AUTORADIOGRAPHIC STUDY OF CORNEAL NEOVASCULARIZATION INDUCED BY ALKALINE BURN

Ali Ghadiri,* MD and Yin Shuguo,** MD

Abstract—Corneal neovascularization (CNV) can cause significant visual loss. The kinetics of endothelial cells during microvascular growth were studied using a model of alkaline cauterization-induced neovascularization of the 40 rabbits cornea. Changes in DNA synthetic activity of endothelial cells during neovascularization were assessed by autoradiographic study of ³H-TdR incorporation, In vivo and In vitro. Microscopic study demonstrated that migration and redistribution of existing endothelial cells from the limbal vessels enabled vascular sprouting and elongation with cellular proliferation.

Acta Medica Iranica 33 (1&2): 12-17; 1995

Key words: corneal neovascularization; endothelial cells; ³H-TdR

INTRODUCTION

Over the past years, numerous investigators have studied the process of neovascularization using the cornea as a model (1,2,3,4,5,6). The normal cornea having the great advantage of avascularity, transparency, and accessibility to biomicroscopical examination, has been widely employed as an experimental tissue in these investigations.

This study demonstrates changes in DNA synthetic activity of endothelial cells during corneal neo-vascularization (CNV).

*Resident-in-Training, Farabi Eye Hospital, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran; **Associate Professor, Department of Ophthalmology, the Second Affiliated Hospital of China Medical University, Shenyang, China.

MATERIALS AND METHODS

Animals and chemical agent

We used 40 New Zealand white rabbits weighing 2.5-3.5 Kg. The corneas were examined with an operating microscope to exclude the possibility of existing neovascularization. The experiments were done under the effect of topical anesthesia with dicaine 0.5% in each cornea to prevent the corneal reflex prior to cauterization.

Cautery of the cornea was performed by filter paper 6 mm in diameter; the filter was soaked in 1 Mol NaOH and then placed on the corneal surface for 10 seconds. A dense white lesion, approximately 5 mm in diameter appeared immediately. Next, the corneas were flushed with 2 ml of sterile saline and 0.25% chloromycine, after which the rabbits were observed (8). Ten days following stimulation of cornea, careful assessment of the neovascularization was made by magnification of 3×. Forty eyes were cauterized and 12 uninjured corneas served as controls.

Exposure to labeled isotopes

In vivo: Approximately 0.5 ml or 20 μCi of ³H-methyl thymidine (Chinese National Nuclear Center, Beijing, specific activity 20 mCi mMol) was injected into conjunctival sacs of both 20 experimental and 6 uninjured control eyes. Four hours later all animals were deeply anesthetized with intraperitoneal injection of 30% urethane (1g per Kg).

In vitro: Animals (20 experimental and 6 uninjured control eyes) were anesthetized by intraperitoneal injection of 30% urethane. Corneas were extracted under aseptic conditions. Each cornea was separately soaked in 2 ml of culture medium containing 4 μCi ³H-TdR. Each

specimen was then placed in an incubator at 37°C for 90 minutes.

Tissue processing

Corneal tissues fixed in 10% neutral buffered formalin were dehydrated in ascending grades of ethanol, cleared in xylene-cedarwood oil, and embedded in paraffin. Six to eight-mm thick sections were cut on a Spencer rotary microtome (12).

Autoradiography

Autoradiography of paraffin sections were prepared by dipping the slides in nuclear emulsion (Chinese made) and exposing them in a dark cabinet for 2 weeks at 4°C. At the end of the exposure period, slides were removed from the cabinet and developed. After washing, the section was stained with hematoxylin and eosin. The presence of DNA synthesis was inferred by the presence of five or more silver grains overlying a nucleus. These grains were distinguished from the scattered silver nitrate deposits by their small and uniform size, concentration over nuclei, and presence in the corneal epithelial cells of noncauterized animals(1,2,7).

RESULTS

Normal (noncauterized) cornea

The pattern of the vasculature at the limbus of normal corneas has been described in detail by Peter and Burger et al (8), who utilized scanning electron microscopy of vascular casts. In our study, the pericorneal vessels included small arteries, postcapillary venular plexus, a capillary arcade and a flanking veins for the 12 control eyes. No silver grains were concentrated over the nuclei of any endothelial cells or pericytes in these pericorneal vessels in the noncauterized eye. In 8 μm-thick sections, endothelial cells had a squamous appearance with flat elongated nuclei, typical of endothelial cells in unstimulated vessels. No intracorneal vessels were apparent in the control animals. There was 1.5% labeling index (LI) for the control group, all of the silver grains were concentrated over the nuclei of the basal cell layer of the corneal epithelium (Fig. 1 and Table 1).

Neither thymidine labeling nor mitosis was observed in the uniform, elongated fibroblasts flattened between the collagen lamellae of the corneal stroma (1,12).
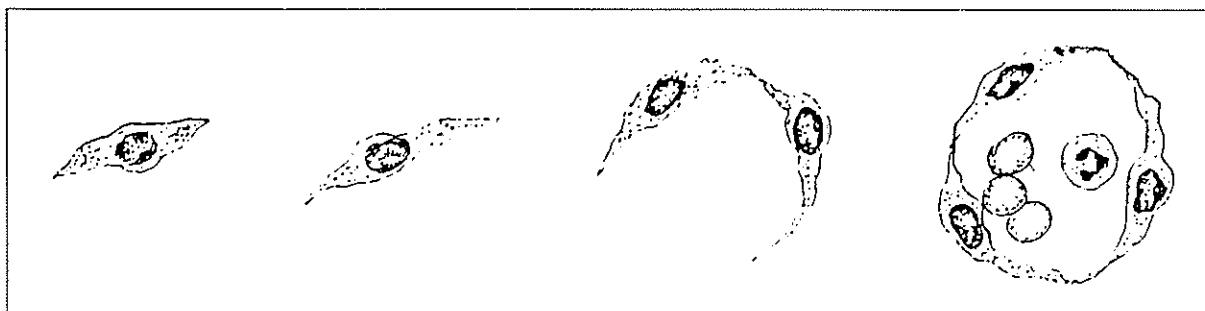

Fig. 1. CNV surrounded by plasma cell and polymorphonuclear leukocytes.

Table 1. Labeling index (percent) of corneal epithelium in the control and experimental groups.

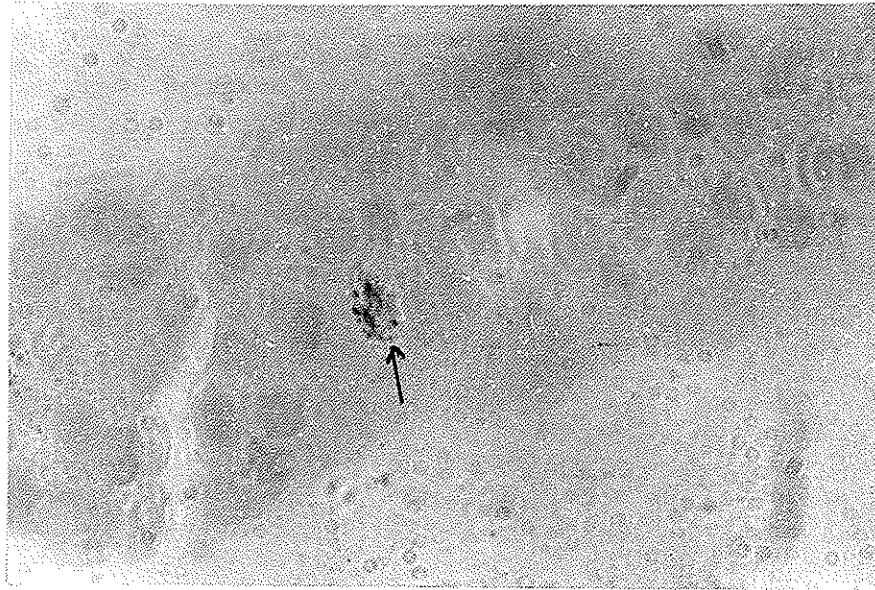| Cornea No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Control group (LI %)	In vivo	1	2	2	2	1	1														
	In vitro	1	1	2	1	2	2														
CNV group (LI %)	In vivo	4	4	3	5	4	5	5	4	4	4	3	6	5	5	4	4	5	4	4	3
	In vitro	3	5	5	2	6	5	5	4	5	5	4	4	3	5	6	5	4	4	4	4

Fig. 2. Control group. All of the silver grains are concentrated over the nuclei of the basal cell layer of the corneal epithelium (arrow) (In vitro, H & E,× 280).
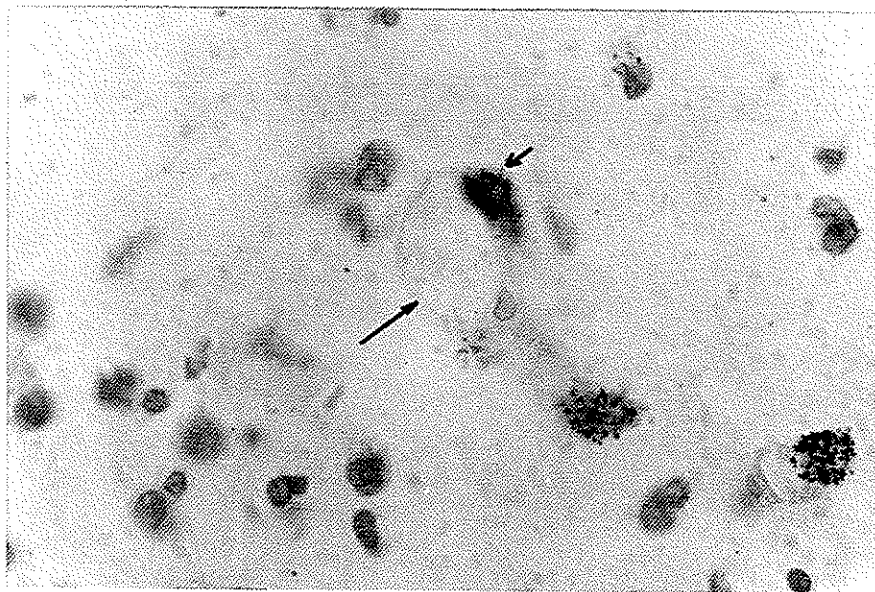

Fig. 3. Experimental group. Intracorneal new vessels at ten days after burn. ³H-TdR-labeled endothelial cells (short arrow) as well as an erythrocyte (long arrow) passed through the lumen (In vivo, H & E,× 280).
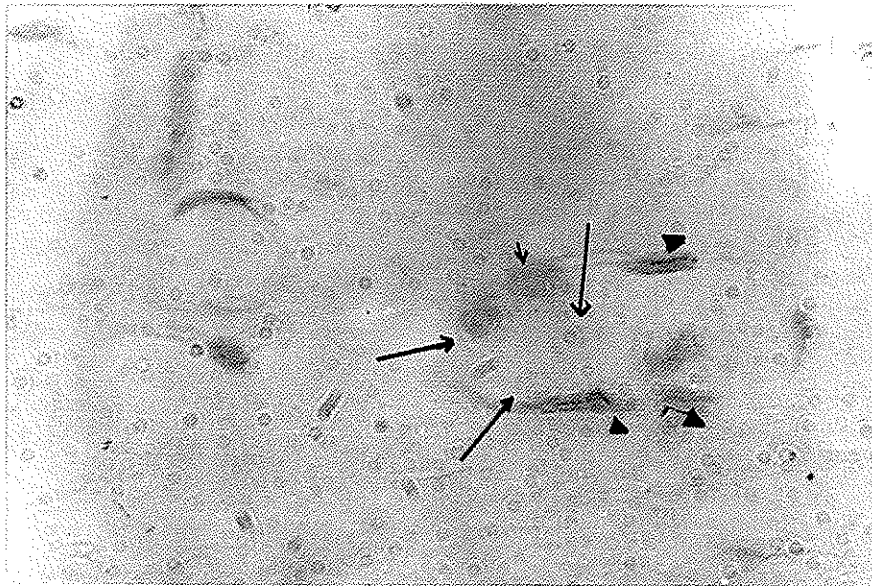
Fig. 4. Experimental group. Intracorneal new vessels at ten days after alkali burn. ^{3}H-TdR-labeled endothelial cells (arrow heads) as well as an endothelial cell in mitosis (short arrow), and also a few erythrocytes (long arrows) which passed through the lumen are present (In vitro, H & E,× 280).
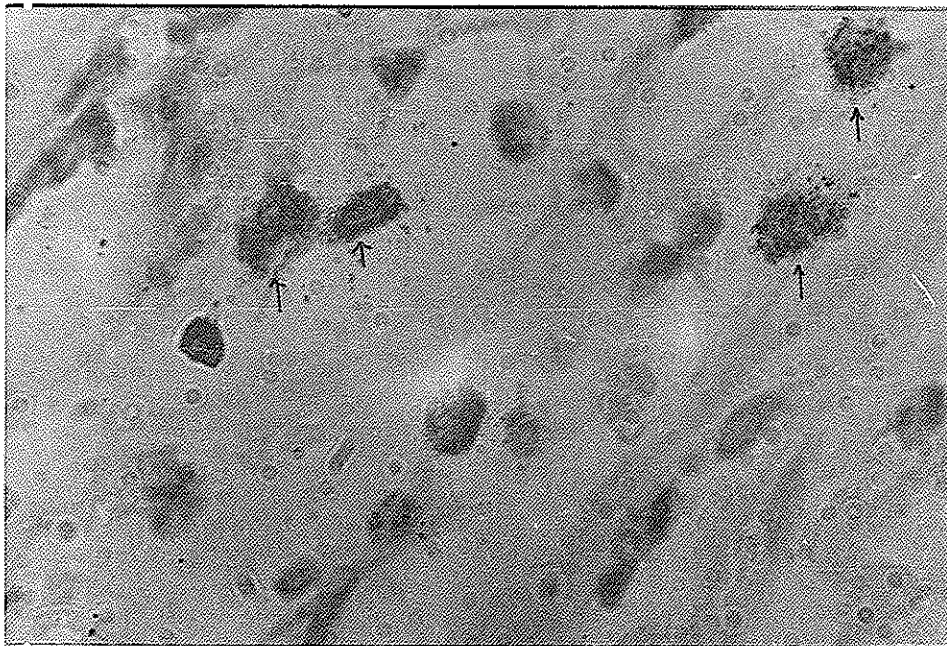

Fig. 5. Experimental group. Corneal stroma at ten days after alkali burn. ^{3}H-TdR-labeled fibroblasts (arrows) as well as different size of stromal collagen fibers are seen (In vitro, H & E, × 280).

Experimental (cauterized) cornea

Corneal epithelium. Cauterization immediately converted the epithelium and underlying corneal stroma into an amorphous white coagulum. In all of the 40 experimental corneas, reepithelization was started the second day of the burn, although the epithelium was thicker and less stratified than in the control corneas. The labeling index of the corneal epithelium between the cautery site and the corneoscleral junction ranged from 2 to 6 percent (average LI=4.25%) (Table 1).

Corneal stroma. Corneal fibroblasts were labeled but were often difficult to identify with certainty by light microscopy after the influx of mononuclear inflammatory cells. The latter cells filled and conformed to the interstices of the corneal stroma and assumed elongated shapes similar to that of normal keratocytes. A labeling index was therefore not determined for this cell type (Fig. 5).

Blood vessels. The pericorneal vasculature, especially the veins and capillaries, were variably dilated and engorged with erythrocytes, mononuclear cells and polymorphonuclear leukocytes.

The earliest morphologic alteration in the vascular endothelial cells and pericytes of these cell types were enlarged and had lost their flattened crescentic profiles. The former bulged into the lumina, and the latter projected into the extravascular space. The nuclei and nucleoli were enlarged and rounded, and the nuclei were often, but not always, labeled. The pericytes often appeared slightly detached from the capillaries and assumed a plate-like polygonal appearance. New vessels extending to the cautery site were clearly evident and identified by their linear configuration and, in contrast to normal capillaries with both endothelial cells and pericytes, had a single layer of mural cells. Most of the new vessels appeared to arise from venules, as opposed to capillaries.

It was our impression that the first fibroblast's division and proliferation happened. In this condition ^{3}H-thymidine labeling over the nuclei was very clear. In the next step two or more divided cells connecting together and new capillary-like sprouts of corneal neovascularization appeared (Fig. 2).

Vascular differentiation. The large intracorneal vessels were continuous with either a pericorneal artery or vein. Those connected to a pericorneal artery had the more uniform caliber, prominent and pointed endothelial cells, and longitudinal furrows that characterized the normal pericorneal arteries.

There were new vessels extending between the pericorneal plexus and the site of the burn in all of the animals. Under the microscope, the intracorneal vessels in the eye were different in size, some were extremely fine, like threads, that only one erythrocyte could pass through the lumen, whereas some vessels observed would allow a few erythrocytes to pass through. (Figs. 3 and 4).

Corneal endothelium. Corneal endothelium cells were morphologically normal by light microscopy, except for the segment immediately below the burn site where the endothelium was focally two cell layers thick.

Inflammatory cells. Acute inflammation, consisted of mid infiltration of the corneal stroma with polymorphonuclear neutrophils.

DISCUSSION

Corneal neovascularization is an important and frequently encountered clinical problem (9,10). It can result from primarily inflammatory insults such as alkali injuries, where the vessels may contribute to tectonic stability and facilitate healing. However, vessels can also develop insidiously when triggered by an immunogenic stimulus and usually have a deleterious effect on vision. CNV occurring in association with herps simplex stromal keratitis, an immunologically mediated disease (11), can lead to lipid exudation and scarring. The presence of a vascularized recipient bed can impair corneal allograft survival, at least in part by enhancing it's exposure to systemic immune reactants (18). Over the past years, numerous investigators have studied the process of neovascularization using the cornea as a model. This study demonstrates changes in DNA synthetic activity of endothelial cells during corneal neovascularization, by assessing the autoradiographic study of ^{3}H-TdR incorporation, in vivo and in vitro.

The pathogenesis of CNV has been reviewed by Klintworth and Burger (1). Campbell and Michaelson first suggested that CNV results from the diffusion of soluble mediators from within the cornea (14,15). The mechanism of CNV was investigated by Cogan, who suggested that reduction in tissue compactness alone was an important precipitating event (16). However, Ashton and Cook (17) did not feel that a loss of tissue compactness was a sufficient stimulus for CNV and postulated that other factors played a role.

In several studies, investigators have recorded the evolution of new intracorneal vessels from the first blind sprouts to the large looping mature vessels (1,8,18). The present study indicates that alkaline burn of the rabbit's cornea, elicits a vasoproliferative response at the corneoscleral limbus. In the present study, the label was not apparent over the nuclei of vascular endothelial cells or pericytes of 12 control animals. Also, the labeling index of 1.5 percent in control animals was reached at 4.25 percent in the experimental group in corneal

sprouts to the large looping mature vessels (1,8,18). The present study indicates that alkaline burn of the rabbit's cornea elicits a vasoproliferative response at the corneoscleral limbus. In the present study, the label was not apparent over the nuclei of vascular endothelial cells or pericytes of 12 control animals. Also, the labeling index of 1.5 percent in control animals was reached at 4.25 percent in the experimental group in corneal epithelium. This change in the labeling index percent and the absence of mitotic figures, suggest that vascular cells at the corneoscleral limbus do not replicate often under normal conditions.

The mechanism by which new vessels are assembled has yet to be defined precisely, but it is likely that both migration and mitosis of endothelial cells and perhaps pericytes are operative. Previous ultrastructural studies of the response to chemical cautery have shown that the first morphologic change in parent pericorneal vessels is hypertrophy of vascular endothelial cells and pericytes nucleolar prominence and dispersal of ribosomes into their free form (19). Such changes in other experimental situations have been termed activated 'regenerative' or 'recovering' by several investigators, and because of the frequency of associated thymidine labeling, have been felt to indicate a proliferating endothelium (1,20). In our study the endothelial cells at the tip of growing new vessels progressed towards the site of the injury, whereas mitoses were confined to the cells more proximal on the capillary bud (1). Because of these observations, it is suggested that the initial event in neovascularization is a migration and formation of provisional lumina by vascular endothelial cells and these changes antecede endothelial mitoses. The connections between the new intracorneal blood vessels and the pre-existing vascular plexus at the corneoscleral limbus, characterized the surface features of these new vessels. The intracorneal vessels that connected with the pericorneal arterioles had features characteristic of arterioles, such as prominent impressions of endothelial cell nuclei and short longitudinal grooves. Those new vessels that connected directly with veins near the corneoscleral limbus, on the other hand, had venous attributes, such as irregular lumina and inconspicuous endothelial cell impressions.

REFERENCES

1. Burger CP et al. Autoradiographic study of corneal neovascularizaton induced by chemical cautery. Lab Inves. **45:** 328-335; 1981.

2. Sholley MM et al. Mechanisms of neovascularization. Lab Inves. **51:** 624-634; 1984.

3. Frucht J et al. Topical indomethacin effect on neovascularization of the cornea and on prostaglandin E_2 levels. Brit J Ophthalmol. **68:** 656-659; 1984.

4. Benezra D. Neovasculogenic ability of prostaglandin, growth factors and synthetic chemoattractants. Am J Ophthalmol. **86:** 455-461; 1978.

5. Avery LR et al. Systemic amiloride inhibits experimentally induced neovascularization. Arch Ophthalmol. **108:** 147-1476; 1990.

6. Schanzlin JD et al. Histopathology of corneal neovascularization. Arch Ophthalmol. **101:** 472-474; 1983.

7. Abercrombie M. Estimation of nuclear population from microtome sections. Anat Res. **94:** 239; 1946.

8. Burger CP et al. Corneal neovascularization as studied by scanning electron microscopy of vascular casts. Lab Inves. **48:** 169-180; 1983.

9. Epstein RJ et al. Corneal neovascularization pathogenesis and inhibition. Cornea **6:** 250-257; 1989.

10. Klintworth GK .et al. Neovascularization of the cornea, current concepts of its pathogenesis. Int Opthalmol Clin. **23:** 27-39; 1983.

11. Hendricks RD et al. The effect of cellular immunotolerance to HSV-1 antigens on the immunopathology of HSV-1 Keratitis. Invest Ophthalmol Vis Sci. **30:** 105-115; 1989.

12. Moore WJ et al. Comparison of the neovascular effects of stimulated macrophages and neutrophils in autologous rabbit corneas. Am J Pathol. **120:** 87-98; 1985.

13. Epstein JR et al. Photodynamic therapy for corneal neovascularization. Cornea **10** (5): 424-432; 1991.

14. Klintworth CK et al. Neovascularization of cornea. Int Ophthalmol Clin. **23:** 27-39; 1983.

15. Campbell FW et al. Blood-vessel formation in the cornea. Brit J Ophthalmol. **33:** 248-255; 1949.

16. Cogan DG. Vascularization of the cornea. Arch Ophthalmol. **41:** 406-416; 1949.

17. Ashton N et al. Mechanism of corneal vascularization. Brit J Ophthalmol. **37:** 193-209; 1953.

18. Klinworht GK. The cornea in structure and macromolecules in health and disease. Am J Pathol. **89:** 719-782; 1977.

19. McCracken SJ et al. Morphologic observation on experimental corneal vascularization in the rat. Lab Inves. **41:** 519-530; 1979.

20. Polverini PJ et al. Endothelial proliferation in the delated hypersensitivity reaction. J Immunol. **118:** 529-532; 1977.

■■