Histol Histopathol (1999) 14: 471-478

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Comparative histochemical and biochemical studies on acid ß-galactosidase activity in the experimentally injured rabbit cornea and tear fluid using the sensitive substrate ß-galactoside-4-trifluoromethylumbelliferyl (HFC)

J. Čejková[1], J. Zvárová[2], Z. Andonová[1] and T. Ardan[1]

[1]Department of Eye Histochemistry, Institute of Experimental Medicine, Academy of Sciences of Czech Republic, Prague, Czech Republic and [2]EuroMise Center of Charles University and Academy of Sciences of Czech Republic, Prague, Czech Republic

Summary. Comparative histochemical and biochemical studies on acid ß-galactosidase activity in the rabbit eye after various experimental injuries were performed using the same sensitive fluorogenic substrate ß-galactoside-4-trifluoromethylumbelliferyl (HFC). The aim of the study was to examine whether the severity of corneal damage corresponds with the level of the enzyme activity in the tear fluid. As until recently the substrate ß-galactoside-4-HFC had not been used for the histochemical detection of acid ß-galactosidase in the cornea, results obtained with this substrate in a fluorescent method were compared in parallel cryostat sections with results obtained using the substrate 5-bromo-4-chloro-3-indoxyl ß-galactoside in the indigogenic method (previously shown to be very sensitive for the detection of acid ß-galactosidase activity in the cornea). Both methods revealed similar localization and changes in enzyme activity; using ß-galactoside-4-HFC an acceptable cellular localization was achieved. For the measurement of acid ß-galactosidase activity in the tear fluid a semiquantitative biochemical method was elaborated using filter paper punches with the substrate (ß-galactoside-4-HFC) soaked with tears and incubated at 37 °C. The time of the first appearance of a greenish-yellow fluorescence (enzyme positivity) was recorded by UV lamp and compared with the appearance of fluorescence in calibrated punches containing known acid ß-galactosidase activities. The results show that ß-galactoside-4-HFC is useful for the biochemical assessment of acid ß-galactosidase activity in the tear fluid. Comparing histochemical and biochemical results, it can be concluded that increased enzymatic activity in tears parallels the severity of corneal damage. Further studies are necessary to evaluate whether the detection of acid ß-galactosidase activity in tears might be useful for diagnostic purposes in humans.

Key words: Acid ß-galactosidase, Enzymatic marker, Corneal injury

Introduction

Acid ß-galactosidase belongs to the acid glycosidases, which are an important constituent of lysosomes. They are ubiquitous in mammalian tissues. The presence of acid glycosidases can be demonstrated biochemically and histochemically. The histochemical approach is unique in that it can reflect the heterogeneous distribution of enzymes. Various methods for the in situ detection of acid glycosidases have been summarized by Gossrau et al. (1991). Acid ß-galactosidase is best detected histochemically in lysosomes in freeze-dried cryostat sections. The technique of unfixed cryostat sections (on semipermeable membranes and a gel incubation medium with the substrate) allows the total activity of acid ß-galactosidase to be demonstrated. The leakage of the enzyme activity into the incubation medium is prevented. This technique was introduced by Lojda (1973) for the detection of acid ß-galactosidase using the indigogenic method with the substrate 5-bromo-4-chloro-3-indoxyl ß-D-galactoside. Lojda (1996a,b) proposed the technique of semipermeable membranes and a gel incubation medium for the histochemical assessment of lysosomal hydrolases using sensitive fluorogenic substrates with 7-amino-4-trifluoromethylcoumarin (AFC) or 4-trifluoromethylumbelliferyl (HFC) leaving groups (Enzyme Systems Products, Dublin, CA, USA). This was in contrast to the statements of Raap (1986) and Smith et al. (1992) who

Offprint requests to: Prof. Jitka Čejková, MD, PhD, DSc, Head, Department of Eye Histochemistry, Institute of Experimental Medicine, Vídeňsák· 1083, 14220, Prague 4, Czech Republic

considered these substrates to be unsuitable for this purpose due to their high water solubility. Van Noorden and Butcher (1991) mention with respect to the findings of Gossrau (1983) that AFC substrates can be used for the localization of proteases in monolayers provided that the substrates are incorporated in agar-gel. Lojda (1996b) pointed out that the technique of semipermeable membranes and an agar-gel incubation media, introduced by McMillan 1967) and modified by Lojda (1972, 1973) and Meijer (1972, 1973) for the demonstration of enzymes that are not firmly associated with the structure (Lojda et al., 1979; Meijer, 1980), was not used. This procedure not only prevents the leakage of enzymes but also reduces the diffusion of the primary reaction product. As discussed by Lojda (1996b), this enables the use of AFC or HFC substrates for the localization of various enzymes (proteases, acid glycosidases) in tissue sections. However, because some diffusion artifact cannot be excluded, Lojda (1996b) recommended AFC or HFC substrates for comparative histochemical and biochemical studies using the same substrate or for cases in which no other reliable method for histochemical localization of the enzyme activity was available (e.g. enzymes of pericellular proteolysis: plasmin, plasminogen activators).

In this paper the sensitive substrate ß-D-galactoside-HFC was used for comparative histochemical and biochemical studies in the rabbit eye, with the aim of examining whether the level of the enzyme activity in the tear fluid corresponded with the severity of corneal disorders, in which case the measurement of the enzyme activity in the tear fluid might be useful for diagnostic purposes. For the detection of acid ß-galactosidase activity in the cornea, a histochemical approach using a substrate with a HFC leaving group as recommended by Lojda (1996a,b) was employed. As until recently ß-D-galactoside-HFC has not been used for the localization of acid ß-galactosidase activity in the cornea, histochemical results obtained with the substrate ß-D-galactoside-HFC in a fluorescent method were compared in parallel cryostat sections with results obtained with the substrate 5-bromo-4-chloro-3-indoxyl ß-D-galactoside in the indigogenic method. The indigogenic method was previously shown to be very sensitive for the demonstration of acid ß-galactosidase activity in the cornea (Čejková et al., 1975a, 1988, 1993; Čejková and Lojda, 1994).

At present AFC derivates have been used primarily in flow cytometry (van Noorden and Butcher, 1991) and overlay membranes (dry cellulose strips with AFC substrates are available as "Enzyme Overlay Membranes" from Enzyme Systems Products, Dublin, CA, USA). Another expanding field in which AFC substrates are used are methods for the semiquantitative determination of enzyme activities in the tear fluid or bodily fluids in which several μl of the fluid are dropped onto dry filter paper punches with the respective substrate. The incubation takes place in a thermostat (37 °C), and the time of the appearance of fluorescence is recorded and compared with the appearance of fluorescence in calibrated punches containing a known concentration of the respective enzyme (for references see Čejková et al., 1992, 1993; Lojda and Čejková, 1993; for review see Čejková, 1998). Smith et al. (1992) used a colorimetric method instead of a fluorescent one; after the addition of a solution of p-dimethylamino-cinnamaldehyde a reddish dye is formed by the liberated AFC.

In this paper the substrate ß-D-galactoside-HFC is used for the measurement of enzyme activity in the tear fluid; for this purpose a semiquantitative method with this substrate was elaborated using a similar methodological approach as previously described for the extra situm detection of plasmin (Čejková et al., 1992, 1993) and plasminogen activator of the urokinase type (Lojda and Čejková, 1993).

Materials and methods

Animals and experimental injuries

Chinchilla rabbits weighing 2.5-3.0 kg were used in our experiments. Histochemical and biochemical examinations of acid ß-galactosidase activity were performed in normal eyes and eyes under various experimental conditions (irradiation of the cornea with UVB rays, alkali burns, contact lens wear). The procedures used in this study were consistent with the ARVO Resolution on the Use of Animals in Research: according to the World Medical Association Declaration of Helsinki, Finland, 1964 and revised by the World Medical Assembly in Hong Kong in 1989.

1) Contact lens wear

The eye-lids were slightly distended and sterile contact lenses (SCL) were applied to the cornea of both eyes. SCL were prepared in the Institute of Macromolecular Chemistry of the Czech Academy of Sciences to fit the rabbit eye (7.5 mm curvature radius, 0.2 mm thickness, 18 mm diameter). The water content of the lenses was 68%. The lenses remained continuously on the eyes of rabbits for 6 days. After this time interval SCL were removed. In the tear fluid of 5 rabbits (10 eyes) the concentration of acid ß-galactosidase was examined using our semiquantitative biochemical method. Afterwards rabbits were killed by thiopental narcosis and 10 corneas were examined histochemically and histologically.

2) Repeated irradiation of the eye with UVB rays

Rabbits were anesthetized intravenously by an i.m. injection of Rometar (Xylazinum hydrochloricum, 2%, 0.2 ml/1kg body weight). The open eyes of 5 rabbits (10 eyes) were irradiated from a distance of 0.3 m with a Bioblock UV lamp (Scientific, Illkrich, Cedex, France) which generates UV rays of 312 nm wavelength (UVB

rays), for 5 min once a day for 6 days. On day 6 the lenses were removed and the acid ß-galactosidase activity was measured in the tear fluid of 10 eyes. Afterwards rabbits were sacrificed by thiopental anesthesia and 10 corneas were examined histochemically and histologically.

3) Alkali burns

The rabbits were anesthetized by an i.m. injection of Rometar (Xylazinum hydrochloricum, 2%, 0.2 ml/1kg body weight). The eyes of rabbits were proptosed and 1.0M NaOH was applied on the corneas using a plastic tube (9 mm diameter) for 15 s. After quick removal of alkali with a cotton swab the eyes were rinsed with tap water. For histochemical and histological examination the animals were sacrificed on day 14, 21 or 28 (10 eyes at each time point). On day 21 and 28 (before sacrificing the animals) the activity of acid ß-galactosidase was measured in the tear fluid (10 eyes at each time point).

Histochemical examination

The eyes of normal animals served as controls. The normal and injured corneas were quenched in light petroleum chilled with acetone-dry ice mixture, and sections were cut in a cryostat (Jung, Leica Instruments GmbH, CM 1900, Heidelberg, Germany).

Acid ß-galactosidase activity was examined by two methods: the fluorescent method using the substrate ß-D-galactoside-4-HFC as recommended by Lojda (1996a,b) and by the indigogenic method using 3 mg 5-bromo-4-chloro-3-indoxyl-ß-D-galactoside as the substrate (see Lojda, 1973; Čejková et al., 1975a, 1992; Lojda et al., 1979; Čejková and Lojda, 1994 for details).

Sections of corneas were cut in a cryostat and transferred to semipermeable membranes. Tubing manufactured for renal dialysis (Nephrophan, Wolffen, FRG) was used. Moist tubing was stretched across one end of a hollow plastic incubation vessel, secured with elastic bands and allowed to dry. Unfixed cryostat sections were transferred on the upper surface of the membrane (see Lojda et al., 1979 for details).

The detection of acid ß-galactosidase by the fluorescent method:
Incubation medium: 2 mg of substrate ß-D-galactoside-4-trifluoromethylumbelliferyl (HFC) (Enzyme Systems Products, Dublin, CA, USA) were dissolved in 0.15 ml of dimethylformamide and mixed with 1 ml of citric acid-phosphate buffer, pH 5.0. The solution was mixed and 1 ml of 2% warm Agar (Difco) (previously prepared by dissolving 2g of Agar/100 ml of citric acid-phosphate buffer, pH 5.0 at 37 °C for 48 hrs) was added. The incubation solution was shaken well, poured into the inverted end of the incubation vessel, allowed to gel (for 3 min) in a refrigerator and incubated in a thermostat (37 °C) for several hours. Membranes surrounding sections were cut away, mounted in glycerine or in Vectashield mounting medium (Vector Laboratories, Burlingame, Ca, USA), immediately examined in a fluorescence microscope (Leitz, Vario-Orthomat 2, 200W mercury ultra-high pressure lamp, the filter set for FITC) and photographed.

The detection of acid ß-galactosidase by the indogogenic method: Three mg 5-bromo-4-chloro-3-indoxyl-ß-D-galactoside (Serva, Feinbiochemica, Heidelberg, Germany) was dissolved in 0.15 ml N,N-dimethyl-formamide. To this solution 5 ml of 0.1M citric acid-phosphate buffer, pH 5.0 with 0.7 ml of 1.65% potassium ferricyanide and with 0.7 ml of 2.11% potassium ferrocyanide was added. The solution was mixed well and 5 ml of warm 2% Agar (Difco) in 0.1M citric acid-phosphate buffer was added. The final solution was poured into the incubation vessels, allowed to gel in a refrigerator for 3 min and incubated in a thermostat (37 °C) for several hours.

Histological examination

In all experimental groups some cryostat sections of corneas were post-fixed in formol-calcium chloride and stained with Haematoxylin-eosin.

Biochemical investigation

The assessment of acid ß-galactosidase activity (calibrated punches)

Punches of Whatman 1 filter paper (5 mm diameter) were put into the substrate solutions, then removed by forceps, transferred onto a glass plate and dried. Dry substrate punches were preserved in the refrigerator in small covered Petri dishes. The substrate solutions were prepared as follows: 1 mg of the substrate ß-galactoside-HFC (Enzyme Systems Products, Dublin, CA, USA) was dissolved in four drops of DMF (N,N,dimethyl-formamide). Then 1 ml of 0.1M citric acid-phosphate buffer, pH 5.0 was added. For the semiquantitative assessment of acid ß-galactosidase activity, solutions containing ß-galactosidase (Sigma, St. Louis, USA, from bovine liver, 0.15 units/mg protein) at various concentrations (3.0, 1.5, 0.75, 0.345, 0.187, 0.094, 0.047, 0.023, 0.012, 0.006, and 0.003 IU) were prepared. Five μl of each sample were dropped onto dry substrate punches ("calibrated punches"). Punches were then incubated in a wet chamber in a thermostat at 37 °C and observed under UV light (UVGL-25, manufactured by CVP, Inc., San Gabrioel, CA, USA) at 1 min intervals during the first 15 min and at 5 min intervals thereafter. The time of the appearance of yellow-greenish fluorescence was recorded. This procedure was performed with five ß-galactosidase specimens (from bovine liver, Sigma). With each specimen two measurements for all concentrations (3.0-0.003 IU) were performed.

The activity of acid ß-galactosidase in tears was assessed in the following way: substrate punches were soaked with tears either by a short touch to the respective site of the cornea or by dropping 5 μl of tear fluid collected with a micropipette. Subsequently they

were incubated in a wet chamber together with calibrated punches. The appearance of positive fluorescence in the punches under investigation was compared with its appearance at various concentrations in "calibrated punches" (freshly prepared substrate punches were used and substrate punches were stored in a refrigerator for two weeks). Tears of normal rabbit eyes (eyes before experimental injuries) and tears of eyes after experimental injuries were examined; in each experimental group 10 measurements were performed.

Statistics

From the data obtained on five specimens (ß-galactosidase, from bovine liver, 0.15 units/mg protein, Sigma), each with two mesurements for all concentrations (3.0IU -0.003 IU) using freshly prepared substrate punches for the first mesurement and substrate punches after two-week storage for the second mesurement, parameters of the calibration curve were estimated for the acid ß-galactosidase activity. The following mathematical form of the calibration curve was used: y=exp (exp (a+b.log(log(3) - log(x)/log(2))), where y is the time of the appearance of green-yellowish fluorescence (enzyme positivity) in seconds and x the enzyme concentration in IU. The mathematical form of the calibration curve was estimated from measured data in such a way that we transformed the data to reach the homoscedascity and then used the least square criterium for the parameter estimation. Thus we can use the calibration curve for the estimation of concentration from the measured time and also calculate the lower and upper bounds of the 95% confidence interval for the concentration.

Results

Histochemical and histological examination

The localization of acid ß-galactosidase activity in the normal cornea as well as changes in the enzyme activity under various experimental conditions, as obtained by the indogogenic method and fluorescent method, were similar. The indigogenic method revealed excellent cellular localization; using fluorescent method an acceptable cellular localization was achieved. In comparison to the normal corneal epithelium (Figs. 1, 4), after contact lens wear the enzyme activity was increased in the superficial layers of the corneal epithelium (Figs. 2, 5) and, after repeated irradiation of the eye with UVB rays, the acid ß-galactosidase activity was increased in all layers of the corneal epithelium (Figs. 3, 6). After severe alkali burn of the cornea, inflammatory cells present in the corneal stroma were highly enzymatically active (Figs. 7, 8). The amount of enzymatically active inflammatory cells in the corneal stroma gradually increased with longer time intervals after the alkali burn (Fig. 9 - day 14 after the alkali burn; Fig.10 - day 21 after the alkali injury). During the 4th week after alkali burn of the cornea, the enzyme activity was released from cells of the inflammatory infiltrate into the substantia propria of the corneal stroma (Figs. 11, 12). At this time interval corneal ulcers appeared.

Biochemical examination

Calibrated punches

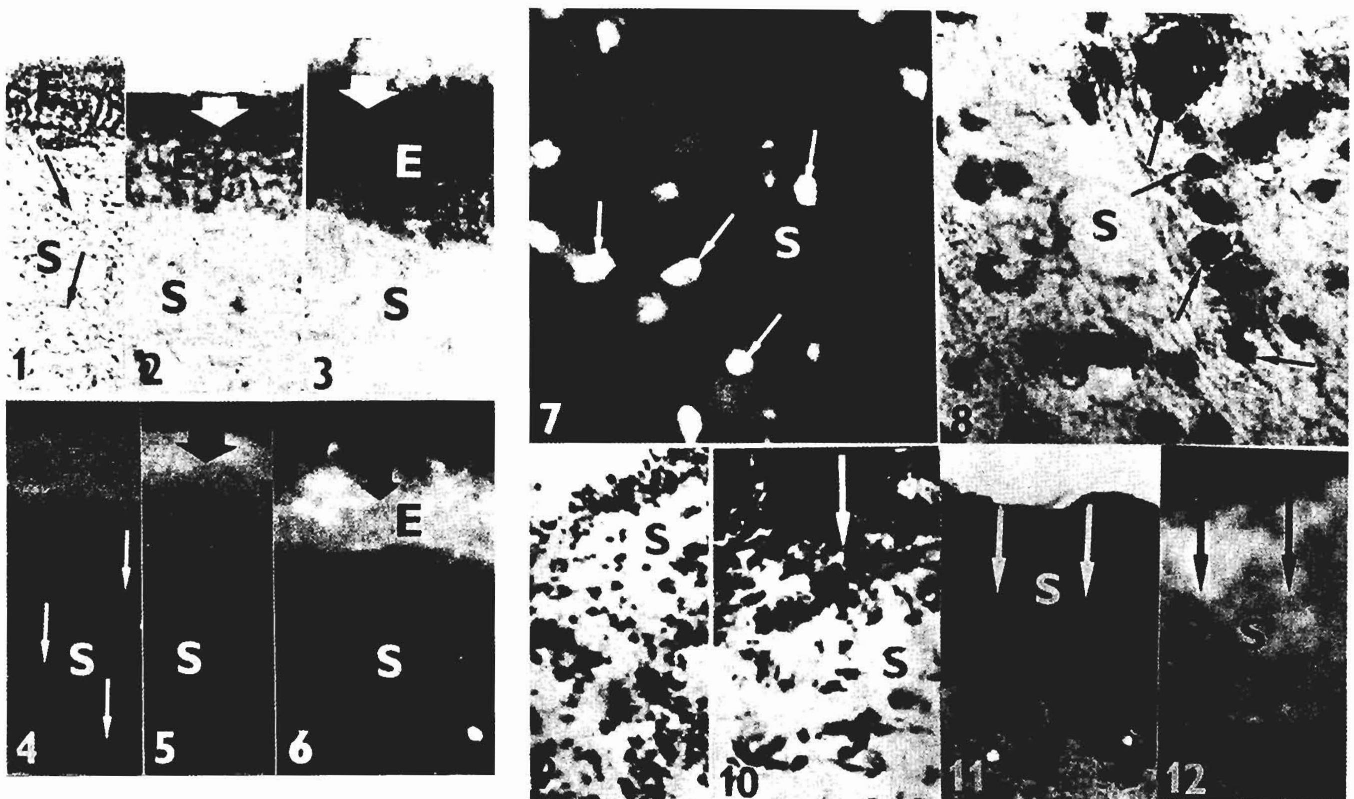
Punches soaked with standard substrate solutions for the assessment of acid ß-galactosidase activity (ß-galactoside-HFC) displayed a positive fluorescence with acid ß-galactosidase solutions containing 3 IU immediately after dropping them on substrate punches. The positive fluorescence of acid ß-galactosidase at a concentration of 1.5 IU appeared after 5 min incubation, that of 0.75 IU after 10 min, 0.375 IU after 15 min, 0.187 IU after 25 min, 0.094 IU after 30 min, 0.047 IU after 50 min, 0.023 IU after 70 min, 0.012 IU after 4 h and 20 min, 0.006 IU after 10 h, and 0.003 IU after 22 h incubation. The concentration of 0.001 IU revealed no positive fluorescence. Using fresh substrate punches, similar results were obtained with five ß-galactosidase specimens (from bovine liver, Sigma). The storage of substrate punches in a refrigerator for two weeks prolonged the time until the appearance of positive fluorescence; therefore storage of substrate punches for a maximum of two weeks can be recommended for the measurement of acid ß-galactosidase concentrations. The storage of the substrate punches in a refrigerator up to two weeks resulted in the appearance of acid ß-galactosidase at a concentration of 1.5 IU after 5min - 5 min 50 s incubation, that of 0.75 IU after 10 min - 10 min 30, 0.375 IU after 15 min - 15 min 25 s, 0.187 IU after 25 min - 27 min, 0.094 IU after 30 min - 36 min, 0.047 IU after 50 min - 55 min, 0.023 IU after 70 min - 90 min, 0.012 IU after 4 h and 20 min - 4 h and 50 min, 0.006 IU after 10 h - 12 h, and 0.003 IU after 22 h - 24 h incubation (Fig.13).

In comparison to the normal eye (eyes before experiments) the concentration of acid ß-galactosidase activity in the tear fluid paralleled the increase in corneal disorders as examined histochemically (compare Fig.1-12 with Fig.13). After contact lens wear that evoked an increase in acid ß-galactosidase activity in the superficial layers of the corneal epithelium only, the level of acid ß-galactosidase activity was slightly elevated (0.094-0.187 IU). Repeated irradiation of the eye with UVB rays evoked a higher acid ß-galactosidase activity (0.187-0.375 IU). This was coincidental with the incerease of acid ß-galactosidase in the whole corneal epithelium. The highest enzyme activity (0.75-1.5 IU) occurred after severe alkali burns, accompanied by the release of enzyme activity from cells of the inflammatory infiltrate into the substantia propria of the corneal stroma. Histologically as well as macroscopically, corneal ulceration or perforation was observed.

Discussion

The activities of acid glycosidases (including acid ß-galactosidase) have been detected in the normal cornea biochemically (Hayasaka, 1974; Bolková and Čejková, 1977; Hayasaka and Sears, 1978; Schive and Volden, 1982; Shino et al., 1986) as well as histochemically (Čejková et al., 1975a ; Bolková and Čejková,1977). These enzymes were suggested to participate in the metabolism of glycosaminoglycans and glycoproteins and to play an important role during corneal wounding and remodelling (Basu and Hasany, 1975; Čejková et al., 1975b,c; van Haeringen et al., 1986; Mishima et al., 1992; Panjwani et al., 1995) and in the process of inflammation, as in alkaline and acid burns of the cornea (Chayakul and Reim, 1982; Pahlitz and Sinha, 1985, Čejková et al., 1988, 1993).

Glycosidase activities were found to be present in the tear fluid of normal human as well as rabbit eyes (van Haeringen and Glacius, 1976; van Hoof et al., 1977; Kitaoka et al., 1985). The authors studying acid glycosidases (and also some other lysosomal hydrolases) in the human eyes of normal adult volunteers (Kitaoka et al., 1985) or the eyes of infants for the identification of several inborn errors of metabolism (van Haeringen, 1981) suggested that the main source of enzymes in the tear fluid was the lacrimal gland and that the corneal and conjunctival epithelium might provide a secondary source for these enzymes. However, in the tear fluid of rabbit eyes experimentally injured (mechanical or chemical corneal injury, irradiation with UVB rays) (van Haeringen et al., 1986; Liu et al., 1987; Čejková et al.,



Figs. 1-12. Histochemical pattern of acid ß-galactosidase activity in cryostat sections of the rabbit cornea obtained by the indigogenic method with the substrate 5-bromo-4-chloro-3-indoxyl-ß-D-galactoside **(1, 2, 3, 8, 9, 10, 11)** as compared with the histochemical pattern of the enzyme activity achieved in parallel cryostat sections by the fluorescent method using the substrate ß-D-galactoside-HFC **(4, 5, 6, 7, 12)**.

Figs. 1, 4. Normal cornea. The enzyme activity is present in the corneal epithelium (E) and keratocytes (arrows) of the corneal stroma (S). x 160

Figs. 2, 5. Cornea after continuous contact lens wear for 6 days. The acid ß-galactosidase activity is increased in superficial layers (arrows) of the corneal epithelium (E). In the corneal stroma (S) the enzyme activity is at a normal level. Fig. 2, x 140; Fig. 5, x 180

Figs. 3, 6. Cornea after the repeated irradiation of the eye with UVB rays for 6 days. UVB rays evoke an increase in acid ß-galactosidase in the whole corneal epithelium (E) with the most profound enzyme activity in the superficial epithelial layers (arrows). In the corneal stroma (S) the activity is also increased in keratocytes of the corneal stroma (S) beneath the corneal epithelium which is better seen in the fluorescent method (6). x 180

Figs. 7, 8. Alkali burned cornea. On day 14 after severe alkali injury the corneal stroma (S) is full of inflammatory cells active for acid ß-galactosidase (tangential section of the corneal stroma). x 210

Figs. 9, 10. Alkali burned cornea. On days 14 (9) and 21 (10) after severe alkali burn, the number of enzymatically active inflammatory cells is increased in the corneal stroma (S), particularly in the superficial region (arrow) (sagittal section of the corneal stroma). x 140

Figs. 11, 12. Alkali burned cornea. On day 28 after alkali injury, acid ß-galactosidase is released from inflammatory cells into the substantia propria of the corneal stroma (S). The most pronounced activity is seen in the superficial parts of the corneal stroma (arrows) without corneal epithelium. x 160

1992, 1993, Čejková and Lojda, 1995) or human eyes with corneas that had been injured or had underlying pathology (Salonen et al., 1987; Tervo et al., 1988, 1989, 1994a,b; Tozser et al., 1989; Virtanen et al., 1994, 1997), increased activities of some serine proteases (plasmin, plasminogen activator) were found (for a review see Čejková, 1988), suggesting that they were secreted in larger amounts by damaged corneal cells (mainly cells of the corneal epithelium) and leaked into the tear fluid. Similar findings were obtained in this study with acid ß-galactosidase. Using comparative histochemical and biochemical assessments with the same fluorescent substrate, we can show that the concentration of acid ß-galactosidase activity in the tear fluid parallels the severity of corneal disturbances (Figs. 1-13). A slightly increased level of the enzyme concentration in the tear fluid is associated with an increase in acid ß-galactosidase activity in the superficial layers of the corneal epithelium. A moderate activity of the enzyme in tears is accompanied by an increase in enzymatic activity in the whole corneal epithelium. The most pronounced activity in the tear fluid is found when the enzyme activity is released from inflammatory cells into the substantia propria corneae during corneal ulceration.

Acid ß-galactosidase activity in the tear fluid was determined with a semiquantitative fluorescent method, which was elaborated in this study using a similar methodological approach as described previously (for references, see Čejková et al., 1992, 1993; Lojda and Čejková, 1993; for review, see Čejková, 1998). This method is based on the use of filter paper punches containing sensitive fluorogenic substrates - derivates of 7-amino-3-trifluoromethylcoumarin (AFC) or 4-Trifluoromethylumbelliferyl (HFC) - onto which several

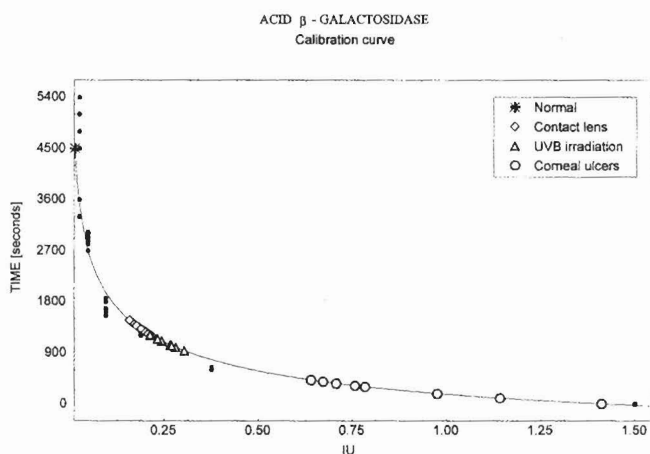


Fig. 13. Calibration curve of acid ß-galactosidase activity obtained from the appearance of enzyme positivity at various time intervals (time in seconds) of "calibrated punches" containing various enzyme concentrations (IU). In the calibration curve we demonstrate the level of the enzyme activity in the tear fluid of normal rabbit eyes (normal), tears after repeated irradiation with UVB rays (UVB irradiation) and the tear fluid of eyes with corneal ulcers appearing during the 3rd and 4th week after severe alkali burn.

μl of tears are dropped. The incubation of the filter paper punches takes place in a thermostat at 37 °C together with so called "calibrated punches" containing known concentrations of the respective enzyme (in this study acid ß-galactosidase, Sigma). Using a hand fluorescent lamp, the time of the first green-yellowish fluorescence (enzyme positivity) is recorded in the punches under investigation and compared with the positivity of an appropriate concentration of "calibrated punches". Although various biochemical methods and substrates have been employed for the detection of lysosomal hydrolases in tears and the enzyme activity determined spectrophotometrically (Kitaoka et al., 1985; van Haeringen and Glacius, 1976; van Hoof et al., 1977; van Haeringen et al., 1986), fluorometrically (Tervo et al., 1994a,b) or by means of the method of radial caseinolysis (Salonen et al., 1987; Tervo et al., 1989), the advantage of our semiquantitative method is its sensitivity and simplicity; it does not require any expensive laboratory equipment.

In contrast to the assessment of acid ß-galactosidase activity in the tear fluid of experimentally injured rabbit eyes, first investigated in this study, the histochemical as well as the biochemical picture of corneas experimentally injured have been previously described in detail for various enzymes, including acid glycosidases after contact lens wear (Čejková et al., 1988, 1992), irradiation of the eye with UVB rays (Čejková and Lojda 1994), chemical injury (Chayakul and Reim, 1982; Schive and Volden, 1982; Pahlitzsch and Sinha, 1985; Čejková et al., 1988,1993) and immunogenic keratitis (van Haeringen et al., 1986).

In the above-mentioned histochemical studies, the indigogenic method using the substrate 5-bromo-4-chloro-3-indoxyl-ß-D-galactoside was found to be very sensitive for the detection of acid ß-galactosidase activities in corneas; therefore in this paper the results obtained with this method were compared in parallel cryostat sections with those achieved by the fluorescent method using the substrate ß-galactosidase HFC. This fluorescent method was introduced by Lojda (1996a,b); however, until recently, it has not been employed in the cornea.

Using both methods, similar localization and activity of acid ß-galactosidase was seen in the normal rabbit cornea (Figs. 1, 4) as well as in corneas after contact lens wear (Figs. 2, 5), irradiation with UVB rays (Figs. 3, 6) or after severe alkali burns (Figs. 11, 12). The indigogenic method revealed excellent cellular localization; with the fluorescent method an acceptable cellular localization was achieved.

The results of comparative histochemical and biochemical studies on acid ß-galactoside activity using the same sensitive substrate ß-galactoside-HFC show that this substrate is useful for the biochemical assessment of acid ß-galactosidase activity in the tear fluid. The increased level of acid ß-galactosidase activity in the tear fluid corresponds with the severity of corneal damage (Figs. 1-13). We suggest that acid ß-

galactosidase measured in the tear fluid may be a good enzymatic marker of corneal disorders. This might be very important for the evaluation of early enzymatic changes of the corneal epithelium, e.g. during contact lens wear. Early enzymatic disturbances related to contact lens wear appear in the superficial layers of the corneal epithelium. In this location the acid ß-galactosidase activity is increased, accompanied by an elevated concentration of this enzyme in the tear fluid. Further studies are necessary to evaluate whether the measurement of acid ß-galactosidase activity in the tear fluid will be useful for clinical practice in humans.

Acknowledgements. This study was supported by the grant G/16-3 from GA Ministry of Health (CR) and by grants No. 304/96/0908 and 307/96/K226 from GA of CR.

References

Basu P.K. and Hasany S.M. (1975). Histochemical localization of hydrolytic enzymes during corneal graft reaction. Can. J. Ophthalmol. 10, 239-247.

Bolková A. and Čejková J. (1977). Species variation of some acid hydrolases in the normal cornea. Ophthalmic Res. 9, 155-161.

Čejková J. (1998). Enzyme histochemistry of corneal wound healing. Histol. Histopathol. 13, 553-564.

Čejková J. and Lojda Z. (1994). The damaging effect of UV rays (with wavelength shorter than 320 nm) on the rabbit anterior eye segment. I. Early changes and their prevention by catalase-aprotinin application. Acta Histochem. 96, 281-286.

Čejková J, and Lojda Z. (1995). The damaging effect of UV rays below 320 nm on the rabbit anterior eye segment. II. Enzyme histochemical changes and plasmin activity after prolonged irradiation. Acta Histochem. 87, 183-188.

Čejková J., Lojda Z. and Havránková E. (1975a). Distribution of acid phosphatase, ß-glucuronidase, N-acetyl-ß-D-glucosaminidase and ß-galactosidase in cornea of albino rabbit. Histochemistry 44, 337-343.

Čejková J., Lojda Z., Obenberger J. and Havránková E. (1975b). Alkali burns of the rabbit cornea. I. A histochemical study of ß-glucuronidase, ß-galactosidase and N-acetyl-ß-D-glucosaminidase. Histochemistry 45, 65-69.

Čejková J., Lojda Z., Obenberger J. and Havránková E. (1975c). Alkali burns of the rabbit cornea. II. A histochemical study of glycosaminoglycans. Histochemistry 45, 71-75.

Čejková J., Lojda Z., Brünová B., Vacík J. and Michálek J. (1988). Disturbances in the rabbit cornea after short-term and long-term wear of hydrogel contact lenses. Histochemistry 89, 91-97.

Čejková J., Lojda Z., Vacìk J., Digenis G.A. and Dropčová S. (1992). Histochemical changes in the rabbit cornea and plasmin activity in the tear fluid during contact lens wear. Favourable influence of protease inhibitors (aprotinin, PC5, elastatinal). Histochemistry 97, 69-76.

Čejková J., Lojda Z., Dropčová S. and Kadlecová D. (1993). The histochemical pattern of mechanically or chemically injured rabbit cornea after aprotinin treatment: relationships with the plasmin concentration of the tear fluid. Histochem. J. 25, 438-445.

Chayakul V. and Reim M. (1982). Enzymatic activity of ß-N-acetylglucosoaminidase in the alkali-burned rabbit cornea. Graefe's Arch. Clin. Exp. Ophthalmol. 218, 149-152.

Gossrau R. (1983). Fluorescence histochemical detection of hydrolases in tissue sections and culture cells. Histochemistry 79, 87-94.

Gossrau R., Lojda Z. and Stoward P.J. (1991). Glycosidases. In : Histochemistry. Theorerical and applied. Vol. 3. Stoward P.J. and Pearse A.G.E. (eds). 4th ed. Churchill Livingstone. Edinburgh, London, Melbourne, New York and Tokyo. pp 241-280.

Hayasaka S. (1974). Distribution of lysosomal enzymes in the bovine eye. Jpn. J. Ophthalmol. 18, 233-239.

Hayasaka S. and Sears M.L. (1978). Distribution of acid phosphatase, beta glucuronidase, and lysosomal hyaluronidase in the anterior eye segment of the rabbit eye. Invest. Ophthalmol. Vis. Sci. 17, 982-987.

Kitaoka M., Nakazama M. and Hayasaka S. (1985). Lysosomal enzymes in human tear fluid collected by filter paper strips. Exp. Eye Res. 41, 259-265.

Liu J., Hong B.-S. and Price J. (1987). Correlation of corneal epithelial wound healing and plasminogen activator activity in rabbit tears. Invest. Ophthalmol. Vis. Sci. 28 (Suppl), 330.

Lojda Z. (1972). Aktuelle Probleme der Cytochemie der lysosomal Hydrolasen. Acta Morphol. Acad. Sci. Hung. 22, 269-293.

Lojda Z. (1973). Histochemical methods for acid ß-galactosidase. Technics for semipermeable membranes. Histochemie 37, 375-378.

Lojda Z. (1996a). The use of very sensitive fluorogenic substrates: derivates of 7-aminio-4-trifluoromethylaminocoumarin (AFC) and trifluoromethylumbelliferol (HFC) in the biochemical and histochemical assessment of hydrolases. Histochem. J. 28, 299-323.

Lojda Z. (1996b). The use of substrates with 7-amino-3-trifluoromethyl-coumarine (AFC) leaving group in the localization of protease activities in situ. Acta Histochem. 98, 215-228.

Lojda Z. and Čejková J. (1993). Biochemical and histochemical studies of plasminogen activator of urokinase type. I. A simple rapid semiquantitative fluorescent method for its detection in the tear fluid. Acta Histochem. 95, 232-237.

Lojda Z., Gossrau R. and Schiebler T.H. (1979). Enzyme histochemistry. A laboratory manual. Springer. Berlin, Heidelberg, New York.

McMillan PJ. (1967). Differential demonstration of muscle and heart type lactic dehydrogenase of rat muscle and kidney. J. Histochem. Cytochem. 15, 21-23.

Meijer A.E.F.H. (1972). Semipermeable membranes for improving the histochemical determination of enzyme activities in tissue sections. I. Acid phosphatase. Histochemie 30, 31-39.

Meijer A.E.F.H. (1973). Semipermeable membranes for improving the histochemical determination of enzyme activities in tissue sections. III. Lactate dehydrogenase. Histochemie 35, 165-172.

Meijer A.E.F.H. (1980). Semipermeable membrane technics in quantitative enzyme histochemistry. In: Trends in enzyme histochemistry and cytochemistry. Ciba Foundation Symposium 73, 103-120.

Mishima H., Ueda A., Nishida T. and Otori T. (1992). Increased lysosomal enzyme activity of keratocytes after endocytosis of foreign particles. Jpn. J. Ophthalmol. 36, 84-90.

Pahlitzsch T. and Sinha P. (1985). The alkali-burned cornea: electron microscopical, enzyme histochemical, and biochemical observations. Graefe's Arch. Clin. Exp. Ophthalmol. 223, 178-286.

Panjwani N., Ahmad S. and Raizman M.B. (1995). Cell surface glycoproteins of corneal epithelium. Invest. Ophthalmol. Vis. Sci. 36, 355-363.

Raap A.K. (1986). Localization properties of fluorescence cytochemical enzyme procedures. Histochemistry 84, 317-21.

Salonen E.-M., Tervo T., Torma E., Tarkkanen A. and Vaheri A. (1987). Plasmin in tear fluid of patients with corneal ulcers: basis for new therapy. Acta Ophthalmol. 65, 3-12.

Shino T., Hayasaka S. and Mizuno K. (1986). Acid hydrolases in the bovine corneal epithelium. Graefe's Arch. Clin. Exp. Ophthalmol. 224, 467-468.

Schive K. and Volden G. (1982). Characterization and quantification of acid phosphatase and glycoside hydrolases in rabbit cornea. Acta Ophthalmol. 60, 590-598.

Smith R.E., Reynolds C.J. and Elder E.A. (1992). The evolution of proteinase substrates with special reference to dipeptidylpeptidase IV. Histochem. J. 24,637-647.

Tervo T., Salonen E.-M., Vahen A., Immonen I., van Setten G.B., Himberg J.J., Tarkkanen A. (1988). Elevation of tear fluid plasmin in corneal disease. Acta Ophthalmol. 66, 393-399.

Tervo T., van Setten G.-B., Andersson R., Salonen E.-M., Vaheri A., Immonen I. and Tarkkanen A. (1989). Contact lens wear is associated with the appearance of plasmin in the tear fluid - preliminary results. Graefe's Arch. Clin. Exp. Ophthalmol. 227, 42-44.

Tervo T., Virtanen T., Honkanen N., Härkönen M, and Tarkkanen A. (1994a). Tear fluid plasmin activity after excimer laser photorefractive keratectomy. Invest. Ophthalmol. Vis.Sci. 35, 3045-3050.

Tervo T., Honkanen N., van Setten G.B., Virtanen T., Tarkkanen A. and Harkonen M. (1994b). A rapid fluorometric assay for tear fluid plasmin activity. Cornea 13, 148-155.

Tozser J., Berta A. and Punyiczk M. (1989). Plasminogen activator activity and plasminogen-independent amidolytic activity in the tear fluid from healthy persons and from patients with anterior eye segment inflammation. Clin. Chim. Acta 183, 323-331.

van Haeringen N.J. and Glacius E. (1976). The origin of some enzymes in tear fluid determined by comparative investigation with two collection methods. Exp. Eye Res. 22, 267-272.

van Haeringen N.J. (1981). Clinical biochemistry of tears. Surv. Ophthalmol. 26, 84-96.

van Haeringen N.J., Bolková A. Oosterhuis J.A. and van Delft J.L. (1986). Enzyme activities in the rabbit cornea during immunogenic keratitis. Graefes Arch. Clin. Exp. Ophthalmol. 224, 288-290.

van Hoof F., Libert J., Aubert-Tulkens G. and Serra M.V. (1977). The assay of lacrimal enzymes and ultrastructural analysis of conjunctival biopsies: New techniques for the study of inborn lysosomal diseases. Metab. Pediatr. Ophthalmol. 1, 165-171.

van Noorden C.J.F. and Butcher R.G. (1991). Quantitative enzyme histochemistry. In: Histochemistry. Theoretical and applied. 4th ed. Vol.3. Stoward P.J. and Pearse A.G.E. (eds). Churchill Livingstone. Edinburgh, London, Melbourne, New York and Tokyo, pp 355-432.

Virtanen T., Honkanen N., Harkonen M., Tarkkanen A. and Tervo T. (1994). Elevation of tear fluid plasmin activity of contact lens wearers studied with rapid fluorometric assay. Cornea 13, 210-213.

Virtanen T., Konttinen Y.T., Honkanen N., Härkönen M. and Tervo T. (1997). Tear fluid plasmin activity of dry eye patients with Sjogren's syndrome. Acta Ophthalmol. Scand. 75, 137-141.

Accepted October 1, 1998