

## TYPE I COLLAGEN COMBINED WITH A RECOMBINANT TGF- $\beta$ SERVES AS A SCAFFOLD FOR MESENCHYMAL STEM CELLS

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A mesenchymal stem cell (MSC) can be defined by its ability to maintain its population at an existing state or level, and to give rise to a number of differentiated cell types, including osteocytes, chondrocytes, myocytes, adipocytes, fibroblasts, and marrow stromal cells. Osteogenic cell lines are believed to be derived from a common MSC, probably resident of the bone marrow (1). Difficulties in isolating the MSC arise from the fact that they display no known morphological or biochemical markers (2). A number of studies have demonstrated the pivotal role of collagen in modulating cell growth, differentiation, expression of the bone cell phenotype (3), and formation of mineralized matrix (4). MSCs are capable of self-renewal and undergo expansion in the presence of transforming growth factor-beta 1 (TGF- $\beta$ 1), a pleiotropic cytokine with autocrine and paracrine functions (5). In order to capture and expand a population of cells with osteogenic potential, we have devised an *in vitro* culture system with a mesenchymal cell compatible collagen framework, containing a recombinant TGF- $\beta$ 1 fusion protein (rTGF- $\beta$ 1-F2). The analysis of certain markers as alkaline phosphatase (AP), osteocalcin, calcium phosphate, and the morphological and histological features, have been used to know the *in vitro* and *in vivo* osteogenic differentiation.

Collagen matrices were prepared using rat type I collagen as previously described (6). Bone marrow cells were cultured at a density of  $2 \times 10^6$  cells/150  $\mu$ l collagen/well in 48-well dishes (Falcon<sup>TM</sup>, Oxnard, CA, U.S.A.) inside the collagen matrix. During the first ten days cells were cultured in MEM (Gibco BRL, Gaithersburg, MD, U.S.A.) with 0.5% FBS (Gibco BRL) in order to kill the hematopoietic cells (selection period, SP). To selectively keep alive MSCs we added the following GFs: hTGF- $\beta$ 1 (R&D Systems, 0.5 ng/ml), and a genetically engineered recombinant TGF- $\beta$ 1 fusion protein, bearing an auxiliary von Willebrand's factor-derived collagen-binding domain (rTGF- $\beta$ 1-vWF, 1 ng/ml). In controls, GFs were omitted. At day 10 the media was changed to 10% FBS (rescue and amplification period, R-AP) and cells were cultured during six more days. During the last two days, the cells were exposed to dexamethasone  $10^{-8}$ M (Sigma Chemical, Poole, Dorset, UK) and  $\beta$ -glycerophosphate 2mM (Sigma) (induction period, IP). Every 3 days the media was changed and GFs added. At the end of SP and IP samples were analyzed (7) for DNA content, AP activity and osteocalcin expression. After the *in vitro* procedure, cells were released from the collagen gel by mechanical disruptions and then implanted in demineralized bone marrow (DBM) and diffusion chambers (0.45 and 30  $\mu$ m pores, and 130  $\mu$ l of volume), into 7 months old male rats under the dorsal skin. DBM chambers were made as previously described (7) and diffusion chambers were assembled from commercially available components (Millipore, UK Ltd, Harrow, UK). The implants were harvested 28 days later (implantation period, IMP) and analyzed by X-ray. The contents of the chambers were carefully removed from within the membrane filters and analyzed for AP activity, osteocalcin and calcium content. We also performed histological studies by light microscopy examination.

Figure 1 summarizes the results obtained under *in vitro* experimental conditions. It is clear that during the selection period, cells decreased in numbers maintaining a low proliferating rate. These cells were able to produce AP but not osteocalcin. The rTGF- $\beta$ 1-F2 group showed higher cell number than commercial hTGF- $\beta$ 1, especially during the rescue and induction period. At the end of this period, the effects of GFs were enhanced. Figure 2 shows the selected cells throughout the *in vitro* experimental procedure. In control plates only few round cells survived. Only the cells treated with rTGF- $\beta$ 1-F2 were able to form colonies during the rescue and amplification period (Figs. A,B,C), and after addition of inducers, visible calcium deposition around cell nodules could be detected (Fig. D). After the implantation period, chambers were examined by X-ray, revealing radioopaque zones in the case of chambers implanted with cells treated with rTGF- $\beta$ 1-F2. Light microscopy study of

those areas showed cartilage and bone-like nodules; within the chambers, the cell layers were observed alongside the interior surface of the membrane filters, and mineralized nodules were formed among the cell layers. In the case of cells treated with hTGF- $\beta$ 1, those areas were made up of large amount of cartilage (data not shown).

The present study was designed to test if a primitive population of MSC could be selected and expanded by their proliferative responses to TGF- $\beta$ , and to examine the potential utility of these cells in *in vivo* implantation systems. Type I collagen has been suggested to be a necessary but not sufficient component of the ECM for the promotion of the osteoblast phenotype (8). Here we report the first demonstration that a population of cells present in rat bone marrow, with many characteristics of osteoprogenitor MSCs, can be selected and expanded within a collagen matrix by modified GFs, induced *in vitro*, introduced into chambers, and when implanted into mature syngeneic rats are able to produce large amounts of cartilage and bone tissue. The strategy followed was to starve the cells within a supporting collagen matrix in the presence of bound GFs, a condition which seems to favour their growth and inhibit that of other cells.

This bioactive osteoinductive-conductive matrix, combined with the targeted delivery of fusion proteins, with mitogenic and inductive potentials, as well as enhanced matrix binding properties, should be able to stimulate osteogenesis at sites of bone repair or remodelling.

## References

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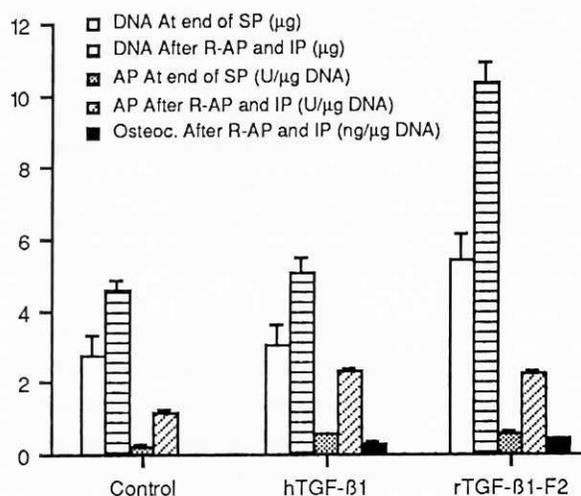


Figure 1. Analysis of DNA, alkaline phosphatase (AP), and osteocalcin (Osteoc.), after the selection period (SP), rescue and amplification period (R-AP), and induction period (IP).

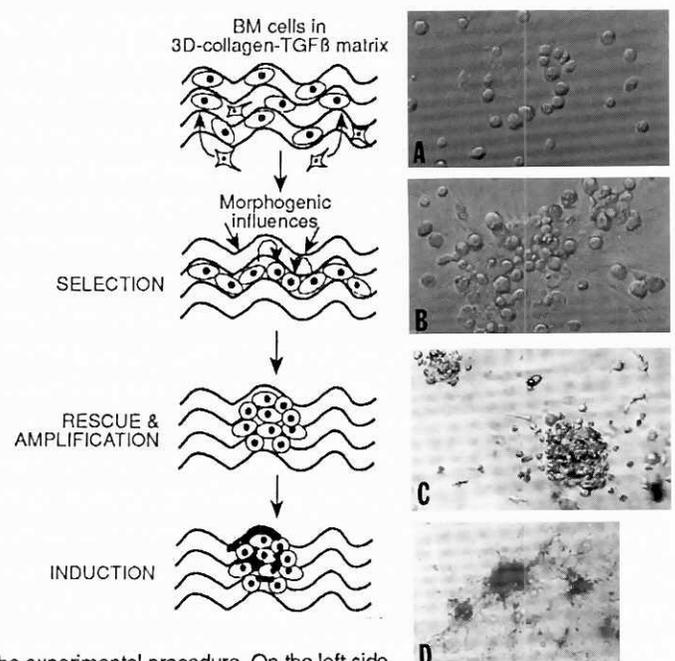


Figure 2. *In vitro* cultures of bone marrow cells into collagen matrix during the experimental procedure. On the left side, we represent an interpretation of the pictures.