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EFFECTS OF ELECTROMAGNETIC STIMULATION ON OSTEOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STROMAL CELLS SEEDED ONTO GELATIN CRYOGEL

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Bone tissue engineering typically uses biomaterial scaffolds, osteoblasts or cells that can become osteoblasts, and biophysical stimulations to promote cell attachment and differentiation. In this study, we investigated the effects of an electromagnetic wave on mesenchymal stromal cells isolated from the bone marrow and seeded upon gelatin cryogel disks. In comparison with control conditions without electromagnetic stimulus, the electromagnetic treatment (magnetic field, 2 mT; frequency, 75 Hz) increased the cell proliferation and differentiation and enhanced the biomaterial surface coating with bone extracellular matrix proteins. Using this tissue-engineering approach, the gelatin biomaterial, coated with differentiated cells and their extracellular matrix proteins, may be used in clinical applications as an implant for bone defect repair.

A key component in tissue engineering for bone regeneration is the biomaterial scaffold that serves as a template for cell colonization, cell differentiation, and the deposition of bone extracellular matrix, thus providing structural support to the newly formed tissue (1, 2).

Gelatin cryogel is a promising new biomaterial owing to its biocompatibility (3-6). The *in vitro* modification of gelatin cryogel, with osteogenic signals of the transforming growth factor-β (TGF-β) superfamily and with bone morphogenetic proteins (BMPs), enhances the tissue regeneration *in vivo* (7), suggesting that the modification of gelatin cryogel could play an important role in bone tissue engineering.

Besides the preceding material modifications, in order to enhance the osteointegration of a bone implant, many strategies have been developed, including the optimization of implant material, implant design, and surface morphology (8); in addition, to improve the bone healing around implanted biomaterials, biophysical stimulation methods have been applied: for instance, the electric and electromagnetic fields which were initially developed to accelerate the fracture healing (8-10).

In particular, the electromagnetic fields regulate the synthesis of proteoglycans and collagen, increase the bone formation in models of endochondral ossification, increase the union rates in fractures, and produce results equivalent to bone graft methods (11). Investigations have begun to clarify how osteoblastic cells respond to biophysical stimuli in terms of signaling molecules and gene expression for structural proteins (12-15).

In this study, we have electromagnetically stimulated the mesenchymal stromal cells (MSCs) isolated from the bone marrow because they are regarded as suitable cells for the bone regeneration (16, 17). Moreover, MSCs can

Keywords: mesenchymal stromal cells, bovine gelatin cryogel, electromagnetic bioreactor, bone matrix proteins.

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be easily manipulated and *ex vivo* expanded to large numbers without losing multipotency properties, and their differentiation into bone-forming cells is the core of bone regeneration strategies.

As consequence, the main aim of this research was to investigate the differentiation effects of a novel culture method consisting of the application of an electromagnetic wave onto MSCs seeded upon gelatin cryogel: using this tissue-engineering approach, the gelatin biomaterial, coated with differentiated cells and their extracellular matrix proteins, may be used in clinical applications as an implant for bone defect repair.

MATERIALS AND METHODS

Gelatin cryogel disks

Bovine gelatin cryogel disks (diameter, 10 mm; height, 2 mm) were kindly provided by Polymer Chemistry and Biomaterials Research Group, University of Ghent (Ghent, Belgium) (3-5) (Fig. 1).

To anchor the gelatin cryogel disks to standard well-plates, 3% (w/v) agarose solution was prepared and sterilized in autoclave, and during cooling, at 45°C, 100 µl of agarose solution were poured inside the wells to hold the placed gelatin disks and to fix them after completed cooling. The well-plates with the biomaterial disks were sterilized by ethylene oxide at 38°C for 8 h at 65% relative humidity. After 24 h of aeration in order to remove the residual ethylene oxide, the disks were ready.

Cells from bone marrow aspirates and cell seeding

Mononuclear cells were isolated from bone marrow aspirates (30 ml) by density gradient centrifugation in Ficoll (density, 1.077 g/ml) (Lymphoprep, Nycomed Pharma) and plated in non-coated 75-175 cm^2 polystyrene culture flasks at a density of 160000 cell/cm^2 (18). The culture condition was based on the basal medium Mesencult (Stem Cell Technologies) supplemented with 2 mM L-glutamine, 50 µg/ml gentamicin, and 10% fetal calf serum. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 48 h, non-adherent cells were discarded and culture medium was replaced twice a week. After reaching 80% confluence as a minimum, the cells were harvested and re-plated for expansion at a density of 4000 cell/cm^2 until 5th passage. The colony-forming unit-fibroblast (CFU-F) formation was examined after incubation for 12 days in a humidified atmosphere (37°C, 5% CO_2); the clonogenic efficiency was calculated as the number of colonies per 10^6 bone marrow mononuclear cells seeded. According to the International Society for Cellular Therapy on the nomenclature of mesenchymal progenitors, the cells cultured for this study were defined as multipotent stromal cells.

The obtained bone marrow stromal cells were then cultured in α-MEM (Invitrogen) supplemented with 10% fetal bovine serum, 50 µg/ml penicillin-streptomycin, and 1% L-glutamine. After reaching 80% confluence as a minimum, the cells were harvested and re-plated for expansion at a density of 2.5×10^4 cell/cm^2. The cells were cultured at 37°C with 5% CO_2, three fifths of the medium were renewed every 3 days, and then the cells were routinely trypsinized, counted, and seeded onto the gelatin cryogel disks as follows: a suspension of 1.5×10^5 bone marrow stromal cells in 400 µl was added onto the top of each disk and, after 0.5 h, 600 µl of culture medium were added to cover the disks.

We have utilized two types of culture medium. For two weeks, we have used the proliferative medium (i.e. α-MEM supplemented with 10% fetal bovine serum, 50 µg/ml penicillin-streptomycin, and 1% L-glutamine) and then, for the following two weeks, the differentiation medium (i.e. the proliferative one supplemented with 50 µg/ml ascorbic acid, 10^{-7} M dexamethasone, and 5 mM β-glycerophosphate).

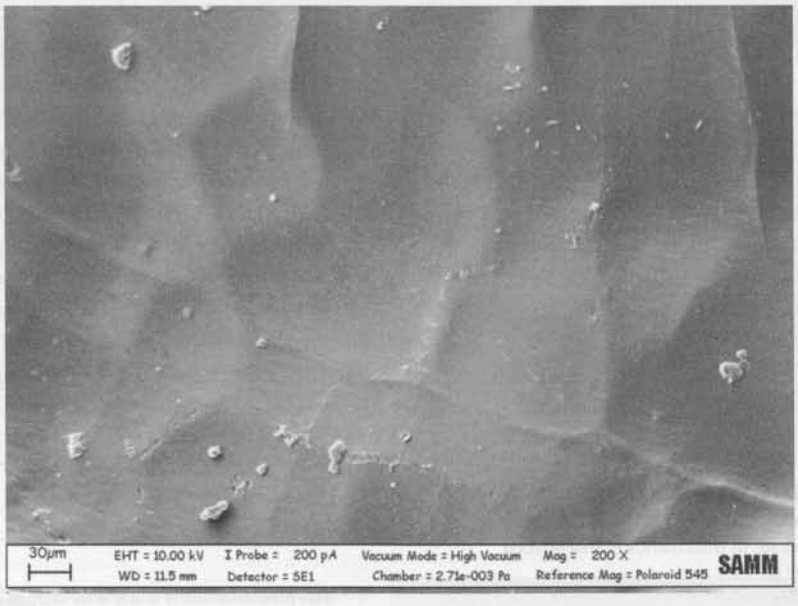

Fig. 1. *Unseeded gelatin cryogel (3-5).*

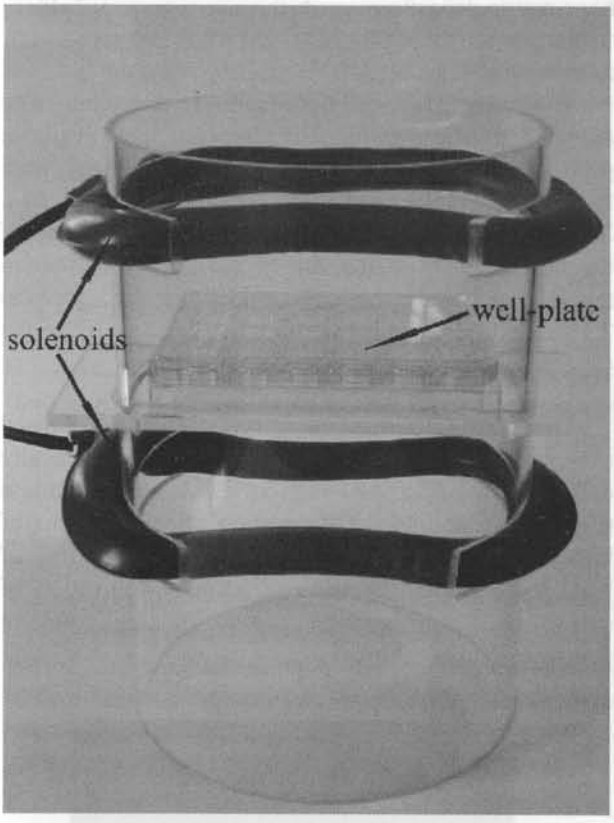

Fig. 2. *Electromagnetic bioreactor.*

The control culture (in differentiation medium) was placed for other 28 days into a standard cell culture incubator with an environment of 37°C and 5% CO_2, where the electromagnetic stimulation was not detectable (the medium was changed every 3 days).

The electromagnetic culture (in differentiation medium) was placed for other 28 days into the electromagnetic bioreactor (the medium was changed every 3 days).

Electromagnetic bioreactor

The electromagnetic bioreactor (14, 15, 19-23) consisted of a carrying structure machined in a polymethylmethacrylate tube: the windowed tube carried a well-plate and two solenoids, the planes of whom were parallel (Fig. 2). The gelatin disks were 5 cm distant from each solenoid plane, and the solenoids were powered by a Biostim SPT pulse generator (Igea, Carpi, Italy), a generator of pulsed electromagnetic fields (PEMFs).

Given the position of the solenoids and the characteristics of the pulse generator, the electromagnetic stimulation had the following parameters: intensity of the magnetic field equal to 2 ± 0.2 mT, amplitude of the induced electric tension equal to 5 ± 1 mV, signal frequency of 75 ± 2 Hz, and pulse duration of about 1.3 ms.

The electromagnetic bioreactor was placed into a standard cell culture incubator with an environment of 37°C and 5% CO_2. The electromagnetic culture was stimulated by the PEMF for 20 min/day.

Scanning electron microscope (SEM) analysis

At the end of the culture period, the gelatin disks were treated as previously described (24). Briefly, gelatin cryogel disks were fixed with 2.5% (v/v) glutaraldehyde solution in 0.1 M Na-cacodylate buffer (pH=7.2) for 1 h at 4°C, washed with Na-cacodylate buffer, and then dehydrated at room temperature in a gradient ethanol series up to 100%. The samples were kept in 100% ethanol for 15 min, and then critical point-dried with CO_2. The specimens were sputter coated with gold and observed at 200× magnification with a Leica Cambridge Stereoscan 440 microscope.

DNA content

At the end of the culture period, the cells were lysed by a freeze-thaw method in sterile deionized distilled water and the released DNA content was evaluated with a fluorometric method (Molecular Probes). A DNA standard curve (24), obtained from a known amount of cells, was used to express the results as cell number per disk.

Set of rabbit polyclonal antisera

Fisher L.W. (National Institutes of Health, Bethesda, MD) presented us, generously, with the following rabbit polyclonal antibody immunoglobulins G: anti-osteocalcin, anti-type-I collagen, and anti-osteopontin (antisera LF-32, LF-67, and LF-166).

Set of purified proteins

Osteocalcin (immunoenzymatic assay kit, BT-480, Biomedical Technologies), osteopontin (immunoenzymatic assay kit, 900-27, Assay Designs), and type-I collagen (Sigma-Aldrich).

Confocal microscopy

At the end of the culture period, the disks were fixed with 4% (w/v) paraformaldehyde solution in 0.1 M phosphate buffer (pH=7.4) for 8 h at room temperature and washed with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH=7.4) three times for 15 min. The disks were then blocked by incubating with PAT (PBS containing 1% [w/v] bovine serum albumin and 0.02% [v/v] Tween 20) for 2 h at room temperature and washed. Fisher's antisera were used as primary antibodies with a dilution equal to 1:1000 in PAT. The incubation with the primary antibodies was performed overnight at 4°C, whereas the negative controls were based upon the incubation, overnight at 4°C, with PAT instead of the primary antibodies. The disks and the negative controls were washed and incubated with Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Molecular Probes) with a dilution of 1:500 in PAT for 1 h at room temperature. At the end of the incubation, the disks were washed in PBS, counterstained with Hoechst solution (2 μg/ml) to target the cellular nuclei, and then washed. The images were taken by blue excitation with the TCS SPII confocal microscope (Leica Microsystems) equipped with a digital image capture system at 40× magnification.

Quantification of bone matrix proteins

At the end of the culture period, in order to evaluate the amount of the extracellular matrix constituents over the gelatin surface, the disks were washed extensively with sterile PBS

(137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH=7.4) in order to remove the culture medium, and then incubated for 24 h at 37°C with 1 ml of sterile sample buffer (1.5 M Tris-HCl, 60% [w/v] sucrose, 0.8% [w/v] sodium dodecyl sulphate, pH=8.0). At the end of the incubation period, the sample buffer aliquots were removed. The calibration curves to measure osteocalcin, osteopontin, and type-I collagen were performed by an ELISA assay with Fisher's antisera (24). The amount of extracellular matrix constituents onto the disks is expressed as fg/(cell×disk).

Statistics

Results are expressed as mean ± standard deviation. In order to compare the results between the culture systems, one-way analysis of variance (ANOVA) with *post hoc* Bonferroni test was applied, electing a significance level of 0.05.

RESULTS

The MSCs were seeded onto the surface of gelatin cryogel disks and then cultured with or without an electromagnetic stimulus. These culture methods allowed to study the differences in terms of cell differentiation and bone matrix deposition.

Microscope analysis

SEM images revealed that, due to the electromagnetic stimulation, the cells proliferated on the available gelatin surface (Fig. 3B), whereas control cells were few and essentially organized in a monolayer (Fig. 3A). These observations were confirmed by the measure of the DNA content at the end of the culture period: in the control culture the cell number per disk grew to $2.1\times10^5 \pm 8.2\times10^3$, whereas to $3.2\times10^5 \pm 8.4\times10^3$ in the electromagnetic culture ($p<0.05$).

The immunolocalization of type-I collagen showed a more intense fluorescence in the electromagnetically cultured disks (Fig. 4B) than in the control condition (Fig. 4A), revealing the stimulation effects in terms of higher cell proliferation and more intense building of bone extracellular matrix. The immunolocalization of osteocalcin and osteopontin confirmed a similar culture structure (data not shown).

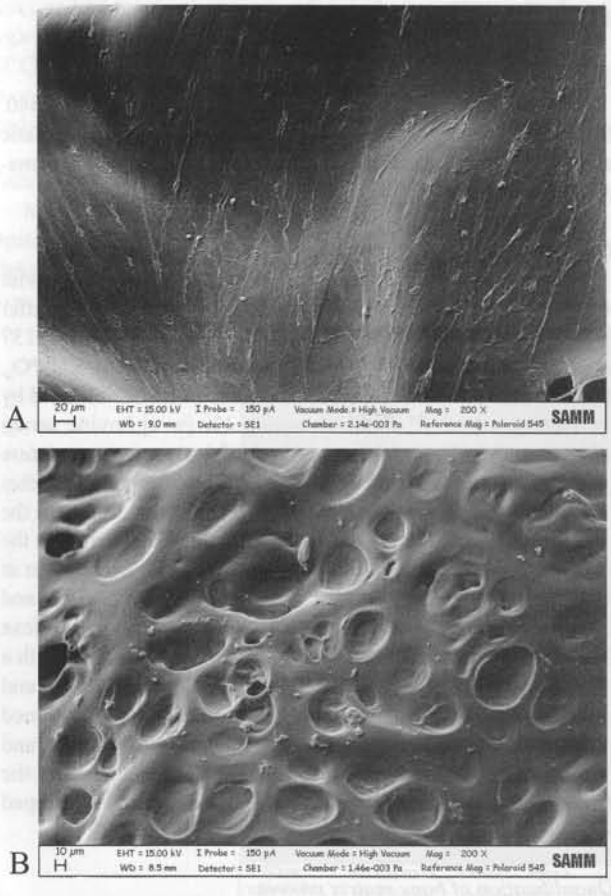

Fig. 3. *SEM observations of seeded gelatin disks coated with differentiated cells in the control* ***(A)*** *and electromagnetic* ***(B)*** *cultures (200× magnification).*

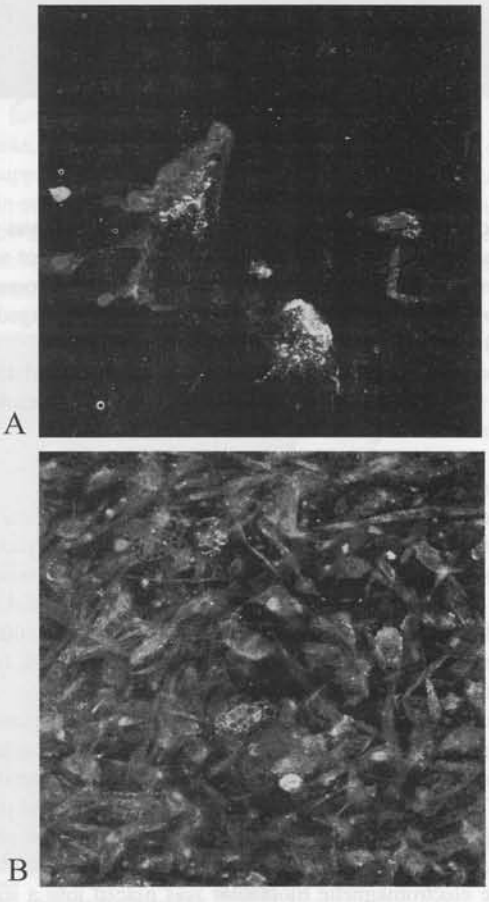

Fig. 4. *Immunolocalization of type-I collagen (light) and localization of cellular nuclei (dark) in the control (A) and electromagnetic (B) cultures (40× magnification).*

Table I. *Amount of extracellular matrix constituents onto gelatin disks.*

Total matrix coating in fg/(cell×disk)			
	Control culture	Electromagnetic culture	Electromagnetic/Control
Osteocalcin	3.53 ± 0.12	11.20 ± 0.06	3.17-fold
Osteopontin	4.70 ± 0.09	5.69 ± 0.07	1.21-fold
Type-I collagen	48.10 ± 0.10	196.00 ± 0.20	4.07-fold

Table note: $p<0.05$ in all "Control" vs. "Electromagnetic" comparisons.

Extracellular matrix extraction

In order to evaluate the amount of bone extracellular matrix onto the gelatin cryogel disks, an ELISA of the extracted matrix was performed: at the end of the culture period, in comparison with the control culture, the electromagnetic stimulation significantly increased the surface coating with osteocalcin, osteopontin, and type-I collagen ($p<0.05$) (Table I).

DISCUSSION

In this *in vitro* study, we have shown the effects of a pulsed electromagnetic wave on mesenchymal stromal cells isolated from the bone marrow and seeded upon gelatin cryogel disks.

The electromagnetic wave enhanced cell adhesion, proliferation, and differentiation with consequent increased deposition of extracellular matrix proteins, in other words, it caused a better surface modification of the biomaterial under a biomimetic viewpoint (6, 19, 21, 23). In particular, the electromagnetic stimulus improved the synthesis of type-I collagen, osteopontin, and osteocalcin, which are fundamental constituents of the physiological bone matrix.

Type-I collagen is the most important and abundant structural protein of the bone matrix; osteopontin is an extracellular glycosylated bone phosphoprotein secreted at the early stages of the osteogenesis before the onset of the mineralization, it binds calcium, it is likely to be involved in the regulation of the hydroxyapatite crystal growth, and, through specific interaction with the vitronectin receptor, it promotes the attachment of the cells to the matrix; osteocalcin is secreted after the onset of mineralization and it binds to bone minerals.

In this study, the differentiation method obtained the biomimetic modification of the material, whose surface was coated by differentiated osteoblasts and by a layer of bone matrix. The use of autologous bone marrow stromal cells showed the potential of the method and the worth of the new gelatin cryogel for total immunocompatibility and for complete biocompatibility with the patient, respectively.

In conclusion, using this biomimetic tissue-engineering approach, the gelatin biomaterial, coated with differentiated cells and their extracellular matrix proteins, may be used in clinical applications as an implant for bone defect repair.

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