

Invited Review

Enzyme histochemistry of corneal wound healing

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Summary. The usefulness of enzyme histochemical methods for the localization of enzymes as catalysts of molecular interactions in the cells and tissues of healing corneal wounds is shown in rabbits. The current data on corneal wound healing in humans as well as in rabbits with particular reference to serine proteases are reviewed. Some inflammatory mediators are also discussed. Plasmin is a serine protease which is absent (or present only in very low concentration) in the tear fluid, and its activity appears under various pathological conditions in humans or following experimental injuries in rabbits. The role of increasing plasmin activity in the tear fluid in the depending upon the severity of corneal injury is evaluated. Great attention is devoted to conditions leading to long-lasting elevated levels of plasmin activity in the tear fluid correlated with corneal ulceration. The differences between the histochemical pattern of untreated corneas or corneas treated with some serine protease inhibitors are shown, and the efficacy of these drugs is discussed in light of present knowledge.

Key words: Cornea, Enzymes, Histochemistry, Wounds, Healing

Introduction

Ulcerative and other destructive processes of the cornea are catastrophic complications of ocular lesions that are difficult to manage and can result in the loss of vision. The pathogenesis of these processes (occurring as persistent corneal epithelial defects or deeper ulcers) remains largely unsolved. In published papers, an association with various direct or indirect insults to the eye, such as trauma (alkali burn or irradiation of the eye; e.g. with UV rays), immunological derangement (rheumatoid arthritis), ocular infection (bacterial or fungal infections) and vitamin A deficiency (keratomalacia) has been described (Carubelli et al., 1990). The role of various inflammatory mediators has also been

considered. The majority of destructive processes are resistant (or only slightly sensitive) to conventional therapy (based on the use of anti-inflammatory drugs and/or antibiotics) (Burns et al., 1989). Recently, the uncontrolled action of proteolytic enzymes, metalloproteinases (Fini et al., 1992; Matsumoto et al., 1993), enzymes of pericellular proteolysis (Tervo et al., 1992; Vaheri et al., 1992) and reactive oxygen species (ROS) (i.e., hydrogen peroxide, singlet oxygen and oxygen-free radicals such as superoxide anion and hydroxyl radical) (Fantone and Ward, 1982; Kehrer, 1993) generated from environmental pollutants or produced during intermediary metabolism by the action of various oxidases has been supposed to play the key role in the pathogenesis.

The harmful action of proteases and ROS led to attempts at using various pharmacological agents, including inhibitors of proteolytic enzymes (Burns et al., 1989; Čejková et al., 1989, 1992; Fini et al., 1992; Tervo et al., 1992; Tervo and Setten, 1992; Wentworth et al., 1992; Paterson et al., 1994) and scavengers of ROS (Nirankari et al., 1981; Čejková and Lojda, 1994). The efficacy of these drugs (in vitro or in experimental animals) can be investigated by biochemical methods, and much information can be accumulated using this approach. However, biochemical methods cannot furnish any information about possible focal disturbances in individual tissue layers; e.g., corneal layers. On the other hand, classical histological methods, which have usually been employed, cannot reveal metabolic alterations of the cornea. Thus, sensitive histochemical tests are required. For the detection of enzymes, histochemical or immunohistochemical methods can be used (see Lojda et al., 1991 for details). Immunohistochemical methods are very important; however, because they detect enzymes as proteins, an active and inactive enzyme (proenzyme) cannot be distinguished and therefore a method for the localization of enzyme activities is indispensable. Enzyme activities can be revealed in situ by methods of enzyme histochemistry (catalytic histochemistry). The detection of active enzymes is of decisive importance because only active enzymes can be involved in various processes, such as angiogenesis, chemotaxis, inflammation and activation of proenzymes.

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Enzyme histochemical methods (using enzymes as markers of metabolic disturbances) are useful for better understanding the mechanisms of corneal damage and repair. The histochemical pattern of the experimentally-injured rabbit cornea is shown in relationship with plasmin activity in the tear fluid. Plasmin is a serine protease which is absent (or present only in very low concentration) in the normal eye. However, under various pathological conditions in humans or following experimental injuries in rabbits, plasmin activity appears in the tear fluid. Elevated plasmin activity is thought to be associated with corneal inflammation and the development of corneal ulcers or other destructive processes. On the other hand, some plasmin activity is presumed to be necessary for the healing process. Therefore, we compared plasmin activity in the tear fluid with the histochemical pattern of untreated corneas or corneas treated with aprotinin (an inhibitor of plasmin and some other serine proteases) evaluating the healing as "efficient" or "inefficient". Cases of experimental corneal injury, in which "efficient" corneal healing is achieved by leukocyte elastase inhibitors and ROS scavengers, are also mentioned.

Histochemical methods used are summarized in Table 1 (see Lojda et al., 1991 for details; Xanthine oxidase activity was detected as recommended by Gossrau et al., 1989, 1990). The localization and activities of individual enzymes in the normal cornea and during the healing process after experimental injury have been described by Čejková et al. (1975a-c), Čejková and Lojda (1978, 1988a,b), Čejková et al. (1988, 1989, 1992, 1993), and Čejková and Lojda (1994, 1995a,b, 1996).

In this review, results in experimental animals are compared with clinical findings. The current data on corneal healing and the theoretical basis for therapeutic

interventions are also discussed briefly.

Plasmin activity in the tear fluid and simple methods of its detection

Plasmin is a serine protease which appears after the conversion of plasminogen by plasminogen activators (Berman et al., 1983; Saksela, 1985; Wang et al., 1985; Vaheri et al., 1992). Berman et al. (1988), Čejková et al. (1989) and Hayashi et al. (1991) have shown that plasmin activity in the tear fluid increases in corneal ulcerative processes. Plasmin activity in the tear fluid can be measured by various methods. However, two simple methods exist which do not require expensive laboratory equipment (important for use in clinical practice): the method of radial caseinolysis (Saksela, 1981) as modified by Salonen et al. (1987); or a semi-quantitative fluorescence method which we developed (Čejková et al., 1989, 1992). This method uses filter paper punches soaked with D-Val-Leu-Lys-7-amino-4-Trifluoromethyl Coumarin (AFC) (Enzyme Systems Products, Livermore, CA, USA) as a substrate for plasmin detection. Three μl of tears or other fluids are dropped onto dry filter paper punches containing the substrate. Incubation takes place at 37 °C, and the time of the first appearance of the yellow fluorescence (evaluated under UV light) is recorded and compared with the appearance of yellow fluorescence in calibrated punches with a known activity (concentration) of plasmin (Sigma, Munich, Germany). In calibrated punches, a plasmin concentration of $>3.0 \mu\text{g/ml}$ was detectable after 15 min incubation. For this concentration the designation "very high plasmin activity" is used. The fluorescence of samples with "high plasmin activity" ($2.0\text{-}3.0 \mu\text{g/ml}$) appeared after 30 min incubation. Samples with "moderate activity" ($1.0\text{-}2.0$

Table 1. Enzyme histochemical methods used in our experiments

| ENZYME | METHOD |
|--|--|
| Na ⁺ -K ⁺ -dependent adenosinotriphosphatase (Na ⁺ -K ⁺ -dependent ATPase) | ATP + Pb ²⁺ (inhibitor ouabain) |
| γ -glutamyl transpeptidase (GGT) | γ -glutamyl-MNA + FBB |
| Aminopeptidase M (APM) | alanyl-MNA + FBB |
| Dipeptidyl peptidase I (DPP I) | gly-arg-MNA + NSA (or FBB) |
| Dipeptidyl peptidase II (DPP II) | lys-pro-MNA or ala-pro-MNA + FBB (or NSA) |
| Dipeptidyl peptidase IV (DPP IV) | glycyl-prolyl-MNA + FBB |
| Alkaline phosphatase | naphthol AS-MX-phosphate + FBBB |
| acid phosphatase | naphthol AS-BI-phosphate + HPR |
| β -glucuronidase | naphthol-AS-BI- β -D-glucuronide + HPR or |
| β -galactosidase | 4-Cl-5-Br-3-indolyl- β -D-galactoside |
| Acetyl- β -D-glucosaminidase | naphthol AS-BI-N-acetyl- β -D-glucuronide + HPR |
| succinate dehydrogenase (SDH) | succinate in water medium + NBT+menadione |
| lactate dehydrogenase (LDH) | lactate in medium + PVA + NBT |
| catalase | H ₂ O ₂ + DAB (aminotriazole as inhibitor) |
| xanthine oxidase | hypoxanthine + Ce ³⁺ + DAB + Co ²⁺ |

Abbreviations: for aminoacids according to "IUPAC-IUB Commission on Biochemical Nomenclature", Eur. J. Biochem. (1972), 27, 20. FBB: Fast Blue B; FBBB: Fast Blue BB salt; HPR: hexazo-p-rosaniline; DAB: diaminobenzidine; MNA: methoxy-b-naphthylamide; NBT: Nitro Blue Tetrazolium; NSA: Nitrosalicyl Aldehyde; PVA: polyvinyl alcohol. Methods according to Lojda, 1973; Lojda et al., 1979, 1991 and method for xanthine oxidase activity as recommended by Gossrau et al., 1989, 1990; see for details.

$\mu\text{g/ml}$) fluoresced after 1 h incubation. "Low activity" (0.4-1.0 $\mu\text{g/ml}$) was detectable after 1.5 h incubation. "Very low activity" (0.2-0.4 $\mu\text{g/ml}$) was found after 2 h incubation. A plasmin level of $<0.2 \mu\text{g/ml}$ exhibited very low yellow fluorescence and therefore this concentration was regarded as negative.

Radial caseinolysis requires a greater amount of tears, and thus, tear collection is necessary. In our method it is sufficient for clinical purposes to soak the filter paper punches with tears by means of a touch of the punch to the eye surface. The fluorescent method using filter paper punches is preferable for its greater sensitivity. It uses very sensitive substrate (D-Val-Leu-Lys-AFC) and enables us to measure plasmin activity at the same place on the corneal surface during the entire healing period. This is very important, for example, in alkali burns or in some other cases of corneal injury, e.g., the irradiation of the eye with UVB rays. The differences in plasmin activity were found depending on the precise area of the corneal surface where tear samples were taken. The highest plasmin activity occurred in the transition area between the burned and unburned cornea (Čejková et al., 1993). This is in accordance with the findings of Hayashi et al. (1991), that, in alkali-burned cornea, the resurfacing corneal epithelium secretes elevated levels of plasminogen activator of urokinase type (u-PA). Very similar findings were observed in other experiments when rabbit corneas were irradiated with UVB rays (wavelength 312 nm). In the corneal epithelium, active u-PA appeared, accompanied by plasmin activity in the tear fluid (Lojda and Čejková, 1993; Čejková and Lojda, 1995a,b). Plasminogen activator converts plasminogen (Wang et al., 1985) adsorbed to the subepithelial fibrin-fibronectin matrix (Berman et al., 1980) to plasmin, which appears in the tear fluid in higher amounts. Elevated plasmin activity in

the tear fluid is considered to be harmful from the point of view of the initiation (Wang et al., 1985) as well as the development of corneal destructive processes (Berman et al., 1988).

The dependence of plasmin activity on the severity of corneal damage

Although extensive information is available concerning the role of plasmin activity in the tear fluid in ulcerative processes in the human cornea (Berman et al., 1980; Wang et al., 1985; Salonen et al., 1987; Hayashi et al., 1991), there is no information dealing with the dynamics of the pathological process in relation to plasmin activity in the tear fluid. Well-established animal models of various injuries were thus essential for elucidating the process by which the damaged cornea is either healed or destroyed. Animal models allow comparison between the microscopic pattern of the cornea and plasmin activity in the tear fluid at different time intervals after injury. This was studied in our experiments with various models of corneal injury: mechanical injury (single or repeated de-epithelization of the whole cornea), chemical injury (mild alkali burn, repeated mild alkali burn); severe alkali burn of varying extent, and irradiation of the eye (single or repeated irradiation with UVB rays). In this connection we also examined contact lens wear (Table 2).

It was found that plasmin activity appeared in various cases of corneal injury. However, there were remarkable differences in the levels of plasmin activity in the tear fluid, which depended on the severity of corneal damage.

Very low plasmin activity (0.2-0.4 $\mu\text{g/ml}$) in the tear fluid was seen after short-term (1-2 h) contact lens wear. Low plasmin activity (0.4-1.0 $\mu\text{g/ml}$) in the tear fluid

Table 2. Experimental injuries of the rabbit cornea and contact lens wear.

| | |
|--|--|
| De-epithelization of the cornea (Čejková et al., 1993) | The whole cornea was de-epithelized by means of Graefe's knife |
| Repeated de-epithelization of the cornea (Čejková et al., 1993) | The whole cornea was de-epithelized twice - with an interval of one week |
| Mild alkali burn (Čejková et al., 1993) | The cornea was burned with 1M NaOH by means of a plastic tube, 8 mm diameter, 15 s exposure |
| Repeated mild alkali burn (Čejková et al., 1993) | The cornea was similarly burned twice with one week interval |
| Severe alkali injury - small extent (Čejková et al., 1975a-c, 1993) | The cornea was burned with a filter paper strip, 10 x 2 mm, soaked with 1M NaOH, 15 s exposure |
| Severe alkali injury - large extent (Čejková and Lojda, 1988; Čejková et al., 1988, 1993) | The cornea was burned with 1M NaOH by means of a plastic tube, 8 mm diameter, 15 s exposure |
| Irradiation of the eye with UVB rays (Čejková and Ojda, 1995a,b, 1996) | Rabbit eyes were irradiated with UV lamp, 312 nm, 6W, distance 0.3 m (once daily for 5 min over 10 days) |
| Soft contact lens wear (Čejková et al., 1988, 1992) (lenses manufactured to fit the rabbit eye, 7.5 mm curvature radius, 0.2 mm thickness, 15 mm diameter) soft contact lenses with higher oxygen permeability (68% water content), soft contact lenses with lower oxygen permeability (37% water content) | Continuous wear of contact lenses for 1, 2, 3, 43, 10, 14, 21 and 28 days |

Drugs used for local application (dropwise) on the eye surface (2-8 times daily). Enzyme inhibitors: aprotinin (Traysol, Bayer, Leverkusen, Germany), 60 IU/ml saline; elastatinal (Sigma, Munich, Germany), 50 $\mu\text{g/ml}$ saline; tetrapeptide carbamate (synthesized by Prof. Digenis, Lexington, KY, USA) 250 $\mu\text{g/ml}$ of 1.5% propylene glycol in saline.

was found after longer time intervals of contact lens wear (2 h - 7 days), de-epithelization of the whole cornea, single irradiation of the eye with UVB rays and at early time intervals after mild alkali injury. Moderate plasmin activity (1.0-2.0 $\mu\text{g/ml}$) in the tear fluid was observed after repeated de-epithelization, after mild alkali burn (during later time intervals after the injury), after repeated irradiation of the eye with UVB rays, after severe alkali burn (small extent), after severe alkali injury (large extent - during early time intervals after the injury), and prolonged continuous contact lens wear (7-14 days).

High plasmin activity (2.0-3.0 $\mu\text{g/ml}$) in the tear fluid was found after severe alkali burn (large extent) during the 2nd week after the burn, after repeated irradiation of the eye with UVB rays and after 14-28 days of continuous contact lens wear.

Very high plasmin activity (>3.0 $\mu\text{g/ml}$) occurred during corneal ulceration (after severe alkali injury (large extent)), repeated irradiation of the eye, and prolonged wearing of contact lenses (in a majority of cases when bacteria were involved).

The relationship of plasmin activity in the tear fluid with the histochemical pattern of injured corneas

When very low plasmin activity was detected in the tear fluid, the activities of $\text{Na}^+\text{-K}^+$ -dependent ATPase and GGT were decreased in corneal cells. $\text{Na}^+\text{-K}^+$ -dependent ATPase seems to be an essential component of the active fluid transport mechanism (Geroski and Edelhauser, 1984). A decrease in its activity is associated with an increase in corneal hydration (Trenberth and Mishima, 1968; Čejková and Lojda, 1978; Čejková et al., 1988, 1992). GGT is thought to participate in the translocation of amino acids across cellular membranes as a consequence of its role in the γ -glutamyl cycle (Orlowski and Meister, 1970; Meister and Tate, 1976). A decrease in its activity in the corneal endothelium may indicate impaired amino acid transport (Čejková et al., 1988a,b). Low plasmin concentrations were associated with a decreased activity in succinate dehydrogenase activity and an increase in lactate dehydrogenase activity. These enzymes are good markers of glucose metabolism in the corneal epithelium (their enzyme activities are changed under anaerobic conditions at the surface of the cornea) (Čejková et al., 1989). Also, increased activities of lysosomal hydrolases (acid glycosidases, lysosomal proteases) were seen in the corneal epithelium. Acid glycosidases were found to be associated with the metabolism of glycoproteins and glycosaminoglycans (see Čejková et al., 1975a-c, 1989 for details). Of lysosomal proteases, DPP I is a very potent exopeptidase, cleaving dipeptides from the aminoterminal of many peptide chains. DPP II is a potent serine exopeptidase occurring in lysosomes. Increased activities of DPP I and II point to enhanced intralysosomal and extralysosomal proteolysis (Čejková and Lojda, 1988 a,b; Lojda et al., 1991). When moderate

plasmin activity was found, the activities of lysosomal hydrolases in the corneal epithelium were greatly increased. Keratocytes in the corneal stroma (particularly beneath the epithelium) were also highly enzymatically active). Inflammatory cells (mainly polymorphonuclear leukocytes) appeared in the corneal stroma. Plasmin activity increased as the inflammatory reaction developed. High levels of plasmin were connected with large numbers of inflammatory cells with pronounced lysosomal hydrolase activities (acid glycosidases, acid phosphatase, lysosomal proteases). As destructive corneal processes develop, high levels of DPP IV activities appear in the corneal stroma (Čejková et al., 1989). DPP IV is a serine protease that cleaves dipeptides with proline in the penultimate position from the aminoterminal of a polypeptide chain (Lojda et al., 1991). This enzyme is present in the normal cornea only in some keratocytes beneath the epithelium (Čejková and Lojda, 1988a). However, during destructive processes of the corneal stroma, its activity is high in the corneal stroma pointing to a process of collagen degradation (Čejková et al., 1989, 1993). The evidence for its role in collagen degradation is strengthened by the observation that DPP IV shows a specificity for cleaving peptide bonds after proline residues, which are numerous in the collagen molecule (Pahlitzsch and Sinha, 1985). When very high activities of plasmin were found, extracellular release of enzyme activities from inflammatory cells into the substantia propria of the corneal stroma took place and was accompanied by corneal ulceration. ROS together with destructive proteases play the most important role in corneal melting. Stimulated inflammatory cells not only release various proteases into the cell surroundings, but produce ROS during the respiratory burst that contribute to and amplify the degradation of the extracellular matrix (Carubelli et al., 1990).

The efficacy of aprotinin intervention

From our experiments it is evident that aprotinin favourably influences the healing of corneal injuries marked by a significant inflammatory reaction, such as repeated de-epithelization, repeated mild alkali burn, repeated irradiation of the eye with UVB rays, severe alkali burn and prolonged wearing of contact lenses. The severity of the injury influences the inflammatory response and the plasmin activity in the tear fluid. Indeed, the increases in both plasmin activity in the tear fluid and also the inflammatory reaction are proportional to the severity of the injury (Čejková et al., 1989, 1992, 1993; Čejková and Lojda, 1994, 1995 a,b).

When aprotinin treatment was applied dropwise immediately after severe injury, plasmin activity in the tear fluid appeared later and reached a lower concentration. The number of inflammatory cells (as well as their enzyme activity) decreased. In contrast, no significant effect on corneal healing was observed when aprotinin was applied to the corneal surface following milder lesions (associated with low plasmin activity in

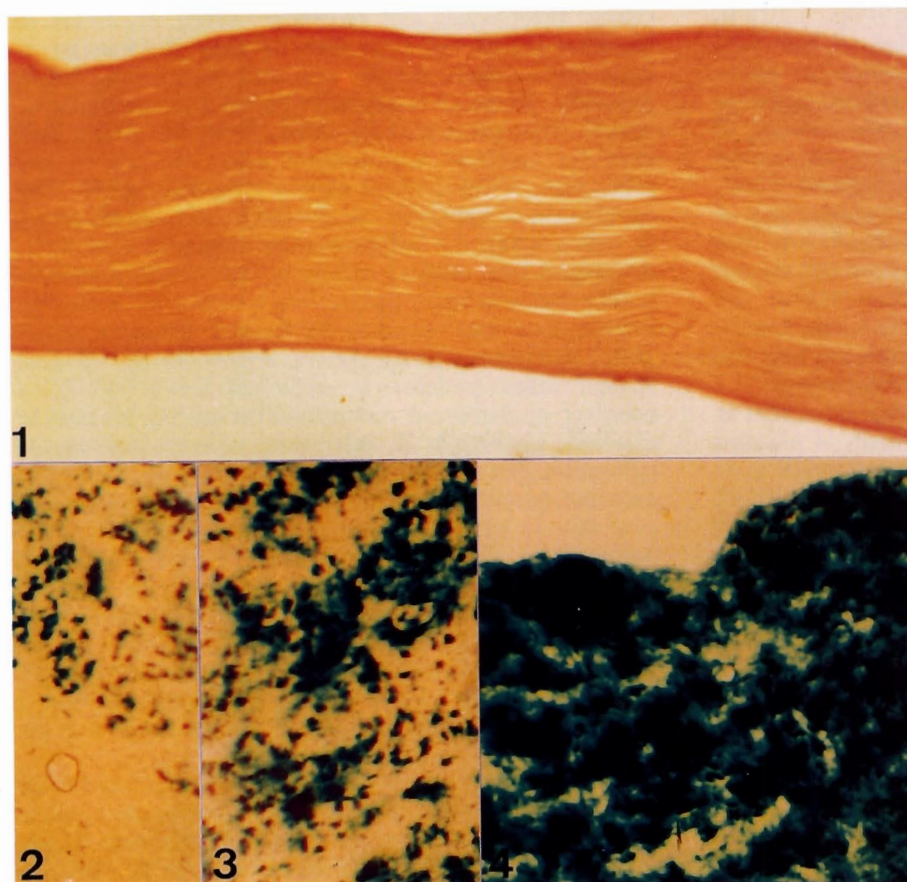
the tear fluid) (Čejková et al., 1993). Healing was neither accelerated nor protracted. Similar results have been reported by Boissoly et al. (1990). This is contrary to the findings of Zieske and Bukusoglu (1991) with aprotinin in "in vitro" experiments (scraped cornea) in which aprotinin inhibited corneal re-epithelization. Different findings between "in vivo" and "in vitro" results may be explained, at least in part, by the frequency of aprotinin application onto the corneal surface: in "in vivo" studies 2-6 times daily; in "in vitro" situations cells are permanently exposed to aprotinin. Moreover, some of the mechanisms which operate "in vivo" do not operate "in vitro". The increased amount of fibronectin in tears after corneal injury (Jensen et al., 1985) and the presence of epidermal growth factor (van Setten et al., 1989) as a component of the tear fluid are of great importance. Moreover, aprotinin does not inhibit plasminogen activators (Berman et al., 1980, Čejková, 1996) so that a low amount of plasmin may be present during the healing process.

Conditions influencing the favourable effect of aprotinin on corneal healing

The degree to which corneas experimentally injured with severe alkali finally heal is dependent not only on

the concentration of alkali and the length of exposure but also on the extent of alkali injury. Severe injury (small extent) evokes the appearance of "high plasmin activity" in the tear fluid (which quickly decreases); however "very high activity" (associated with ulceration) has never been observed. The corneas heal without ulceration (untreated or treated with aprotinin). In contrast, after severe alkali burn (large extent), the majority of untreated corneas are ulcerated within a month (which is accompanied by long lasting elevated plasmin activity in the tear fluid). In this case the aprotinin treatment favourably influenced corneal healing. However, the efficacy of aprotinin was highly dependent on the time interval (after the injury) prior to aprotinin intervention. Best results were obtained when the aprotinin treatment was started immediately or very early after the injury (Čejková et al., 1993). When aprotinin treatment was started later (during a developed inflammatory response), the efficacy of aprotinin was highly limited (Čejková et al., 1993; Kadlecová and Čejková, 1994).

Corneal ulcerations are always associated with a large number of inflammatory cells, mainly neutrophils (Kenyon et al., 1979; Chayakul and Reim, 1982; Pfister et al., 1984; Pahlitzsch and Sinha, 1985). After the burning of a large area of the cornea (the burned area is



Figs. 1-4. Acid β -galactosidase activity (and haematoxylin-eosin staining) in the rabbit cornea burned with severe alkali (large extent). Haematoxylin-eosin staining and acid β -galactosidase activity detected by the indigogenic method with 4-Cl-5-Br- β -galactoside as the substrate. **1.** Haematoxylin-eosin staining. Immediately after burn the cornea is necrotic (without living cells). **2-4.** Acid β -galactosidase activity. During the prolonged time interval after the burn - (2) the second week (3), the third week and (4) the fourth week - the amount of invading inflammatory cells and their enzyme activities are increasing in the corneal stroma. (During this time interval inflammatory cells are the only viable cells in the burned area). During the 4th week (4) the activity of acid β -galactosidase is seen in the corneal stroma (released from inflammatory cells into the substantia propria of the corneal stroma). x 140

necrotic immediately after the injury), the cells of the inflammatory infiltrate are the only viable cells in the burned area for 2 or 3 weeks (Čejková and Lojda, 1988b). Enzymes (acid glycosidases, lysosomal proteases) are released into their surroundings during the 4th week. (Figs. 1-4). The initial healing, which takes place from the unburned periphery, is stopped after one week when the number of enzymatically active infiltrating cells increases. On the contrary, in eyes treated with aprotinin (Čejková et al., 1989, 1993) (applied immediately after the injury and for one month), the healing starts from the zone of highly activated corneal cells in the undamaged areas at the periphery. The sliding of epithelial cells continues quickly. In the regenerated epithelium, lactate and succinate dehydrogenase activities appear. The activities of GGT, Na^+ - K^+ -dependent ATPase and alkaline phosphatase are restored quickly. This is in contrast to animals not treated with aprotinin where these enzymes cannot be demonstrated in epithelial cells. The activities of acid glycosidases are initially decreased and become normalized later, in contrast to the high activities of lysosomal hydrolases in untreated corneas. After aprotinin treatment the burned area of the corneal stroma is rebuilt from the metabolically activated keratocytes displaying high acid glycosidase, DPP I, DPP II, and APM activities. Significantly increased activities of these enzymes in keratocytes of the corneal stroma are very good markers of corneal rebuilding (Figs. 5-10). In the corneal endothelium the activities of Na^+ - K^+ -dependent ATPase and GGT reappear when the endothelial layer is rebuilt. In corneas which become transparent at the end of the 4th week, the staining of glycosaminoglycans is restored. This is in agreement with previous findings (Anseth 1972; Čejková et al., 1975a-c, 1988a,b; Yee et al., 1985). The transparency of the cornea depends on normal hydration which is in turn dependent on the glycosaminoglycan content and normal transport functions across the endothelium. Hence, for the restoration of corneal transparency, the normalization of active transport across the endothelium and the rebuilding of the glycosaminoglycans are necessary.

The healing of corneas after aprotinin treatment is likely to be due first of all to the inhibition of plasmin and some other serine proteases. Plasmin is thought to be formed in tissues after alkali burn (Berman et al., 1982; Wang et al., 1985), after the irradiation of the eye with UVB rays, and during prolonged contact lens wear. Plasmin has been directly demonstrated in the tears of patients with corneal ulcerations (Salonen et al., 1987), and also under various pathological conditions (Salonen et al., 1988; Tozser et al., 1989; Barlati et al., 1990; Berta et al., 1990). Plasmin activity in the tear fluid was also found during contact lens wear in humans (Tervo et al., 1989, 1992; Vannas et al., 1992). Plasmin can degrade fibronectin (Vartio et al., 1981, Vaheri and Salonen, 1988), a multifunctional glycoprotein critically involved in corneal epithelization (Fujikawa et al., 1981; Tervo et al., 1986; Salonen et al., 1987, 1988; Watabe et al., 1987;

Nishida, 1988). Moreover, plasmin can activate latent collagenase and other proteinases capable of degrading extracellular matrix components (Dano et al., 1985; Pöllänen et al., 1988). This is thought to lead to the degradation of the corneal stroma and to ulceration. However, the damage is also affected by inflammatory cells invading the corneal stroma and other parts of the anterior eye segment due to chemotactic fragments of proteins generated by the action of plasmin (Berman et al., 1982).

When aprotinin was applied immediately after severe injury (large extent), the number of neutrophils was greatly decreased in comparison to placebo-treated eyes (Čejková et al., 1992, 1993). It is also noteworthy that after aprotinin therapy, GGT activity was restored very early, starting in the corneal epithelium and endothelium. Later, this enzyme also appeared in keratocytes, first beneath the regenerated epithelial cells. High GGT activity in keratocytes is a very good marker of "efficient" corneal healing. GGT plays an integral role in the γ -glutamyl cycle in which glutathione (GSH) is a central metabolite (Meister and Anderson, 1983). It is hypothesized that a lack of GGT prevents the hydrolysis of GSH, so that a greater quantity of the oxidized form (GSSH) is present (Čejková et al., 1993). This hypothesis is supported by the finding of an increased amount of GSSH in alkali-burned cornea (Reim et al., 1982). GSSH activates a latent collagenase by disulfide interchange (McCartney and Tschesche, 1980). Moreover, in the absence of GGT an increased production of 5-hydroxyeicosatetraenoic acid and leukotriene B₄ is possible. These substances are powerful chemotactic agents of the prostaglandin series (Fleischer, 1988). This may be another mechanism contributing to the infiltration of the corneal stroma with neutrophils. The application of aprotinin can diminish these disturbances. In cases when the inflammatory response was well-developed (the corneal stroma is full of enzymatically active inflammatory cells), the efficacy of aprotinin intervention was found to be limited (Čejková et al., 1993; Kadlecová and Čejková, 1994). In the corneal stroma various proteases are activated. It is known that a number of different enzymes (including leukocyte elastase, Cathepsin B, kallikrein) convert proenzyme (plasminogen activator of urokinase type) to active enzyme (converting plasminogen to plasmin), and plasmin in turn can activate procollagenases and macrophage elastase (Vaheri et al., 1992). In cases of a developed inflammatory reaction, leukocyte elastase inhibitors (elastatinal and tetrapeptide carbamate) were found to be helpful (Čejková et al., 1992). Other authors have used metalloproteinase inhibitors (Burns et al., 1989; Paterson et al., 1994), in some cases combined with inhibitors of metalloproteinase synthesis (Fini et al., 1992).

During contact lens wear many questions have to be considered. Contact lens wear in humans may cause disturbances of the corneal epithelium (Hamano et al., 1983; Hayashi et al., 1985; Holden et al., 1986; Madigan

et al., 1987), acute and chronic keratitis (Zantos and Holden, 1978), ulceration (Adams et al., 1983; Weissman et al., 1984) and vascularization of the cornea (Nirankari et al., 1983). Hypoxia (Masters, 1988), mechanical stress (Kilp et al., 1985), bacterial contamination (Kersley et al., 1977) and other factors (Lohman, 1986) are thought to play a role in these processes. Contact lens wear in experimental animals evokes very similar corneal disturbances to those in humans. The histochemical pattern of contact lens wear in rabbits has been studied and compared with histological and biochemical findings of other authors (see Čejková et al., 1988, 1992 for details).

During contact lens wear, the occurrence of plasmin activity and the development of corneal disturbances were highly dependent on the water content of the soft contact lenses (which parallels oxygen permeability), duration of contact lens wear, mechanical stress, and bacterial contamination. Mechanical irritation is considered to be the main factor leading to the appearance of plasmin activity in the tear fluid (Čejková et al., 1989, 1992). Our experiences with aprotinin intervention were very similar to those in other injury models. When aprotinin was applied immediately after fitting contact lenses with high oxygen permeability, the epithelial disturbances appeared later and no plasmin activity appeared in the tear fluid. When aprotinin was applied immediately after fitting contact lenses with high oxygen permeability (and afterwards 4 times daily), in the later stages the amount of enzymatically active PMNs decreased along with their enzyme activities. This was accompanied by decreased plasmin activity in the tear fluid. Aprotinin had no effect on corneal vascularization (Čejková et al., 1992). In humans, plasmin activity in the tear fluid was also found to be related to contact lens wear (reviewed by Tervo et al., 1992).

After the irradiation of the eye with UVB rays, the development of anterior eye segment inflammation was diminished after aprotinin treatment when aprotinin application was started immediately after the irradiation and continued during repeated irradiation (up to five days). Later, the favourable effect of aprotinin on the decrease of corneal disturbances was limited (Čejková and Lojda, 1994).

Repeated irradiation of the eye with UVB rays evoked a profound imbalance between enzymes generating and cleaving ROS, first in the corneal epithelium and later also in the corneal endothelium and lens epithelium (Čejková and Lojda, 1996). This was described in the case of catalase (an enzyme belonging to ROS-cleaving enzymes) and xanthine oxidase (an enzyme belonging to ROS-generating enzymes). In contrast to the normal cornea, where the activities of both enzymes are balanced and present at high levels, after irradiation of the eye with UVB rays, the activity of catalase is decreased and afterwards lost, while the activity of xanthine oxidase is increased. These changes are accompanied by increased activities of lysosomal

hydrolases in the corneal cells and elevated plasmin activity in the tear fluid. In this situation, intervention with aprotinin decreases plasmin activity in the tear fluid; however, better results are achieved if catalase is added. After catalase treatment (dropwise) the imbalance between xanthine oxidase and catalase is diminished in the corneal epithelium. This is very important because under profound catalase/xanthine oxidase imbalance, ROS generated by xanthine oxidase are insufficiently cleaved by catalase, which contributes to the corneal damage (Čejková and Lojda, 1994, 1995a,b, 1996).

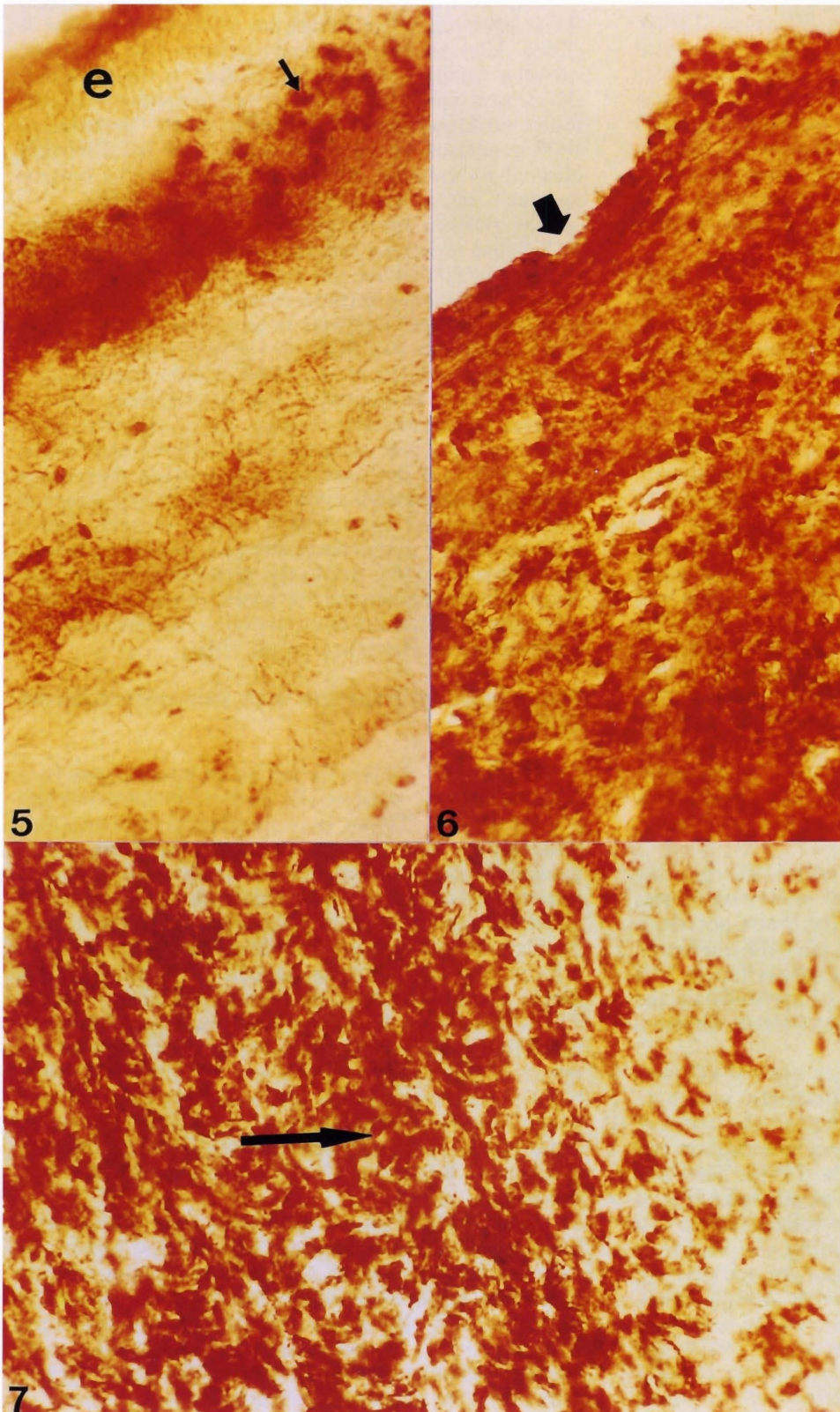
Of antioxidant enzymes, superoxide dismutase, glutathione reductase and peroxidase are also present in the corneal epithelium and endothelium. However, until recently no reliable method existed for the detection of activities of these enzymes *in situ*. The distribution of these enzymes has been described immunohistochemically (Atalla et al., 1987, 1988). Antioxidant enzymes have an important role in protecting the eye against the oxidative damage (e.g., against ROS generated by UVB radiation, see Čejková and Lojda, 1994, 1995a,b for details). Superoxide dismutase eliminating superoxide anion inhibits lipid oxidation and prevents cataract formation. Catalase protect against oxidative injury eliminating hydrogen peroxide (Bhuyan and Bhuyan, 1978). Similarly, glutathione peroxidase reduces hydrogen peroxide to water, as well as breaking peroxidative chain reaction involving organic and fatty acid hydroperoxides (Atalla et al., 1988).

Therapeutic intervention with leukocyte elastase inhibitors

Leukocyte elastase (a serine protease), attacks a wide variety of structurally important proteins and glycoproteins. These include elastin, fibronectin, proteoglycan, and collagen fibres (e.g. Sandhaus, 1986). Leukocyte elastase has been implicated in the abnormal turnover of connective tissue proteins associated with the development of both pulmonary emphysema and rheumatoid arthritis (Janoff, 1985). It is also responsible for the degradation of important controlling agents during severe inflammatory processes (Čejková, 1997). Therefore, there has been considerable interest in producing inhibitors for therapeutic use to combat the effects of this enzyme (Travis and Fritz, 1991). Several types of leukocyte elastase inhibitors have been reported in the literature. These include elastatinal (Sigma) or tetrapeptide carbamate, developed by Prof. Digenis, Lexington, Kentucky, USA (e.g. Banks et al., 1990, Stone et al., 1992).

Leukocyte elastase inhibitors (for therapeutic intervention of corneal wound) were used at first in our experiments. Elastatinal, and particularly tetrapeptide carbamate, inhibited invading PMNs and vascularization of the cornea (Čejková et al., 1992). The corneal healing was improved. In contrast to aprotinin, leukocyte elastase inhibitors enabled corneal healing also if applied later (when the inflammatory reaction was developed).

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Figs. 5-8. DPP II activity in the rabbit cornea burned with severe alkali (large extent) treated with placebo (saline) or with aprotinin-tetrapeptide carbamate mixture. DPP II activity is detected by the simultaneous azocoupling method using Lys-pro-MNA as the substrate and FBB as the coupling agent. **5.** The 4th week after the burn (the corneal center). The cornea is treated with aprotinin-tetrapeptide carbamate mixture for 4 weeks. Efficient healing takes place, the burned area is re-epithelized (e: epithelium), and the corneal stroma is rebuilt by keratocytes from the unburned corneal stroma. Only rare inflammatory cells are seen in the corneal stroma beneath the epithelium (arrow). In the same location keratocytes are highly enzymatically active. Remodelling of the corneal stroma takes place. **6.** The 4th week after the burn (the corneal center). Placebo-treated cornea (saline). Initial corneal ulceration is seen in the superficial portion of the corneal stroma. Arrow points to the remaining part of the corneal stroma where highly enzymatically active inflammatory cells are present and some DPP II activity is released from inflammatory cells into the substantia propria. Vascularization of the corneal stroma in the deeper part of the corneal stroma is also seen. (Compare with (5) where the cornea is healed after the aprotinin-tetrapeptide carbamate treatment). x 160

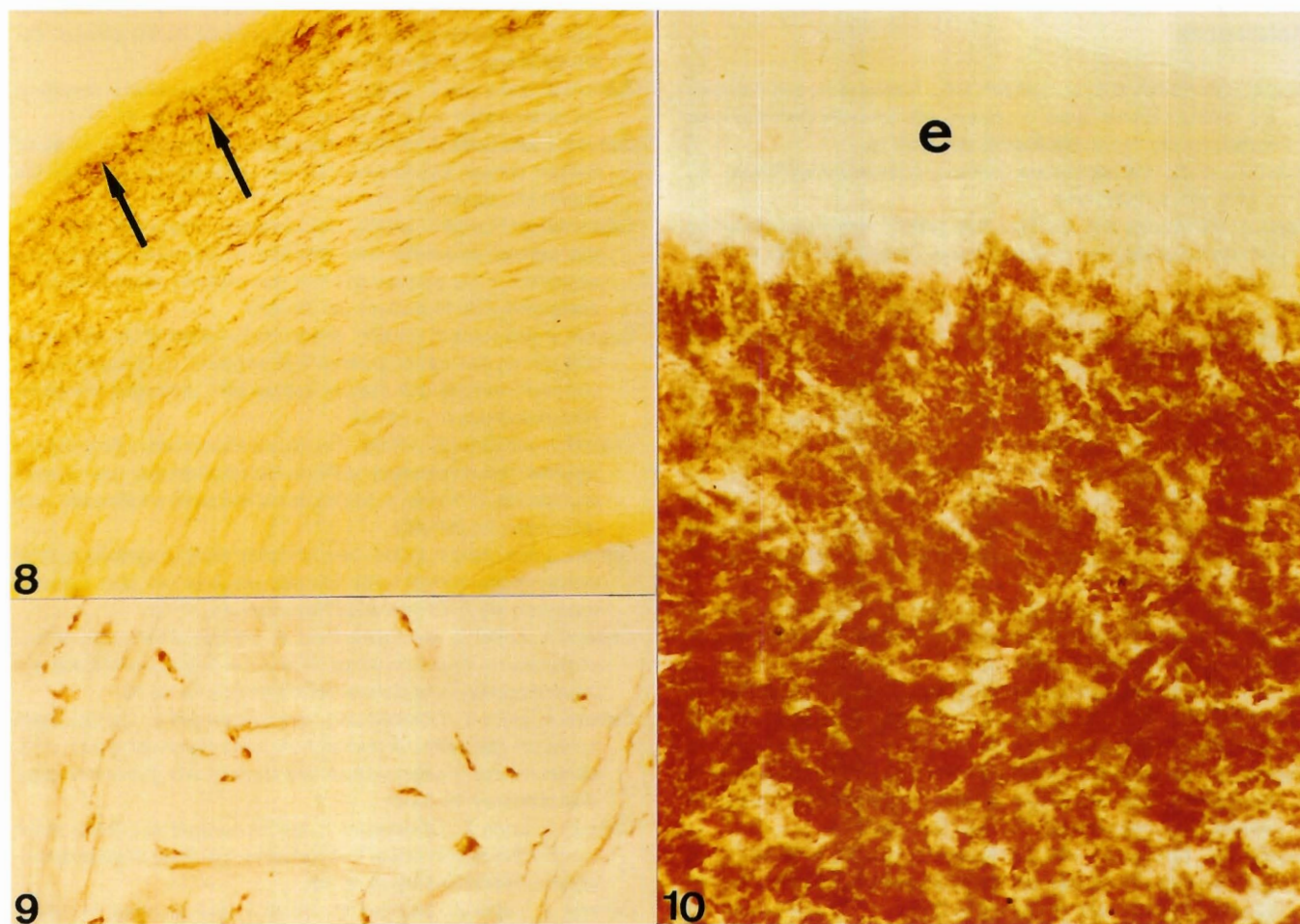
Leukocyte elastase inhibitors do not inhibit plasmin activity in the tear fluid. However, after their application on the eye surface, plasmin concentration in the tear fluid occurs later and reaches lower levels in comparison to untreated cases. Moreover, a combination of aprotinin and leukocyte elastase inhibitor (tetrapeptide carbamate) potentiates the inhibitory effect of individual drugs (see Cejková et al., 1992a) and improves the healing processes (Figs. 5-7).

Conclusion

Using methods of catalytic histochemistry, we have briefly summarized findings in corneal wound healing, either untreated or treated with selected serine protease inhibitors and compared with clinical observations or findings obtained using biochemical methods and mono-

and polyclonal antibodies to enzymes of pericellular proteolysis (e.g., Hayashi et al., 1991; Vaheri et al., 1992).

In our studies, the efficacy of inhibitors was evaluated using enzymes as markers of metabolic disorders: $\text{Na}^+\text{-K}^+$ -dependent ATPase and GGT - enzymes associated with the transport of water ions and metabolites across the cornea; lactate and succinate dehydrogenase - enzymes involved in glucose metabolism in the corneal epithelium; lysosomal hydrolases (acid glycosidases and lysosomal proteases) - as markers of the metabolism of glycosaminoglycans and glycoproteins; and catalase and xanthine oxidase - enzymes belonging to oxidases which either cleave (catalase) or generate (xanthine oxidase) ROS. However, for evaluation of the inhibition of plasmin or leukocyte elastase in situ by inhibitors, specific methods for the



Figs. 8-10. Aminopeptidase M (APM) activity in the normal cornea and in the cornea burned with severe alkali injury (small extent) and treated with aprotinin (within 3 weeks after the injury). APM activity detected by the simultaneous azocoupling method with Alanyl-MNA as the substrate and FBB as the coupling agent. **8.** In the normal cornea APM activity is present in keratocytes, mainly beneath the epithelium. Epithelium and endothelium are negative. **9.** The 2nd week after the injury. The burned area is being rebuilt by keratocytes from the immediate vicinity of unburned cornea. Young keratocytes have long processes. **10.** The 3rd week after the burn. Remodelling of the cornea takes place. Keratocytes are highly enzymatically active. The cornea is re-epithelized (e: epithelium). Remark: Although in the case of placebo-treated cornea (small severe injury), the cornea also heals and ulcers are never observed, after aprotinin treatment the healing is accelerated and in the majority of cases corneal transparency is restored. x 160

detection of active proteases in situ (at the site of their activation) are necessary. Until recently, these methods were not available. Lojda (1996a,b) recommended sensitive substrates (synthesized by Enzyme Systems Products, Livermore, CA, USA, see Smith et al., 1992 for details) containing 7-amino-4-trifluoromethylcoumarine (AFC) leaving groups for the localization of these proteases in situ. Our first findings with this approach (Čejková and Lojda, 1995b, 1996; Čejková, 1997) show that this new methodological approach will be useful for the better understanding of the circumstances leading to corneal damage or repair.

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