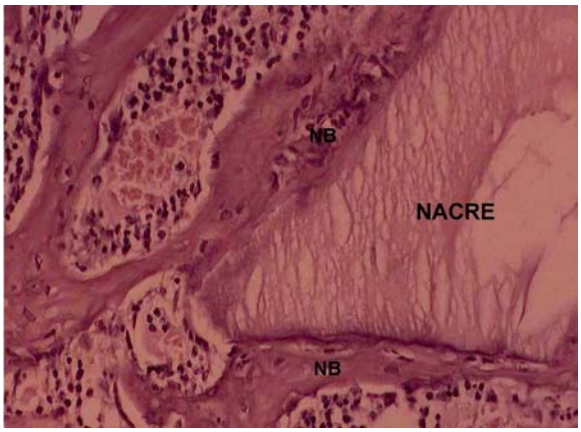

Figure 4. Newly formed bone apposed on Nacre treated with Ca(OH)2, within the DBM cylinder. NB is newly formed bone apposed on Nacre.

On x-raying the mineralized new bone, we observed that even in the best combination of polymer scaffold constructs (50:50 ratio) there was a delayed bone formation in comparison to DBM's without polymer (24). Furthermore, it is argued that stable, non-degrading porous biomaterials, like those used in this study, provide an important tool to expand our comprehension of the role of biomaterials in scaffold-based tissue engineering approaches. Also, we tested a Nacre mineral treated with Ca(OH)2 and non treated in the DBM bone generation model (19). We found that the calcium carbonate mineral of Nacre was surrounded by fibrous tissue, separating it from newly formed bone (19). While, when treated with Ca(OH)2 the newly fomed bone apposed directly on the Nacre surface (Figure 4). This DBM bone generation model is able to evaluate in detail biocompatability or bioactivity of scaffolds surface. Furthermore, the delay in bone generation in comparison to control DBM's can indicate on their expected clinical performance. The cell-scaffold interaction is most probably controling the timing of bone generation.

4. *IN VITRO* SELECTION AND EXPANSION OF PROGENITOR CELLS AND THEIR TRANSPLANTATION TO FORM BONE, *IN VIVO*

In the adult, the ability of bone tissue to regenerate and replace the lost bone is dependent on the existence of sufficient amounts of stem cells or progenitors that upon division form differentiated progeny. However, the low estimated frequency of precursor cells may influence the modeling capacity in the adult or where large amount of bone loss occurs. Apart from embryonic stem cells established from blastocyst, the bone marrow is the predominant source for progenitors, in the adult.

Mesenchymal stem cells, bone marrow stromal cells, periosteal cells and osteoblasts have been successfully used for the generation of bone tissue (29). Also, recent studies suggest that periosteum (the bone envelope) and perivascular cells contain fraction of progenitors which by appropriate stimulus proliferate and differentiate into bone tissue, in the adult.

Novel techniques propose to grow cell populations from explants of specific tissues including bone marrow and periosteum, in culture (30-32). The outgrowing populations of cells proliferate *in vitro* and the cell populations could be dramatically expanded. The expanded cell populations are capable of producing specific tissues like bone *in vitro*, by adding dexamethasone, BMPs, PGE2 and other treatments. *In vivo*, they are placed in defects, using biocompatible carriers and scaffolds. In most cases this enrichment procedures *in vitro* are not selective for cells with osteoprogenitor characteristics and therefore very limited amount of bone tissue is developed *in vivo* by their transplantation (30,31). Moreover, the presence of progenitor cells alone is not sufficient to guarantee suitable repair or regeneration. In addition, these cells must be able to respond appropriately to local extracellular matrix (ECM) molecules, cytokines and physical activity which regulate the regenerative process. Also, they need to be able to produce a mineralized matrix and bone structure which has the optimal properties required for bone function and communication with the host bone.

We have previously described a two dimensional culture method (34) which supports the growth of osteogenic cells responsive to PTH, on plastic surfaces by using low Ca medium including 10% fetal calf serum (FCS). This culture method enables maintenance of the osteoblast phenotype without affecting their proliferating capacity, while inhibiting the growth of fibroblasts which depends on higher concentrations of calcium in the medium. It was also shown that cells grown in low Ca medium did not loose their osteoblastic phenotype even after several passages (34). The low calcium medium to enrich for osteoprogenitors was also confirmed by other authors (35) who showed that cells of osteoblast phenotype developed *in vitro*.

Using flow cytometry cell sorting technique we were able to separate from confluent cultures two cell populations, a population of small, non-cycling cells with low cytoplasmic granularity that display progenitor characteristics (designated as PS cells) and large mature cells (designated as DL cells) that display rich cytoplasm and large nucleus reminiscent of differentiated cells, as was previously described (36,37). The PS cell fraction was shown to have low protein content, high proliferative rate, self renewal capacity, in contrast to the highly differentiated DL cells, having a limited proliferation capacity (36, 37).

Here we show that low calcium medium increased by 10 folds the amount of PS that display early progenitor characteristics and decreased the amount of DL large mature cells, in comparison to cells grown in regular calcium in the medium (Figure 5). Under regular calcium culture conditions, the PS fraction is 8-12% (R1 bracket in Figure 5) of total cell count and the DL fraction is around 18-24% (R2 bracket in Figure 5) of the total. It was noted that most of the cell population consists of medium size

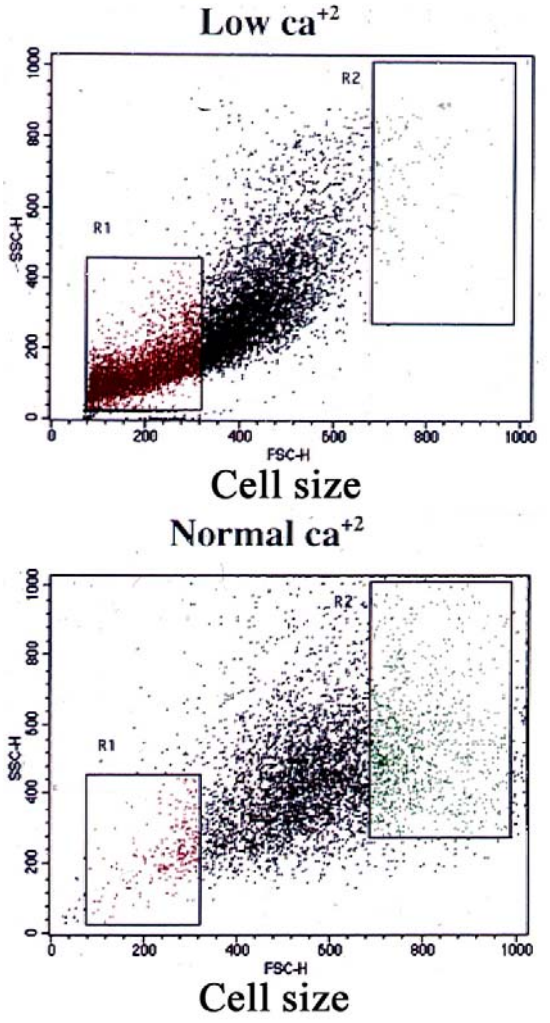

Figure 5. Flow cytometry of cells cultured in low calcium (Low Ca) and in normal (1mM) calcium (Normal Ca) in the medium. R1 is for small cell fraction (PS cells) and R2 is for large cell fraction (DL cells).

cells with active cytoplasm and a nucleus which is two times larger than the nucleus of PS cells. We found that the population of PS is amplified *in vitro* by the low calcium medium (Figure 5). Finally, we have shown that intramembranous bone develops on the inner surface of DBM cylinders when PS cell population when placed within the DBM cylinders and implanted at thoracic subcutaneous sites of DA rats, reminiscent of normal bone (Figure 6 a, b, c). While, DL cells developed granulation tissue rich of blood vessels (Figure 6d).

In brief, a concentrate of cells, 300.000 cells / 10ul medium were placed gently into DBM cylinders. The DBM's including the cells were gently put in a sterile dish in the incubator to allow attachment of the cells to DBM inner surface. DBM's filled with marrow were used as positive controls, as described above. Also, parental cells and cells of primary cultures which were grown in low calcium and regular calcium medium were transplanted for comparison. After four weeks the DBM cylinders were surgically removed from their subcutaneous site and were processed for microradiography (Figure 6a), histology (Figure 6 b, c and d).

On microradiography, in 6/8 of the specimens of PS cells, mineralized tissue filled most of the DBM inner surface. Unlike the bone marrow, the PS cells bone tissue (Figure 6 a,b,c) was deposited onto the walls of the DBM surface, very much like intramembranous bone (Figure 6b). It consisted of cortical bone apposed to the DBM surface and trabecular bone which extends toward the inner space of the DBM cylinder. Bone marrow like tissue, including blood vessels, blood cells and stroma like fibers develops in between the bone trabecullii (Figure 6 b and c). In contrast to PS cells, the DL cells did not form bone in DBM cylinders, *in vivo* (Figure 6d). Cells prepared from primary cultures under low calcium or under normal calcium produced bone *in vivo* in DBM cylinders, much less than the PS-cell cultures (data not shown). It was noted that all DBM which included cultured cells the bone was mainly apposed to the DBM surface, reminiscent of intramembranous bone. Also, bone did not form on the external surface of the DBM surface.

5. CONCLUSIONS

For the restoration of structural and functional deficits of human body tissues, beyond their natural healing capacity, the external regenerative resources include cells, scaffolds, and growth/trophic factors (GF) that are provided either in combination or as single constituents. In order to test a variety of scaffolds or cells that already are employed in the clinic or those which are constantly being developed for tissue engineering of bone, there should be an animal model where cell matix interaction can mimic the normal cascade of bone development in a non-osseous environment. Here, we describe an animal model where tissue engineering of bone in a non-osseous environment at subcutaneous thoracic site of DA rats generates (18-21). In this model, bone is developing into a well organised ossicle like structure including newly formed bone marrow, during 3-4 weeks. Histogenesis of cartilage, bone and bone marrow is closely related to changes in gene expression (23). This model is most competent to compare the effectiveness and bioactivity of different scaffolds for tissue engineering of bone, as well as evaluating cell-scaffold interactions. We tested different scaffolds, like MBM, HA, polymers and Nacre and their interaction with mesenchymal stem cells present in fresh bone marrow (24,25). In another series of experiments we tested mesenchymal populations separated from cultures of calvaria and periosteum for their ability to interact with demineralized bone matrix (DBM) to form bone. Finally, this rat model can elucidate the very early interactions between ECM and cells which lead to differentiation of bone. Furthermore, the delay in bone generation in comparison to control DBM's can indicate on their expected clinical performance. The cell-scaffold interaction is most probably controling the timing of bone generation. Moreover, how cells and matrices direct the development and generation of endochondral or intramembranous bone could be also studied in this model

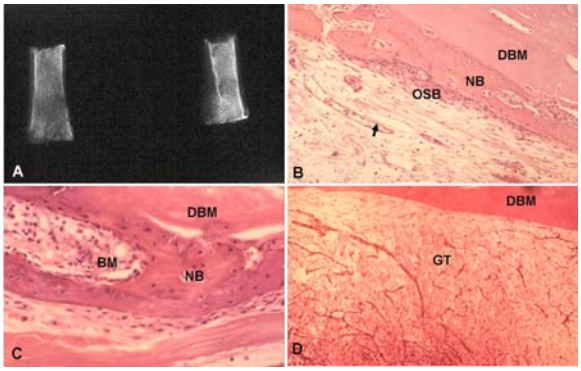

Figure 6. (A) microradiography of PS cells placed in DBM cylinders, 4 weeks after implantation. (B) layers of newly formed bone (NB) appose on inner surface of DBM, surrounding bone marrow tissue (BM). Next, young connective tissue is present. Cuboidal osteoblasts (OSB) are covering the bone matrix. See, prominent blood vessel (arrow). (C) Trabecular bone (NB) and bone marrow (arrow). (D) Young connective tissue and many blood vessels consist most of DBM volume (GT), 4 weeks after implantation of DL cells.

6. ACKNOWLEDGMENT

We acknowledge that the authors I.B., H.B and A.Y. contributed eaqually to this manuscript. The author D.B. contributed to the molecular studies and R.Z. contributed to separation of cellular fractions of bone cell cultures.

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