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Research Article

Influence of extracellular matrix on the proliferation and adhesion properties of stem cells derived from different sources

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ABSTRACT

One of the most important issues in regenerative medicine is the development of culture conditions mimicking the natural ones, which allows obtaining a large number of cells and their long-term maintenance in undifferentiated state. *In vivo*, cells are surrounded by a specific microenvironment called extracellular matrix (ECM), which plays an important role in the regulation of processes such as proliferation, migration, differentiation or apoptosis. In this study we assessed the influence of different extracellular matrix components (fibronectin, laminin, collagen IV, poly-D-lysine) on the *in vitro* adhesion and proliferation of stem cells isolated from bone marrow, adipose tissue and hair follicles. Our results showed that stem cells derived from different sources present various responses to ECM components. None of the tested extracellular proteins reduced the proliferation of bone marrow as well as adipose-derived mesenchymal stem cells, with the exception of laminin. This demonstrates the biocompatibility of such modified surfaces and possibility of using them for culturing these types of stem cells. Different results were obtained for hair follicle stem cells. The presented results indicate that ECM is an important component of the cellular niche in the tissue. It is also possible that ECM is required for the reconstitution of the niche of stem cells *in vitro*.

Keywords: Stem cells; Bone marrow; Adipose tissue; Hair follicles; Extracellular matrix.

1. INTRODUCTION

Tissue engineering methods offer new possibilities for the regeneration of diseased and damaged tissues and thus find an increasing attention in clinical practice. In tissue engineering, a variety of different cell types are used. However, the most attractive type are stem cells, particularly mesenchymal stem cells (MSCs).

One of the most important issues in the use of stem cells is the development of culture conditions mimicking the natural ones, which allows obtaining a large number of cells and their long-term maintenance in undifferentiated state. The proper growth and functioning of cells *in vivo* and *in vitro* depends on many factors, which result not only

from the interaction between cells (cell-cell type), but also from the interaction between cells and the extracellular environment (cell-matrix type) [1-3]. *In vivo*, cells are surrounded by a specific micro-environment called extracellular matrix (ECM), which plays an important role in the regulation of processes such as proliferation, migration, differentiation or apoptosis.

Although, fundamentally, ECM is composed of water, proteins and polysaccharides, every tissue has ECM with a unique composition and topology that is generated during tissue development through a dynamic and reciprocal biochemical and biophysical dialogue between the various cellular components and the evolving cellular and protein microenvironment. The ECM components can be divided into three major groups of molecules: insoluble (such as collagen, laminin, elastin, fibronectin), soluble (e.g. growth factors, chemokines, cytokines) and surface proteins of neighboring cells (cadherins). However, the composition and amount of all matrix molecules depends on cell type and location [2]. The selection of suitable extracellular matrix components may have a significant influence on *in vitro* cell growth. Moreover, appropriately selected ECM molecules often allow cell culturing in serum-free medium and/or without growth factors [4]. Such approach can minimize the risk of differentiation under *in vitro* conditions.

To date, both biological and synthetic materials have been used as ECM for *in vitro* cultures. However, materials derived from natural sources (e.g. collagen, laminin, fibronectin) appear to be preferable due to the presence of cell surface receptors that recognize these molecules [1].

The aim of this study was to assess the influence of different ECM components on the *in vitro* adhesion and proliferation of stem cells isolated from bone marrow, adipose tissue and hair follicles.

2. MATERIALS AND METHODS

The Local Bioethical Commitee of Nicolaus Copernicus University approved all procedures. In all studies, male Wistar rats (n=10) were used.

2.1. Isolation and culturing of bone marrow mesenchymal stem cells

Isolation of bone marrow was conducted using the Lennon and Caplan method [5]. Briefly, isolated rat femurs were washed with PBS supplemented with penicillin/streptomycin (100 μg/ml) and amphotericin B (5 μg/ml) (PAA, Austria). Distal parts of the femurs were cut off and bone marrow was flushed out using DMEM/Ham's F12 supplemented with 1% antibiotics solution. Subsesquently, the bone marrow was washed twice with PBS and centrifuged at 350 x g for 10 min. Isolated cells were cultured in the above medium containing additionally 10% FBS (PAA, Austria), 10 ng bFGF (Sigma, Germany) and L-glutamine (PAA, Austria).

2.2. Isolation and culturing of adipose mesenchymal stem cells

Adipose tissue was washed in PBS with antibiotics: penicillin/streptomycin (100 μg/ml) and amphotericin B (5 μg/ml). Subsequently, the tissue was purified from blood vessels and incubated in collagenase type I solution (1 ml/g of tissue) (Sigma, Germany) for 30 min in 37°C with shaking every 5 minutes. The digestion process was inhibited by adding an equal volume of culture medium. After that, the tissue was filtrated using a 100 μm cell strainer (BD Bioscience, USA). Thus obtained filtrate was centrifuged at 350 x g for 10 min and the cell pellet was washed twice with the culture medium. The cells were cultured in DMEM/Ham's F12 supplemented with 10% FBS (PAA, Austria), 10 ng bFGF (Sigma, Germany), amphotericin B (5 μg/ml), penicillin/streptomycin (100 μg/ml) and L-glutamine (PAA, Austria).

2.3. Isolation and culturing of follicle stem cells

Follicle stem cells were isolated from the hair follicles of rat sensory whiskers. A fragment of the skin was separated from the subcutaneous adipose and connective tissue and then washed in PBS with antibiotics: penicillin/streptomycin (100 μg/ml) and amphotericin B (5 μg/ml). Subsequently, the tissue was incubated in a dispase solution (10 mg/ml) (Gibco, USA) at 4°C for 16 h.

Hair follicles were isolated using micro-tweezers and the bulge regions were cut. Thus obtained hair follicle fragments were incubated in a solution of collagenase type P (1 mg/ml) (Roche, Switzerland) and dispase (1 mg/ml) for 0.5 h at 37ºC, followed by 0.05% trypsin solution (Biomed, Poland) for additional 1.5 h. After the incubation period, the solution was centrifuged (350 x g for 10 min). Cell culture was set up on a feeder layer (3T3 cell line) in Keratinocyte Serum-Free Medium (KSFM) (Lonza, Switzerland) supplemented with penicillin/streptomycin (100 μg/ml) and amphotericin B (5 μg/ml).

2.4. Phenotype analysis of isolated cells

Isolated stem cells were analyzed for the presence of specific surface markers by flow cytometry. Bone marrow stem cells were characterized with the use of CD90 and CD34 marker, adipose mesenchymal stem cells with the use of CD90, CD44, CD34 and CD45, while follicle stem cells with the use of cytokeratins 7, CD34 and p63. All analysis were performed according to the protocols previously described [6-8].

2.5. Evaluation of the influence of extracellular matrix proteins on the growth of stem cells

Stem cells isolated from three sources were cultured on 6-well plates coated with different ECM components such as: fibronectin, poly-D-lysine, laminin and collagen IV (BD Bioscience, USA). The number of seeded cells was 5 x 10^4/per well. Cells seeded on the polystyrene 6-well plate, not coated with any of the extracellular matrix components, served as a control. The cultures were run in media suitable for each type of stem cells at 37°C and 5% CO_2. Every 2-3 days, the medium was changed. The cells were incubated in these conditions for 7 days. Cell viability was analyzed using the MTT assay.

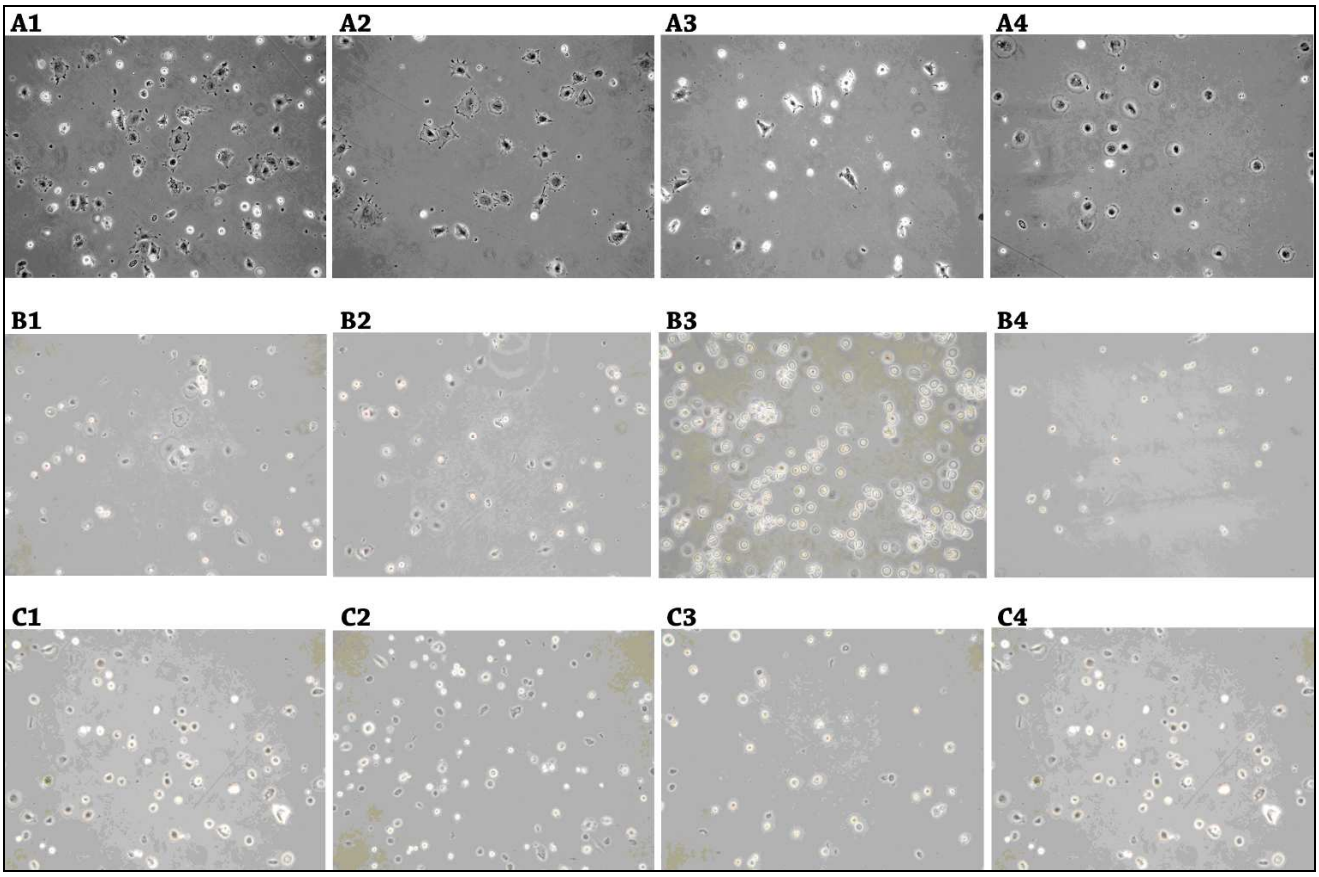

Figure 1. Stem cells isolated from bone marrow (A), adipose tissue (B), hair follicle (C) 30 minutes after seeding on plates coated with fibronectin (1), collagen IV (2), laminin (3) and poly-D-lysine (4).

3. RESULTS

For phenotypic characterization bone marrow, mesenchymal stem cells were assessed for the expression of CD90 and CD34. Expression of CD90 was at high level, while staining for CD34 was negative [6]. Adipose mesenchymal stem cells showed the high expression of CD90 and lower CD34 and CD44. The presence of CD45 was not detected [7]. Follicle stem cells expressed epithelial markers and were slighly positive for CD34 and p63 [8].

The analyzed stem cells showed significant differences in the ability of adhesion to the growth surface. The adhesion of bone marrow mesenchymal stem cells to the growth surface coated with fibronectin and collagen IV was 90% in 30 min after seeding (Fig. 1A1 and A2). Both modified surfaces supported the formation of a regular monolayer of spindle-shaped cells. However, at the same time, in the cultures on laminin- and poly-D-lysine-coated surfaces, the adhesion of cells was only 60% (Fig. 1A3 and A4).

Adipose-derived mesenchymal stem cells demonstrated a 90% adhesion during 30 min after seeding on plates coated with fibronectin, collagen IV and poly-D-lysine (Fig. 1B1, B2 and B4). However, the adhesion of the same cells cultured at the same time on laminin-coated surface was only 45% (Fig. 1B3). Hair follicle stem cells cultured on collagen IV- and laminin-coated plates showed a 50% adhesion during 3 h after seeding on the modified surfaces (Fig. 1C2 and C3). However, the adhesion of these cells to the culture plates coated with fibronectin and poly-D-lysine at the same time was only 10% (Fig. 1C1 and C4). The rate of cell proliferation was determined by MTT assay after 7 days of culture. The proliferation of bone marrow mesenchymal stem cells was the fastest on plates coated with fibronectin and collagen IV compared to the control culture. The slowest growth of these cells was observed on laminin-coated surface (Fig. 2).

The best results regarding the proliferation of adipose-derived mesenchymal stem cells were observed on the control surface, as well as the surface coated with collagen IV. The slowest growth of these cells was observed on plates coated with laminin (Fig. 3).

The proliferation of hair follicle stem cells was the fastest on the control surface that was not coated with any of analyzed extracellular matrix components. On each modified surface, cell growth was about 4 times slower (Fig. 4).

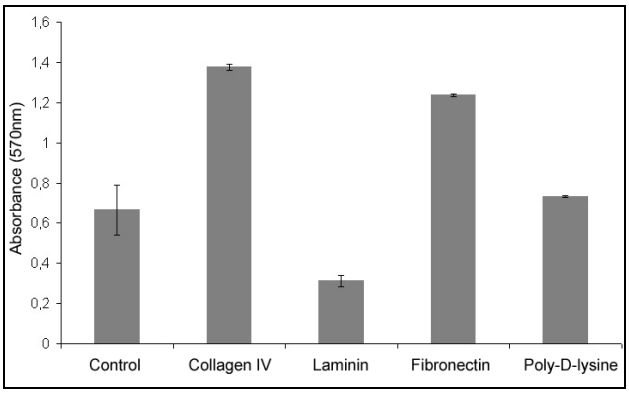

Figure 2. Proliferation rate of bone marrow mesenchymal stem cells on surfaces coated with different components of extracellular matrix.

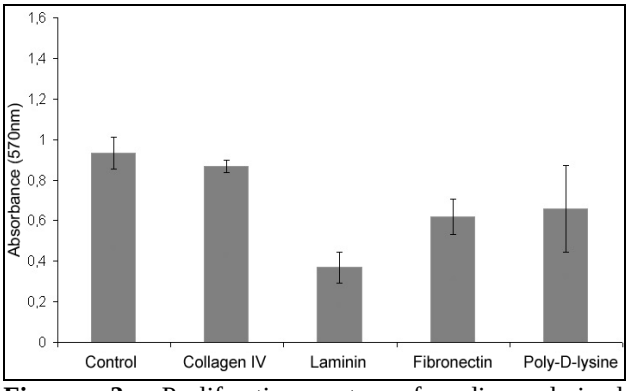

Figure 3. Proliferation rate of adipose-derived mesenchymal stem cells on plates coated with different components of extracellular matrix.

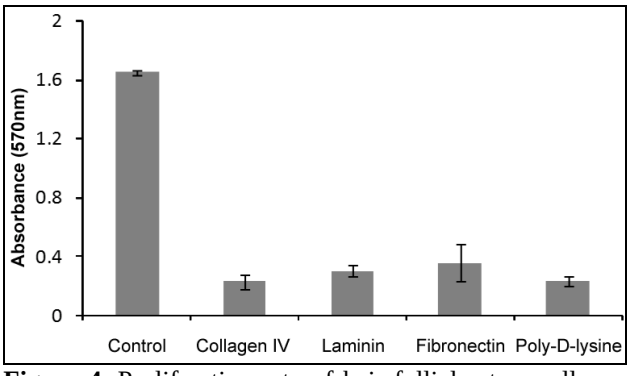

Figure 4. Proliferation rate of hair follicle stem cells on plates coated with different components of extracellular matrix.

4. DISCUSSION

The success of an *in vitro* culture often depends on the creation of environment that mimics the *in vivo* conditions. To date, a lot of biomaterials have been tested to provide a matrix for the proper cell adhesion and proliferation. Many materials such alginate, collagen or fibrin have been used. These molecules should support cell-cell and cell-matrix interactions, as well as regulate cell proliferation, migration, matrix remodeling and tissue organization - similarly to the *in vivo* conditions. Moreover, materials that are used should be biocompatible: show low antigenicity, biodegradability, non-toxicity, and should be able to maintain stem cells in their undifferentiated phenotype and promote differentiation only after induction. Due to these features of ideal micro-carriers, all attention is directed to the natural ECM components [3].

Stem cells are located in different niches that serve as their reservoir in physiological conditions. That is why it seemed very interesting to demonstrate how stem cells derived from three different sources would respond to various extracellular matrix components. In order to investigate the influence of ECM proteins on the ability of adhesion and proliferation rate of the stem cells from bone marrow, adipose tissue and hair follicles, collagen IV, fibronectin, laminin and poly-D-lysine were used.

The cell membrane of mesenchymal stem cells (MSCs), isolated inter alia from bone marrow, amniotic fluid, skin and adipose tissue, has receptors of adhesion molecules, such as ICAM-1, VCAM-1 and subunits of integrins [9, 10]. MSCs produce ECM proteins, such as collagen type I and III, laminin, vimentin and osteonectin [11]. Therefore, interaction of these cells with the matrix proteins appears to be essential for their differentiation. Lanfer et al. observed that cells grown on standard polystyrene culture vessels lose their original organization observed in physiological conditions [12].

Our results show that none of the tested substances reduced the proliferation of bone marrow, as well as adipose-derived mesenchymal stem cells, with the exception of laminin. This demonstrates the biocompatibility of such modified surfaces and possibility of using them for culturing these types of stem cells. In the case of stem cells isolated from rat bone marrow, the best proliferation rate was observed on plates coated with fibronectin and collagen IV, which also favored maintaining spindle-shaped, fibroblast-like cell morphology. Salasznyk et al. also showed that 80% of cells demonstrated adhesion during 30 min after seeding on a surface coated with fibronectin [13]. They also observed a slower proliferation of MSCs on culture dishes coated with laminin, which is consistent with the results obtained in this study. Similar findings were reported by Cool and Nurcombe [14], who demonstrated that human bone marrow mesenchymal stem cells attached to culture plates coated with fibronectin grew much better than those attached to other analyzed modified surfaces. Our studies showed that a similar effect is obtained with fibronectin, which promotes cell adhesion and proliferation.

Culture plates coated with collagen IV also showed a positive effect on the adhesion and proliferation of rat adipose-derived stem cells. However, in this case, favorable results were also observed on control plates that were not modified. This is in agreement with the suggestion by van Dijk et al. that MSCs from different sources show strong affinity to plastic culture plates [15].

We obtained different results regarding hair follicle stem cells. The adhesion of these cells to culture surfaces was much slower than in other cell types. Moreover, from all analyzed matrix proteins, the adhesion of hair follicle stem cells was the fastest on the control polystyrene surface, not coated with any of analyzed matrix components. Much poorer adhesion of these cells was observed on all analyzed modified surfaces. These results do not coincide with the previous data. Studies using mouse hair follicle stem cells, as well as limbus epithelial cells, showed that collagen IV was the best substrate for the culture of those cells [16, 17]. Adams and Watt showed a low level of adhesion of epithelial cells to culture plates coated with laminin [18]. In our studies, we noticed slow adhesion and proliferation rate not only on collagen IV and fibronectin, but also on laminin. Such weak adhesion rate of rat hair follicle stem cells to surface coated with collagen IV and fibronectin may be due to the lack of appropriate integrin receptors (α1β1,

α2, α3, α3β1, α4, α5) on the cell surface [19, 20]. It does not seem that the exposure of cells to ECM proteins could induce them to synthesize the corresponding receptors. However, the ubiquity of laminin and collagen IV in the basement membrane of hair follicles, described by Jahoda et al., may explain quite rapid adhesion of hair follicle stem cells to these substrates [21].

Extracellular matrix is a multifunctional network of fibrous, which provides structural and biochemical support to all tissues. These proteins have been implicated in many cellular processes, such as migration, proliferation, differentiation or apoptosis [21].

Regardless of the tissue type, ECM consists of different components and growth factors. More recently, it has been shown that stem cells are able to respond to the mechanical properties of the matrix. Cells can respond to microenvironment and change ECM expression, which resulting in remodeling f the matrix [22]. Besides its obvious role in determining the architecture and mechanical properties, ECM strongly influences the different cell functions [21]. However, the structure of ECM in most tissues is not well understood.

5. CONCLUSIONS

Our results showed that, depending on the origin of stem cells, their response to ECM components is different and that stem cells derived from distinct sources present differences in adhesion and proliferation rates with reference to the ECM components used. This indicates that ECM is an important component of the cellular niche in the tissue, supplying critical biochemical and physical signals to initiate or sustain cellular functions. It is also possible that ECM is required for the reconstitution of the niche of stem cells *in vitro*.

ETHICAL APPROVAL

All procedures conducted in the experiments involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

AUTHORS' CONTRIBUTION

AB - study design/planning, data collection/entry, data interpretation, literature analysis/search, wrote the initial draft of the manuscript. DP - data collection/entry, data interpretation. KR - literature analysis/search, data interpretation, statistical analysis, preparation of manuscript. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare that they have no conflict of interest.

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