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Skin extracellular matrix components accelerate the regenerative potential of Lin⁻ cells

Research Article

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Abstract: Due to their unique properties, bone marrow-derived Lin⁻ cells can be used to regenerate damaged tissues, including skin. The objective of our study was to determine the influence of the skin tissue-specific microenvironment on mouse Lin⁻ cell proliferation and migration *in vitro*. Cells were analyzed for the expression of stem/progenitor surface markers by flow cytometry. Proliferation of MACS-purified cells in 3D cultures was investigated by WST-8 assay. Lin⁻ cell migration was evaluated by *in vitro* scratch assay. The results obtained show that basement membrane matrix is more effective for Lin⁻ cell proliferation *in vitro*. However, type I collagen matrix better enhances the re-epithelization process, that depends on the cell migratory properties. These studies are important for preparing cells to be used in transplantation.

Keywords: *Lineage negative cells • BALB/c mouse • Proliferation • Migration • In vitro*

1. Introduction

The use of stem cells (SCs) in therapy is promising because of their unique ability to self-renew and generate several differentiated cell types [1]. Most adult tissues contain populations of primitive cells that maintain normal homeostasis. A number of studies have demonstrated the ability of different SC types to regenerate many tissues. However, there is no consensus among researchers regarding the microenvironmental influence on cell proliferation, differentiation and migration; further, the best SC source for therapeutic use still remains unclear [2].

Bone marrow contains two discrete populations of SCs. The first being hematopoietic stem cells (HSCs) responsible for production of all blood cell types, and the second – mesenchymal stem cells (MSCs), which can differentiate into several nonhematopoietic cell lineages [3]. Beside these undifferentiated multipotent SCs, bone marrow also contains a heterogeneous population of progenitor cells at various stages of differentiation. There is a growing interest in bone marrow-derived endothelial progenitor cells (EPCs) due to their important role in the formation of new blood vessels during the tissue regeneration [4]. Progenitor cells, similar to SCs, are characterized by the absence of lineage markers (Lin⁻ cells), these populations have also a self-renewal capacity and multilineage differentiation potential [5].

Because of their unique properties, bone marrow-derived Lin⁻ cells can be used to regenerate damaged tissues, including skin [6]. Delayed cutaneous wound healing is frequently associated with a decreased migration and proliferation of cells involved in the sufficient tissue renewal. In adult organism, these processes are controlled by the local microenvironment in which the cells reside. An important role in the regulation of different cell behavior is played by the tissue-specific extracellular matrix components [7]. The evaluation of the individual process *in vivo* is difficult because of multiple overlapping reactions that occur in

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wound healing [8]. *In vitro* models of cell proliferation and migration can be useful to obtain valuable information for the preparation of cells for transplantation *in vivo* [9]. The literature contains numerous studies of the regenerative capacities of different cells *in vitro*, including epithelial cells, fibroblasts, endothelial cells, and MSCs [10-12]. However, there are limited data on the behavior of Lin^- cell population, although these cells play an important role in skin regeneration. The objective of our study was to determine the influence of skin tissue-specific microenvironment on bone marrow-derived Lin^- cell proliferation and migration *in vitro*.

2. Experimental Procedures

2.1 Animals

Female BALB/c mice (20–25 g) were used. Animals were housed at 22±2°C under a 12 h light-dark cycle and with a free access to food and water. All procedures were carried out in accordance with the guidelines of the European Union and were approved by the Lithuanian Ethics Committee on the use of the laboratory animals under State Veterinary service.

2.2 Bone marrow cell isolation and lineage depletion

Bone marrow cells were isolated from long bones of BALB/c mice 8 weeks of age. Mice were sacrificed by cervical dislocation. Bone marrow was obtained from femurs and tibias by the perfusion method. The bone marrow cells were collected in sterile PBS and washed three times by centrifugation for 6 min at 300 x *g*. The cells were purified using magnetic cell sorting (MACS) techniques with the BD IMag™ mouse hematopoietic progenitor enrichment set (composed of BD IMag™ Streptavidin Particles Plus – DM and biotin-conjugated monoclonal antibodies: anti-mouse CD3e, clone 145-2C1; anti-mouse CD11b, clone M1/70; anti-mouse CD45R/B220, clone RA3-6B2; anti-mouse Ly-6G and Ly-6C (Gr-1), clone RB6-8C5; anti-mouse TER-119, clone TER-119) (all from BD Biosciences, San Jose, CA, USA) applied as recommended by the manufacturer.

2.3 Phenotypic characterization of purified cells

Bone marrow lineage-depleted cells were analyzed by flow cytometry. The following monoclonal antibodies were used: phycoerythrin (PE)-labeled anti-mouse Sca-1, fluorescein isothiocyanate (FITC)-labeled anti-mouse CD117, PE-labeled anti-mouse CD90.2 and FITC-labeled anti-mouse CD133. The cells stained with PE- or FITC-labeled rat anti-mouse IgG served as controls. The cells were incubated with appropriate amounts of antibodies for 30 min at 4°C in the dark, and then analyzed by FACSCallibur™ flow cytometer (BD, USA).

2.4 Cell culture conditions

Bone marrow Lin^- cells were cultured under both 2D and 3D conditions. The growth medium for purified cells consisted of 90% Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS) and 1% antibiotics.

In the 3D culture method 85 µl of Geltrex (Gibco, USA) or 35 µl of 4 mg mL^{-1} rat tail collagen type I (Gibco, USA) was added per well of 96-well plate and incubated at 37°C for 30 minutes to promote gelling of matrix. Purified Lin^- cells were suspended in growth medium containing 2% of Geltrex or 2% of 5 mg mL^{-1} collagen type I solution, and 10^4 cells/well were plated.

For surface coating, Geltrex was diluted to 0.5 mg mL^{-1} concentration in ice-cold serum-free medium, and collagen type I was diluted to 50 µg mL^{-1} concentration in 0.02 mol L^{-1} acetic acid. The sufficient amount of prepared solution was added into each well of a 24-well plate to cover the entire growth area. Coated plate was incubated at 37°C for 60 minutes. Before cell seeding, collagen-coated wells were rinsed three times with media to remove the acid. Purified Lin^- cells were plated at a density 5×10^5 cells/well in 1 ml of growth medium.

For the control study, Lin^- cells were transferred directly to 96-well or 24-well microplates without type I collagen or Geltrex and were maintained under same conditions as the other groups.

All cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

2.5 Cell proliferation assay

Cell proliferation was assayed with the Cell Counting Kit-8 (CCK-8) according to the manufacturer's protocol (Dojindos Laboratories, Japan). Bone marrow Lin^- cells were plated in 96-well plates as described above. CCK-8 reagent was added at 24, 48, 72 and 96 hours after seeding and incubated at 37°C for 4 hours. The optical density (OD) values were read at 450 nm on a microplate reader TECAN Sunrise (Austria).

2.6 Cell migration assay

Purified Lin^- cells were cultured in 24-well plates as described above. Cells were grown to confluence. A single stripe was scraped on the cell-coated surface with a 200 µl disposable plastic pipette tip, and detached cells were removed by washing with medium. Wound closure was monitored by phase-contrast microscopy at the indicated time points. The images were captured with a Nikon DS-2MBW camera connected to a Nikon

ECLIPSE TE2000-U microscope. Wound closure percent was determined by the following formula:

Wound closure (%) = [(Wound area$_{0h}$ – Wound area$_{xh}$)/Wound area$_{0h}$] × 100

2.7 Statistical analysis

All assays were repeated at least in three independent experiments. Data were expressed as mean ± SD. Statistical significance was determined using Student's t-test; $P<0.05$ was considered significant.

3. Results

3.1 Phenotypic analysis of Lin$^-$ cells

Bone marrow lineage negative (Lin$^-$) cells used in our experiments were isolated by magnetic-activated cell sorting (MACS). Purified cells comprised 3.5 ± 1.5% of the total bone marrow-derived cell population. Lin$^-$ cells were analyzed by flow cytometry for the expression of specific stem/progenitor cell markers. The results showed that most of the cells in Lin$^-$ population expressed high level of Sca-1 and CD117 (Table 1).

3.2 Lin$^-$ cells proliferation in different extracellular environments

The influence of extracellular matrix on the proliferation of Lin$^-$ cells was examined in 3D microenvironment consisting of type I collagen or basement membrane proteins. In control experiments, Lin$^-$ cells were grown on tissue culture plastic plates. Cell proliferation rates in different microenvironments are presented in Figure 1.

The results revealed that during the test period cell proliferation was more intense in type I collagen and basement membrane components containing microenvironments when compared with the control group. Lin$^-$ cells in the control group lose their ability to proliferate after 72 h while in both experimental groups cells maintained the proliferative potential. The marked increase in Lin$^-$ cell proliferation compared to the initial cell number in cultures with type I collagen and basement membrane matrix was established after 72 h and 96 h, respectively.

3.3 Effects of extracellular matrix proteins on Lin$^-$ cells migration *in vitro*

The re-epithelization process *in vitro* was observed until complete wound closure. The first wound closure was noticed in Lin$^-$ cell monolayers cultured on type I

Cell surface marker	Percentage of cell population
Sca-1$^+$CD117$^+$	74.36±4.64
Sca-1$^+$CD117$^-$	9.71±3.51
Sca-1$^-$CD117$^+$	8.13±1.59
CD90.2$^+$CD133$^+$	1.27±0.35
CD90.2$^+$CD133$^-$	6.17±1.43
CD90.2$^-$CD133$^+$	3.17±0.84

Table 1. Bone marrow Lin$^-$ cells bearing stem/progenitor cell surface markers.

CD – cluster of differentiation; Sca-1 – stem cell antigen 1. Values are means±SD; n=8 experiments.

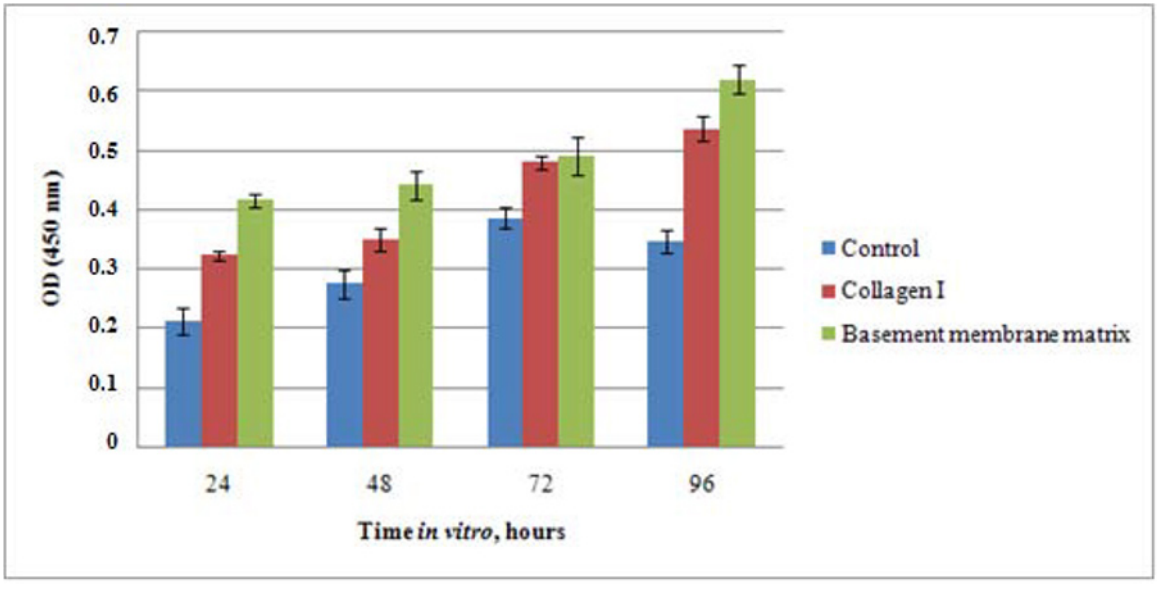

Figure 1. Proliferation of purified Lin$^-$ cells in different extracellular environments. Results are expressed as mean ± SD of four independent experiments. Statistical significance was determined with Student's t-test ($P<0.05$).

collagen (Figure 2). In this group re-epithelization was complete by 72 h, but no closed wounds were seen in basement membrane matrix group or control group. Figure 3 represents the data of cell migration kinetic analysis. First migratory cells were detected after 8 h on type I collagen surface. In this group wound closure was 9.34%. After 24 h the wound closure percentage was higher on basement membrane matrix than on type I collagen coating, respectively 49.22% and 36.38%. However, after 72 h full coverage of the scratched area by migrating Lin⁻ cells was determined on type I collagen coating. After 72 h in basement membrane components containing microenvironment wound closure percentage was 75.11%. Complete wound closure in this group was observed at 96 h post-wounding. After 72 h and 96 h no completely closed scratches were seen in control group.

4. Discussion

The present study was designed to examine the influence of extracellular matrix components on the proliferation and migration of bone marrow-derived Lin⁻ cells *in vitro*. We chose collagen type I and basement membrane matrixes which are important regulators of cell functions, including proliferation and migration, during cutaneous wound healing.

Successful cell therapy depends to quite a great extent on the methods of purification of specific cell populations. Several stem and progenitor cell populations, expressing significant regenerative properties, are found in the bone marrow [13]. To obtain pure cell populations is still a challenge as the primitive cells do not possess a well defined phenotype; the same marker could be expressed on the surface of different stem/progenitor cells [14-16]. Bone marrow cells bearing no surface markers, specific for mature cells (Lin⁻ cells)

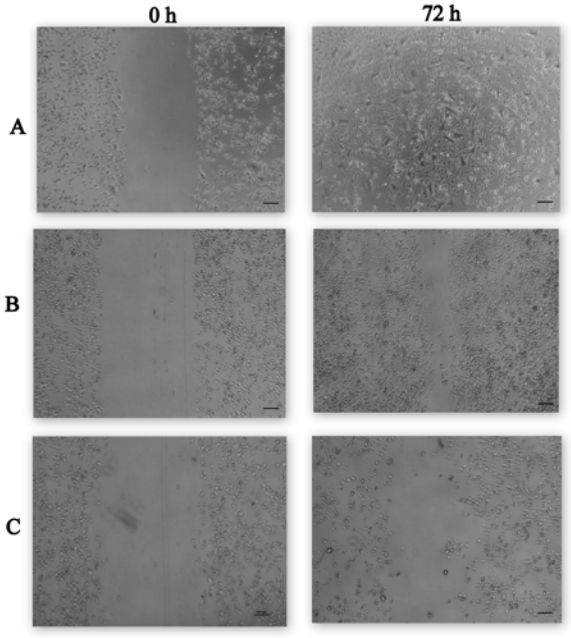

Figure 2. *In vitro* scratch assay. Representative phase-contrast micrographs of Lin⁻ cell migration on type I collagen (A), basement membrane matrix (B) and plastic (C) at 0 h and 72 h. Original magnification 4x. Scale bar is 100 μm.

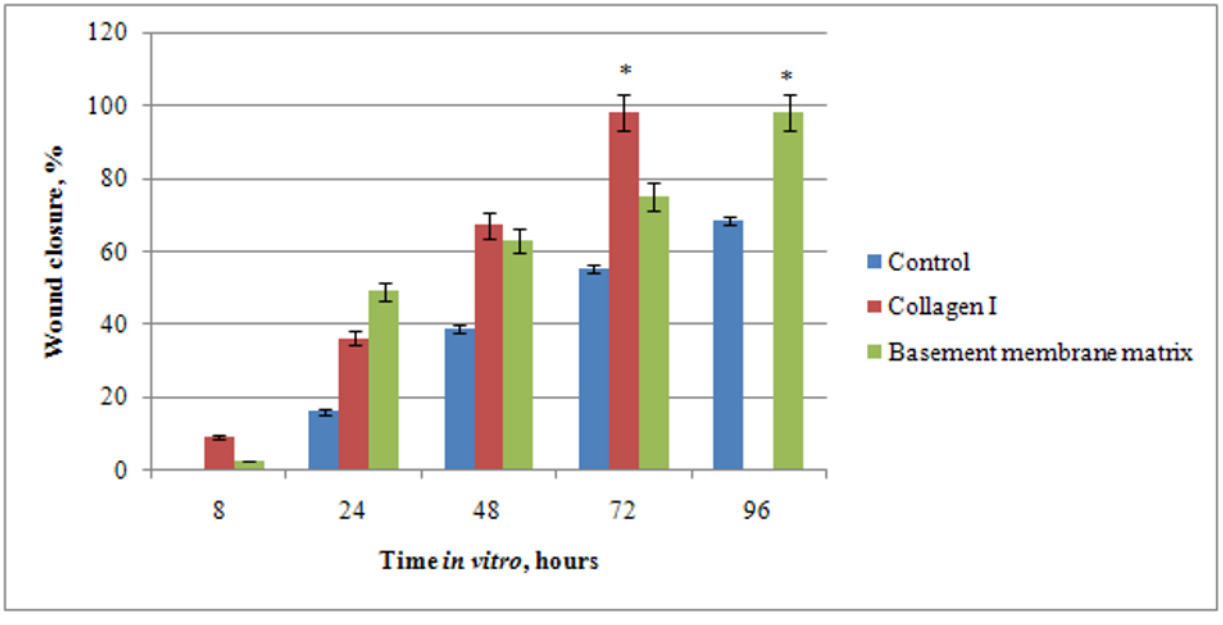

Figure 3. Kinetic analysis of Lin⁻ cell migration. Data are presented as mean ± SD (n = 5). *P<0.05, compared with the control.

were used in our study. There is no specific marker to define most primitive stem/progenitor cells. However, several studies have indicated a higher multilineage differentiation potential of bone marrow cells, expressing different levels of surface markers CD117, Sca-1, CD90.2 and CD113 [17-19]. Our results revealed the highest percentage of Sca-1 and CD117 positive cells in Lin^- population while CD90.2 and CD133 positive cells were low. The earlier studies have proven a positive effect of bone marrow Lin^- cells on the restoration of damaged tissue in different organ injury models, such as heart, lungs, kidney and skin [20-22]. However, for a successful application of Lin^- cell population in therapy, a thorough study is necessary; cell behavior mechanisms should be elucidated, the influence of the microenvironment on the cell regenerative properties must be explained.

The regeneration of wounded tissue *in vivo* mainly depends on the functional properties of stem cells and progenitors. High cell proliferative activity is necessary to ensure their sufficient amount *in vitro* as well as their integration at the site of inflammation and the participation in the healing process [23]. Though traditional two-dimensional (2D) cultures play a significant role in different spheres of investigation, the behavior of cells in three-dimensional (3D) culture is closer to the process in their natural microenvironment [24]. In our study, the proliferative activity of Lin^- cell population was observed under standard conditions on tissue culture plastic plates as well as in 3D type I collagen / basement membrane matrix gels. The results obtained show that in the extracellular substance bearing microenvironment, Lin^- cells start to proliferate more intensively after a different rest period. The data of other investigators revealed that different proteins of extracellular origin *in vitro* may also influence the proliferative activity of other cell types involved in the tissue repair process, such as fibroblasts [25], keratinocytes [26] and separate stem cell populations, in particular mesenchymal stem cells [27]. In our experimental groups, where the cells were cultivated in collagen type I and basement membrane matrixes, the proliferation activity increased by 29% after 72 h and by 30% after 96 h, respectively. Segal *et al.*, [28] found that for optimal keratinocyte proliferation the basement membrane proteins are necessary. Experiments by Tsai *et al.* [29] on the human mesenchymal stem cells (hMSC) analyzing the influence of different extracellular matrix proteins on cell proliferation properties *in vitro* proved that collagen type I might influence a later growth increase [29]. The intensive proliferation of the hMSC in these experiments was observed during the first 96 hours in the presence of fibronectin, laminin and poly-L-lysine, later it was decreased. However, with collagen type I, the growth stimulation continued until day 7. There is data in the literature that hMSC cultivation using different basement membrane components or their mixture provides not only better cell viability *in vitro* but also stimulates a more expressed proliferation activity [30]. In our experiments, the proliferation of Lin^- cell population was most active in the matrix of the basement membrane.

Cell migration is another essential process that ensures not only tissue development and maintenance of homeostasis but also provides a successful wound healing process [31]. The *in vitro* scratch assay is a suitable model for the preliminary studies, providing the possibility to investigate the influence of separate extracellular matrix components on the cell migration properties [9]. Because of its unquestionable influence in maintaining the tissue structure and cell functional properties in normal development and pathology, collagen type I and basement membrane matrixes are the most often chosen materials in such experiments [32-34]. The migration of the Lin^- cell population in our study was also investigated using the matrix of these extracellular components. It was determined that the fastest re-epithelization process occurred when cells were cultivated on the surface of a collagen type I matrix. There is evidence in the literature on the influence of different extracellular matrix components on migration of various cell types *in vitro*. O'Toole [35] demonstrated increased keratinocyte migration *in vitro* using type I collagen and type IV collagen, but a decrease in the presence of some laminins. Boleman *et al.* [36] determined *in vitro* that dermal fibroblasts and dental pulp stem/progenitor cells migrate most rapidly on a surface matrix comprising a mixture of basement membrane components; unlike a surface covered by separate individual components. Although the first migrating cells in our experiment were detected in the matrix of collagen type I after 8 h (a wound closure of 9.34%), a higher degree of wound closure was observed after 24 h on the surface of the basement membrane matrix (49.22%) compared to that of the type I collagen cultivated monolayer (36.38%). Intensive studies of cell migration *in vitro* are performed with dermal fibroblasts, possessing the ability to influence the whole wound healing process. There is data in the literature that under *in vitro* conditions, type I collagen stimulates fibroblast migration without the additional use of growth factors [37]. However, Hakkinen *et al.* [34] showed that basement membrane matrix enhances dermal fibroblast migration twice as fast as type I collagen. Our data showed a complete coverage of the wound by migrating Lin^- cells earlier on the surface of type I collagen

(after 72 h) and on the surface of basement membrane matrix (after 96 h). At these time intervals, no completely closed wounds were observed in the control group.

In conclusion, our results show that skin tissue extracellular matrix components may differently influence bone marrow stem and progenitor cell proliferation and their migratory properties *in vitro.* Basement membrane matrix is more effective for Lin^- cell proliferation *in vitro*. However, a type I collagen matrix enhances the re-epithelization process, which depends on cell migratory properties. These studies are important for preparing cells to be used in transplantation.

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