Biochemical Effects of Collagen Supports-Coated with Stem Cells on Experimental Skin Wound Healing

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Drug delivery systems based on collagen represent a continuous challenge for chemistry and medicine. Our studies aimed the biochemical effects of biological collagen-coated matrices with rat mesenchymal stem cells on rat skin wound healing in vivo. When compared to control group, or to collagen matrices, the collagen matrices coated with mesenchymal stem cells induced a better repairing response, also from the point of view of macroscopic as well as microscopic examination. The obtained results proved evidence that the coating of collagen type I matrices with rat mesenchymal stem cells would accelerate the wound repairing processes at the level of experimentally damaged rat skin. The inflammatory processes are increased, the proliferative tissue is enhanced, and a large number of fibroblasts are active. Meanwhile, the connective tissue proliferated somehow in an exaggerated manner, inducing also a relative destruction of striated muscle layer. This aspect is equal to less controlled processes. The studies concerning the application of such strategies in therapeutics need further development.

Keywords: collagen, matrices, mesenchymal stem cell, rat, skin, wound repairing

Skin is functioning as a filter with immunological, sensorial and protecting properties, being the biggest organ. It's all along exposed to the environmental aggressive factors and could be damaged or injured with variable intensity and loss of extracellular matrix. That's why there were achieved multiple modalities to treat skin wounds, including but not limiting to allografts, autografts, and tissue-engineered alternatives, wound bandages and nanofiber scaffolding devices. Although such therapeutic methods demonstrated clinical effectiveness, they are associating issues concerning inappropriate vascularization, further morbidity, lack of appendages reproduction techniques, wound focus very low adhesion as well as really high production costs. Actual methods developed starting from nanofiber scaffolds constitute effective alternatives for regenerative and repairing skin medicine, using cellular growth factors, many types of cells, and new biomaterials and advanced bio-producing methods [1].

Such an example is represented by sericin, a cheap glycoprotein resulting as a secondary product in the silk industries. This glycoprotein shows different amino acid and functional groups in peptide chains, resulting in interesting bioactive abilities, especially important for its applications in medicine. Furthermore, sericin is successfully used in tissue engineering and cell culture approaches, having an impressive antioxidant and moisturizing capacity, as well as mitogenic effects on mammalian cells. Bandages with sericin were developed starting from observed positive effects exerted on keratinocytes and fibroblasts, being used as skin tissues repair systems. In addition, sericin is able to induce the nucleation of hydroxyapatite in a manner very close to that of bones structures. Established biomaterials having as base the silk sericin are including films, sponges, and hydrogels. Sericin is also a good candidate for drug delivery as nano- and microparticles taking into account its particular chemical reactivity and *p*H-responsive abilities [2].

The older wounds fail to go through physiological repairing processes, placing the patients outside the consecutive wound healing time stages, increasing the risk for complications, deeply altering the quality of life and involving higher final healthcare costs. Although speaking about physiological or prolonged wound repairing processes, one very important element is the role played by extracellular matrix. Extracellular matrix represents the largest constituent of dermal layers and ensures the cellular and supportive structure absolutely necessary for physiological tissue repairs. Extracellular matrix is highly degraded in chronic wounds, which are characterized by an aggressive inflammatory and proteolytic hotbed. For the treatment of dermal human chronic wounds, the acellular scaffolds are used, providing supportive matrices able to trigger cellular specific functioning. The target for such used approaches is to populate *de novo* the scaffolds with body cells, allowing the regenerative healing, and not fibrous repairing. There are many discussions on scaffold matrices for regenerating tissues, the evidences being critically and particularly analyzed in each case [3].

There are several attempts to develop at least one good model for skin engineering starting from human amniotic mesenchymal stem and epithelial cells, derived from amniotic membrane. FACS and microscopy confirmed the morphologic and phenotypic appearance of the above mentioned cell types. The biomarkers expressed by stem cells as well as keratinocytes were uncovered by real time-polymerase chain reaction and immunofluorescence. The co-culture of the two cell types was developed in the view of characterizing the growing pattern and phenotype expression of amniotic mesenchymal stem and epithelial cells. Histopathological methods were applied to assess the *in vitro* engineered skin. The two amniotic isolated and cultured cell types exhibited morphous fibroblast- and epithelial-like shapes. Moreover, specific markers were expressed on the surface of mesenchymal stem and epithelial cells (CD73, CD90 as well as CD105), whereas others (CD34, HLA-DR) were missing. Real time-

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polymerase chain reaction found the expression of biomarkers as Nanog, c-MYC, K19, β1-integrin and K8 for mesenchymal stem cells. On the other hand, on the surface of human amniotic epithelial cells the real time-polymerase chain reaction uncovered the expression of KLF4, c-MYC, K19, β1-integrin, K14, K5 and K8. The phenotypes of both cell cultures were not changed by the co-culture technique. By the way, it amplified the proliferative capacity of amniotic epithelial cells, and reduced the proliferative ability of amniotic mesenchymal stem cells. The obtained engineered tissue presented a very similar structure to normal one. Thus, it was demonstrated that human amniotic mesenchymal stem cells and human amniotic epithelial cells could be basically used to construct a tissue similar to skin at least *in vitro*. Such engineered tissues are to be applied successfully in regenerative medicine [4].

There are almost none studies on therapeutic means in combination with ageing factors on skin [5]. When speaking about anti-ageing domain it is obvious that adipose-derived stem cells became a popular subject as an attempt to reverse tissue senescence. Thus, the human dermal fibroblasts were treated with B ultraviolet radiations for induction of different levels of senescence and further with collected culture medium of adipose-derived stem cells. After 48 h of exposure, the proliferation of human dermal fibroblasts was assessed, as well as morphologic appearance, apoptotic index, and expression of SA- β-Gal, mRNA collagen I, collagen III and elastin. Human dermal fibroblasts proliferative capacity was reduced by intrinsic ageing factors and B ultraviolet radiation, their senescence ratio was augmented, and the expressed mRNA of collagen I, collagen III and elastin was altered. In addition, when applied, the culture medium of adipose-derived stem cells could improve to a small degree or to a significant one the proliferation of irradiated and non-irradiated human dermal fibroblasts. Meanwhile, the restoration of the physiological functions in these cells was also observed in experimental conditions. Moreover, culture medium obtained from adipose-derived stem cells reduced apoptotic index and senescent status obtained after B ultraviolet radiation treatment, but had no significant effects when intrinsic ageing factors were used. These observations were the same for 3 generations of human dermal fibroblasts and were not dependent on the degree of obtained senescence. Thus, it might be concluded that the secretome of adipose-derived stem cells is able to protect human dermal fibroblasts in variable degrees against ageing damages, although many limitations are obvious [6].

There are many other attempts to study the possibility of applying skin wounds dressing models composed of cultured cells (e.g., human umbilical cord mesenchymal stem cells) and alginate gel scaffolds. The proliferative capacities of human umbilical cord mesenchymal stem cells were followed as compared to their migration and expressed levels of vascular endothelial growth factors when administered into Balb/c mice skin wounds, as a mix with alginate gel scaffolds. The rates of wound healing processes were assessed and immunohistochemistry was applied to examine the microscopic results. Although the growth rate of human umbilical cord mesenchymal stem cells in alginate was fine, their capacity to migrate was reduced, although significantly statistic as compared to control series. Moreover, the expressed vascular endothelial growth factor was also higher for stem cells in gel scaffold after 3-6 days. At 15 days, neovascularization was greater and wound healing rate was more pronounced when human umbilical cord mesenchymal stem cells were administered. The paracrine signaling is to be involved in the skin wound healing beneficial models of human umbilical cord mesenchymal stem cells-alginate gel scaffolds treatments [7].

Our studies aimed the biochemical effects of biological collagen-coated matrices with rat mesenchymal stem cells on rat skin wound healing *in vivo*.

Experimental part

Femural and tibial bone marrow from 180-200 g Wistar male rats (Bãneasa source) were used to isolate mesenchymal stem cells, using an adapted method [8]. Mesenchymal stem cells were grown in α-MEM medium (Sigma-Aldrich) with 10% fetal bovine serum (FBS, Sigma-Aldrich). The appropriate cells, the nucleated ones, were multiplied in Petri culture dishes through incubation at 37°C for 6 consecutive days. After 2 harvestings with 0.25% trypsin-EDTA (Sigma-Aldrich) and consecutive resuspension in the above complete medium, the cells were further cultured for 3 days. The obtained monolayers were equated with rat mesenchymal stem cells [8, 9]. The phenotype of resulted cells was analyzed using fluorescein isothiocyanate (FITC)-conjugated primary antibodies against CD34 and CD44. The secondary necessary antibodies were represented by goat anti-rat antibody (Sigma-Aldrich). The analysis was performed using flow cytometry using a FACS caliber flow cytometer (Becton Dickinson Immunocytometry Systems).

The biological collagen type I matrices were prepared in accordance with the technique previously described [10].

The rats (Wistar from Bãneasa source) for developing wound healing skin model were bred in identical laboratory conditions, under a day-night cycle of 12 h. The rats were anesthetized one by one using thiopental administered i.p., waxed in dorsal area on a surface of about 10 cm^2 using depilatory cream *Farmec* and a polished wooden wand. 24 h later there were induced the lesions in the middle of waxed area, using a pair of scissors and sterile forceps. Each incision having a length of 1 cm was a deep one without affecting the underlying layers.

Collagen matrices coated with stem cells were applied immediately after bleeding stopping, so that to cover the entire induced lesion, and were fixed with plaster. The comparison was made with the control groups, which received only collagen matrices or no treatment. Macroscopic evaluation (visual inspection) and histopathological (microscopic) healing were conducted 5 days after the application of dressings. The absolute majority of rats kept the matrices on the defect during this time. After macroscopic evaluation, the rats were anesthetized and killed with an adequate dose of thiopental, after that the damaged skin region was excised and fixed in 10% formaldehyde solution. Hematoxylin-eosin staining was used.

The microscopic setup was based on an inverted Nikon Eclipse TE-300 microscope, using 20 x objectives, as well as the Nikon software (NK Remote v.2.2.3.). The collected images were processed through the help of free ImageJ (National Health Institute, U.S.A.) [11-14].

The protocols involving Wistar rats were previously approved by the Ethics Committee of the Grigore T. Popa University of Medicine and Pharmacy from Iasi.

Results and discussions

Microscopic examination of control group receiving no treatment (fig. 1) revealed ulceration of the epidermis covered with hematic crust, permeating deeply the dermis. Acute inflammation with polymorphonuclear cells is

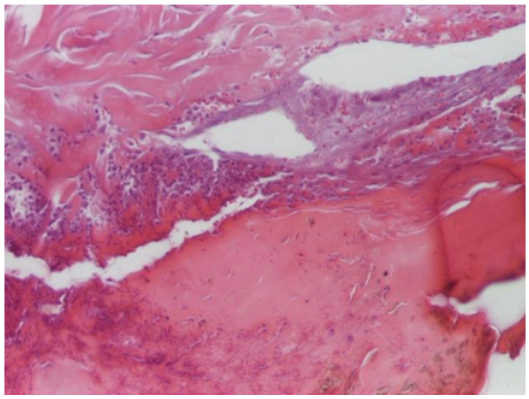

Fig. 1. Representative image of microscopic examination of control group receiving no treatment. Acute inflammation is limited and fibrosis is a beginning one (20X)

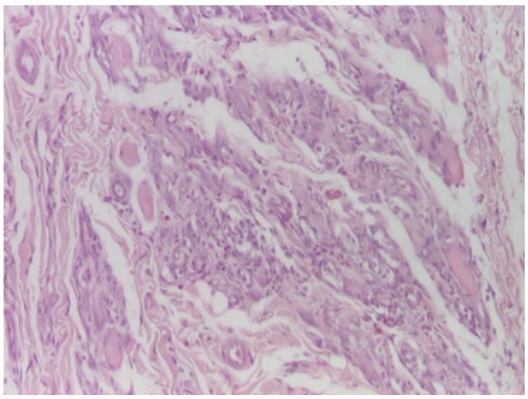

Fig. 2. As compared to control group, the microscopic appearance was relatively better when treatment consisted of collagen matrices (20X)

limited and leukocytes margination is evident in a small number of deep dermis vessels. Fibrosis is a beginning one with lymphocytes and plasma cells, very rare mast cells and rare multinucleated giant cells.

When applied only collagen matrices the macroscopic healing is relatively better that in group with no treatment. Microscopically (fig. 2), the epithelium was ulcerated, with large areas of muscle layer destruction, muscle cells being replaced by connective fibers and macrophages, weak inflammatory reaction with polymorphonuclear cells and very rare eosinophils.

Collagen matrices coated with mesenchymal stem cells induced a better response, also from the point of view of macroscopic examination. Microscopic examination (fig. 3) showed ulceration of squamous epithelium, proliferated connective tissue with active fibroblasts, young vessels, and rare inflammatory cells. Striated muscle layer destruction and penetration of proliferated connective tissue below the muscle layer were also observed.

The obtained results proved evidence that the coating of collagen type I matrices with mesenchymal stem cells would accelerate the repairing processes at the level of experimentally damaged rat skin. The inflammatory processes are augmented, the proliferative tissue is enhanced, and a large number of fibroblasts are active. Meanwhile, the connective tissue proliferated somehow in an exaggerate manner, inducing also a destruction of striated muscle layer. This aspect is equal to less controlled processes.

Moreover, our results might be in accordance with recent findings suggesting the paracrine secretion of mesenchymal stem cells being involved as effector of these cells activation and functioning in different environments [9, 15].

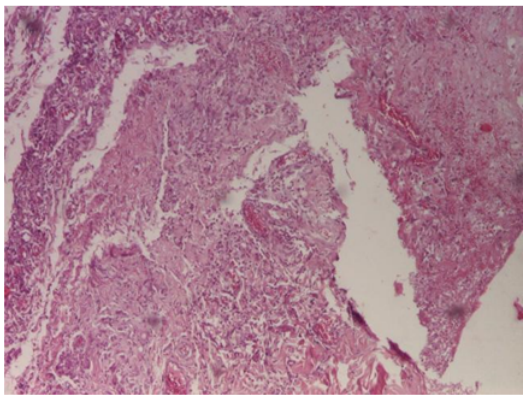

Fig. 3. Coating of collagen matrices with mesenchymal stem cells improved the skin reparatory processes (20X)

Collagen-drug delivery systems represent a continuous challenge for chemistry and medicine. For example, there are many tries to obtain composites of collagen and vinblastine in spongious form as a promising pharmacological mean for Kaposi's sarcoma treatment. These composites are absorbing high quantities of water, a process dependent on crosslinking and drug concentrations. The antitumoral drug is released with high velocity in the first hour and then slower, allowing the inhibition of tumoral cells growth and the consolidation of effects [16].

Another attempt to obtain biosystems with antioxidant capacities was represented by collagen and tannic acid composites to be used topically or as bandages for skin wound healing. As bases could be used collagen hydrogels or matrices, both of them being able to release tannic acid in a controlled manner [17].

Soft infections could be treated with hydrogels administered topically, based on collagen and minocycline. The composites are to be crosslinked or uncrosslinked, showing thixotropic behaviours, as well as pseudoplastic and shear thinning [18].

Plasma-coated surfaces with high degree of collagen adsorption were obtained starting from films formed by poly(ethylene terephthalate) and a direct current helium discharge. These surfaces allowed the sustained growth of human aortic endothelial cells as a monolayer *in vitro* [19].

Extensive burns need skin surrogates to be functionally applied in a conceivable period of hospitalization since the repairing processes are allowed within a narrow time. That's why there were fabricated such surrogates starting from nanofibers and cells assemblies and applied in experimental wound healing of mice. The three-dimensional constructs included as sandwiches components (layer by layer) keratinocytes, polycaprolactone nanofibers, and fibroblasts. These aggregates evolved as structures similar to skin with fine strength and expressed biomarkers specific to keratinocytes. Moreover, deposits of extracellular matrix, characteristic for connective tissue, were developed by these sandwiches. When compared, the assemblies including autografts were less effective than cultured skin surrogates to treat the experimental wounds produced on the mice back. For the last mentioned devices the epithelialization was completely achieved. The clear conclusion is that cultured skin surrogates for 14 days could mimic the physiological environment needed for large wounds as burns to be efficiently treated [20].

Another tissue where surrogates were tried is represented by oral mucosa. These surrogates were developed starting from Wharton's jelly stem cells. The derived stem cells were combined in surrogates with

keratinocytes from oral mucosa. The clear target of the studies was the degree of differentiation of stroma. The results clearly pointed out that the engineered oral mucosal stromas presented an appropriate fibrillar network. *De novo* collagen fibers were detected in high amounts and *in vivo* applying generated an augmented synthesis of collagen. The lack of elastic and reticular fibers was obvious. Synthesis of glycoproteins was increased, found to be really dependent on the *in vivo* surroundings and was strongly related to existence of a good epithelium on top [21].

Conclusions

Collagen matrices coated with mesenchymal stem cells induced a better response, also from the point of view of microscopic as well as macroscopic examination when administered as a treatment for experimental skin damages in rats.

Moreover, our results might be in accordance with recent findings suggesting the paracrine secretion of mesenchymal stem cells being involved as effectors of these cells activation and functioning in different environments.

Acknowledgements: Gianina Elena Bogza is a recipient of Ph.D. studies at the Grigore T. Popa University of Medicine and Pharmacy of Iasi, 16, University Str.,700115, Romania

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Manuscript received: 3.02.2016