Tissue Transglutaminase in Apoptosis of Photoreceptor Cells in Rat Retina

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Purpose. The possible involvement of tissue transglutaminase (tTG) in apoptosis during photoreceptor degeneration was examined in retinal photic injury in rats and in retinal dystrophy of Royal College of Surgeons (RCS) rats.

Methods. Retinal photic injury was induced in 48 male Lewis albino rats by exposure to green fluorescent light of 300 to 320 foot-candles. The retinal tTG was examined by enzyme assay, immunohistochemistry, and Western blot analysis after 9, 12, or 24 hours of exposure or at 6 or 24 hours of dark adaptation after 24 hours of light exposure. Retinas from RCS rats at various stages of degeneration also were examined with similar methods.

Results. There was a progressive increase in retinal tTG activity after 300 to 320 ft-c of light exposure, reaching a peak after 24 hours of light exposure. In the RCS rats, tTG activity increased with age. Western blot analysis revealed an immunoreactive band at 80 kDa, which increased in accordance with the transglutaminase activity in both models. In normal rat retinas, tTG immunolabeling was present only in the outer segments. There was an increased number of immunolabeled photoreceptor nuclei from 12 hours of light exposure to 24 hours of light exposure. In the RCS rat, increasing numbers of immunopositive photoreceptor nuclei from 20 to 50 days of age were noted.

Conclusions. The data associated increased retinal tTG activity and enzyme levels with photoreceptor cells undergoing apoptosis. The tTG-dependent irreversible cross-linking of intracellular protein may play an important role in causing the structural changes in cells undergoing apoptosis in the retina. Invest Ophthalmol Vis Sci. 1996;37:1793-1799.

Apoptosis, a self-directed, multistep, energy-dependent process of cell death, plays an important role in embryogenesis, tissue remodeling, tumor regression, hormone-induced atrophy, and effector-mediated cytolysis in the immune system. In the retina, it occurs during normal development or as a consequence of specific mutations in photoreceptor proteins after photic injury in rats or in the hereditary dystrophic retina of Royal College of Surgeon (RCS) rats.

Tissue transglutaminase (tTG) is a Ca^{2+}-dependent enzyme that catalyzes an acyl transfer reaction between protein-bound glutamyl residues and the primary amino groups of a lysine residue of another protein or other primary amino compounds, such as polyamines, to form a highly cross-linked protein aggregate. It has been shown to be present in neural tissues, for example, during development of the central and peripheral nervous systems. Although the activity in these systems often has been associated with proliferation of glial cells, the developing rat cerebellum had high activity levels and immunoreactivity in the axons of the external layer. In addition, tTG was identified as a retinoid-inducible protein in the developing rat spinal cord. Immunolocalization showed that tTG was present in the motor neurons of the ventral horn.
when some cells undergo apoptosis. Similar observations were reported in developing chick spinal chord.

Recent studies have linked the increased activity and/or expression of the cytosolic tTG to the formation of cross-linked detergent-insoluble proteins of the apoptotic body. Induction of tTG also has been reported in neuronal cell culture systems undergoing apoptosis. However, other studies were unable to identify the activation or induction of this enzyme during apoptotic death in the brain. Hence, the universal role for tTG in apoptosis is uncertain. The involvement of tTG activity and enzyme levels in normal and apoptotic photoreceptor cells is unknown. This study examined the activity, enzyme content, and localization of tTG in two models of retinopathy that showed apoptotic photoreceptor degeneration: photic injury of Lewis rats and retinal dystrophy of RCS rats. The results demonstrated that both tTG activity and its content were elevated during apoptosis of photoreceptor cells.

MATERIALS AND METHODS

Chemicals

The chemical [1,4(N)-3H] putrescine dihydrochloride (16.3 Ci/mmol) was obtained from Amersham (Arlington Heights, IL), and N',N'-dimethylcasein, bovine serum albumin, and 3,3'-diaminobenzidine tetrahydrochloride were obtained from Sigma Chemical (St. Louis, MO). Additionally, 125I-protein A (68 mCi/mg) was obtained from ICM Biomedicals (Irvine, CA), and nitrocellulose membrane (0.2 μm) was obtained from Bio-Rad Laboratories (Hercules, CA). Peroxidase-conjugated streptavidin was purchased from Biogenex Laboratories (San Ramon, CA), and biotinylated goat anti-rabbit IgG(H + L) was obtained from Vector Laboratories (Burlingame, CA). An affinity-purified, monospecific, anti-human tTG rabbit IgG against soluble tTG of human red blood cells was obtained from Dr. L. Fesus (Debrecen University, Debrecen, Hungary).

Animals and Treatment

Forty-eight 35- to 40-day-old male Lewis albino rats (Harlan, Indianapolis, IN) were reared in 12-hour cycles of light (5 foot-candles) and darkness for 14 days, followed by dark adaptation for 24 hours. Twenty-four rats were exposed to green fluorescent light (300–320 ft-c; 490–560 nm) at room temperature. Immediately after 9, 12, or 24 hours of continuous light exposure, four rats were killed with a single intraperitoneal injection of 2 ml of 5% pentobarbital sodium. Additional groups of four rats were killed 6 or 24 hours after 24-hour light exposure. Four animals were kept in 5 ft-c of light and killed at identical schedules for control.

Pink-eyed RCS (RCS-rdy-p) rats were obtained from the National Institutes of Health (Bethesda, MD) and were bred and reared in our vivarium in 12-hour cycles of light (5 ft-c) and dark. The retinas of the animals were studied at 7, 14, 29, 35, 40, 50, and 60 days after birth. A minimum of four eyes were used under each experimental condition. Six albino Lewis rats, two each at 35, 42, and 60 days of age, served as controls. The care and maintenance of the RCS and Lewis rats conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Histopathology and Immunohistochemistry

For histopathologic and immunohistochemical studies, both eyes of each animal were removed and immediately fixed in Davidson's fixative (95% ethanol, 10% neutral buffered formalin, glacial acetic acid, distilled water at a concentration of 3:2:1:33) for 24 hours.
Eyes were processed and then embedded in paraffin. Morphologic evaluation was performed by light microscopy on 5 μm sections of all specimens. Incubation with the primary antibody (1:75) was carried out in a humidified chamber at 4°C overnight. A biotinylated goat anti-rabbit IgG was used as a secondary antibody followed by a peroxidase-conjugated streptavidin complex. The reaction was developed using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as chromogen substrate and 0.01% H₂O₂. Endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol, and background staining was minimized by incubating sections with 5% nonimmune goat serum and 0.1% bovine serum albumin (BSA). Adjacent serial sections were processed similarly, but normal rabbit serum was used instead of the tTG antiserum for a negative control.

Transglutaminase Activity Assay

Four or eight retinas from a single time point of each model were dissected free of retinal pigment epithelium (RPE) and choroid, frozen on dry ice, and stored at −80°C. The frozen retinas were thawed in 0.5 ml of buffer containing 150 mM Tris-HCl, 30 mM NaCl, 10 mM dithiothreitol (DTT), 1 mM EDTA, and 1 mM PMSF (pH 8.3), and they were sonicated at 4°C for 2 minutes. After centrifugation at 13,000g for 10 minutes, the supernatant fractions were pooled for transglutaminase activity and Western blot analysis.

Transglutaminase activity was measured by detection of the incorporation of [3H] putrescine into N,N'-dimethylcasein. The incubation mixture contained 150 mM Tris-HCl, 30 mM NaCl, 10 mM DTT, 5 mM CaCl₂, 1 mM EDTA, 1 mM PMSF, 2.5 mg/ml N,N'-dimethylcascin, 0.2 mM putrescine (containing...
FIGURE 4. Immunolabeling of tissue trans-glutaminase in injured retinas of Lewis rats. Photomicrographs were taken from the most representative areas of the temporal retinas of each group. The immunolabeling procedure was described in Materials and Methods. (A) In unexposed control retina, note mild staining in the outer segment (OS) (arrow). (B) After 12 hours of light exposure, many positively labeled photoreceptor nuclei showed a ring configuration (arrow). Staining also was observed in the retinal pigment epithelium (RPE) and choroid. (C) At 24 hours after light exposure, intense labeling occurred in a majority of photoreceptor cells in the ONL. INL = inner nuclear layer; IS = inner segments.

Western Blot Analysis
Aliquots of the supernatant fraction of the retinal homogenates (50 to 100 μg of total protein) were mixed with an equal volume of buffer containing 5% sodium dodecyl sulfate (SDS), 2.5% DTT, 0.002% Bromophenol blue, 20% sucrose, and 0.3% Tris-base (pH 6.7). They were boiled for 5 minutes and then separated electrophoretically on a 10% SDS-polyacrylamide gel. Transfer of proteins to nitrocellulose membrane was performed with a Semi-Dry Transfer Cell (Bio-Rad Laboratories) at a constant voltage of 10 V for 2 hours. The nitrocellulose membrane was blocked with 3% dry milk in phosphate buffered saline (PBS) containing 0.1% Tween 20 (PBST) overnight at 4°C and incubated in tTG antibody (1:500 in PBST containing 0.1% BSA). The membrane was washed with 3% dry milk in PBST. The bound tTG antibody was detected by autoradiography (Kodak X-OMAT x-ray film; Eastman Kodak, Rochester, NY) after incubation with 125I-protein A and washing with PBST.

RESULTS
Retinal tTG Activity
The tTG enzyme activity in light-exposed retinas increased progressively with the time of light exposure, reaching a maximal level that was 2.5-fold higher than normal at 24 hours of light exposure and returning to a normal level at 24-hours after the 24-hour light exposure (Fig. 1A). In contrast, retinas exposed to 5 ft-c of light showed no significant change in tTG activity (Fig. 1B).

With the use of either 7-day-old retinas from RCS rats or normal retinas of 60-day-old Lewis rats as references, the tTG activity from RCS rat retinas increased progressively according to age and remained elevated at 60 days of age (Fig. 2A). In contrast, retinas from 35-, 42-, or 60-day-old Lewis rats showed no significant difference in the retinal tTG activity (Fig. 2B).

Determination of tTG Protein Levels by Western Blot Analysis
Western blot analysis showed a single band at 80 kDa corresponding to the tTG protein (Fig. 3) in all nor-
FIGURE 5. Immunolabeling of tissue transglutaminase in the retinas of Royal College of Surgeons rats at different ages. (A) At 20 days, note intense staining in the outer segment (OS) and faint, diffused labeling in the outer nuclear layer (ONL) (arrows). (B,C) In retinas of 30- and 35-day-old rats, respectively, many nuclei of the ONL were labeled (arrows), and the ONL was reduced in thickness. (D,E) In retinas of 50- and 60-day-old rats, respectively, few remained, and the entire shortened OS was intensely labeled (arrows). INL = inner nuclear layer.

normal and light-exposed retinas. The level of tTG in light-exposed retinas increased progressively, showing prominent bands at 12 and 24 hours of light exposure, and decreased at 6 and 24 hours of dark adaptation after the 24-hour light exposure (Fig. 3A). Therefore, the changes in tTG activity appeared to correspond with those of the enzyme content after light exposure.

In the dystrophic RCS rat retinas, the tTG band was barely detectable at 7 and 14 days after birth, whereas a progressively prominent tTG band was noted at 29, 35, 40, 50, and 60 days (Fig. 3B). These changes also corresponded to the changes in tTG activities.

Immunohistochemical Studies
The tTG immunostaining was noted only in the outer segment (OS) of the photoreceptor cell in the normal retinas of Lewis rats. After 12 hours of light exposure, positive staining was noted in the outer nuclear layer (ONL), the RPE, and the choroid, in addition to the OS. The intensity of immunostaining in the ONL further increased with 24 hours of light exposure, whereas positive staining remained in the RPE, OS, and choroid (Fig. 4).

A similar pattern was observed with the dystrophic retinas of the RCS rats. Only the OS was positively stained in 7- and 11-day-old RCS rat retinas (data not shown). Scattered staining of the ONL appeared on 20-day-old retinas (Fig. 5A). By 30 days, although the ONL was reduced to eight to nine nuclei in thickness, there was scattered but intense perinuclear immunostaining in the ONL (Fig. 5B). At 35 days, when the ONL at the posterior pole was reduced to five to seven nuclei in thickness, immunostaining for tTG continued to show scattered but intense staining of the ONL (Fig. 5C). By 50 to 60 days of age, the OS and the remaining few rows of photoreceptor cells in the ONL were intensely stained (Figs. 5D, 5E).

DISCUSSION
In previous studies, we reported that photoreceptor cells underwent apoptosis after photic injury and in
the inherited retinal dystrophy of RCS rats. In this study, we show that the normal rat retina expressed constitutive tTG activity in the OS of the photoreceptor cell. The activity and content of tTG increased after photic injury and in the retinal dystrophy of RCS rats, whereas immunolocalization revealed scattered perinuclear staining in the ONL in both retinal degenerative models. To our knowledge, the above observations represent the first evidence of tTG activity and its immunolocalization in photoreceptor cells.

Retinal extracts from both models of photoreceptor cell degeneration showed increased enzyme activity coincident with apoptosis of photoreceptor cells. Western blot analysis of the Lewis and RCS rats showed a single 80 kDa band, which is in agreement with published reports for tTG. Furthermore, the blots indicated that elevated levels of tTG protein may account in part for the increased tTG activity. Interestingly, the elevated enzyme activity and protein levels of tTG in the photic-injured retina decreased after 6 hours of recovery in the dark and returned to a normal level after 24 hours of dark adaptation. In contrast, neither the activity nor the protein level of tTG decreased in the 60-day-old dystrophic RCS retina, even though few photoreceptor cells remained. Perhaps the apoptotic bodies containing tTG are degraded more rapidly by the neighboring cells or invading macrophages in photic-injured retinas of Lewis rats than in the dystrophic retina of RCS rats. In addition, because the RCS rat RPE fails to phagocytose the OS of the photoreceptor, the removal of apoptotic bodies may be inefficient, resulting in an accumulation of tTG.

Both models of photoreceptor degeneration shared a similar pattern of immunolocalization. The immunostaining was present only in the OS of the normal retinas of Lewis rats or of retinas of 11-day-old RCS rats. However, many positively labeled cells in the ONL with perinuclear staining appeared at 12 hours of light exposure in Lewis rats and at postnatal day 30 in the RCS dystrophic retina. The most intense immunostaining was detected in the ONL at 24 hours of light and in dystrophic retinas at 35 to 50 postnatal days, the time corresponding to the peak of enzyme activities and protein level in either model of photoreceptor cell degeneration.

The exact role of tTG in apoptosis is unclear; however, tTG may lead to the formation of detergent-insoluble, cross-linked protein envelopes in cells undergoing apoptosis through the formation of epsilon (glutamyl) lysine isopeptide bonds and/or glutamyl-bis spermidine cross-linking. The assembly of this insoluble envelope in apoptotic cells may prevent the release of harmful intracellular components (e.g., lysosomal enzymes) and thereby avoid inflammatory responses and scar formation, making tTG an effector rather than a regulator of apoptosis. Recently, however, cells transfected with a full-length cDNA for tTG showed a drastic reduction in proliferative capacity paralleled by a large increase in apoptotic cell death. These results linked tTG expression to apoptosis and suggested that elevated levels of this enzyme actually may trigger the process.

In conclusion, our results demonstrated that tTG normally is expressed in the OS of rat photoreceptor cells, where its function is unknown. In addition we found that tTG and enzyme activity increased in the retinas with apoptotic photoreceptor cells. The increase in enzyme activity coincided with a shift in the immunolocalization pattern of tTG to scattered perinuclear staining in the ONL. These observations are consistent with the notion that tTG plays an important role in apoptosis of photoreceptor cells.

Key Words
apoptosis, photic injury, retina, retinal degeneration, tissue transglutaminase

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