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A case of successful interaction between cells derived from human ovarian follicular liquid and gelatin cryogel for biotech and medical applications

Claudia Omes, Lorenzo Fassina, Sandra Van Vlierberghe, Giovanni Magenes, *Member, IEEE*, Peter Dubruel, Patrizia Vaghi, Marcella Reguzzoni, and Federica Riva

Abstract— **Significant research efforts have been undertaken in the last decade to develop specific cell-based therapies and, in particular, adult multipotent mesenchymal stem cells (MSCs) hold great promise toward such regenerative strategies. Biomaterials have been widely used in reconstructive bone surgery to heal critical-size bone defects due to trauma, tumor resection, and tissue degeneration. In particular, gelatin cryogel scaffolds are promising new biomaterials owing to their biocompatibility. There is an increasing demand for MSC-based regenerative approaches in the musculoskeletal system. Combining stem cells with biomaterial scaffolds provides a promising strategy for tissue engineering. Our previous studies showed the possibility to obtain MSCs from the human ovarian follicular liquid (FL) that is usually wasted during *in vitro* fertilization (IVF). In this study, we tested the ability of these FL cells to grow on gelatin cryogel in comparison with MSCs derived from human bone marrow. Samples and controls were analyzed with confocal and scanning electron microscopes. Results demonstrated that FL cells could grow on the biomaterial not only on the top but also in the layers below till 60 µm of deepness. Data suggested that the observed cells were mesenchymal since positive for vimentin and CD-44, typical MSC markers. Successful growth of putative MSCs derived from follicular liquid on 3D gelatin cryogel opens potential developments in biotech and medical applications.**

I. Introduction

The strategies to repair tissue defects are of growing importance due to the increasing number of elderly people with degenerative diseases. In addition to aging and diseases, tissue defects can also occur due to trauma. The regeneration of damaged musculoskeletal tissues is a great challenge in regenerative medicine which aims to repair and replace lost or damaged tissues [1,2].

Current tissue engineering uses 3D biomaterials in combination with stem cells, since mature cells are often not available in sufficient amounts or quality. Biomaterial scaffolds are developed not only as cell carriers providing mechanical support, but also as promoters of cell attachment and proliferation. A great variety of advanced biomaterials is used in regenerative medicine [3]. To design a scaffold for a specific medical application, the material's composition, architecture, structural mechanics, surface topology, and degradation properties have to be considered.

In particular, for the reconstruction of bone and cartilage, a scaffold biomaterial should possess suitable mechanical strength, stiffness, or elasticity to replace the damaged tissue [4]. In addition to biocompatibility, scaffolds are usually designed biodegradable thus avoiding additional surgery: ideally, the scaffold is remodeled by isomorphous tissue replacement, so that the scaffold residence time must be nearly equal to the time required to synthesize a mature tissue via regeneration.

Furthermore, 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" [5].

Another important choice is the cell source. Adult mesenchymal stem cells (MSCs) pose less ethical questions and are less prone to tumor formation compared to embryonic stem cells. MSCs are multipotent, have a great ability for self-renewal, and can differentiate into different cell types including osteoblasts, chondrocytes, and adipocytes [6]. These properties led to use MSCs in regenerative medicine.

Moreover, gelatin cryogel seems to be a promising new biomaterial for the differentiation of bone marrow stromal cells: onto gelatin, in a previous study, we followed a biomimetic strategy where differentiated human bone marrow stromal cells built their extracellular matrix [7,8]. In other study, we found a new source of MSCs derived from human ovarian follicular liquid (FL) that is usually wasted during *in vitro* fertilization (IVF) [9,10].

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C. Omes is with the IRCCS Fondazione Policlinico San Matteo of Pavia, Centro di Procreazione Medicalmente Assistita, Ostetricia e Ginecologia (e-mail: claudia.omes@unipv.it).

L. Fassina is with the University of Pavia, Dip. di Ingegneria Industriale e dell'Informazione, C.I.T. (e-mail: lorenzo.fassina@unipv.it).

S. Van Vlierberghe is with the University of Ghent, Polymer Chemistry & Biomaterials Group (e-mail: sandra.vanvlierberghe@ugent.be).

G. Magenes is with the University of Pavia, C.I.T., Dip. di Ingegneria Industriale e dell'Informazione (e-mail: giovanni.magenes@unipv.it).

P. Dubruel is with the University of Ghent, Polymer Chemistry & Biomaterials Group (e-mail: peter.dubruel@ugent.be).

P. Vaghi is with the University of Pavia, Centro Grandi Strumenti (e-mail: patrizia.vaghi@cgs.unipv.it).

M. Reguzzoni is with the University of Insubria, Dip. di Scienze Chirurgiche e Morfologiche (e-mail: marcella.reguzzoni@uninsubria.it).

F. Riva is with the University of Pavia, Dip. di Sanità Pubblica, Neuroscienze, Medicina Sperimentale e Forense (Sez. di Istologia ed Embriologia Generale), Centro di Ingegneria Tissutale (C.I.T., http://cit.unipv.it/cit), via Forlanini 10, 27100 Pavia, Italy (phone: +390382987272; fax: +390382528330; e-mail: federica.riva01@unipv.it).

In this work, our aim was to show the combination between multipotent MSCs derived from FL and gelatin cryogel scaffold as promising strategy for tissue engineering.

II. Materials and Methods

A. Gelatin cryogel disks

Bovine gelatin cryogel disks (diameter, 10 mm; height, 2 mm) were kindly provided by Polymer Chemistry & Biomaterials Group, University of Ghent (Belgium) [7,8,11] (Fig. 1).

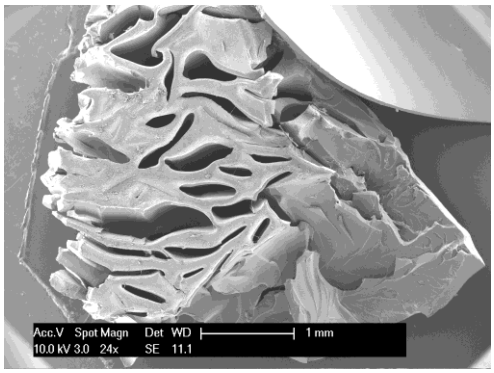

Figure 1. Unseeded gelatin cryogel at SEM. Bar equal to 1 mm, 24× magnification.

B. Cell cultures

By transvaginal ultrasound-guided aspiration, ovarian follicular liquids were collected during oocyte retrieval from 5 women of heterogeneous age (33.4 ± 4.7 years) (Prot. # 20080002153, Bioethics Committee of IRCCS Fondazione Policlinico San Matteo of Pavia). After the removal of the cumulus oophorous-oocyte complexes, follicular aspirates were centrifuged in density gradient (Lymphoprep, Nycomed Pharma, Oslo, Norway) for 30 min at 1800 rpm in order to eliminate red blood cells and debris. In the middle layer is clearly visible the buffy coat containing the follicular liquid cells, which is recovered, twice washed in 10 ml of sterile PBS, and centrifuged for 10 min at 1200 rpm for the final cell collection.

The gelatin cryogel disks were placed in Petri dishes and covered with 2 ml of 0.9% NaCl solution. After 24 h, this solution was replaced by DMEM culture medium (Sigma-Aldrich, USA) supplemented with 10% FBS, 2 mM L-glutamine, 100 mg/ml penicillin, and 100 µg/ml streptomycin (EuroBio, France). After 24 h, 5×10^5 FL cells were seeded onto each gelatin disk in 400 µl of culture medium; after 30 min, sufficient medium to completely cover the disks was added. Identical culture conditions were applied for human bone marrow stromal cells used as a positive control [8]. Cultures were maintained at 37°C and 5% CO_2. After 48 h, non-adherent cells were discarded and the culture medium was changed twice a week.

C. Immunostaining and confocal microscopy analysis

After 15 days of culture, cells were fixed with 4% paraformaldehyde for 3 h at room temperature, washed three times in PBS, and incubated for 20 min in PTA blocking solution (1% BSA and 0.02% Tween 20 in PBS). Cells were then incubated for 1 h at room temperature with a mouse primary antibody (diluted 1:100 in PTA) (monoclonal anti-vimentin or monoclonal anti-CD-44, Biogenex, USA), washed three times in PTA, and then incubated for 30 min at room temperature with anti-mouse FITC-conjugated secondary antibody (diluted 1:100 in PTA) (Sigma-Aldrich). Then, the cells were washed in PBS, counterstained for DNA with 0.5 µg/ml Hoechst 33258 (Sigma-Aldrich). Finally, the cells were observed by a confocal laser scanning microscope (Leica TCS SP2, Leica Instruments, Germany) acquiring images every 1.5 µm till 100 µm of depth.

D. Scanning electron microscopy (SEM) analysis

After 15 days of culture, gelatin cryogel disks were fixed with 2.5% 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 1200× magnification with a Philips XL30 FEG SEM (Koninklijke Philips Electronics N. V., The Netherlands) at secondary electron mode.

III. Results

A. Immunostaining and confocal microscopy analysis

Cells were seeded onto gelatin cryogel disks and cultured for 15 days. Adherent cells showed cytoplasmic positivity for vimentin (a cytoskeleton protein in mesenchymal cells) (Fig. 2) and surface positivity for CD-44 (a marker of mesenchymal cells) (Fig. 3). These data also showed that FL cells were able to grow till 60 µm of deepness (Fig. 3) in similar manner as bone marrow MSCs.

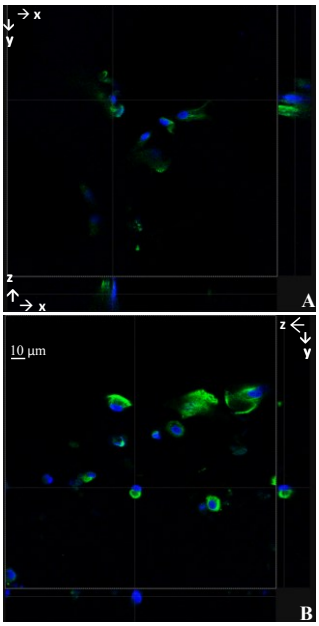

Figure 2. Bone marrow MSCs (*A*) and FL cells (*B*) cultured onto gelatin cryogel and immunostained for vimentin (green fluorescence). Nuclei were counterstained with Hoechst 33258 (blue fluorescence). Bar equal to 10 µm.

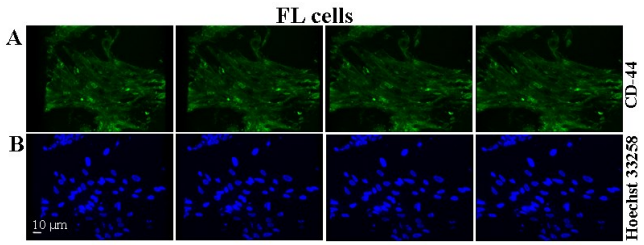

Figure 3. FL cells cultured onto gelatin cryogel and immunostained for CD-44 (green fluorescence, *A*). Nuclei were counterstained with Hoechst 33258 (blue fluorescence, *B*). Bar equal to 10 µm.

B. SEM analysis

Similarly to bone marrow MSCs, FL cells grew not only on the surface of the biomaterial, but also in the layers below and in the biomaterial gaps (Fig. 4).

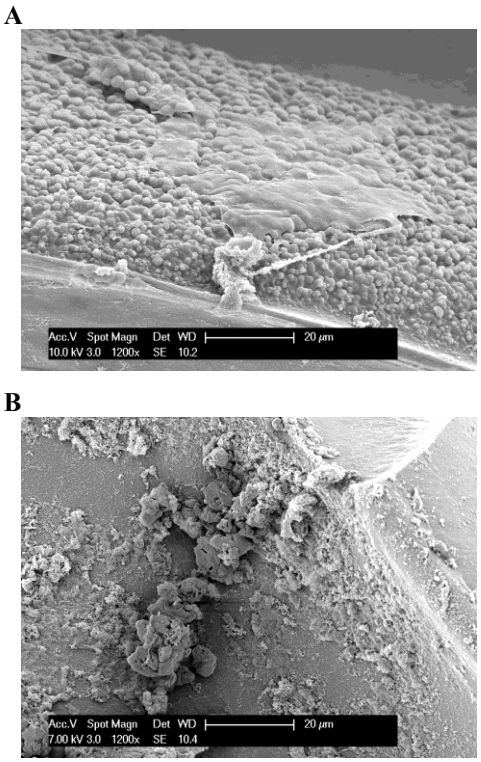

Figure 4. Bone marrow MSCs (*A*) and FL cells (*B*) cultured onto gelatin cryogel and observed by SEM. Bar equal to 20 µm.

IV. Discussion

Human MSCs can be isolated from several sources, including bone marrow and adipose tissue [12,13]. MSCs proliferate onto the surfaces with fibroblastic morphology and can differentiate into osteoblasts [14], chondrocytes [15], and adipocytes [16]. MSCs can be seeded onto biomaterials and transplanted into tissue defects [8].

In this study, we used two different mesenchymal cell populations derived from human ovarian follicular liquid and from human bone marrow. Results showed that mesenchymal FL cells grew on gelatin cryogel till 60 µm of deepness and had an excellent compatibility with the biomaterial surface in terms of expression of vimentin and CD-44 which are important markers of stemness. Bone marrow MSCs, used as control, showed a similar behavior.

V. Conclusion

In conclusion, FL cells showed promising affinity with the gelatin cryogel. These data suggest to consider the follicular liquid as a new source of mesenchymal stem cells. In future work, we intend to use this cell-biomaterial construct as a tissue engineering product for the bone repair and also to test the ability of FL cells to produce bone extracellular matrix [8] under physical stimuli [17,18,19].

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