Polymorphonuclear Leukocytes Inhibit Proliferation of Epithelial Cells of Rabbit Cornea. (Fig. 3, Table 2)

Chikako Katakami*, *, Todd Perkins*, Neil Dorfman***
Abbot G. Spaulding* and Winston W.-Y. Kao*, **
Departments of Ophthalmology, and Biochemistry and Molecular Biology**,*
University of Cincinnati College of Medicine.
*Department of Ophthalmology***,*
School of Medicine, Kobe University.

Abstract

Injuries inflicted upon corneas cause infiltration of polymorphonuclear leukocytes (PMN) into the tissues. Persistence of PMN in the injured cornea retards re-epithelializatien of epithelial defects. Our results indicate that the cessation of epithelial cell proliferation is concomitant with the appearance of PMN in lacerated corneas. It is possible that PMN may modulate epithelial cell proliferation. To examine this hypothesis, corneas were co-cultured with PMN, labeled with [^{3}H] thymidine, and then subjected to radioautography. These results indicated that PMN significantly inhibited the corneal epithelial cell proliferation when the production of oxidants, e.g., superoxide, H_2O_2, HOCl, by PMN was prevented by addition of catalase or methionine to the culture medium. It is suggested that PMN produce factors inhibiting epithelial cell proliferation in injured corneas. Our observations provide an alternative explanation as to why the presence of PMN retards corneal re-epithelialization. (Acta Soc Ophthalmol Jpn 92 : 798—805, 1988)

Key words: polymorphonuclear leukocytes, corneal wound healing, inhibition of epithelial cell proliferation, re-epithelialization

要　約

角膜に感染，外傷等の侵襲が加わった場合，多核白血球(PMN)の浸潤が最初に惹起される．しかし，角膜創傷治癒過程における PMN の役割は十分明らかではない．損傷角膜における PMN の存在が，しばしば角膜上皮の再生遅延と関連することが報告されているが，その機序は不明な点が多い．我々は，早期の角膜創傷治癒過程を検索する目的で，家兎角膜に穿孔創を加え，0～7日後に角膜を摘出し，〔^{3}H〕thymidine (10μCi/ml)で標識して autoradiography 及び組織学的検索を行った．その結果，受傷後1日目の角膜に多数の PMN の集積が見られ，かつ autoradiogram にて上皮の基底細胞による〔^{3}H〕thymidine の取り込みが見られず，上皮細胞の増殖が停止している所見が認められた．そこで受傷後1～16時間の角膜について PMN の集積と上皮細胞の増殖の停止の相関を調べたところ，明らかな相関が認められた．以上の所見より，PMN が上皮細胞の増殖を抑制する因子を分泌している可能性が示唆された為，家兎 PMN を分離し，PMN の存在下で家兎正常角膜を16時間培養した後，〔^{3}H〕thymidine で標識し autoradiography を行った結果，PMN と共に培養した角膜の上皮細胞の増殖は control に比べ著明に抑制されていた．以上の結果より，PMN が角膜上皮細胞の増殖を抑制する因子を分泌していることが強く示唆された．この知見は，PMN が角膜上皮の再生を遅延させる機序の一端を解明するものである．(日眼　92：798—805，1988)

キーワード：多核白血球，角膜創傷治癒，上皮再生，上皮細胞増殖抑制

Reprint requests to: Winston W.-Y. Kao, Ph.D. Department of Ophthalmology, University of Cinncinnati, College of Medicine, 231 Bethesda Avenue, Cinicinnati, Ohio 45267-0527 U.S.A.
(Accepted January 28, 1988)

I INTRODUCTION

The infiltration of PMN into injured corneas, e.g., alkali-burn, epithelial defects, laceration, bacterial and viral infection marks the first event of corneal wound healing[1)~10)]. Subsequently, epithelial cells proliferate to restore the multi-cellular layer of epithelium[11)12)]. However, the precise role of PMN in corneal wound healing has not been established. For example, the presence of PMN in injured corneas is frequently associated with delayed re-epithelialization. However, the mechanism by which PMN retard corneal re-epithelialization remains largely unclear. At least two mechanisms may account for this phenomenon: 1. Removal of adhesive macromolecules from the corneal surface by proteases secreted by PMN; 2. Inhibition of epithelial cell proliferation by factors secreted by PMN. Since PMN contain many proteases[13)14)], it is possible that proteases secreted by PMN may remove adhesive maromolecules in the extracellular matrix, e.g., fibronectin, laminin and collagen. Therefore, the migration of epithelial cells along the corneal surface is retarded. This hypothesis is supported by the transient appearance of the components of the extracellular matrix during corneal re-epithelialization using immunofluorescent staining with the antibodies specific to fibronectin, laminin, collagen (IV), and bullous pemphigoid protein, etc[15)16)]. This orderly appearance of macromolecules during re-epithelialization may be disturbed in the presence of PMN. Alternatively, PMN may modulate the proliferatien of epithelial cells during corneal wound healing. In the present studies, attempts were made to examine the latter hypothesis. Our results were consistent with this notion that PMN may secrete factors inhibiting epitheial cell proiferation.

II MATERIALS AND METHODS

Materials: [^{3}H] Thymidine (78.0 Ci/mmol) was obtained from New England Nuclear (Boston, MA.). Dulbecco's minimum essential medium was from M. A. Bioproducts (Walkersville, MD.). Nuclear track emulsion NTB2, developer D-19 and fixer were purchased from Kodak (Rochester, N.Y.). Glycogen (Type II) and catalase were from Sigma Chemical (St. Louis, MO). Adult albino rabbits of both sexes weighing 4—5kg were obtained from Clerco Research Farm (Cincinnati, OH.).

Corneal Laceration: Rabbits were anesthetized by combined administration of ketamin and pentobarbital as described previously[17)]. A penetrating linear incision of unifrom size (8 mm) was made in the center of each cornea with a microsurgical scalpel. A drop of Neosporin ophthalmic solution (Burroughs Wellcome Co.) was then applied to each eye to reduce the risk of infection. Lacerated corneas were allowed to heal for various periods of time at which points the rabbits were sacrificed by a lethal dose of sodium pentobarbital. The corneas were then excised in 10 mm diameter and labeled with [^{3}H] thymidine (10 μCi/ml) in Dulbecco's minimum essential medium (DME)(1 ml/cornea) at 37℃ for 4 h. The ^{3}H-labeled corneas were subjected to histologic studies and radioautography.

Radioautography: Radioautography was performed as described by Kopriwa and Leblond[18)]. The corneas were fixed and sections (6 μm thick) were prepared. The sections were coated with nuclear track emulsion NTB2 and exposed for 1 week. The radioautograms were developed with D-19 and fixed with Kodak fixer. The sections were then stained with 0.5% toluidine blue in 0.5% sodium borate. Cells containing silver grains in each section were counted under a light microscope.

PMN Preparations: PMN were obtained from rabbit peritoneal cavities after glycogen stimulation as described by Meyers and Pettit[19)]. Rabbits were stimulated by the injection of 100 ml of 0.1% glycogen in normal saline into the peritoneal cavity. Four hours later, the peritoneal cavity was irrigated with 400 ml of Ringer's solution containing heparin (2 units/ml). PMN were harvested by the centrifugation of the peritoneal fluid at 1000 rpm for 15 min, By differential cell count, PMN were represented approximately 95% of total cells recovered.

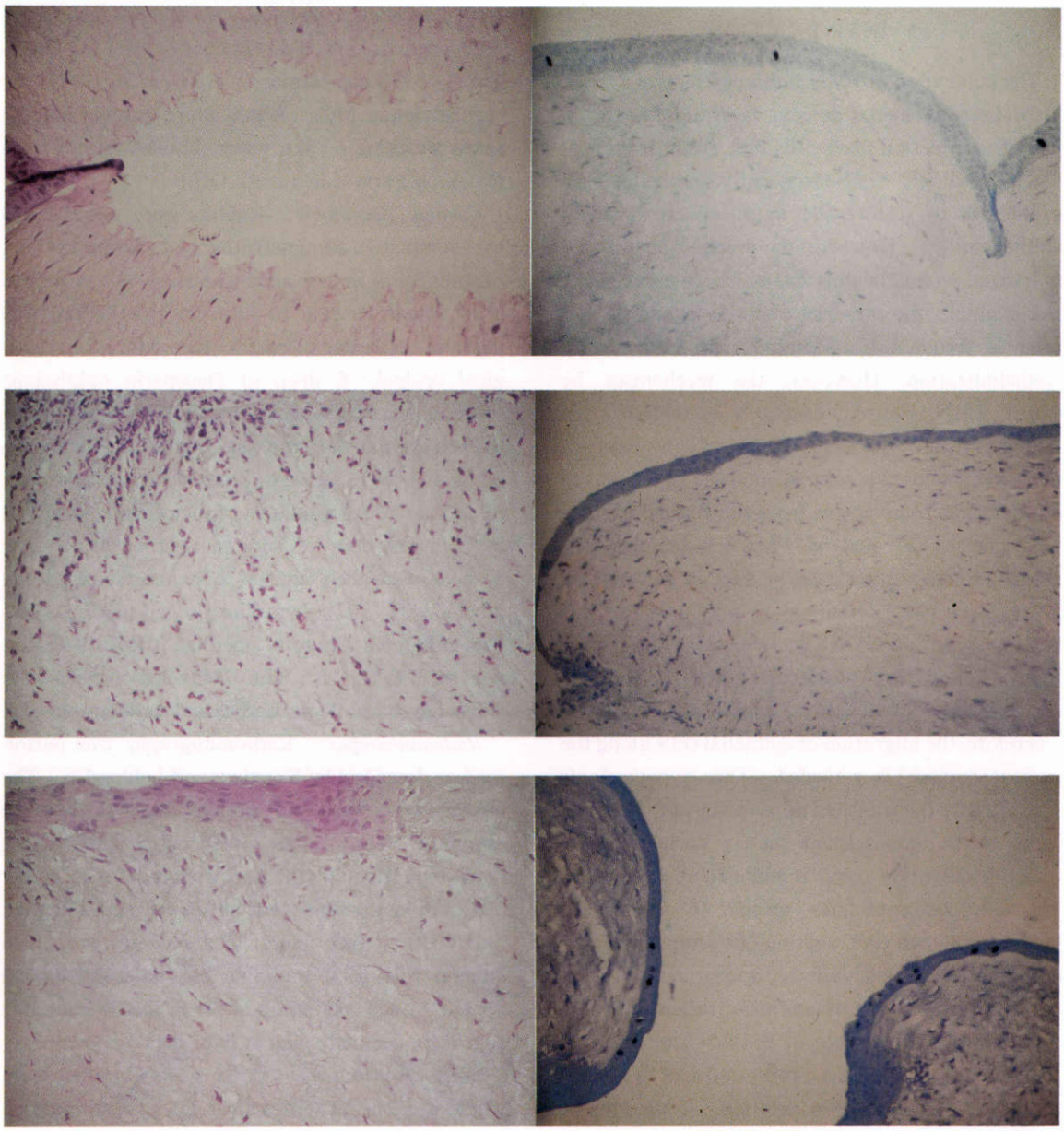

Fig. 1　Radioautograms of Lacerated Rabbit Corneas.
Albino rabbits were anesthetized by combined administration of ketamine and sodium pentobarbital as described previously (17). Linear penetrating incisions (8 mm long) were made on the central portion of the corneas. The wounds were allowed to heal for 0 to 7 days. The rabbits were sacrificed with sodium pentobarbital overdose, and the corneas were excised. The corneas were then labeled with [^{3}H] thymidine (NEN, 10 μCi/ml)in DME for 4 h. The tissues were then processed for histology and radioautography as described by Kopriwa and Leblond (18), Left, Histograms (Magnification 63x); Right, Radioautograms (Magnification 40x) ; Top, 0 day healing ; Middle, 1 day healing ; Bottom, 5 day healing.

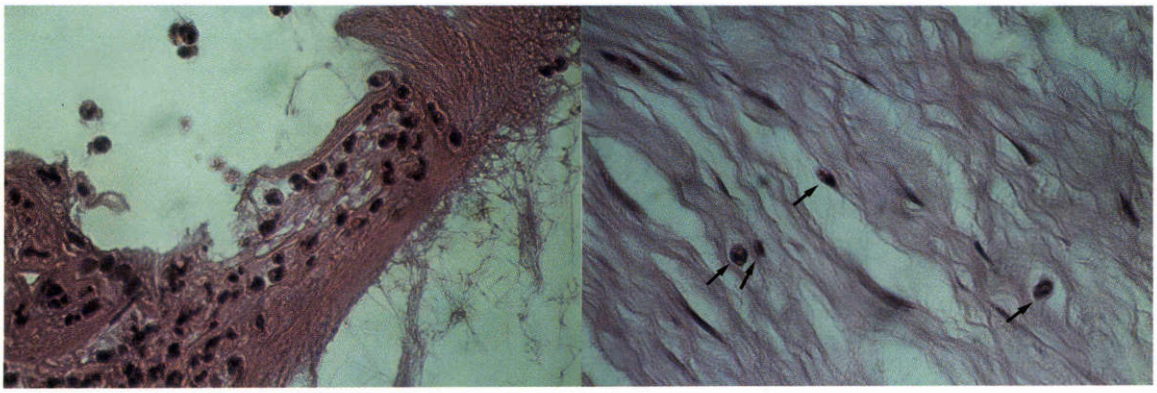

Fig. 2 Histograms of the wound (1 day after laceration)
PMN accumulated within the fibrin plug at the wound (left) and within the stroma adjacent to the wound (right, arrows). PMN have segmented nucleus and contain granules stained by eosin in the cytoplasm. Keratocytes have non-segmented nucleus and no granules. (HE stain, magnification 200×)

Corneal Cultures with PMN: Normal rabbit corneas were excised and cultured in 1 ml of DME containing various concentrations of PMN in the presence of catalase (88 units/ml) or methionine (10 mM) at 37℃ for 16 h. The corneas were then labeled with [^{3}H] thymidine (10 μCi/ml) for another 4 h and autoradiograms were prepared.

III RESULTS

The histologic examination of lacerated corneas indicated that PMN accumulated within the fibrin plug of the wounds 1 day after injury (Fig. 1, Fig. 2) and were no longer seen after 3 days. Fibroblasts appeared in the fibrin plug at day 7. Examinations by radioautography indicated that in normal corneas the basal cells of the epithelium engaged in proliferation continuously (Fig. 1, Table 1). After 1 day of healing, the epithelial cells within the entire epithelium (normal and injured area) of the lacerated cornea stopped engaging in proliferation (Fig. 1, Table 1). At day 3, the epithelial cells located at the margin of the wound began DNA sythesis. These DNA synthesis activities in the regions of the wounded epithelium reached a peak at day 5 and then declined (Table 1). Initially, keratocytes in normal stromas of injured corneas were not actively involved in proliferation. At day 3 and 5, many keratocytes within the stromas adjacent to the wounds contained silver grains in their nuclei. At day 7, fibroblast-like cells containing silver grains were also found in the fibrin plugs instead of the stromas, suggesting the cells within the stroma mgirated into the wounds (Table 1).

To examine whether injured corneas produce factor(s) inhibiting epithelial cell proliferation, the injured corneas which were allowed to heal for one day were co-cultured with normal cerneas for 24 h. The tissues were then labeled with [^{3}H] thymidine and then subjected to radioautography as described above. The results indicated that the lacerated corneas whose epithelial cells remained silent in DNA synthesis did not inhibit the epithelial cell proliferation of normal corneas (data not shown).

An experiment was performed to examine the possible correlation between the appearance of PMN and the cessation of epithelial cell proliferation. The lacerated corneas were allowed to heal for 1-16 h in vivo. The corneas were then excised and labeled with [^{3}H] thymidine, then subjected to histologic examination and radioautography. In Fig. 3, it demonstrated that cessation of epithelial cell proliferation was concomitant with the accumulation of PMN in injured tissues.

To determine whether PMN play any role in

Table 1. Proliferation of Epithelial and Stromal Cells in Lacerated Corneas.
Penetrating incisions were made on rabbit corneas. The corneas were labeled with [^{3}H] thymidine and radioautograms were prepared as described in Fig. 1. The tissue sections were examined and numbers of nuclei containing silver grains were counted in each tissue layer of corneal section (10 mm long). Most endothelial cells were lost during the preparation of tissue sections. The values are the average of four different tissue sections from each sample.

Cornea	Days after healing	No. of Cells Containing Silver Grains			
		Normal Tissues (cell No./section)		Wounds[a] (cell No./section)	
		Epithelium	Stroma	Epithelium	Stroma
Control	—	33.5	3.5	—	—
Lacerated	0	34.3	1.2	0	0
	1	0.5	0	0.2	0
	3	1	0	6.2	53.5[b]
	5	0	1.2	20.3	38.6[b]
	7	0.5	3.0	7.5	41.3[c]

[a] Areas adjacent to the wounds (within 1mm from the wounds).
[b] Cells were found within the stroma adjacent to the wounds.
[c] Cells were found within the fibrin plug.

epithelial cell proliferation, excised normal rabbit corneas were cultured for 16 h with PMN in the presence of catalase (88 units/ml) or methionine (10 mM), then labeled with [^{3}H] thymidine and subjected to radioautography. The data indicated that the number of cells containing silver grains decreased markedly when corneas were cultured with PMN in the presence of catalase or methionine (Table 2), suggesting that PMN may produce factor(s) inhibiting epithelial cell proliferation.

IV DISCUSSION

Persistence of PMN in injured cornea retards re-epithelialization. Two possible mechanisms can account for this phenomenon: 1. Removal of adhesive macromolecules from the corneal surface by proteases secreted by PMN. 2. Inhibition of epithelial cell proliferation by factors secreted by PMN. In the present studies, we examine the possible role of PMN on epithelial cell proliferation in injured

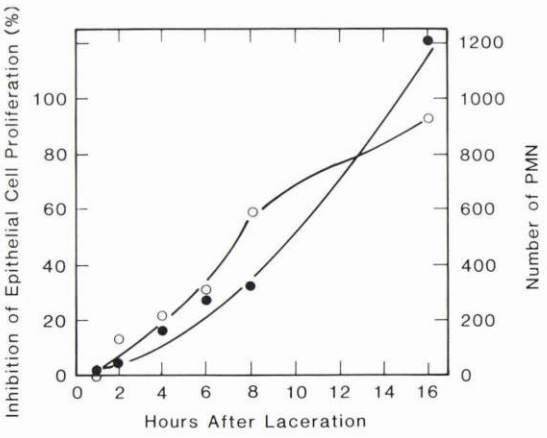

Fig. 3 PMN Accumulation and Inhibition of Epithelial Cell Proliferation in Lacerated Rabbit Corneas.
Penetrating linear incisions were made in the center of rabbit cornea as described in Fig. 1. The wounds were allowed to heal for 1—16 h. The corneas were excised and labeled with [^{3}H] thymidine (10 μCi/ml) in DME for 4 h, then subjected to histology and radioautography. Numbers of PMN which accumulated in each cornea section were counted under microscope. PMN were discriminated from keratocytes as described in Fig. 2. From radioautograms numbers of epithelial cells containing silver grains were counted and presented as % inhibition. Values are the average of 4 sections each of 4 corneas of 2 rabbits. ○——○; Inhibition of epithelial cell proliferation (%), ●——●; Number of PMN.

Table 2. Effects of PMN on Epithelial Proliferation.

PMN were isolated from rabbit peritoneal cavities after glycogen stimulation as described by Meyers and Pettit (19). Normal rabbit corneas were excised and cultured in 1ml of DME containing catalase (88 unit/ml) or methionine (10 mM) in the presence of PMN at 37°C for 16 h. The corneas were then labeled with [^{3}H] thymidine (10 μCi/ml) at 37°C for 4 h and subjected to radioautography.

Cell density No$\times10^{-8}$/ml		Addition to culture medium	Cells containing silver grains[a]	
			No./cm section	% of control
Exp. I	0	—	38 (2)	100
	0	catalase	33 (2)	87
	1	catalase	17 (2)	45
	3	catalase	13 (1)	34
Exp. II	0	—	42 (1)	100
	0	methionine	42 (1)	100
	0.5	methionine	17 (1)	40
	2	methionine	29 (3)	69

[a] The number of cells containing silver grains in each cornea is the average of four tissue sections. The values presented are the averages of the numbers of corneas indicated in parentheses.

corneas.

Our initial observation indicated that upon laceration of cornea PMN infiltrated injured tissue and epithelial cell proliferation was abolished when the lacerated corneas were allowed to heal for 1 day (Table 1 and Fig. 1). One possible explanation is that lacerated corneas may produce factors inhibiting epithelial cell division. But the results of co-culturing normal and lacerated corneas dispute this possibility (data not shown). It should be cautioned, however, that lacerated corneas may produce a small amount of factors and fail to exert its inhibiting effect on normal corneas which were co-cultured with lacerated corneas.

The concomitant appearance of PMN and cessation of epithelial cell proliferation (Fig. 3) strongly suggested that PMN may modulate cell proliferation in injured tissues. When normal corneas were cultured in the presence of PMN, the epithelial cell prolifertion was significantly inhibited (Table 2). Methionine or catalase was included in our cultured medium to minimize the production of oxidants by PMN [20]. Because it was demonstrated previously that oxidants produced by PMN can damage chromatin and may potentially confuse the effects by PMN. Nevertheless, data presented in Fig. 3 and Table 2 are consistant with the notion that PMN may produce factor(s) inhibiting epithelial cell proliferation. Several peptide growth inhibitors had been isolated from serum[23][24] and various cell types, e.g., fibroblasts[25], monocytes[26], and kidney epithelial cells[27]. However, the nature of the inhibitors produced by PMN remains largely unknown and needs to be further investigated. In addition, our data also provide a novel explanation on the retardation of corneal re-epithelialization in injured corneas.

Abbreviation: PMN, polymorphonuclear leukocytes.

ACKNOWLEDGEMENTS

This study was supported in part by NIH grants Ey 04641, Ey 05629, Research to Prevent Blindness, Inc., New York; Fight For Sight, Inc., New York; Ohio Lions Eye Resarch Foundation, Columbus, Ohio.

Chikako Katakami was a Fight For Sight post-doctoral fellow (1984—1985). Wiston W.—Y. Kao is the recipient of Reseach to Prevent Blindness-Robert E. McCormick Scholar (1986). The authors would like to thank Ms. Mary Beth Baker for her assistance in the preparation of the manuscript.

We would like to thank Prof. Misao Yamamoto for his encouragement.

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