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Histology

The Participation of Hemopoietik Bone-Marrow Derived Stem Cells in the Regulation of Damaged Cornea Stroma. Autoradiographical Part

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ABSTRACT. The purpose of this study was to determine the source of the cells participating in regeneration of corneal stroma. The cornea of experimental animals was perforated up to lens by sterile preparative needle. This process was investigated in white adult mice and xenogenic radiation chimeras by means of histological, autoradiographic, immunofluorescence methods. For detection of proliferative activity of corneal stroma cells the pulse and late ^{3}H-thymidine labeling method was used. ^{3}H-thymidine (with specific activity of 52 cu/mM) with the dose of 2 μcu per gram of animal weight was injected into the abdominal cavity of operated animals. Non-operated mice served as controls and they received ^{3}H-thymidine injections according to the same protocol as the operated mice. In both cases material was fixed after 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 days. A great number of the new-formed stromal fibroblasts, which include ^{3}H-thymidine just at the moment of cornea injury (late labeling) did not correspond to the low proliferative potential of stromal cells (pulse labeling) and could not be explained by their multiplication. Results of autoradiographic investigations have shown: labelled fibroblast-like cells are found only in a new-formed stroma, while stromal fibroblasts of intact cornea and stromal fibroblasts of intact parts of injured cornea are not labelled during the whole experiment. We can conclude that precursors of cells involved in regeneration of the cornea do not develop via multiplication of stromal cells of cornea; precursors of cells forming the infiltrate in the injured cornea multiply intensively beyond the borders of damaged area and then migrate into the inflammatory area. *© 2015 Bull. Georg. Natl. Acad. Sci.*

Key words: *stroma, limb, stretches, fibroblast-like cells, bone marrow-derived cells.*

Cornea is a transparent, external part of the supporting layer of the eye and thus is often exposed to traumas due to its topographic position on eye front surface. According to the literature regeneration of injured corneal stroma proceeds at the expense of fibroblasts. Those cells are well known and described in details; however, their origin remains an open question and the source of regeneration of injured corneal stroma is an important problem. It is not clear whether new formation of stroma happens at the expense of innate cells or elements of hematogenic nature. The goal of our work is to elucidate this question.

Recent majority of the works deals with regen-

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eration of epithelium of injured cornea and ways of its treatment**.** These authors mentioned, that the maintenance of a healthy corneal epithelium under both normal and wound healing conditions is achieved by population of stem cells (SC) located in the basal epithelium at the corneoscleral limbus [1-3]. The characteristics of the specific microenvironment of corneal SC, as provided by growth factor activity and basement membrane heterogeneity in the limbal area, could serve as additional tools for their selective enrichment and ***in vitro*** expansion for the purpose of ocular surface reconstruction [4]. The human corneal epithelium is being continuously renewed. Differentiated epithelial cells originate from limbal stem cells (LSCs) located in the periphery of the cornea, the corneoscleral limbus. The population of limbal epithelial stem cells (LESCs) are responsible for maintaining the epithelium throughout life by providing a constant supply of daughter cells that replenish those constantly lost from the ocular surface during normal wear and tear and following injury. LESCs deficiency leads to corneal opacification, inflammation, vascularization and discomfort [5, 6]. Cultured LESCs delivery is one of several examples of successful adult stem cell therapy in patients. The clinical precedence for use of stem cell therapy and the accessibility of the transparent stem cell niche make the cornea a unique model for the study of adult stem cells in physiological conditions as well as in disease [7]. These results indicate that scleral fobroblasts have an increased capacity for myofibroblast formation which appears to negatively affect their ability to support LEP (limbal epithelial progenitor cells) growth. Superior growth of LEPs in the presence of limbal fibroblasts indicates a role for limbal fibroblasts in promoting the proliferation of limbal epithelium during the wound healing [8]. These results indicate that cryopreserved hLESCs (human limbal epithelial stem cells) are non-immunogenic in nature and express negative immunoregulatory molecules which may be critical for their survival in an allogeneic environment [9]. This study confirmed that the small molecular compound pluripotin promoted the proliferation of rabbit limbal epithelial cells by improving the expansion of limbal stem/progenitor cells ***in vitro*** [10]. The present study demonstrates the functional and molecular characterization of P-gp (P-glycoprotein) and peptide transporters in the mitochondrial membranes of rPCECs (rabbit primary corneal epithelial cells). This knowledge of mitochondrial existence of P-gp and peptide transporter will aid in the development of subcellular ocular drug delivery strategies [11]. These results show that TGF-β (transforming growth factor beta) plays an important role in directing local inflammatory responses in ocular surface epithelial cells [12].

Numerous studies show, that fibroblasts play an important role in corneal wound healing [13-15]. When an incisional wound through the epithelium into stroma occurs the keratocytes become hypercellular myofibroblasts. These can later become wound fibroblasts, which provides continued transparency or become myofibroblasts that produce a disorganized ECM (extracellular matrix) resulting in corneal opacity [16]. These data are in agreement with our hypothesis that TSP-1 (thrombospondin-1) localization in the stromal matrix is involved in the transformation of keratocytes into myofoiroblasts [17]. These results suggest that KCM (keratocyte-conditioned medium) can direct human MSCs (mesenchymal stem cells) to differentiate into keratocyte-like cells [18]. Our finding suggests that limbal stromal cells and an intact cell-cell contact help to maintain LSCs (limbal stem cell) in an undifferentiated state ***in vitro*** during expansion [19]. This study provides ***in situ*** evidence that TGF-β and PDGF-B (platelet-derived growth factor-B) have important roles in modulating myofibroblast generation in the mouse cornea after haze-associated injury [20]. These results corroborate the findings of recent ***in vitro*** work that demonstrated an antagonistic effect of TGF-β and IL-1 on myofibroblast viability, and found that IL-1-triggered myofibroblast apoptosis was suppressed by TGF-β. Thus, IL-1 is an important modulator of myofibroblast viability during corneal wound healing [21]. Our results suggest that IL-6, IL-8, and MCP-1 may there-

fore play a key role in the inflammatory response to corneal infection [22]. These data suggest that TGF-β is a paracrine modulator that regulates the generation of myofibroblasts from either corneal fibroblasts or bone marrow-derived cell precursors [23]. Repopulating keratocytes subsequently reorganize the associated fibrotic extracellular matrix deposited in the anterior stroma by the myofibroblasts. Investigations of myofibroblast biology are likely to lead to safer pharmacological modulators of corneal wound healing and transparency [24]. The results demonstrated a powerful action of LXA_4 in protecting corneas with injuries that compromise the stroma by decreasing inflammation and increasing wound healing [25]. Systemically transplanted MSCs (mesenchymal stem cell) can engraft to injured cornea to promote wound healing, by differentiation, proliferation, and synergizing with hemotopoietic stem cells [26]. Stromal keratocyte apoptosis has been well-characterized as an early initiating event of the corneal wound healing response triggering subsequent cellular process that include bone marrow-derived cell infiltration, proliferation and migration of residual keratocyte cells, and in some circumstances, generation of myofibroblast cells [27]. C57BL/6J-GFP chimeric mice were generated through bone marrow transplantation from donor mice that expressed enhanced GFP (green fluorescent protein) in a high proportion of the bone marrow-derived cells. GFP chimeric mice underwent haze-generating corneal epithelial scrape followed by irregular phototherapeutic keratectomy with an eximer laser in one eye. In this mouse model, the majority of myofibroblasts developed from the bone marrow-derived cells [28].

Materials and Methods. To determine the origin of stroma cells involved in regenerating of the cornea white adult mice. The cornea of experimental animals was perforated in the centre up to lens by sterile preparative needle. At different time intervals after damage the lens was extracted and the cornea was cut along the limb. after 3 hours of injury the stretches of cells directed towards wound move off the limb vessels.

Autoradiographic Methods. For detection of proliferative activity of corneal stroma cells ^{3}H-thymidine (with specific activity of 52 cu/mM) with the dose of 2μcu per gram of animal weight was injected into the abdominal cavity of operated animals (group I) 1 hour before sacrificing the animals and fixation (pulse labeling). The second group of animals comprised mice that were injected with ^{3}H-thymidine just after operation (late labeling). This method allows us to compare the number of cells synthesizing DNA in studied tissue, i.e. inserting labeled precursor of DNA in 1 hour (pulse labeling) with the number of labeled cells observed in a tissue in case of preliminary insertion of an isotope-late labeling [29,30].

The material was fixed on 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days after injury.

Non-operated mice served as controls and they received thymidine injections according to the same protocol as the operated mice. Material was fixed 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days after operation. Deparaffinized sections were covered with liquid photoemulsion of "M" type. The sections were exposed for 8 weeks at 4^0C, developed with developer D-19 (methylhydroquinone for radioautography) and stained with hematoxylin-eozin according to Karachi [31]. Percentage of labeled cells on preparations was calculated. Cells with at least 5 silver grains were considered as labeled. Taking into account intensity of the label cells were subdivided into intensively (more than 15 grains) and weakly (from 5 to 15 grains) labeled ones. The obtained results were analyzed statistically.

Results and Discussion. To determine the origin of the pieces of the stretches and the corneal stroma we applied an autoradiographic method using pulse and late ^{3}H-thymidine labeling. This method allows us to compare the number of cells synthesizing DNA in the studied tissue, i.e. inserting labeled precursor of DNA in 1 hour (pulse labeling) with the number of labeled cells observed in a tissue in case of preliminary insertion of an isotope (late labeling). ^{3}H-thymidine insertion has shown that only the cells of the multilayer plane epithelium are labeled, but nuclear

Table 1. Common index of labeling of white adult mouse cornea cells and index of intensity of labeling of these cells in the experiment (pulse labeling)

Terms of fixation (days after operation)	Common index of cell labeling in infiltrate	Common index of cell labeling in stretches	Index of labeling intensity in infiltrate	Index of labeling intensity in stretches
1	–	–	–	–
2	5.6 ± 1.7%	3.2 ± 0.8%	24.0 ± 2.7%	26.0 ± 2.5%
3	5.1 ± 1.6%	2.1 ± 0.4%	26.0 ± 2.0%	25.0 ± 2.2%
4	5.0 ± 0.6%	1.4 ± 0.3%	25.0 ± 3.5%	24.0 ± 0.1%
5	5.2 ± 1.5%	1.7 ± 0.7%	26.0 ± 2.0%	20.0 ± 2.7%
6	6.1 ± 1.1%	2.5 ± 0.1%	25.0 ± 3.5%	20.0 ± 1.0%
7	4.4 ± 1.4%	1.6 ± 0.2%	23.0 ± 2.6%	18.0 ± 1.5%
8	3.9 ± 0.1%	1.3 ± 0.2%	20.0 ± 1.3%	22.0 ± 3.4%
9	2.6 ± 0.1%	1.1 ± 0.4%	20.0 ± 3.0%	20.0 ± 2.0%
10	2.5 ± 0.5%	1.0 ± 0.1%	19.0 ± 1.0%	18.0 ± 1.0%

labeling is not observed in the stroma or in the cells of endothelium of cornea both at pulse and late labeling in norm.

On the second day after operation the labeled cells are detected in the infiltrate filling wound cavity and in stretches.

Results of autoradiographic investigations are given in Tables 1 and 2. It is seen from the data obtained that indices of cell labeling are low both in the infiltrate and stretches at pulse labeling. This process is stable as it is almost preserved during the whole experiment. At pulse labeling only intensively labeled cells are observed; their intensities in the infiltrate and stretches are almost equal and make up on average 23.1±2.4% and 21.4 ±1.8%, respectively (Table 1; Figs. 1, 2).

At late labeling high indices of labeling are recorded in the infiltrate and stretches. At late labeling maximal number of labeled fibroblast-like cells are observed on 3rd and 4th days after injuring in the infiltrate (33.1±3.6%; 31.2±3.4%) and on 2nd and 3rd days in the stretches (13.3±3.3%; 11.6±3.4%). after 4 days of operation the number of labeled cells is reduced rapidly both in the infiltrate and stretches, which is not observed at pulse labeling (Table 2; Figs. 1, 2).

The labeling intensity is maximal at 3rd day in the infiltrate and stretches and equals to 25.0±1.0% and 21.0±2.4% silver grains per nucleus, correspondingly. From 4th day to 10th day gradual reduction of cell labeling occurs in the infiltrate as well as in stretches (Table 2; Figs. 1, 2).

It should be noted that at the later time points, single but intensively labeled cells are also seen both in the infiltrate and stretches. On the 8th day after operation cells with intensity of 11.3±3.0% and 18.0±2.1% grains per nucleus occur in the infiltrate and stretches, respectively (Table 2; Figs. 1, 2).

Results of autoradiographic investigations have shown that at the pulse and late labeling essential distinctions between intensity of nucleus labeling and indices of cell labeling occur in the infiltrate and stretches. Hence, at late labeling the indices of labeled cells both in infiltrate and stretches are on average 3.5 times higher, but the intensities of labeling are on average 2 times lower in infiltrate and 1.5 times lower in stretches than at pulse labeling.

Those autoradiographic data clearly indicate that the precursors of cells involved in regeneration of the cornea do not develop via multiplication of corneal stroma cells.

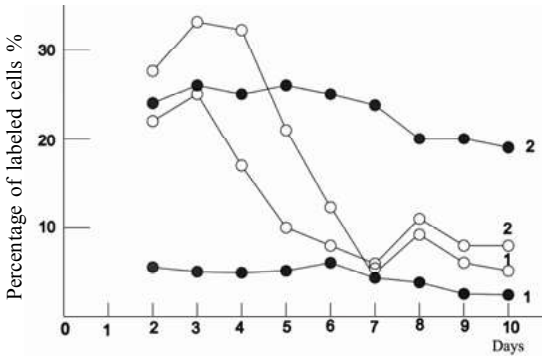

Fig. 1: Number of labeled cells in infiltrate of injured cornea of white adult mice.
On absciss – a time of beginning of aseptic inflammation (days); ordinate – percentage of labeled cells (%).
● ^{3}H-thymidine is injected 1 hour prior to fixation.
○ ^{3}H-thymidine is injected along with injuring the object.
1. Common index of labeled cells.
2. Index of intensity of labeled cells.

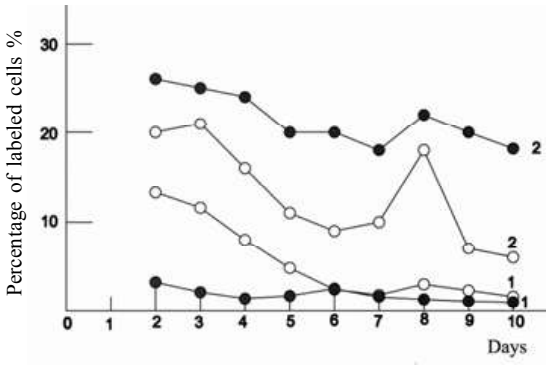

Fig. 2: Number of labeled cells in stretches of injured cornea of white adult mice.
On absciss – a time of beginning of aseptic inflammation (days); ordinate – percentage of labeled cells (%).
● ^{3}H-thymidine is injected 1 hour prior to fixation.
○ ^{3}H-thymidine is injected along with injuring the object.
1. Common index of labeled cells.
2. Index of intensity of labeled cells.

Results of autoradiographic investigations show: data obtained at pulse labeling indicate weak proliferation of innate stromal fibroblasts (fibroblast-like cells).

Thus, in postoperative regeneration area of cornea significant difference is observed between the numbers of cells labeled with ^{3}H-thymidine at pulse or late labeling. Namely, a great number of the new-formed stromal fibroblasts, which include ^{3}H-thymidine just at the moment of cornea injury (late labeling) do not correspond to low proliferative potential of innate stromal cells (pulse labeling) and cannot be explained by their multiplication.

Rapid reduction of the number of labeled cells may suggest that an intense exchange occurs in the cellular infiltrate at the expense of migration of new, multiply fissioned cells. Dilution of a label to that level, which is not registered autoradiographicaly,

Table 2. Common index of labeling of white adult mouse cornea cells and index of intensity of labeling of these cells in the experiment (late labeling)

Terms of fixation (days after operation)	Common index of cell labeling in infiltrate	Common index of cell labeling in stretches	Index of labeling intensity in infiltrate	Index of labeling intensity in stretches
1	–	–	–	–
2	27.6 ± 1.7%	13.3 ± 3.3%	22.0 ± 1.7%	20.0 ± 1.4%
3	33.1 ± 3.6%	11.6 ± 3.4%	25.0 ± 1.0%	21.0± 2.4%
4	31.2 ± 3.4%	8.0 ± 1.8%	17.0 ± 1.0%	16.0 ± 2.0%
5	21.0 ± 1.2%	4.8 ± 1.6%	10.0 ± 0.5%	11.0 ± 2.0%
6	12.3 ± 3.1%	2.4 ± 0.4%	8.0 ± 0.7%	9.0 ± 1.9%
7	5.5 ± 0.7%	1.8 ± 0.1%	6.0 ± 1.0%	10.0 ± 1.0%
8	9.2 ± 2.3%	3.0 ± 0.1%	11.0 ± 3.0%	18.0 ± 2.1%
9	6.1 ± 0.1%	2.3 ± 0.8%	8.0 ± 1.5%	7.0 ± 0.5%
10	5.2 ± 1.0%	1.6 ± 0.3%	8.0 ± 1.4%	6.0 ± 0.1%

could not be explained by cell fission in the inflammatory area. Conclusion about permanently proceeded exchange of cells in inflammatory area is confirmed by the data about insignificant percentage of intensively labeled cells in the infiltrate and stretches at the later stages of observations.

The fact that cells getting into eye are already labeled and these labeled cells in limb blood vessels are single, we can consider that the source of cells actively synthesizing DNA occur out of a limb. Taking into consideration also the fact that labeled fibroblast-like cells are found only in a new-formed stroma, while stromal fibroblasts of intact cornea and stromal fibroblasts of intact parts of injured cornea are not labeled during the whole experiment, we can conclude that precursors of cells involved in regeneration of the cornea do not develop via multiplication of stromal cells of cornea; precursors of cells forming the infiltrate in the injured cornea multiply intensively beyond the borders of damaged area and then migrate into the inflammatory area.

ჰისტოლოგია

ძვლის ტვინის სისხლის ღეროვანი უჯრედების მონაწილეობა დაზიანებული რქოვანას სტრომის რეგენერაციაში. ავტორადიოგრაფიული ნაწილი

ქ. დავითაია

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აღნიშნული კვლევის მიზანი იყო დაზიანებული რქოვანას სტრომის რეგენერაციაში მონაწილე უჯრედების წყაროს დადგენა. ექსპერიმენტული ცხოველების რქოვანა პერფორირებული იყო ბროლამდე სტერილური საპრეპარაციო ნემსით. ეს პროცესი შევისწავლეთ თეთრ სქესმწიფე თაგვებსა და ქსენოგენურ რადიაციულ ქიმერებზე ჰისტოლოგიური, ავტორადიოგრაფიული, იმუნოფლუორესცენტური მეთოდებით. რქოვანას სტრომის უჯრედების პროლიფერაციული აქტივობის დასადგენად გამოვიყენეთ იმპულსური და გვიანი ^{3}H-თიმიდინით მონიშვნის ხერხი, რომლის დროსაც ცხოველის გ/წონაზე ცხოველების ერთი ჯგუფის მუცლის ღრუში შევიყვანეთ ^{3}H-თიმიდინი (ხვედრითი აქტივობით 52 cu/mM) 2 μcu დოზით, მასალის ფიქსაციამდე 1 საათით ადრე - იმპულსური მონიშვნა; ხოლო ცხოველების მეორე ჯგუფში ^{3}H-თიმიდინი შევიყვანეთ ოპერაციის დამთავრებისთანავე - გვიანი მონიშვნა. კონტროლად ითვლებოდნენ არანაოპერაციევი თაგვები, რომლებშიც ^{3}H-თიმიდინი შეგვყავდა იმავე ვადებში, როგორც ნაოპერაციევ ცხოველებში. ორივე შემთხვევაში მასალას ვაფიქსირებდით 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 დღეების შემდეგ. გამოკვლევამ გვიჩვენა, რომ ახლადფორმირებული რქოვანას სტრომის ფიბრობლასტების დიდი რაოდენობა, რომლებიც ჩაირთავენ ^{3}H-თიმიდინს რქოვანას დაზიანებისთანავე (გვიანი მონიშვნა), არ შეესაბამება სტრომის ადგილობრივი უჯრედების მცირე პროლიფერაციულ პოტენციალს და

არ შეიძლება აიხსნას მათი გამრავლებით. ავტორადიოგრაფიული კვლევის შედეგად დავადგინეთ: მონიშნული ფიბრობლასტების მსგავსი უჯრედები წარმოდგენილია მხოლოდ ახლადფორმირებულ სტრომაში, მაშინ როდესაც რქოვანას ინტაქტური სტრომის ფიბრობლასტები და ნაჭრილობევი რქოვანას ინტაქტური ნაწილი არ მოინიშნა მთელი ექსპერიმენტის მანძილზე. აქედან დასკვნა: რქოვანას რეგენერაციაში მონაწილე უჯრედების წინამორბედები არ წარმოიქმნებიან სტრომის უჯრედების გამრავლების ხარჯზე; არნიშნული უჯრედების წინამორბედები, რომლებიც ნაჭრილობევი რქოვანას ინფილტრატის ფორმირებას ახდენენ, მრავლდებიან დაზიანებული ზონის ფარგლებს გარეთ და შემდეგ სისხლის მეშვეობით მიგრირებენ ანთების უბანში.

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