Synthesis of Group II Phospholipase A2 and Lysozyme in Lacrimal Glands

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Purpose. The aim of this study was to demonstrate the synthesis and cellular distribution of group II phospholipase A2 and lysozyme in the main and accessory lacrimal glands.

Methods. The authors studied samples of normal main lacrimal glands of seven autopsied subjects and accessory lacrimal glands of eight patients who underwent ptosis surgery. The specimens were immunostained with a rabbit antiserum against group II phospholipase A2 and a monoclonal antibody against lysozyme. Expression of group II phospholipase A2 gene was shown using Northern hybridization and in situ hybridization.

Results. Lysozyme was present in the secretory granules of most acini, whereas group II phospholipase A2 was seen in a minority of acinar cells, primarily in the central parts of lobules in the main and accessory lacrimal glands. Synthesis of group II phospholipase A2 in the glandular cells was confirmed by Northern hybridization and by in situ hybridization.

Conclusions. There are two specialized cell types in the main and accessory lacrimal glands, one synthesizing group II phospholipase A2 and the other synthesizing lysozyme. These enzymes are important nonspecific antibacterial factors in tears. Invest Ophthalmol Vis Sci. 1996;37:1826-1832.

The main part of tear film is composed of an aqueous layer produced by main and accessory lacrimal glands. Tears play an important role in the nonspecific defense against bacterial and fungal invasion by washing away the microbes from the surface of the eye.1 In addition, tears contain several proteins, many of which have antimicrobial properties.1-4 Lactoferrin, lysozyme, betalysin, complement, and secretory immunoglobulin A are the most important proteins responsible for the natural immune defense against microbes.1-4

Lysozyme is a bacteriolytic enzyme that catalyzes the hydrolysis of N-acetylmuramic acid—N-acetylglucosamidase β1 to β4 linkage of bacterial cell walls with subsequent osmotic lysis of the bacterial protoplast.5 The mean lysozyme content in tears is 1700 μg/ml.3 Inflammatory diseases of the anterior segment increase IgA and other immunoglobulins, lactoferrin, and albumin in the tear film, whereas levels of lysozyme remain constant.2,5

We reported recently the presence of a novel antimicrobial enzyme,6 group II phospholipase A2 in human main lacrimal glands and tears.7 The concentration of this enzyme was measured up to 1450 μg/l in tears of healthy subjects by immunoassay.7 The immunohistochemical localization in lacrimal glands resembled that of lysozyme.7-8

Phospholipase A2 is a lipolytic enzyme that catalyzes the hydrolysis of the acyl ester bond at the sn-2 position of phosphoglycerides. This enzyme is widespread in snake and bee venoms, mammalian cells and secretions, bacteria, and plants.9 Three human genes of phospholipases A2 have been cloned: two coding secretory enzymes and one coding an intracellular enzyme.10-13 The secretory phospholipases A2 are divided into two subgroups on the basis of the amino acid sequence.14 Group I phospholipase A2 is found in mammalian pancreas. Snake venoms contain group I or group II phospholipases A2.15 The human secretory nonpancreatic phospholipase A2 of synovial fluid is classified to group II.11,12

Phospholipase A2 plays a role in the inflammatory process.16 The concentration of pancreatic group I...
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Phospholipase A2 is above normal in serum in acute pancreatitis. The concentration of nonpancreatic group II enzyme is increased, for example, in sepsis and rheumatoid arthritis.

The distribution of phospholipase A2 and lysozyme containing cells were not compared in our previous study. In addition, absorption of group II phospholipase A2 from plasma to lacrimal glands could not be ruled out. In the current study, we show by Northern blot and in situ hybridization that phospholipase A2 is synthesized in the main and in accessory lacrimal glands and that there are specialized cells that secrete either group II phospholipase A2 or lysozyme.

METHODS

The study protocol was approved by the Ethics Committee of the University of Turku and the Turku University Central Hospital. Informed consent was obtained from all patients.

Samples from the main lacrimal glands of the right and the left sides were obtained from seven autopsied subjects (one man, six women; age range, 56 to 84 years; mean age, 76 years) for immunohistochemistry. Eyelid tissue specimens with the accessory glands of Wolfring were obtained for immunohistochemistry and in situ hybridization during surgical resection of congenital or senile ptosis from eight patients (six males, two females; age range, 2 to 71 years; mean age, 32 years). Congenital ptosis was corrected in three children (ages 2, 2, and 10 years) by transconjunctival resection of the levator muscle. Two patients (ages 21 and 25 years) with congenital ptosis demonstrating a fair levator action and three patients with senile ptosis (ages 62, 70, and 71 years) underwent tarsus resection using the technique described by Fasanella and Servat. Normal human small intestine served as a positive control tissue. For Northern hybridization, total RNA was extracted by homogenization in guanidine isothiocyanate and centrifugation in a CsCl gradient. Ten micrograms of total RNA were run on denaturing 1% agarose–formaldehyde gels, stained with ethidium bromide, photographed under ultraviolet light, and transferred into GeneScreen Plus nylon membranes (DuPont–New England Nuclear, Boston, MA) hybridized and washed according to the manufacturer’s instructions. A 0.45 kb cDNA probe (kindly donated by Dr. Stefania Di Marco) covering the whole protein coding area of phospholipase A2 was cloned into Hind III–BamH1 site of pUC 18 plasmid. The insert was labeled with ³²P-dCTP (Amersham, Buckinghamshire, UK) by a random oligonucleotide primer extension to a specific activity of approximately 10⁷ cpm/µg. Filters were washed after the hybridization, and autoradiographic films (X-Omat; Eastman Kodak, Rochester, NY) were exposed at −80°C and developed.

In situ hybridization was performed on sections of formalin-fixed, paraffin-embedded tissues with group II phospholipase A2 anti-sense (test) and sense (control) single-stranded RNA-probes by using a modification of a method described earlier. RNA probes were synthesized by cloning a 0.45 kb cDNA of group II phospholipase A2 described above covering the protein coding area of group II phospholipase A2 into HindIII–BamH1 site of pGEM-3Z transcription vector (Promega, Madison, WI). RNA transcripts were generated by using T7 (anti-sense probe) or SP6 (sense probe) polymerases (Boehringer–Mannheim, Mannheim, Germany) after linearization of the vector with appropriate restriction enzymes. RNAs were labeled by ³²P-UTP (Amersham) according to the manufacturer’s instructions for the transcription kit (Boehringer–Mannheim). The size of the transcripts was controlled by agarose formaldehyde gels. The specific activity of the probes varied from 0.3 to 1 × 10⁶ cpm/µg template DNA. The hybridization cocktail contained RNA benzidine was used as the chromogen, and the sections were counterstained slightly with Mayer’s hematoxylin. Sections containing accessory lacrimal glands of eyelid were doublestained by using the Vectastain ABC peroxidase kit (for phospholipase A2) and the Vectastain ABC alkaline phosphatase kit (for lysozyme). The Vector alkaline phosphatase substrate kit I was used for production of a red chromogen (Vector Red) which, according to the manufacturer’s instructions, is highly fluorescent and can be visualized as a bright red or orange fluorescent precipitate in the rhodamine, fluorescein, or AMCA excitation systems. In addition, a staining in which the labels were reversed was performed.

For histology, sections were stained by hematoxylin and eosin, van Gieson, Alcian blue, and periodic acid-Schiff methods. For Northern hybridization, total RNA was extracted by homogenization in guanidine isothiocyanate and centrifugation in a CsCl gradient. Ten micrograms of total RNA were run on denaturing 1% agarose–formaldehyde gels, stained with ethidium bromide, photographed under ultraviolet light, and transferred into GeneScreen Plus nylon membranes (DuPont–New England Nuclear, Boston, MA) hybridized and washed according to the manufacturer’s instructions. A 0.45 kb cDNA probe (kindly donated by Dr. Stefania Di Marco) covering the whole protein coding area of phospholipase A2 was cloned into Hind III–BamH1 site of pUC 18 plasmid. The insert was labeled with ³²P-dCTP (Amersham, Buckinghamshire, UK) by a random oligonucleotide primer extension to a specific activity of approximately 10⁷ cpm/µg. Filters were washed after the hybridization, and autoradiographic films (X-Omat; Eastman Kodak, Rochester, NY) were exposed at −80°C and developed.

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probes in a concentration of 10^7 cpm/ml. Other modifications to the previous protocol were the addition of an RNase digestion step and a 7-day exposure time of the autoradiographic emulsion. The concentration of RNase A (Sigma Chemicals, St Louis, MO) was 20 μg/ml in 0.5 M NaCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.5. The posthybridization RNase A digestion was performed after hot washes. After autoradiography, the slides were stained with hematoxylin and eosin.

RESULTS

Light microscopic examination disclosed similar structures in the main and the accessory lacrimal glands. Secretory granules both in the main and the accessory lacrimal glands were stained lightly with Alcian blue and periodic acid-Schiff.

Lysozyme was detected by immunohistochemistry in most glandular cells of the main and accessory glands (Fig. 1) although some cells in the central parts of the lobules seemed to be devoid of the immunoreaction. Occasional duct cells contained lysozyme (Fig. 2). A weak reaction occasionally was present in the conjunctival epithelium.

Immunoreaction for group II phospholipase A2 was found in round gland cells in the central parts of lobules in the main and the accessory lacrimal glands (Fig. 3), as well as in the apical cytoplasm of duct epithelial cells (Fig. 4). Elastic fibers were labeled slightly in some specimens.

The cytoplasmic immunoreactions for lysozyme and group II phospholipase A2 appeared granular. Immunohistochemistry of serial sections revealed two distinct gland cell populations: a less numerous group II phospholipase A2 positive centrally located cell population and a much larger population of cells containing lysozyme (Fig. 5). Two cell populations were also evident in double-stained sections (Fig. 6).

Northern blot analysis (Fig. 7) was applied to eyelid and jejunal (control) tissues removed during surgery. An approximately 900 base transcript was detected in the RNA isolated from both the eyelid and the jejunal samples. This corresponds to the known molecular weight of the mRNA of human group II.
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Figure 5. Immunohistochemical serial section investigation of main lacrimal gland. Arrows point to a gland visible in all serial sections. (a) Primary antibody replaced by albumin. No immunoreaction is present, and normal glandular structure is seen in Mayer's hematoxylin counterstain. Magnification, ×200. (b) Most acinar cells in the main lacrimal gland are stained with anti-lysozyme antibody. A minority of cells, however, appears negative. Magnification, ×200. (c) Occasional acini in the main lacrimal gland are stained dark with anti-group II phospholipase A2 antiserum. Magnification, ×200. (d) Immunohistochemical control staining with nonimmune rabbit serum. Magnification, ×200.

Phospholipase A2. The signal from the eyelid was several-fold higher than that from the jejunum. There was also a higher molecular weight (2.4 kb, Fig. 7) form in addition to the 900 base transcript in the eyelid, and a longer exposure of 6 days showed a 4 kb form of mRNA (not shown).

In situ hybridization was applied to tissues removed during surgery and fixed immediately in formalin. In situ hybridization with the anti-sense group II phospholipase A2 probe showed intensive labeling of a number of gland cells, indicating synthesis of the enzyme protein in accessory lacrimal glands (Fig. 8a). Intralobular duct cells and blood vessels tested negative. Negative controls with sense probes did not give any signals (Fig. 8b). Jejunum, used as a positive control, showed a strong signal in Paneth cells (not shown).

Discussion

The existence of different cell types has been proven histochemically, and the content of intracellular organelles and secretory granules has been reported to show pronounced differences in neighboring cells in lacrimal glands. The current study demonstrated at least two specialized gland cell types in the main and accessory lacrimal glands: one secreting lysozyme and the other secreting group II phospholipase A2. The exact cellular localization for the synthesis of group II phospholipase A2 protein was visualized by in situ hybridization.

The observed immunoreaction in the excretory duct epithelium, and especially in the conjunctival epithelium, may be the result of absorption from the tear fluid. However, large excretory ducts were not
FIGURE 6. Double staining for group II phospholipase A₂ and lysozyme in accessory lacrimal gland. (a) Bright-field microscopy shows darkly stained cells (diaminobenzidine as chromogen) containing phospholipase A₂ (arrows) and more lightly stained cells (Vector Red as chromogen) representing lysozyme-containing acinar cells. Mayer’s hematoxylin; magnification, ×250. (b) The same field as in (a) photographed under ultraviolet illumination using a rhodamine excitation filter system to visualize Vector Red used as the label in the immunoreaction for lysozyme-containing cells. The phospholipase A₂-containing cells labeled with diaminobenzidine are negative (arrows). Some background autofluorescence is seen, for example, in the connective tissue outside the epithelial elements. Magnification, ×250.

It was found by immunofluorescence that 20% to 50% of acinar and ductular epithelial cells of main and accessory lacrimal glands secrete lysozyme. However, in the current study, we observed that most glandular cells contain lysozyme, and only a minority contained group II phospholipase A₂. The distribution of lysozyme-containing cells resembled the immunohistochemical localization of lactoferrin, which was detected in 90% of acinar cells by immunofluorescence. In a recent immunohistochemical study, it was found that the distribution patterns of lysozyme and lactoferrin are identical in the major and accessory lacrimal glands, where 50% to 70% of acini, as well as some cells of the intralobular ducts, were labeled. Interestingly, the negative acini or negative single acinar cells primarily were observed in the center of the lobules. It is most likely that those negative cells were the same cells in our study that contained group II phospholipase A₂.

The total antibacterial activity of tears is approximately 200 times greater than could be ascribed to lysozyme alone. The nonlysozyme antibacterial protein fraction has an electrophoretic mobility different from that of lysozyme and, unlike lysozyme, is inactivated by heating to 100°C in acid pH. The nonlysozyme fraction is active against many gram-negative organisms, whereas lysozyme is active against certain gram-positive bacteria. Some of the nonlysozyme antibacterial activity in tears is caused by betalysin. Lysozyme lyses the bacterial cell wall, whereas betalysin attacks the cell membrane of the protoplast.

Group II phospholipase A₂ is a heat-labile protein inactivated at 80°C. It is capable of hydrolyzing membrane phospholipids; therefore, it is feasible to postulate that phospholipase A₂ may act in tears synergistically with lysozyme to degrade bacteria. Gram-negative organisms covered with lipopolysaccharide external to the peptidoglycan layer are resistant to lysozyme but are destroyed, for example, by phospholipase A₂ from mouse intestinum. Lysozyme, however, may act in conjunction with phospholipase A₂, complement, and secretory IgA to lyse gram-negative organisms.

Recently, it was demonstrated that group II phospholipase A₂ kills staphylococci and other gram-posi-
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FIGURE 8. (a) In situ hybridization with anti-sense RNA-probe for phospholipase A2 in accessory lacrimal gland. Radioautographic grains are visible on the gland cells. Magnification, X870. (b) Control hybridization with sense RNA-probe shows no labeling. Magnification, X870.

We conclude that both the main and the accessory lacrimal glands are similar in their microscopic structure, and both secrete the antibacterial enzymes lysozyme and group II phospholipase A2. Group II phospholipase A2 and lysozyme are localized in different specialized cell types of the lacrimal glands. Lysozyme is synthesized in most secretory acini, whereas group II phospholipase A2 seems to be synthesized in more central parts of lacrimal glands near the area of intercalated and intralobular ducts.

Key Words
anti-infective agents, lacrimal apparatus, lysozyme, phospholipase A2, tears

References


