S100β Protein Stimulates Regeneration of the Rabbit Cornea

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The effect of S100β protein on the processes of wound repair in the rabbit cornea is studied. Subconjunctival injection of the protein is found to promote favorable and rapid healing of the corneal injury, evidence of which is early epithelization, a drop of the index of migrating inflammatory cells, and prevention of the development of fibrous subepithelial tissue.

Key Words: *S100β protein; cornea; repair processes*

The study of morphogens, factors of growth and development which are implicated in the process of regeneration in the adult organism, is of great importance [1,3,6,9,10]. Such studies attract attention because of their undeniable theoretical and practical value, notably due to the possibility of obtaining biologically active substances - regulators of healing. In this regard the proteins of the S100 group, which are acid Ca-binding proteins of the nervous tissue of animals of different taxa - from mollusks and insects to mammals and humans - seem to be promising. The primary structure of three major representatives of S100 proteins (S100αα, S100β, S100α) has been determined; these proteins not only differ in their subunit composition and other physico-chemical properties, but also exhibit a cell-specific distribution. It has been found that S100α is localized in neurons, whereas S100β occurs in glial cells [4,7]. However, these data are to be further corroborated, since there have been some reports that S100β proteins have been discovered in tissues other than nervous [4]. For instance, proteins of this group have been found in the anterior and posterior cornea, which is attributed to the presumed crest origin of the corneal stroma [11], i.e., its development, as in the case of nerve and glial cells, from the blastophyllum. There is evidence that S100 proteins are implicated in ontogenesis, contribute to cell differentiation, and exert an effect on the regeneration of the nerve processes in a denervated region of the rabbit cornea [4,7]. It is worthy of note that a number of growth factors which accelerate wound repair, such as epidermal growth factor, nerve growth factor, and neurotrophic factor, have been found in the cornea.

The objective of the present study was to explore the effect of S100β on wound healing in the rabbit cornea.

MATERIALS AND METHODS

The cornea is frequently used as an experimental model in studies of the effect of various biologically active substances on inflammatory and regenerative processes, since the cornea is avascular and transparent and is composed of relatively uniform tissues. Ten chinchilla rabbits weighing 1.5-2.0 kg were used in the experiments. The right eye of animals was the experimental one and the left the control. After preliminary analgesia with dicaine a dosed incision

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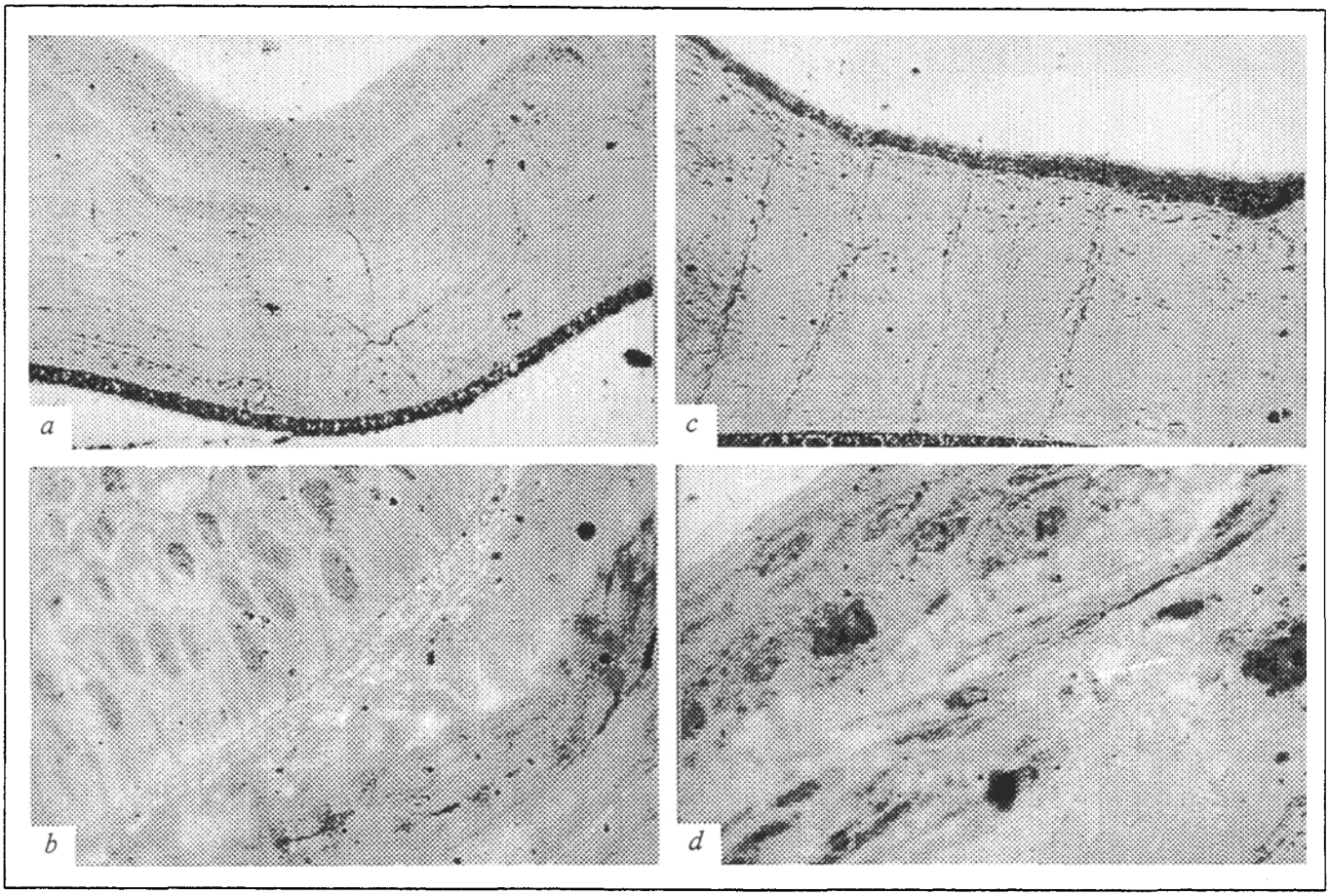

Fig. 1. State of rabbit cornea 25 days after experimental surgical intervention with injection of S100β protein. *a* and *b*) experiment: recovery of stratified squamous epithelium over the entire length; the basement membrane is observed over the entire length; infiltration is absent; corneal layers are parallel (×125); *c* and *d*) control; thickness of epithelial layer is nonuniform; the basement membrane is not observed over its entire length; fibroblast proliferation in subepithelial layers; subepithelial infiltration (×1250).

was performed in the cornea to 2/3 of its thickness with a trepan. The upper part of the trephined zone was removed with a dissecting knife. A single injection of S100β obtained after Selinfreund *et al.* [12] (10^{-8} M in 0.2 ml sterile physiological saline) was performed under the conjunctiva of the right eye on the 1st day after surgical intervention and every day throughout the follow-up period. Physiological saline (0.2 ml) was injected in the control eye. Rabbits were killed by air embolism on days 1, 5, 10, 15, 20, and 25 after the procedure. Both eyes of the animals were then enucleated and placed in 2% glutaraldehyde, this being followed by fixation in paraformaldehyde or in 10% Formalin. The cornea was divided into 4 sectors, and each of them was embedded in paraffin and Epon. Sections 1-2 μ thick were stained with 1% toluidine blue or with 1% toluidine blue + 1% fuchsin (mixed staining) and examined under a light microscope. The total cell count and the number of migrating cells (leukocytes, lymphocytes, and macrophages) and keratocytes/fibroblasts were determined with the aid of an ocular-micrometer in the peripheral and central part of the facette. The results were statistically processed on an IBM PC/AT computer using QuatroPro and Microstat software.

RESULTS

The results of comparative histological analysis are shown in Table 1. It was found that in the presence of S100β epithelization began as early as on day 1 postoperation, and on day 5 the integrity of the basement membrane was restored. On day 10 the cornea was uniformly metachromatically stained without signs of edema. In the control epithelization began on day 2 postoperation, and a discontinuous basement membrane did not form until after day 15; within the first few days necrotic changes of the stroma were noted; they then transformed into edema which was preserved till days 20-25 (Fig. 1).

The time course of the total cell count and of the nature of infiltration in the experiment and in

the control is presented in Fig. 2. The experimental curve of the total cell count has virtually the same shape as in the control. In the experiment, however, on days 1 and 5 this index was higher (which attests to a more rapid onset of the processes of migration) and on days 20 and 25 it was markedly lower. Comparison of this fact with the histological picture suggests that this could be due to an earlier (than in the control) restoration of the intercellular matrix, represented in the cornea by collagen of the corneal layers and glycosaminoglycan (chondroitin and keratin sulfate). This is also shown by the formation of a subepithelial fibroblastic film and irregular coarse collagen fibers in the control on days 20 and 25 (Fig. 1).

A study of the nature of infiltration demonstrated that the curves of lymphocyte infiltration for the effect of S100β protein and in the control were divergent. At the later stages (days 15, 20, and 25) the lymphocyte count dropped in the experiment, while in the control this index sharply rose, especially on day 25 (2-3-fold, as compared with days 5 and 10, $p<0.05$). The time course of monocyte infiltration was typical: its indexes on days 5, 10, and 15 were reliably higher than in the control and sharply dropped toward day 25 postoperation, whereas the control indexes remained high ($p<0.05$). The data obtained suggest a high index of migration of monocyte-macrophagal cells in the focus of lesions during the first 15 days, and then, on days 20-25, a virtual disappearance of migrating magrophagal cells due to restoration of the intercellular matrix. The curve of changes in the fibroblast and keratocyte count (these cells were counted together, since their differentiation is difficult) was the most interesting. In the experiment the fibroblast migration and, evidently, the keratocyte proliferation, peaked on day 5, after which a gradual decrease of their count was observed. Meanwhile, in the control a marked rise of the fibroblast-keratocyte index was noted on day 10, its slight decrease on day 15, and then again a sharp rise on days 20 and 25 ($p<0.05$). Histologically, monocyte-macrophagal and lymphocyte infiltration, disorganization of the corneal layers, stromal edema, and a focal absence of endothelial cells corresponded to the picture in the control (Fig. 2).

The results demonstrate that S100β exerted a pronounced effect on the state of the focus of lesions in the rabbit cornea and caused a more marked monocyte-macrophagal migration and fibroblast and keratocyte proliferation during the first 5 days than in the control. In contrast, on days 10, 15, 20, and 25 a gradual reduction of the total cell

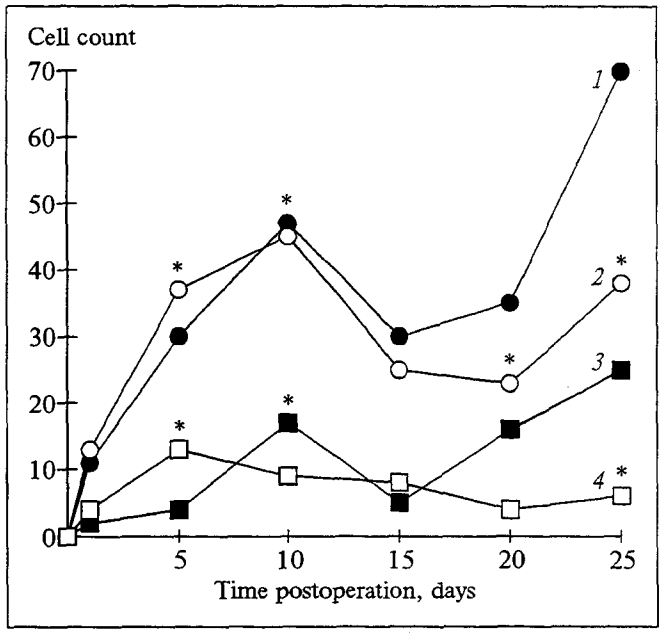

Fig. 2. Time course of total cell count and of fibroblast and keratocyte count in the wound infiltration of rabbit cornea for the effect of S100β proteins. *1*) total cell count in the control; *2*) total cell count in the experiment; *3*) fibroblasts and keratocytes in the control; *4*) fibroblasts and keratocytes in the experiment. An asterisk denotes $p<0.05$.

count was observed, which was most marked on day 25 postoperation, predominantly due to fibroblasts and lymphocytes. The histological picture provided evidence that injection of S100β caused earlier epithelization in the zone of the wound and restoration of the basement membrane on day 5 and of the number of layers and of its thickness on days 20 and 25. Meanwhile, the epithelium remained bilayer, and the monocyte-macrophage infiltration, disorganization of the corneal layers, and formation of the subepithelial film were preserved in the control at the same time points.

Thus, subconjunctival injection of S100β promotes favorable and rapid healing of a corneal wound, evidence of which is early epithelization, a drop of the index of migrating inflammatory cells, and prevention of the development of fibrous subepithelial tissue.

In view of the possible mechanisms of the discovered effect of this protein on the reparative regeneration of a corneal wound it is logical to consider the characteristics of molecular processes and substrates underlying the biological activity of S100β proteins, primarily their capability of Ca binding and of Ca-mediated interaction with a number of proteins and peptides [4]. It is known that Ca is one of the universal regulators of the intracellular metabolic processes, cell-cell interactions, and cell differentiation and development. Ca^{2+} binding is a prerequisite for specific intermo-

TABLE 1. Effect of S100β Proteins (10^{-8} M) on Healing of a Corneal Wound in Rabbits as Assessed by Comparative Histological Analysis

Time after surgical intervention, days	State of zone of surgical intervention	
	experiment	control
1	Epithelium covers edge of facette and extends over the wound surface. Edema and slight infiltration of stroma. Keratocyte nuclei are hyperchromic	Epithelial layer is discontinued at edge of facette. Necrosis of superficial layers of stroma. Pronounced stromal infiltration and edema
5	Complete epithelization of wound surface. Restoration of basement membrane. Absence of infiltration. Fibroblast migration and proliferation	Thickness of epithelium is nonuniform. Basement membrane not differentiated. Stromal edema. Fibroblast and keratocyte proliferation. Stromal infiltration
10	Epithelial plug at edge of facette is narrow. Edema is absent. Uniform stromal metachromasia. Dividing nuclei of keratocytes are hyperchromic. Chaotic distribution of fibers in subepithelial layers. Pinocytic activity of endothelium	Wide epithelial plug, cell-cell contacts are weakened. Basement layer is not differentiated. Stromal edema. Nonuniform metachromasia
15	Epithelial plug comprises 4-5 layers. Cell-cell contacts are restored. Subepithelial edema of fibers	Epithelial plug comprises 8-10 layers. Discontinuous basement membrane. Cell-cell contacts are weakened; multiple intercellular vacuoles. Subepithelial film is formed by proliferating fibroblasts. Edema between fibers. Stromal infiltration.
20-25	Thickness of epithelium is uniform. Number and structure of layers are restored. Parallel arrangement of subepithelial layers. Absence of superficial infiltration. Solitary hyperchromic keratocytes (Fig. 1, *a* and *b*)	Epithelium is bilayer. Subepithelial monocyte-macrophagal infiltration. Fibroblasts; disorganization of corneal layers. Edema in superficial stromal layers; weakening of cell-cell

lecular interaction of S100 proteins with oligopeptides and proteins, including the regulators of cell growth, proliferation, and differentiation [2,4]. In this connection it is interesting that the transforming β growth factor, belonging to the family of epidermal growth factors, promotes healing of corneal-scleral wounds [13].

S100b proteins modulate the activity of some protein kinases and phosphoprotein phosphatases, and are thus able to exert an effect upon phosphorylation/dephosphorylation of a number of cytoplasmic and nuclear protein substrates [4]. These processes are directly associated with the mechanisms of cell proliferation and differentiation, and affect the growth and regeneration of damaged tissues [2,4,14].

S100β proteins exert an effect upon the metabolism and specific regulation of transmitters, some of which, notably biogenic amines, are also factors of growth and development [4,5].

The data available provide evidence that S100β promotes healing of a corneal wound, allowing us to regard the growth and regenerative potentials of this protein as a participant in the general regulatory-trophic system of the organism.

REFERENCES

1. V. N. Kanonov, *Biology of Nerve Growth Factors* [in Russian], Minsk (1986).
2. S. I. Kusen' and R. S. Stoika, *Molecular Mechanisms of Polypeptide Growth Factors* [in Russian], Moscow (1985).
3. N. N. Nikol'skii, A. D. Sorokin, and A. B. Sorokin, *Epidermal Growth Factor* [in Russian], Leningrad (1987).
4. A. B. Poletaev and V. V. Sherstnev, *Usp. Sovr. Biol.*, **103**, № 1, 124-132 (1987).
5. A. R. Chubanov, in: *Morphogenetically Active Substances* [in Russian], Pushchino (1990), pp. 113-125.
6. I. M. Sheiman, in: *Regulators of Morphogenesis and Their Adaptive Role* [in Russian], Moscow (1984).
7. M. B. Shtark, *Brain-Specific Proteins (Antigens) and Neuron Functions* [in Russian], Moscow (1985).
8. K. Y. Choks and R. H. Halchke, *Exp. Eye Res.*, **41**, № 6, 687-699 (1985).
9. A. Dekker, W. H. Gispen, and D. Weed, *Life Sci.*, **41**, № 14, 1667-1678 (1987).
10. M. R. Hanley, *Lancet*, № 8651, 1373-1376 (1989).
11. Abul Kalam M. Samsudin, Virider S. Niranakari, Dallas M. Purnell, and Seung H. Chang, *Ophthalmology*, **93**, 1298-1303 (1986).
12. R. H. Selinfreund, S. W. Barger, W. J. Pledger, and K. L. S. Van Eld, *Proc. Nat. Acad. Sci. USA*, **88**, 3854-3858 (1991).
13. W. E. Smiddy, B. M. Glaser, W. R. Green, *et al.*, *Arch. Ophthalmol.*, **107**, № 4, 577-580 (1989).
14. J. H. P. Skene, *Amer. Rev. Neurosci.*, № 12, 127-156 (1989).