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Research report

Conserved expression of the opioid growth factor, [Met^5]enkephalin, and the zeta (ζ) opioid receptor in vertebrate cornea

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Abstract

In addition to neuromodulation, endogenous opioids serve as growth factors. The naturally occurring opioid peptide, [Met^5]enkephalin, termed opioid growth factor (OGF), has been found to be a potent and tonic inhibitor of processes related to growth and renewal, particularly cell proliferation. OGF mediates its actions through the zeta (ζ) opioid receptor. In order to determine if OGF and/or the ζ receptor are present in human corneal epithelium, immunocytochemistry was utilized. Immunoreactivity with regard to OGF and to the ζ receptor could be detected in the cortical cytoplasm of both basal and suprabasal epithelial cells, but was not associated with the cell nucleus. Investigation of the ubiquity of OGF and ζ receptor in the vertebrate cornea showed that both elements are present in a wide variety of classes of the phylum Chordata, including mammalia, aves, reptilia, amphibia, and osteichthyes. These results suggest that an endogenous opioid system related to growth may have originated as early as 300 million years ago, and that the function of this system in cellular renewal and homeostasis is a requirement of the vertebrate corneal epithelium.

Keywords: Opioid receptor; Eye; Enkephalin; Growth; Cell renewal; Zeta (ζ) receptor; Evolution; Neuropeptide; Immunocytochemistry

1. Introduction

The corneal epithelium forms a vital structural barrier between the external and intraocular environments, and contributes to the maintenance of normal stromal hydration by modulating fluid transport out of the stroma [3]. The corneal epithelium is associated with many ocular diseases [6]. Although growth factors have been postulated to be involved in corneal epithelial homeostasis, a full understanding of the role of these factors is not yet known [15,19,20].

The endogenous opioids originally were believed to be related only to neurotransmission and neuromodulation [1,2]. In 1983, and based on evidence from experimental paradigms utilizing opioid antagonists, it was postulated that endogenous opioids play a role in ontogeny and oncogenesis [23–25]. It is now clear that opioid peptides act as growth regulators in many tissues during development and in normal cellular renewal, as well as in malignant tissues [4,8–10,14,16,18,26–29,33]. In previous studies we have found that the opioid growth factor (OGF), [Met^5]enkephalin, is a potent and tonic modulator of mammalian corneal epithelial growth in tissues and organ culture [13,32]. Recently, we reported that blockade of endogenous opioid–opioid receptor interaction in the wounded rabbit cornea in vivo accelerates epithelial healing [12]. These results demonstrate that opioid peptides participate in the restitution of the cornea both in tissue culture and in the animal.

The role of endogenous opioid systems (i.e. opioid peptides and receptors) in the human corneal epithelium requires elucidation. In this report, we provide the first evidence that the major components of an

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intrinsic opioid system involved with cellular proliferation: OGF (i.e. [Met^5]enkephalin), and its opioid receptor, zeta (ζ), are present in human corneal epithelium. Furthermore, we reveal the ubiquitous nature of the endogenous opioid system associated with growth by documenting the presence of OGF and ζ receptor in the corneal epithelium of a number of major classes of vertebrates.

2. Materials and methods

2.1. Tissues

Human corneal tissues donated to the Central Pennsylvania Lions Eye Bank of The M.S. Hershey Medical Center, not suitable for transplantation, were utilized in this study. Research protocols were ap-

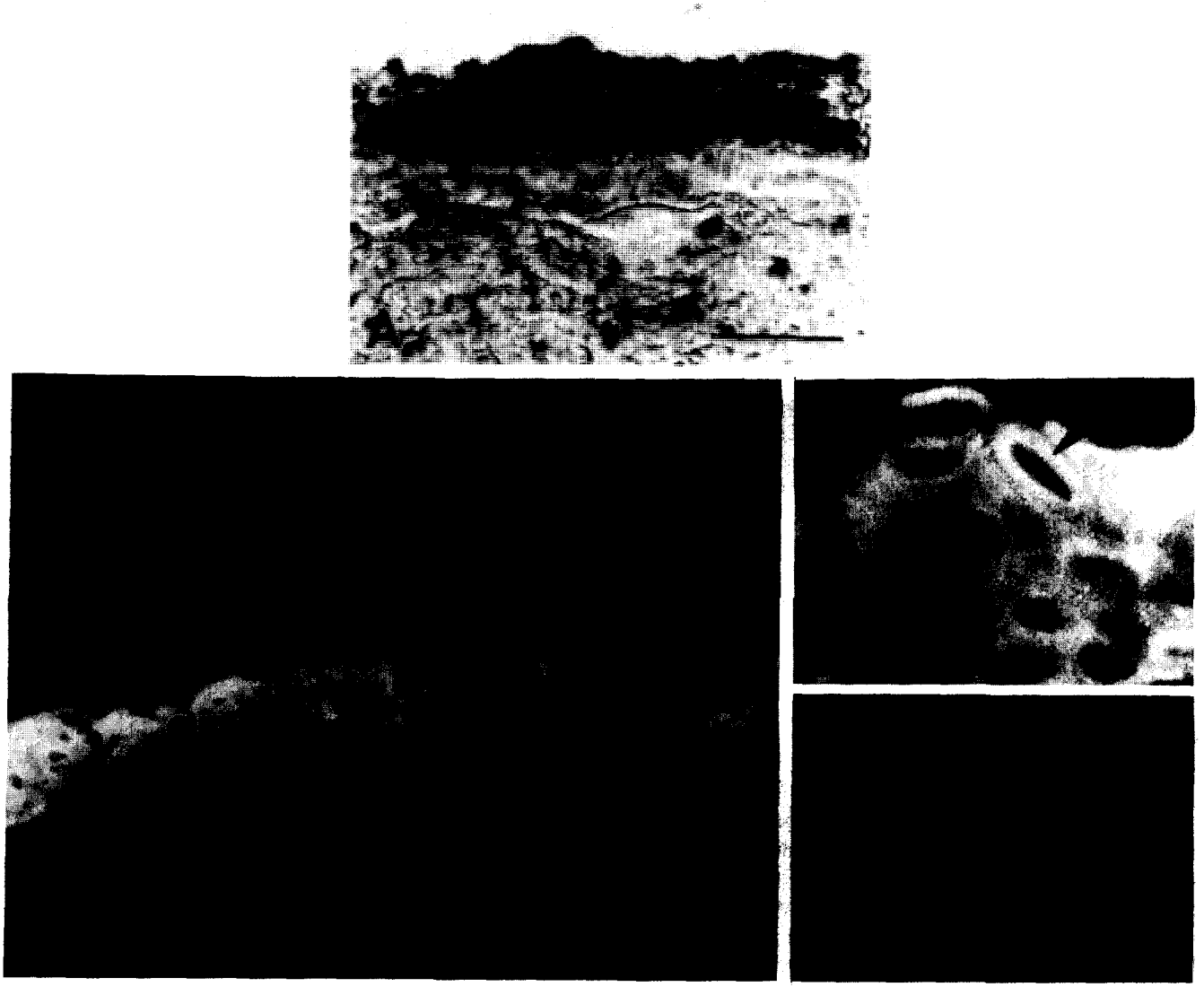

Fig. 1. Photomicrographs of the cornea from a 79-year-old female using brightfield (A) and indirect fluorescent (B–G) optics. A: the epithelium (ep), consisting of both a basal layer and 4–5 suprabasal layers, and the stroma (st) can be observed in this section stained with hematoxylin and eosin. B–D: the location of [Met^5]enkephalin immunoreactivity in the human cornea. B: note the bright staining of the corneal epithelium and the lack of immunofluorescence of the stroma. C: high magnification photomicrograph of the corneal epithelium showing intensely stained cortical cytoplasm (arrows) and unreactive nuclei. D: control section of the cornea stained with antibody pre-absorbed with pure antigen and demonstrating no immunoreactivity in the epithelium. The times of exposure and printing are similar to those in B. E–G: photomicrographs showing the location of ζ opioid receptor immunoreactivity in the human cornea. E: bright immunofluorescence of the corneal epithelium can be recorded, but little staining of the stroma was observed. F: high magnification photomicrograph of the corneal epithelium showed intense immunofluorescent of the cortical cytoplasm (arrows), but unreactive nuclei. G: control section of the cornea stained with antibody pre-absorbed with pure antigen. No immunoreactivity was recorded in the epithelium. The times of exposure and printing are similar to those in E. Bar = 60 μm (A,B,D,E,G) or 19 μm (C,F).

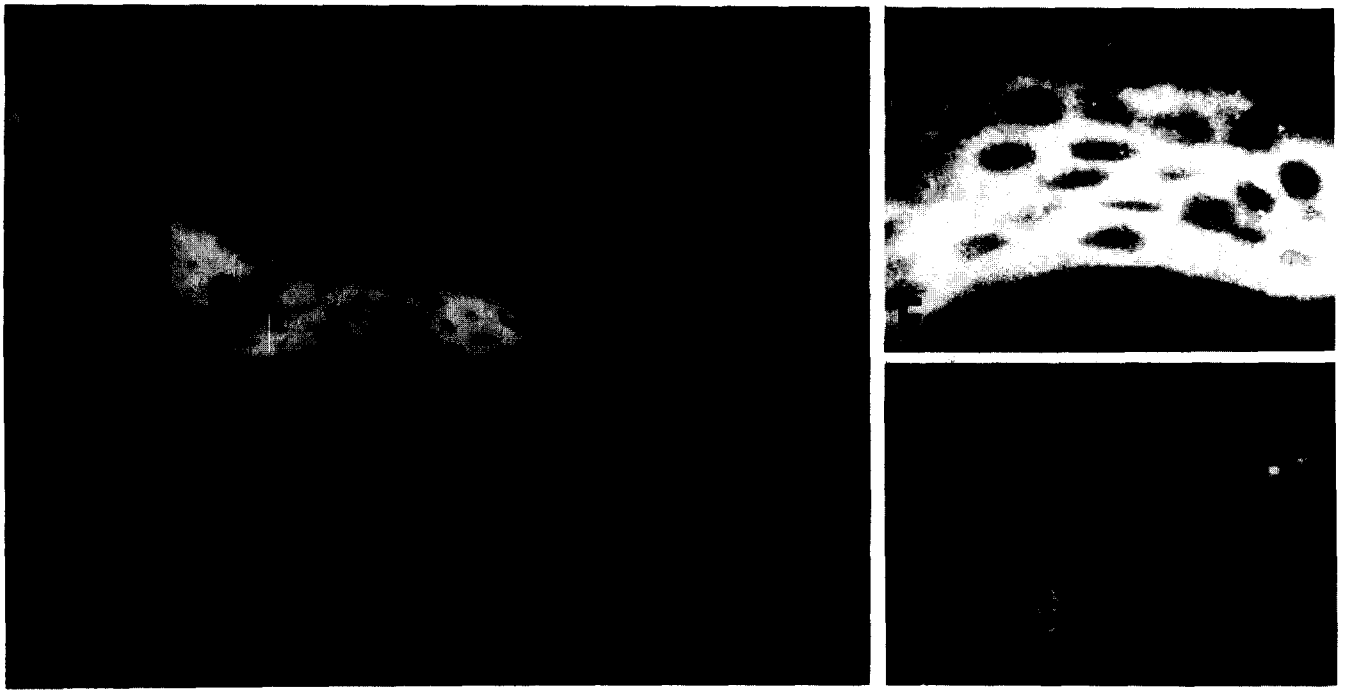

Fig. 1 (continued).

proved by the Clinical Investigation Committee of The M.S. Hershey Medical Center of The Pennsylvania State University. The eyes were enucleated following the protocol used for organ transplantation at The M.S. Hershey Medical Center, placed in ice-cold RPMI 1640 transport medium, and processed for immunocytochemistry within 48 h of death.

All donors were screened serologically for infectious diseases including hepatitis and HIV. Tissues were examined using the biomicroscope to exclude the presence of visible corneal disease. Donors included a 15-month-old male, 19-year-old male, and a 79-year-old female.

A variety of vertebrates were examined, including mouse (*Mus musculus*) (C57BL/6NCrlBR) and rat (*Rattus norvegicus*) (Crl:CD(SD)BR), obtained from Charles River Laboratories (Wilmington, MA), rabbit (*Oryctolagus cuniculus*) (RSI(NZW)SPF) acquired from Robinson Services, Inc. (Winston Salem, NC), and turtle (*Chrysemys picta*) purchased from Kons Scientific Co. (Germantown, WI). Domestic fowl (*Gallus domesticus*) were obtained from Hy-line International (Mt. Joy, PA), and frog (*Rana sylvatica*) and fish (*Carassius auratus*) from a local supplier. All investigations with animals conformed to NIH regulations and the guidelines of the Department of Comparative Medicine of The Pennsylvania State University College of Medicine.

2.2. Immunocytochemistry

Immunocytochemistry using a well-characterized polyclonal antibody to [Met^5]enkephalin (CO-172) was employed to examine for the presence of OGF. This antibody was produced in rabbits against an antigen composed of [Met^5]enkephalin linked with glutaraldehyde to bovine serum albumin. Using a quantitative immunodot assay, a 1:150 dilution of the antisera recognized 25 ng of [Met^5]enkephalin, but did not detect as much as 500 ng of β-endorphin or 1 μg of [Leu^5]enkephalin, [Met^5, Arg^6, Phe^7]enkephalin, [Met^5, Arg^6, Phe^7, Leu^8]enkephalin, or dynorphin A1-8. To localize the ζ opioid receptor, rabbit polyclonal antibody A0-440 generated to the 17 kDa polypeptide of the receptor, and previously characterized [30], was utilized.

Tissues were rinsed in 0.1 M Sorenson's phosphate buffer (SPB) (pH 7.4), immersed in 20% sucrose in SPB, frozen in isopentane cooled by dry ice, and embedded in OCT medium. Cryostat sections (15 μm) were collected on gelatin-coated slides and stored at -20°C with Drierite until use. Sections were placed in ice-cold 95% ethanol (2 min) and acetone (2 min) and rinsed with SPB. Tissues were blocked for 15 min with 3% normal goat serum (NGS) in SPB and 0.1% Triton X-100, pH 7.4. Anti-[Met^5]enkephalin IgG or anti-ζ IgG were diluted in SPB with 1% NGS and 0.1% Triton X-100 and used at a dilution of 1:150 except for the turtle (1:600 for the antibody to [Met^5]enkephalin and 1:300 for the antibody to the ζ receptor). Tissues were incubated in a humidified chamber at 4°C for 16–18 h, rinsed in SPB with 1% NGS, blocked for 15 min in SPB and 3% NGS, and incubated at 4°C with rhodamine-conjugated goat anti-rabbit IgG (1:100) (Cappel Laboratories) for 45 min. Sections were mounted in a 60% glycerol, 40% SPB solution. Immunoreactivity was visualized using an Olympus micro-

scope with a rhodamine interference filter. Controls for specificity included tissues processed with antibodies pre-absorbed with an excess of antigen, and specimens stained only with secondary antibody.

3. Results

Light microscopic and immunocytochemical examination of human corneal tissues from the three donors

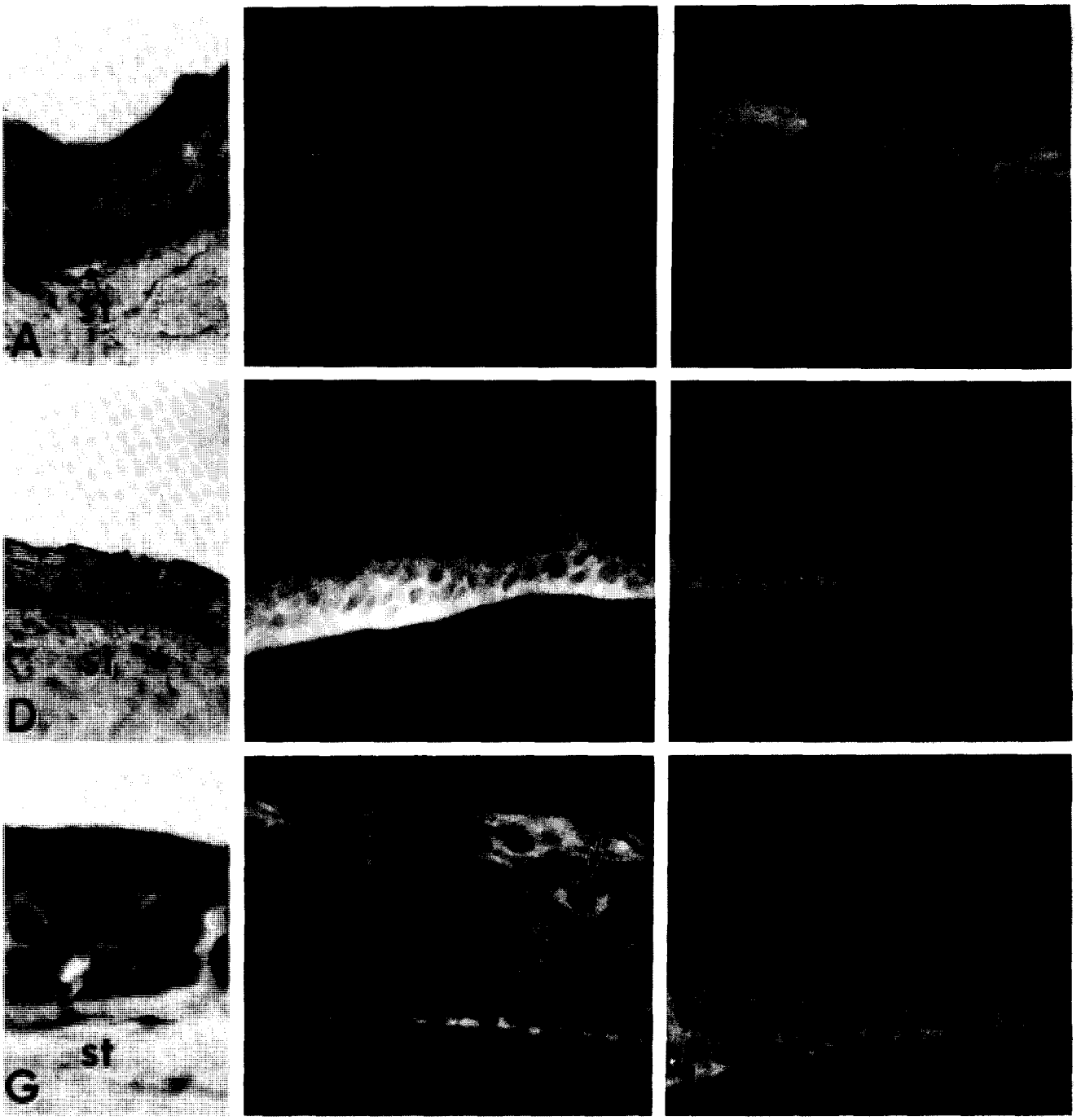

Fig. 2. Photomicrographs of frozen sections of the corneal epithelium of mouse (A–C), bird (D–F), and turtle (G–I) using brightfield (A,D,G) or indirect immunofluorescent (B,C,E,F,H,I) optics. In sections stained with hematoxylin and eosin (A,D,G), basal and suprabasal cell layers in the epithelium (ep) can be observed (st, stroma). Immunofluorescence can be seen in the cortical cytoplasm of both the basal and suprabasal cells with respect to staining with anti-[Met^5]enkephalin IgG (arrows; B,E,H) and anti-ζ receptor IgG (arrowheads; C,F,I); immunoreactivity was not associated with the cell nucleus (B,C,E,F,H,I). Control sections of the cornea stained with antibodies pre-absorbed with pure antigens, showing no immunoreactivity in the epithelium (data not shown). Bar = 27 μm.

yielded similar results. The tissues consisted of a basal layer and 4 to 5 suprabasal layers of corneal epithelium, as well as subjacent stroma (Fig. 1A). Immunocytochemical preparations utilizing an antibody to the OGF, [Met^5]enkephalin, showed intense immunofluorescence associated with both the basal and suprabasal corneal epithelial layers (Fig. 1B). High magnification observations resolved that the cortical cytoplasm of these cells was immunoreactive, but cell nuclei lacked staining (Fig. 1C). Control specimens processed with pre-absorbed antibody (Fig. 1D) or only with the secondary antibody (data not shown) were not stained with anti-[Met^5]enkephalin IgG.

Human tissues stained with antibodies to the ζ receptor exhibited a pattern of immunoreactivity similar to that observed when tissues were processed with anti-[Met^5]enkephalin IgG (Fig. 1E,F). Both the basal and suprabasal corneal epithelial cells were immunofluorescent, in contrast to the absence of immunoreactivity in the stroma (Fig. 1E). Antibody reaction in corneal epithelial cells was associated with the cortical cytoplasm, but the nuclei had no immunoreactivity (Fig. 1F). Control tissues stained with pre-absorbed antibodies (Fig. 1G), or specimens processed only with the secondary antibody (data not shown), were negative.

To examine for the presence of the OGF, [Met^5]enkephalin, and the ζ opioid receptor in the corneal epithelium of lower vertebrates, corneas from animals representing different classes of the phylum Chordata were examined by immunocytochemistry (Figs. 2 and 3). Histological preparations (Figs. 2A,D,G and 3A,D) of specimens that represented the classes Osteichthyes (*Carassius auratus*), Amphibia (*Rana sylvatica*), Reptilia (*Chrysempys picta*), Aves (*Gallus domesticus*), and Mammalia (*Mus musculus*, *Rattus norvegicus*, *Orychtolagus cuniculus*) revealed some variations in the size of cells and the number of layers of suprabasal cells among the vertebrates. Immunocytochemically stained tissues showed that the distribution of both anti-

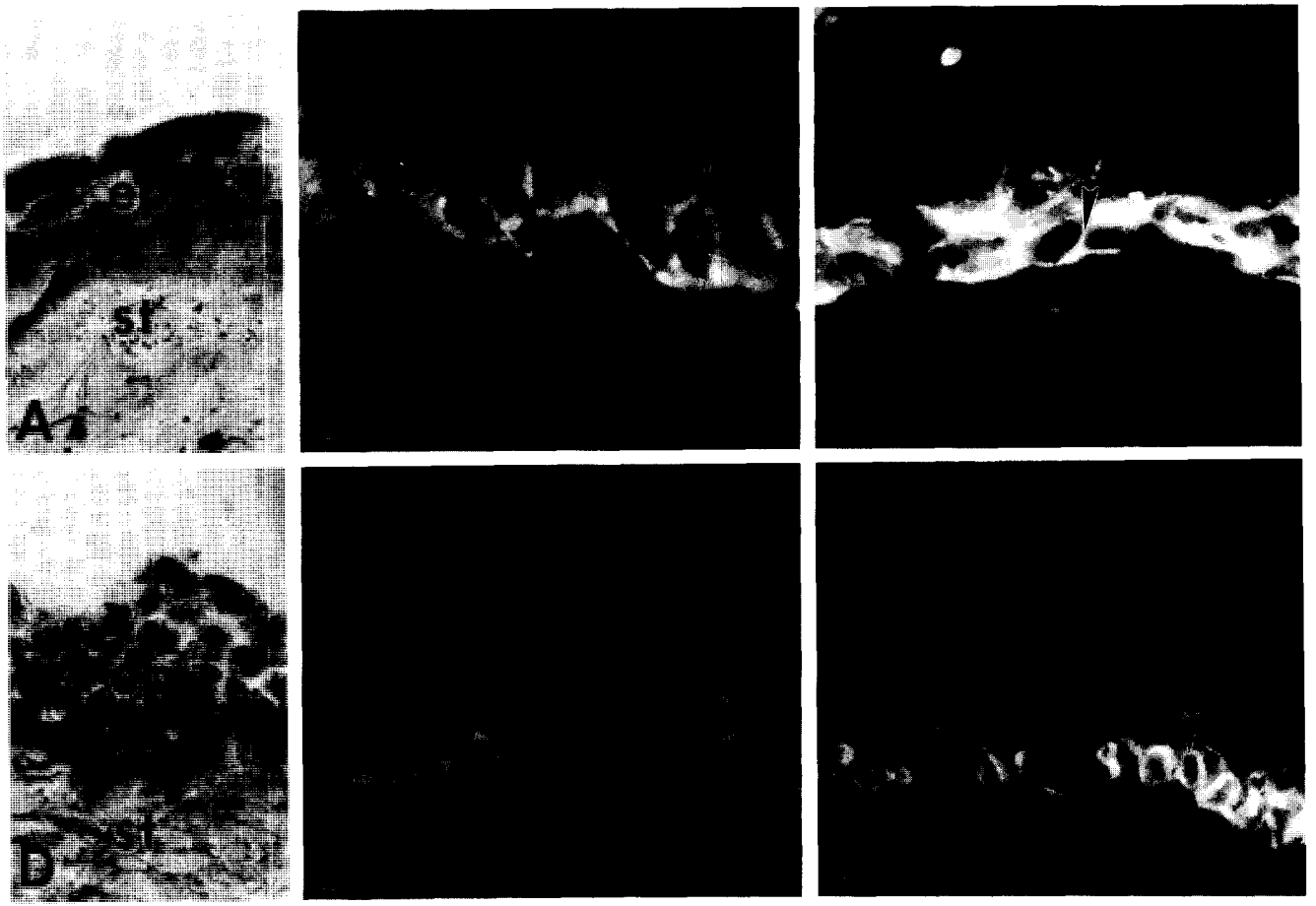

Fig. 3. Photomicrographs of frozen sections of the corneal epithelium of frog (A–C) and fish (D–F) using brightfield (A,D) or indirect immunofluorescent (B,C,E,F) optics. In sections stained with hematoxylin and eosin (A,D), the epithelium (ep) consisting of basal and suprabasal cell layers can be observed (st = stroma). Immunofluorescence can be seen in the cortical cytoplasm of both the basal and suprabasal cells with respect to staining with anti-[Met^5]enkephalin IgG (arrows; B,E) and anti-ζ receptor IgG (arrowheads; C,F); immunoreactivity was not associated with the cell nucleus (B,C,E,F). Control sections of the cornea stained with antibodies pre-absorbed with pure antigens, showed no immunoreactivity in the epithelium (data not shown). Bar = 27 μm.

[Met^5]enkephalin IgG and anti-ζ IgG was similar to that in humans. The cortical cytoplasm in both the basal and suprabasal cells of the corneal epithelium was prominently immunofluorescent using antibodies to [Met^5]enkephalin or the ζ opioid receptor (Figs. 2 and 3; data for rats and rabbits are not shown). No immunoreactivity was detected in the nuclei of epithelial cells. Staining was not observed in sections processed with antibodies to [Met^5]enkephalin or ζ receptor that were preabsorbed with respective antigens (data not shown), or in tissues subjected to the secondary antibody (data not shown).

4. Discussion

Regulation of growth, development, and neoplasia by an endogenous opioid system has been well documented [4,5,8–10,14,16,18,21,23–29,33], including developing neurons and glia, glial cells in culture, myocardial and epicardial cells in the neonatal heart, neural and non-neural cancer cells, epithelium of the tongue, and bacteria. An endogenous OGF, the 573 MW pentapeptide, [Met^5]enkephalin, has been identified in both eukaryotes and prokaryotes, and serves as a tonically active, inhibitory influence [26,27]. This opioid peptide is derived from proenkephalin A, appears to be both autocrine and paracrine produced [22], and is associated with proliferating cells as detected by immunocytochemistry [31,33]. OGF especially modulates cell replication by depressing cell division, but also appears to be important in cellular migration, maturation, survival, and tissue organization [10,13,18,21,26–29,32,33]. Opioid action in regard to growth is stereospecific and blocked by opioid antagonists (e.g. naloxone) [26,27,33], suggesting involvement of opioid receptor mechanisms. The receptor for OGF has been identified and characterized in the developing brain and neural tumor cells, and has been termed the ζ opioid receptor [29]. This receptor is an integral membrane protein associated with the nucleus, consists of 4 binding polypeptides of 32, 30, 17 and 16 kDa, and has been localized to replicating cells. The ζ receptor, however, is not affiliated with mature, non-mitotic cells [30].

A number of studies have begun to explore the role of endogenous opioids in the cornea. Using explant cultures of the rabbit corneal epithelium, Zagon and colleagues [32] have demonstrated that OGF decreased outgrowth by 60% and reduced the number of DNA synthesizing cells by 42% from control levels. The influence of OGF on explant cultures was blocked by concomitant exposure to the opioid antagonist, naloxone, indicating that OGF action occurred at the level of the opioid receptor. Using organ culture of rabbit cornea, Sassani and coworkers [13] have reported that OGF significantly repressed wound healing from control levels. Finally, Sassani et al. [12] found that disruption of opioid–receptor interactions in the rabbit cornea in vivo by daily application (4 times/day) of the potent and long-acting opioid antagonist naltrexone, markedly increased healing time of corneal epithelial abrasions. These results would suggest that OGF functions as a growth factor in both normal and wounded cornea.

In the present study, we show that the key elements of the endogenous opioid system related to growth are in the corneal epithelium of 5 classes of the subphyla Gnathostomata; specimens from the class Chondrichthyes, or the subphyla Agnatha (i.e. class Cyclostomata) were not studied. Both OGF and the ζ opioid receptor were detected in corneal epithelial cells, and were associated with the cortical cytoplasm of these cells but not their nuclei. These results are consistent with earlier observations localizing OGF and the ζ receptor in other tissues [30,31,33]. Furthermore, our data suggest the conserved biochemical composition of the ζ receptor, since an antibody produced against the ζ receptor in rat cerebellar tissue was highly reactive to antigens in a variety of lower vertebrates. Likewise, the biological structure of OGF appeared to be conserved from humans to fish. These findings raise the question of the function of the endogenous opioid system in the vertebrate corneal epithelium. If, indeed, opioids serve as growth factors in a fashion similar to that seen in other epithelial tissues [33], this intrinsic and native peptide may have important contributions in normal epithelial homeostasis and in wound healing.

Phylogenetic studies of opioids, and opioid receptor binding using [^{3}H]naloxone and [^{3}H]dihydromorphine, have demonstrated both opioid peptides and receptors in the brains of organisms representative of all classes of vertebrates [7,11,17]. The present investigation exploring an enkephalin growth factor and its opioid receptor now reveals the conserved expression of these elements in the corneal epithelium of vertebrates. Thus, the discovery that both the OGF and the ζ opioid receptor are detected in such a broad array of classes of animals in the phylum Chordata would suggest the rather ancient nature of this endogenous opioid system. The presence of both OGF and its receptor in the lowest class sampled, Osteichthyes, may indicate a growth-related function by the time of the Devonian and Carboniferous periods in the Paleozoic era. Further work examining the class Chondrichthyes, as well as the Cyclostomata in the subphyla Agnatha, could prove informative and date the evolution of this endogenous opioid system in the vertebrate cornea even earlier than the 300 million years suggested by the present study. Although the primitive nature of this peptide–receptor system in the cornea of vertebrates is impressive, it should be noted that [Met^5]enkephalin

and the ζ opioid receptor have been reported to function and be present in bacteria [28], dating this growth-related opioid complex as early as 2 billion years ago. Finally, the finding that an endogenous opioid system in the cornea has such early origins, may suggest that the function of this system in cellular renewal is a requirement of the vertebrate cornea.

Acknowledgements

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