Stimulation of osteoblast growth by an electromagnetic field in a model of bone-like construct

A. Icaro Cornaglia,[1] M. Casasco,[1] F. Riva,[1] A. Farina,[1] L. Fassina,[3] L. Visai,[2] A. Casasco[1]
[1]Department of Experimental Medicine, Histology and Embryology Unit; [2]Department of Biochemistry; [3]Department of Informatics and Computer Science, University of Pavia, Italy

The histogenesis of bone tissue is strongly influenced by physical forces, including magnetic fields. Recent advances in tissue engineering has permitted the generation of three dimensional bone-like constructs. We have investigated the effects of electromagnetic stimulation on human osteoblast cells grown in a hydrophobic polyurethane scaffold. Bone-like constructs were stimulated by pulsed electromagnetic fields in a bioreactor. Proliferation, bone protein expression and calcified matrix production by osteoblasts were measured using histochemical methods. In stimulated cultures, the number of cells was significantly higher compared to static (control) cultures. In both stimulated and control cultures, cells were immunoreactive to osteoblast markers, including type-I collagen, osteocalcin and osteopontin, thus suggesting that the expression of bone-related markers was maintained throughout the *in vitro* experiments. Morphometric analysis of von Kossa-stained sections revealed that stimulation with electromagnetic field significantly increased matrix calcification. The data lend support to the view that the application of a magnetic field can be used to stimulate cell growth in bone-like constructs in vitro. This finding may be of interest for the production of biomaterials designed for clinical applications.

Key words: osteoblast, SAOS-2 cells, electromagnetic field, bioreactor, bone-like tissue, tissue engineering, histochemistry

Correspondence: Antonia Icaro Cornaglia,
Department of Experimental Medicine, Histology and Embryology Unit, via Forlanini 10,
University of Pavia, Pavia, Italy
Tel.: +39.382.987272.
Fax: +39.382.528330.
E-mail:icaro@unipv.it

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Bone tissue is made up of cells, namely osteocytes and osteoblasts, and a calcified extracellular matrix rich in inorganic and organic compounds that determine bone structural function (Bloom and Fawcett, 1994; Bonucci, 2005). The histogenesis of bone tissue is strongly influenced by mechanical and physical forces, such as gravity and magnetic fields, and by a complex local mechanical environment consisting of tension, compression, stretch and shear stress which modulate cell activity and tissue remodelling (Ingber, 1997; Burger Klein-Nulend, 1999). In order for bone cells to respond in a natural environment, the biomaterial scaffolds must guarantee cell adhesion, cell proliferation and correct cell differentiation (Zdrahala and Zdrahala, 1999). Recently, advances in tissue engineering and the development of bioreactors have permitted the formation *in vitro* of bone-like artificial equivalents (Ciombor and Aaron, 1993). In particular, synthetic porous biomaterials can be adequate in supporting cell survival and mechanical properties and can be efficiently used as a scaffold for three dimensional osteoblast cultures under specific and controlled conditions (Matsunaga *et al.,* 1996). Moreover, the influence of magnetic fields on biological systems has been a topic of considerable interest for many years (see Pagliara *et al.,* 2005 and quotations herein). The aim of this study was to investigate the effects of magnetic fields on a tissue engineered model of bone tissue, made up of osteoblasts grown in a polyurethane scaffold.

Materials and Methods

Cell cultures

SAOS-2 cells, a human osteosarcoma cell line (HTB85, ATCC, Rockville, USA), were cultured in McCoy's 5A modified medium, supplemented with ascorbic acid and 15% fetal bovine serum. The cells were cultured at 37°C with 5% CO2, routinely trypsinized, counted and seeded onto a three-dimensional polyurethane foam.

Generation of three dimensional bone-like constructs

Crosslinked polyurethane foam was synthesized as previously described (Fassina *et al.,* 2005) and used as a three-dimensional scaffold for cell cultures. These scaffolds (Ø =15 mm; h=5 mm) were sterilized by ethyl oxide (at 38°C for 8 h at 65% relative humidity) and placed inside a culture system consisting of a standard well-plate inside the bioreactor. A cell suspension of 1×10^6 cells in 400 μl was added onto the top of each scaffold and after 30 min sufficient McCoy's medium was added and changed on alternate days.

Electromagnetic bioreactor

An electromagnetic bioreactor was built. It consisted of a carrying structure custom-machined in a tube of polymethyl methacrylate. The windowed tube carried a well-plate and two solenoids, the planes of which were parallel. The surfaces of polyurethane scaffolds were 5 cm distant from each solenoid plane. In this experimental setup, the magnetic field and the induced electric field were perpendicular and parallel to the scaffold surfaces, respectively. The solenoids were powered by a generator of pulsed electromagnetic field (PEMF), with intensity equal to 2 mT and frequency of 75 Hz. The electromagnetic bioreactor was placed in a standard cell culture incubator in an environment of 37°C and 5% CO_2. The stimulated (dynamic) cultures were subjected to the PEMF 24 h per day for 22 days, while the unstimulated (static) cultures consisted of a standard well-plate, far from the bioreactor. Both cultures were submitted to the same growth conditions of temperature, humidity, pH, gas and pressure.

Histochemistry

Samples were fixed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 8 h at room temperature, washed with PBS, dehydrated through graded alcohols and routinely embedded in paraffin. Sections were obtained at 5-10 μm thickness, rehydrated and stained with either haematoxylin-eosin or with the von Kossa method (BioOptica, Milan, Italy). Von Kossa staining, which substitutes calcified matrix Ca^{2+} ions with Ag atoms, was used to reveal calcified matrix as black dots. Other sections were processed for the immunohistochemical detection of bone-specific proteins, according to the indirect streptavidin-biotin immunoperoxidase technique (Hsu *et al.,* 1981). Briefly, rehydrated sections were incubated serially with the following solutions: 1) 0.3% hydrogen peroxide for 30 min to remove endogenous peroxidase activity; 2) rabbit polyclonal antibodies to type I collagen, osteocalcin, osteopontin and decorin diluted 1:3000, 1:4000, 1:2000 and 1:2000 respectively, overnight at 4°C; 3) biotinylated goat anti-rabbit IgG (BioGenex, Super Sensitive kit, San Ramon, CA, USA), for 1 h at room temperature; 4) streptavidin-biotinylated peroxidase complexes (BioGenex, Super Sensitive kit) for 1 h at room temperature; 5) 0.03% 3,3'-diaminobenzidine tetrahydrochloride solution to which 0.02% hydrogen peroxide was added just before use. Each solution was prepared in 0.05 M *Tris* buffer, pH 7.4, containing 0.1 mol/L NaCl (0.15 M *Tris* buffer saline), and between each step of the immunostaining procedure the sections were washed in the same buffer. Immunostained sections were finally rehydrated, mounted and observed in a Zeiss microscope equipped with a Nomarski differential interference contrast device and a digital image capture system (Nikon Corporation, Tokyo, Japan).

Polyclonal antisera to bone proteins (namely type I collagen, osteocalcin, osteopontin and decorin) have been previously characterized (Fisher *et al.,* 1995). Specificity controls included omission of the primary antibody and the substitution of the primary antibody with non-immune sera.

Cell counts

The number of cells was compared in static vs. dynamic cultures. Cell numbers were taken by three independent observers (AIC, AC, FR) by counting the nuclei in haematoxylin-eosin-stained sections (magnification 16 x). At least three different sections of different samples (n=5) were screened by each observer. Statistical analysis was made by a simple t-test between data obtained from static vs. dynamic cultures.

Morphometric evaluation of von Kossa staining

Morphometric-computerized analysis was performed by means of an interactive computerized image analysis system (Kontron Zeiss IBAS 2) using a high geometric linearity TV camera directly applied to a Zeiss microscope (magnication 63x) The ratio between the stained area (as revealed by black dots) and total image area was measured in the static and dynamic cultures.

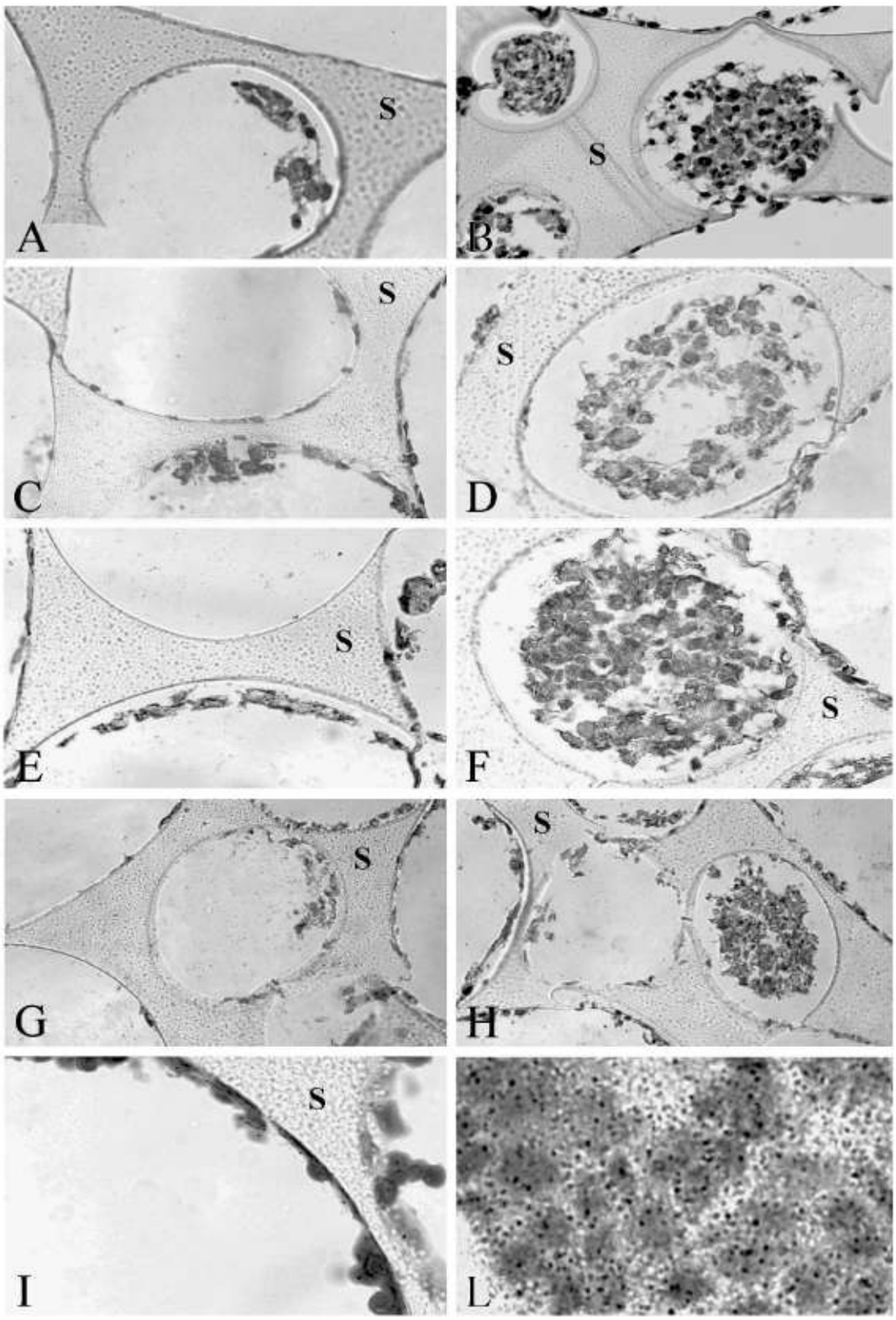

Figure 1. Histological sections of three dimensional bone-like constructs: cultures stimulated by pulsed electromagnetic fields (dynamic cultures; B, D, F, H, L) are compared to unstimulated cultures (control, static cultures; A, C, E, G, I). The number of bone cells was significantly higher in dynamic cultures compared to static cultures (B, D, F, H, L vs. A, C, E, G, I). In both static and dynamic bone-like constructs, cells were immunoreactive for type-I collagen (C, D), osteocalcin (E, F) and osteopontin (G, H). Stimulation with electromagnetic field increased matrix calcification in dynamic cultures, as revealed by Von Kossa staining (black dots in I, L). s = porous polyurethane scaffold. Haematoxylin–eosin staining (A, B); immunoperoxidase staining (C, D, E, F, G, H) and Von Kossa staining (I, L). Magnification 200x (A, B, C, D, E, F, G, H) and 500 x (I, L).

Results

In the static cultures, osteoblasts were adherent to pore surfaces and organized in a monolayer. Conversely, in the dynamic cultures, cells displayed a multilayer organization and almost no pore surfaces were cell-free. The number of cells was significantly higher in stimulated cultures (Figure 1 B, D, F, H, L), compared to the static (control) cultures (Figure 1: A, C, E, G, I). Cell counts on histological sections demonstrated an increase of cell proliferation of about 2-fold. Indeed, the mean number of cells (±standard error) per microscopic field was 18.5±1.8 and 37.4±3.1 in the static and dynamic culture, respectively ($p<0.001$).

In both static and dynamic bone-like constructs, cells were immunoreactive to osteoblast markers, including type-I collagen, osteocalcin and osteopontin (Figure 1 C, D, E, F, G, H); cultured cells also displayed decorin- immunoreactivity (*data not shown*).

Morphometric analysis of von Kossa-stained sections revealed that stimulation with the electromagnetic field increased the matrix calcification around 5-fold ($p<0.05$). In particular, the ratio between the stained area and the total image area was 2.05±0.7% in the static culture and 10.18± 3.7% in dynamic conditions (Figure 1 I vs 1L).

Discussion

In this study, we have investigated the effects of a magnetic field applied in a bioreactor on cell proliferation and differentiation in a three-dimensional model of bone-like tissue. It is well known that bone histogenesis is strongly influenced by physical stimuli, such as mechanical stress and magnetic fields (Ingber, 1997; Burger and Klein-Nulend, 1999). In particular, *in vivo* experiments have demonstrated that a continuous exposure for 30 days to a pulsed electromagnetic field stimulates bone repair in the healing process of transcortical holes in adult horses (Cane *et al.,*1993).

Our data lend support to the view that the application of a magnetic field could be used to stimulate osteoblast growth in bone-like constructs *in vitro*. Indeed, in stimulated cultures, the growth of bone cells was significantly higher compared with control unstimulated cultures. Electromagnetic fields have been shown to influence gene expression in osteoblast cells *in vitro* (Diniz *et al.,* 2002; Otter *et al.,* 1998). However, the molecular mechanisms responsible for the increase in cell growth after stimulation by electromagnetic fields remain to date elusive.

In our experiments, the application of electromagnetic fields induced a 5-fold increase in matrix calcification, as observed by von Kossa staining. It has been shown that electromagnetic stimulation raises Ca^{2+} flux in human osteoblast cells (Fitzsimmons *et al.,* 1994) and that the increase of cytosolic Ca^{2+} may trigger the expression of bone-specific matrix genes (Pavalko *et al.,* 2003). It may be tentatively presumed that this mechanism may be important in increasing matrix calcification in stimulated cultures. Accordingly, biochemical data have shown that production and secretion of bone-specific matrix proteins are increased in our culture system (Fassina *et al.* in press).

From the immunohistochemical stainings, it was seen that the SAOS-2 cells, which are a human osteosarcoma line, displayed osteocalcin and osteopontin positivity also after electromagnetic stimulation, suggesting that the expression of bone cell-markers was maintained throughout the experiments.

In conclusion, the data suggest that the application of magnetic fields may be used to stimulate the growth of bone-like equivalents *in vitro* within a polyurethane porous scaffold. This aspect may be of interest in developing new protocols for the production of engineered bone tissue.

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