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EXPERIMENTAL AUTORADIOGRAPHY AUTORADIOGRAPHICAL STUDY OF THE REGENERATION OF DAMAGED CORNEA STROMA

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ABSTRACT

The purpose of this study was to determine the source of the cells participating in regeneration of damaged corneal stroma by means of autoradiographic method. This process was investigated in white adult mice. The cornea of experimental animals was perforated up to lens by sterile preparative needle. 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: 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.

KEYWORDS: Stroma, limb, stretches, fibroblast-like cells, bone marrow-derived cells.

1. INTRODUCTION

Numerous studies have shown, that fibroblasts play an important role in corneal wound healing.[1-3] 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.[4] 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.[5] These results suggest that KCM (keratocyte-conditioned medium) can direct human MSCs (mesenchymal stem cells) to differentiate into keratocyte-like cells.[6] 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.[7] This study provides *in situ* evidence that TGF-β (transforming growth factor beta) and PDGF-B (platelet-derived growth factor-B) have important roles in modulating myofibroblast generation in the mouse cornea after haze-associated injury.[8] These results corroborate the findings of recent *in vitro* work that demonstrated an antagonistic effect of TGFβ and IL-1 on myofibroblast viability, and found that IL-1-triggered myofibroblast apoptosis was suppressed by TGFβ. Thus, IL-1 is an important modulator of myofibroblast viability during corneal wound healing.[9] Our results suggest that IL-6, IL-8, and MCP-1 (monocyte chemoattractant protein) may therefore play a key role in the inflammatory response to corneal infection.[10] These data suggest that TGFβ is a paracrine modulator that regulates the generation of myofibroblasts from either corneal fibroblasts or bone marrow-derived cell precursors.[11] 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.[12] The results demonstrated a powerful action of LXA_4 (Lypoxin A_4) in protecting corneas with injuries that compromise the stroma by decreasing inflammation and increasing wound healing.[13]

Systemically transplanted MSCs (mesenchymal stem cell) can engraft to injured cornea to promote wound healing, by differentiation, proliferation, and synergizing with hemotopoietic stem cells.[14] 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.[15] 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.[16] These observations demonstrated that human corneal stromal stem cells (hCSSCs) showed a much greater potential, under proper substrate and growth factor guidance, to facilitate the generation of a biological human cornea equivalent. Unlike hCSSCs, human corneal fibroblass (hCFs) were less responsive to these environmental cues and under identical culture conditions generated an extra cellular matrix (ECM) that poorly mimicked the native, functional tissue structure and composition.[17] This study defines optimal in vitro conditions to monitor the development of mouse corneal stromal fibroblasts (MSF) into myofibroblasts. The combined effects of transforming growth factor (TGF) β and plateled-derived growth factor (PDGF) promote the full development of V+A+D+ myofibroblasts from MS.[18] TGFβ – Transforming growth factor beta and PDGF regulate corneal myofibroblast development from bone marrow-derived precursor cells and keratocyte/corneal fibroblast-derived precursor cells.[19] The purpose of this article is to review the factors involved in the maintenance of corneal transparency and to highlight the mechanisms involved in the appearance, persistency and regression of corneal opacity after stromal injury. The development of corneal opacity involves complex processes mediated by cytokines, growth factors, and chemokines—and corneal epithelial-stromal interactions that involve the epithelial basement membrane—that may lead to myofibroblast generation, a decrease in cellular corneal crystallins, and loss of stromal structural components. A better understanding of cells and molecules involved in this process may lead to new treatment options to restore corneal transparency and prevent corneal scar formation.[20] Taken together, these observations suggest melanocytes could play an important role in the maintenance of LESCs in the native human limbal stem cell niche.[21] Mature myofibroblasts that become established in the anterior stroma are a barrier to keratocyte/ corneal fibroblast contributions to the nascent EBM. These myofibroblasts, and the opacity they produce, often persist for months or years after the injury. Transparency is subsequently restored when the EBM is completely regenerated, myofibroblasts are deprived of TGFβ and undergo apoptosis, and the keratocytes re-occupy the anterior stroma and reabsorb disordered extracellular matrix. The aim of this review is to highlight factors involved in the generation of stromal haze and its subsequent removal.[22] Thus, it appears that IL-20 plays a beneficial and direct role in corneal wound healing while negatively regulating neutrophil and platelet infiltration.[23] This study suggests that the EBM has a critical role in modulating myofibroblast development and fibrosis after keratitis—similar to the role of EBM in fibrosis after photorefractive keratectomy. Damage to EBM likely allows epithelium-derived transforming growth factor beta (TGFβ) to penetrate the stroma and drive development and persistence of myofibroblasts. Eventual repair of EBM leads to myofibroblast apoptosis when the cells are deprived of requisite TGFβ to maintain viability. The endothelium and Descemet's membrane may serve a similar function modulating TGFβ penetration into the posterior stroma—with the source of TGFβ likely being the aqueous humor.[24] In this article, we concisely review the available literature regarding the role of HGF in corneal wound healing. We highlight the influence of HGF on cellular behaviors in each corneal layer. Additionally, we suggest the possibility that HGF may represent a therapeutic tool for interrupting dysregulated corneal repair processes to improve patient outcomes.[25]

The aim of this study was to determine whether bone marrow-derived fibrocytes migrate into the cornea after stromal scar-producing injury and differentiate into alpha-smooth muscle actin (αSMA) + myofibroblasts. Chimeric mice expressinggreen fluorescent protein (GFP) bone marrow cells had fibrosis (haze)-generating irregular phototherapeutic keratectomy (PTK). After irregular PTK in the strain of C57BL/6—C57/BL/6-Tg (UBC-GFP) 30Scha/J chimeric mice, however, more than 95% of fibrocytes and other hematopoietic cells underwent apoptosis prior to the development of mature αSMA+ myofibroblasts. Most GFP+ CD45+ αSMA+ myofibroblasts that did develop subsequently underwent apoptosis—likely due to epithelial basement membrane regeneration and deprivation of epithelium-derived TGFβ requisite for myofibroblast survival.[26]

2. MATERIALS AND METHODS

2.1 Materials

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. 3 hours after injury stretches of cells directed towards wound move off the limb vessels.

2.2 Autoradiographic method

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). A 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.[27,28]

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. De-paraffinized sections were covered with liquid photoemulsion of "M" type. The sections were exposed for 8 weeks at 4^{0}C, developed with developer D-19 (methylhydroquinone for radioautography) and stained with hematoxylin-eozin according to Karachi.[29] 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.

3. RESULTS

Data of autoradiographical studies

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 cells of the multilayer plane epithelium are labeled, but nuclear 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 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 in average 23.1±2.4% and 21.4 ±1.8%, respectively (Table 1; Fig. 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%). 4 days after operation the number of labeled cells is reduced rapidly both in the infiltrate and stretches, which is not observed at pulse labeling (Table 2; Fig. 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; Fig. 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; Fig. 1, 2).

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%

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%

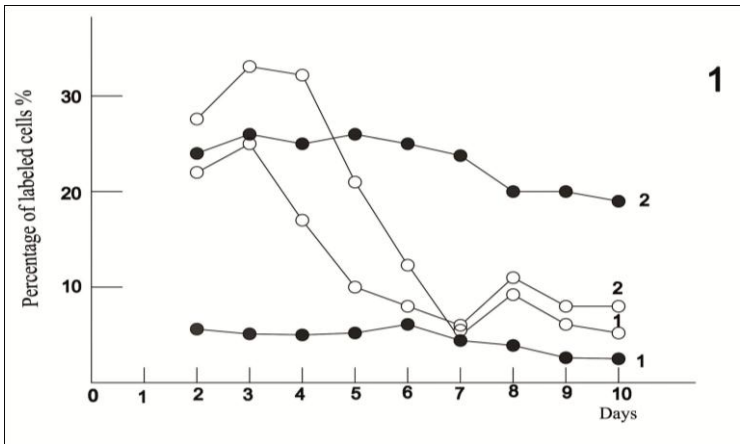

Fig. 1: Number of labeled cells in infiltrate of injured cornea of white adult mice.
On absciss – a time of beginning of aseptic inflammation (days); on ordinate – percentage Of labeled cells (%).
^{3}H-thymidine is injected 1 hour prior to fixation.
^{3}H-thymidine is injected along with injuring of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

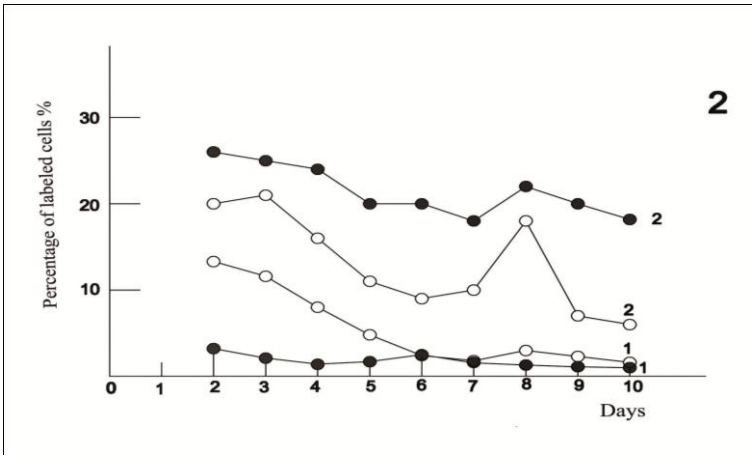

Fig. 2: Number of labeled cells in stretches of injured cornea of white adult mice.

On absciss – a time of beginning of aseptic inflammation (days); on ordinate – percentage of labeled cells (%).
^{3}H-thymidine is injected 1 hour prior to fixation.
^{3}H-thymidine is injected along with injuring of an object.
1. Common index of labeled cells.
2. Index of intensity of labeled cells.

4. CONCLUSION

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.

Results of autoradiographic investigations have shown: 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 can not 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, 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.

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