Differentiation of human bone marrow stromal cells onto gelatin cryogel scaffolds

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Abstract— **Biomaterials have been widely used in reconstructive bone surgery to heal critical-size long bone defects due to trauma, tumor resection, and tissue degeneration. In particular, gelatin cryogel scaffolds are promising new biomaterials owing to their biocompatibility; in addition, the *in vitro* modification of biomaterials with osteogenic signals enhances the tissue regeneration *in vivo*, suggesting that the biomaterial modification could play an important role in tissue engineering. In this study we have followed a biomimetic strategy where differentiated human bone marrow stromal cells built their extracellular matrix onto gelatin cryogel scaffolds. In comparison with control conditions without differentiating medium, the use of a differentiating medium increased, *in vitro*, the coating of gelatin cryogel with bone proteins (decorin, osteocalcin, osteopontin, type-I collagen, and type-III collagen). The differentiating medium aimed at obtaining a better *in vitro* modification of gelatin cryogel in terms of cell colonization and coating with osteogenic signals, like bone matrix proteins. The modified biomaterial could be used, in clinical applications, as an implant for bone repair.**

Keywords— **Gelatin cryogel, bone marrow stromal cell, cell proliferation, bone extracellular matrix, surface modification, biomimetics.**

I. Introduction

One of the key challenges in reconstructive bone surgery is to provide living constructs that possess the ability to integrate in the surrounding tissue. Bone graft substitutes, such as autografts, allografts, xenografts, and biomaterials have been widely used to heal critical-size long bone defects and maxillofacial skeleton defects due to trauma, tumor resection, congenital deformity, and tissue degeneration. The biomaterials used to build 3D scaffolds for bone tissue engineering are, for instance, the hydroxyapatite [1], the partially demineralized bone [2], and biodegradable porous polymer-ceramic matrices [3].

The preceding osteoinductive and osteoconductive biomaterials are ideal in order to follow a typical approach of the tissue engineering, an approach that involves the seeding and the *in vitro* culturing of cells within a porous scaffold before the implantation.

Gorna and Gogolewski [4, 5] have drawn attention to the ideal features of a bone graft substitute: it should be porous with interconnected pores of adequate size allowing for the ingrowth of capillaries and perivascular tissues; it should attract mesenchymal stem cells from the surrounding bone and promote their differentiation into osteoblasts; it should avoid shear forces at the interface between bone and bone graft substitute; and it should be biodegradable.

In this study, following the preceding "golden rules" of Gorna and Gogolewski, we have elected gelatin cryogel [6-8] as bone graft substitute and, applying a differentiating medium to bone marrow stromal cells, we have attempted to populate it with extracellular matrix and differentiated osteoblasts.

Gelatin cryogel [6-8] is a promising new biomaterial owing to its biocompatibility. The *in vitro* modification of gelatin cryogel, with osteogenic signals of the transforming growth factor-β superfamily and with bone morphogenetic proteins, enhances the tissue regeneration *in vivo* [9] suggesting that the modification of gelatin cryogel could play an important role in tissue engineering.

As consequence, aiming, in a future work, at accelerated and enhanced bone regeneration *in vivo*, in the present study of tissue engineering, we show a particular "biomimetic strategy" that consists in the *in vitro* modification of gelatin cryogel with differentiated bone marrow stromal cells and their extracellular matrix produced *in situ*. In other words, using a differentiating medium, our aim was to enhance a bone marrow cell culture onto a gelatin cryogel, that is, to coat the gelatin cryogel with physiological and biocompatible cell-matrix layers. Using this approach, the *in vitro* cultured material could be theoretically used, in clinical applications, as an osteointegrable implant.

II. 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) [6-8] (Fig. 1).

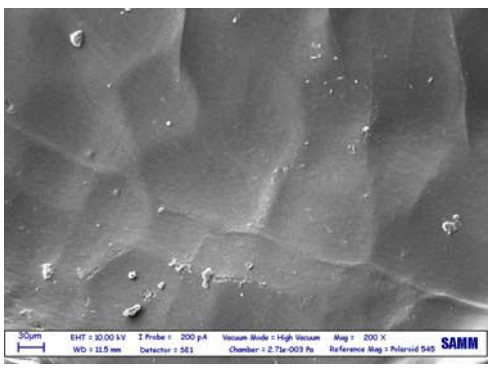

Fig. 1 Unseeded gelatin cryogel [6-8]

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Cells from bone marrow aspirates: 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 (Corning Costar, Celbio) at a density of 160000 cell/cm^2 [10]. The culture condition was based on the basal medium Mesencult (Stem Cell Technologies) supplemented with 2 mM L-glutamine, 50 μg/ml gentamycin, 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 5^{th} passage. The colony-forming unit-fibroblast assay (CFU-F) was performed as described previously [11]. 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 [12].

Cell culture: To study the osteogenic differentiation potential, 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.

Cell seeding: 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.

A suspension of 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 was added to cover the disks.

We have utilized two types of culture medium. For the control well-plate, we have used the "proliferative" medium i.e. α-MEM supplemented with 10% fetal bovine serum, 50 μg/ml penicillin-streptomycin, 1% L-glutamine. Whereas, for the "differentiating" well-plate, we have utilized the proliferative medium only for two weeks and then the "differentiative" one, i.e. the same as above to which ascorbic acid (50 μg/ml), dexamethasone (10^{-7} M), and β-glycerophosphate (5 mM from day 21) were added.

The duration of the control and differentiating cultures was 6 weeks and the culture media were changed every 3 days.

Scanning electron microscopy (SEM) analysis: 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 500× magnification with a Leica Cambridge Stereoscan 440 microscope at 8 kV.

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 [13], obtained from a known amount of cells, was used to express the results as cell number per disk.

Set of rabbit polyclonal antisera: L.W. Fisher (National Institutes of Health, Bethesda, MD) presented us, generously, with the following rabbit polyclonal antibody immunoglobulins G: anti-osteocalcin, anti-type-I collagen, anti-type-III collagen, anti-decorin, and anti-osteopontin (antiserum LF-32, LF-67, LF-71, LF-136, and LF-166, respectively) [14].

Set of purified proteins: Decorin [15], osteocalcin (immunoenzymatic assay kit, BT-480, Biomedical Technologies), osteopontin (immunoenzymatic assay kit, 900-27, Assay Designs), type-I collagen [16], and type-III 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. L.W. 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 100× magnification.

Extraction of the extracellular matrix proteins from the cultured disks and ELISA assay: 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 and the total protein concentration in the two culture systems was evaluated by the BCA Protein Assay Kit (Pierce Biotechnology). The total protein concentration was 198 ± 35 μg/ml in the control culture and 332 ± 51 μg/ml in the differentiating culture ($p<0.05$). The calibration curves to measure decorin, osteocalcin, osteopontin, type-I collagen, and type-III collagen were performed by an ELISA assay with L.W. Fisher's antisera. The amount of extracellular matrix constituents onto the disks is expressed as fg/(cell×disk).

Statistics: The disks number was 24 in each repeated experiment (12 disks in the control culture and 12 disks in the differentiating culture). The experiment was repeated 4 times. Results are expressed as mean ± standard deviation. In order to compare the results between the two culture media, one-way analysis of variance (ANOVA) with *post hoc* Bonferroni test was applied, electing a significance level of 0.05.

III. Results

The human bone marrow stromal cells were seeded onto gelatin cryogel disks, and then cultured without or with a differentiating stimulus for 6 weeks. These culture methods permitted the study of the cells as they modified the biomaterial through the proliferation and the coating with extracellular matrix. The cell-matrix distribution was compared between the two culture media.

Microscope analysis: In comparison to control condition, SEM images revealed that, due to the differentiative medium, the bone marrow stromal cells differentiated and built their extracellular matrix over the available gelatin surface (Fig. 2). At the end of the culture period, control culture showed few cells essentially not surrounded by extracellular matrix, therefore wide biomaterial regions remained devoid of cell-matrix complexes (Fig. 2A). In contrast, the differentiative medium caused a wide-ranging coat of the biomaterial surface: several stromal cells differentiated and the biomaterial was tending to be hidden by cell-matrix layers (Fig. 2B).

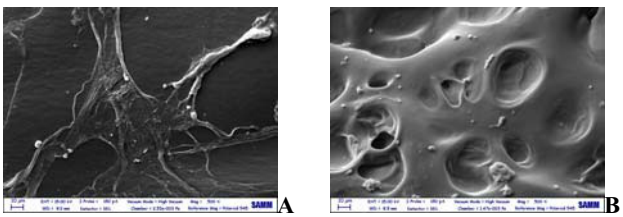

Fig. 2 SEM images of the control (A) and differentiating (B) cultures, bar equal to 10 μm, 500× magnification

The immunolocalization of type-I collagen and decorin showed the differentiating effects in terms of more intense building of the extracellular matrix (Figs. 3 and 4). The immunolocalization of osteocalcin, osteopontin, and type-III collagen revealed similar results (data not shown).

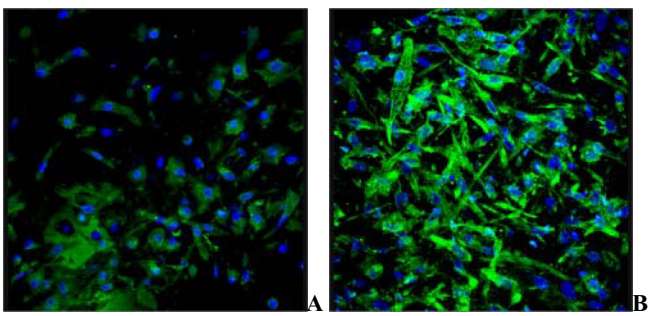

Fig. 3 Immunolocalization of type-I collagen in the control (A) and differentiating (B) cultures, 100× magnification

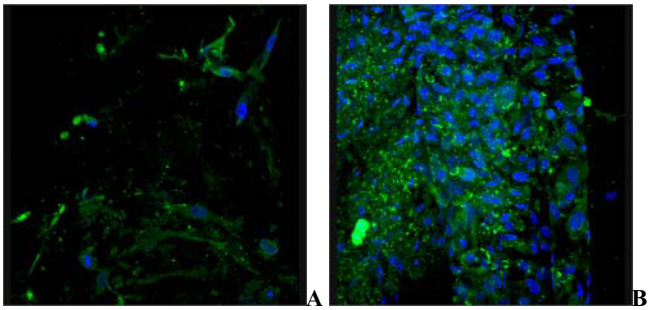

Fig. 4 Immunolocalization of decorin in the control (A) and differentiating (B) cultures, 100× magnification

The differentiative properties of the medium 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 $5.72\times10^5 \pm 4.3\times10^4$ and in the differentiating culture to $5.17\times10^5 \pm 3.7\times10^4$ with $p>0.05$.

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 differentiative stimulation significantly increased the surface coating with decorin, osteocalcin, osteopontin, type-I collagen, and type-III collagen ($p<0.05$) (Table 1).

Table 1 Bone matrix constituents onto gelatin cryogel [fg/(cell×disk)]

	Control culture (C)	Differentiating culture (D)	D/C
Decorin	0.45 ± 0.11	3.01 ± 0.12	6.68
Osteocalcin	0.10 ± 0.34	5.20 ± 0.17	52.00
Osteopontin	0.38 ± 0.23	9.30 ± 0.33	24.47
Type-I collagen	7.26 ± 0.14	21.60 ± 0.21	2.97
Type-III collagen	1.32 ± 0.27	4.17 ± 0.25	3.15

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

IV. Discussion

The aim of this study was the *in vitro* modification of a gelatin cryogel with extracellular matrix and osteoblasts differentiated from bone marrow stromal cells in order to make the biomaterial more biocompatible for the bone repair *in vivo*.

A discussion about the concept of "biocompatibility" is necessary. When a biomaterial is implanted in a biological environment, a non-physiologic layer of adsorbed proteins mediates the interaction of the surrounding host cells with the material surface. The body interprets this protein layer as a foreign invader that must be walled off in an avascular and tough collagen sac. Therefore, the biomedical surfaces must be developed so that the host tissue can recognize them as "self". Castner and Ratner think the "biocompatible surfaces" of the "biomaterials that heal" as the surfaces with the characters of a "clean, fresh wound" [17]: these "self-surfaces" could obtain a physiological inflammatory reaction leading to normal healing. In this study we have followed a biomimetic

strategy where the seeded bone marrow stromal cells built a biocompatible surface made of bone matrix [18].

To enhance the coating of the biomaterial surface, a differentiative stimulus was applied to the seeded biomaterial. The differentiating medium didn't increase the cell proliferation, however significantly enhanced the synthesis of type-I collagen, decorin, osteopontin, osteocalcin, and type-III collagen, which are fundamental constituents of the physiological bone matrix: in particular type-I collagen is the most important and abundant structural protein of the bone matrix; decorin is a proteoglycan considered a key regulator for the assembly and the function of many extracellular matrix proteins with a major role in the lateral growth of the collagen fibrils, delaying the lateral assembly on the surface of the fibrils [19]; 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 gelatin cryogel crystal growth, and, through specific interaction with the vitronectin receptor, it promotes the attachment of the cells to the matrix [20]; osteocalcin is secreted after the onset of mineralization and it binds to bone minerals [21].

In this study the differentiating 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 differentiative medium and the worth of the new gelatin cryogel scaffold for total immunocompatibility and complete biocompatibility with the patient, respectively.

In conclusion, we theorize that the cultured "self-surface" could be used fresh, that is, rich in autologous cells and matrix, or after sterilization with ethylene oxide, that is, rich only in autologous matrix. In future work, we intend to use our constructs, which are rich in autologous matrix, as a simple, storable, tissue-engineering product for the bone repair.

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