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Presence of EGF growth factor ligands and their effects on cultured rat conjunctival goblet cell proliferation

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Abstract

The amount of mucin on the ocular surface is regulated by the rate of mucin synthesis, mucin secretion, and the number of goblet cells. We have previously shown that cholinergic agonists are potent stimuli of mucin secretion. In contrast, there have been no studies on the control of goblet cell proliferation. In this study we investigate the presence of the EGF family of growth factors and their receptors in rat conjunctiva and cultured rat conjunctival goblet cells as well as their effects on activation of signaling intermediates and goblet cell proliferation. Rat conjunctival goblet cells were grown in organ culture and identified as goblet cells by their morphology and positive staining for the lectin UEA-1 and cytokeratin 7. In the rat conjunctiva, the presence of the EGF family members epidermal growth factor (EGF), transforming growth factor α (TGF-α), heparin binding EGF (HB-EGF), and heregulin was determined by RT-PCR. The receptors for these ligands, EGF receptor (EGFR), erbB2, erbB3, and erbB4 were detected in both rat conjunctiva and goblet cells by Western blot analysis. Immunofluorescence microscopy of conjunctival tissue determined that EGFR was present as punctate staining in the cytoplasm of conjunctival goblet cells while ErbB2 was present in the basolateral and lateral membranes of goblet cells. ErbB3 was localized to the cytosol of rat conjunctival goblet cells. In cultured goblet cells, EGFR and ErbB2 were present in the perinuclear area of the cells. ErbB3 was widely distributed throughout the cytoplasm of the cells. ErbB4 was not detected in either the conjunctiva or goblet cells by immunofluorescence microscopy. Using a multiplex assay system we measured phosphorylation (activation) of p44/p42 mitogen-activated protein kinase (MAPK), also known as ERK, Jun N-terminal kinase (JNK), p38 MAPK and AKT (also known as protein kinase B), molecules known to be activated by EGF receptor members. EGF, TGF-α and HB-EGF activated the signaling intermediate proteins whereas heregulin did not. No EGF family member significantly activated AKT. Consistent with these findings, EGF, TGF-α and HB-EGF each stimulated goblet cell proliferation as measured by WST-1 assay or immunofluorescence microscopy using an antibody against Ki-67, a protein expressed in dividing cells. Heregulin did not cause goblet cell proliferation. We conclude that multiple members of the EGF family, EGF, TGF-α and HB-EGF, and heregulin are present with three of the four erbB receptor subtypes. EGF, TGF-α and HB-EGF all stimulated the activation of signaling intermediates and caused goblet cell proliferation.

Keywords: goblet cell; proliferation; secretion; conjunctiva

1. Introduction

The EGF family of ligands interacts with a family of receptor tyrosine kinases known as ErbB receptors. There are four ErbB receptors, the EGF receptor (EGFR, HER1, or ErbB1), ErbB2 (HER2/neu), ErbB3, and ErbB4. The ligands for ErbB receptors can be divided into three groups. The first group is comprised of epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), betacellulin, and amphiregulin, which bind to

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EGFR. Heparin-binding EGF (HB-EGF) and epiregulin, which bind to EGFR and ErbB4, represent the second group. The third group consists of heregulin (HG) isoforms and neu differentiation factor, which bind to ErbB3 and ErbB4. Ligands bind to the ErbB receptors resulting in the formation of homo- and heterodimers and autophosphorylation of specific tyrosine residues in the cytoplasmic domain of the receptor (Iwamoto and Mekada, 2006). Tyrosine phosphorylated ErbB receptors bind to adaptor proteins, which mediate a variety of signal transduction pathways that elicit downstream functional responses. Downstream adaptors include Shc/Grb2, phosphoinositide-3 kinase (PI3K), phospholipase C γ (PLCγ), Src, Vav, Nck, Grb7, and Crk (Yarden and Sliwkowski, 2001). Downstream adaptors activate cascades of enzymes such as protein kinase C (PKC), AKT (also known as protein kinase B), extracellular regulated kinase (ERK 1/2, also known as p44/p42 mitogen-activated protein kinase (MAPK)), Jun N-terminal kinase (JNK), and Abelson tyrosine kinase (Abl) that stimulate nuclear transcription factors to activate functions such as proliferation, apoptosis, migration, adhesion and differentiation. In this study, we explored the role of members of each group of the EGF family of growth factors, EGF, TGF-α, HB-EGF, and heregulin in stimulating conjunctival goblet cell signaling pathways leading to proliferation.

In most vertebrate tissues, the different EGF ligands bind to the appropriate ErbB receptors with varying degrees of preference causing formation and activation of distinct homo- and heterodimers. Each type of ErbB receptor dimer has different sets of phosphorylated tyrosines attracting distinct sets of adaptor proteins causing ligand specific responses. The existence of an ErbB receptor, ErbB2, for which no ligand has yet been identified and an ErbB receptor, ErbB3, with no kinase activity adds an additional level of complexity. Furthermore, certain types of heterodimers are preferentially formed, especially those with ErbB2 as a partner, and certain heterodimers (Erb2/Erb3) have enhanced activity. Thus, the EGF family of ligands can differentially stimulate a given function or stimulate diverse functions. For examples, TGF-α and EGFR are expressed in lung, ovary, and colon tumors (Yarden and Sliwkowski, 2001). ErbB2 is associated with breast cancer (Meric-Bernstam and Hung, 2006). ErbB2/ErbB3 heterodimers are found in prostate cancer (Li et al., 2006). Some epithelial tumors express ErbB4/ErbB2 (Normanno et al., 2005). In addition, HB-EGF's activation of ErbB2 is needed for the maintenance of homeostasis in the heart, whereas its activation of EGFR is required for cardiac development (Iwamoto and Mekada, 2006). In this study we investigated if representatives of the EGF ligands that bind to and activate the four ErbB receptors cause differential effects on goblet cell proliferation.

Goblet cells are present in all wet-surfaced epithelia and synthesize, store, and secrete high molecular weight glycoproteins, including mucins that function to protect these epithelia from changes in the external environment (Perez-Vilar and Mabolo, 2007). The conjunctiva is no exception. This epithelium, with the cornea, forms the ocular surface. Goblet cells in the conjunctiva secrete the mucin MUC5AC into the inner mucous layer of the tear film providing a physical and chemical barrier to maintain a healthy ocular surface (Dartt, 2002). A mucus layer that is optimum in amount and composition is critical to the maintenance of the ocular surface, as either an increase or a decrease in the amount of mucin induces disease. Mucin deficiency is a consequence of diseases such as dry eye syndromes, ocular cicatricial pemphigoid, Stevens-Johnson syndrome, alkali burns, herpes simplex keratitis, and neurotrophic keratitis (Tseng et al., 1984). In contrast, overproduction of mucin leads to the ocular surface disease that occurs in atopy, seasonal allergic conjunctivitis, and mucus fishing syndromes (McCulley et al., 1985; Roat et al., 1993; Dogru et al., 2005). Both an increase and a decrease in mucin production cause disease suggesting that mucin production is tightly regulated.

The amount of mucin on the ocular surface is regulated by controlling the rate of mucin synthesis, the rate of mucin secretion, and the number of goblet cells. We previously found that cholinergic agonists are potent and effective stimuli of goblet cell mucin secretion and work by increasing the intracellular Ca^{2+} concentration and activating PKC isoforms that stimulate the non-receptor tyrosine kinases Pyk2 and Src (Dartt et al., 2000; Kanno et al., 2003). These kinases in turn transactivate the EGFR receptor thereby inducing the ERK pathway. In contrast to secretion there are no studies on the control of conjunctival goblet cell number i.e. goblet cell proliferation. It has been demonstrated that the number of conjunctival goblet cells changes in diseases of mucus over- and underproduction (Lemp, 1992). Conjunctival goblet cell proliferation has been indirectly linked to EGF as Pflugfelder et al. (1999) found that the tear EGF concentration was decreased in patients with Sjogren's syndrome compared to normal controls and that EGF concentration was correlated with conjunctival goblet cell number. There are no studies on the role of other members of the EGF family of growth factors on conjunctival goblet cell function. Further study of goblet cell proliferation and the role of EGF and its family members has been hampered by the lack of an appropriate model that allows easy, accurate study of the proliferation of goblet cells uncontaminated by other conjunctival cell types. Our laboratory has developed a method to culture both rat and human goblet cells in primary culture (Shatos et al., 2001, 2003). We extensively characterized these cells using multiple markers of goblet cell secretory products and cell bodies. In the present study we investigated the regulation of the number of conjunctival goblet cells by studying goblet cell proliferation in culture. We found that EGF, TGF-α, and HB-EGF are: (1) present in conjunctival goblet cells; (2) interact with their appropriate receptors that are present; (3) differentially activate p44/p42 MAPK, p38 MAPK, and JNK, and possibly AKT, signaling pathways; and (4) stimulate goblet cell proliferation. Although heregulin and the ErbB receptor subtypes to which it binds are present in the conjunctiva and in goblet cells, heregulin does not activate any of the above signaling pathways, nor does it stimulate goblet cell proliferation.

2. Materials and methods

2.1. Materials

EGF and TGF-α were from PeproTech, Inc. (Rocky Hill, NJ); HB-EGF and Heregulin were from Sigma-Aldrich