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Activation of the epidermal growth factor receptor by hydrogels in artificial tears

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Abstract

Most formulations of artificial tears include high-molecular weight hydrophilic polymers (hydrogels) that are usually thought to serve to enhance viscosity and to act as demulcents. A few reports have indicated that application of some of the polymers accelerates healing of wounds in epithelia. Since activation of the epidermal growth factor (EGF) receptor is critical for spontaneous corneal epithelial wound healing, we tested commonly used hydrogels for their ability to activate the EGF receptor and enhance closure of wounds. Five structurally unrelated hydrogels used in artificial tears were found to activate the EGF receptor. Importantly, two of the hydrogels enhanced wound healing in an organ culture model. We propose that the efficacy of hydrogels in treating dry eye may be related to their ability to activate the EGF receptor, and that hydrogels are inexpensive, safe agents to promote healing of wounds in the cornea and possibly in other tissues.

Keywords: corneal wound healing; hydrogel; carboxymethyl cellulose; hydroxypropylmethyl cellulose; methyl cellulose; povidone; polyvinyl alcohol

1. Introduction

Application of artificial tears is the most common, and often adequate, therapy for the dry eye group of ocular surface disorders (Johnson and Murphy, 2004). Artificial tears are aqueous solutions of electrolytes, usually containing high-molecular weight hydrophilic polymers (hydrogels). The hydrogel components are typically listed as the ''active'' ingredients, and they are thought to act by providing viscosity, increased oncotic pressure, and possibly some ill-defined demulcent action. A few reports have indicated that hydrogels, such as methyl cellulose and carboxymethyl cellulose, enhance healing of wounds in the cornea (Garrett et al., 2007; Gaton et al., 1998; Lin and Boehnke, 1999).

The epidermal growth factor receptor is a transmembrane tyrosine kinase receptor, that can be activated by a multitude of diverse mechanisms such as binding to extracellular ligands, phosphorylation by non-receptor tyrosine kinases such as p60src, and through inhibition of tyrosine phosphatases that dephosphorylate the receptor (Bazley and Gullick, 2005; Fischer et al., 2003; Warren and Landgraf, 2006; Wells, 2000). The activated, tyrosine phosphorylated receptor provides binding sites for many signaling molecules, and it triggers numerous intracellular signaling pathways, including phospholipase C-gamma, phosphatidylinositol 3′-kinase, and extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Oda et al., 2005). The EGF receptor is activated upon wounding of the corneal epithelium, and this is absolutely required for the subsequent initiation of migration and healing (Block et al., 2004; Nakamura et al., 2001; Xu et al., 2004; Zieske et al., 2000). Given the importance of EGF receptor activation for motility in corneal epithelial cells after wounding, we examined whether hydrogels used in artificial tears might activate the receptor, and we analyzed their effects on corneal epithelial cell motility in an organ culture model.

2. Materials and methods

2.1. Materials

HCLE cells were kindly provided by Dr. Ilene Gipson. Hydroxypropylmethyl cellulose (4000 centipoise, 2% solution),

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dextran (average molecular weight 255 kDa) and povidone (embryo tested) were from Sigma–Aldrich, carboxymethyl cellulose (high viscosity), methyl cellulose (4000 centipoise, 2% solution), and propylene glycol were from MP biomedicals, polyvinyl alcohol (86–89% hydrolyzed) and polyethylene glycol 400 were from Alfa Aesar. Antibodies against phospho-ERK1/2, the EGF receptor phosphorylated on tyrosine 1173, and against a C-terminal epitope (to detect total EGF receptor) were from Santa Cruz Biotechnology. Rabbit eyes were from Pel-Freez Biologicals.

2.2. Cell culture, cell treatments and Western blotting

HCLE cells were grown to confluence in keratinocyte serum-free medium (Gibco–Invitrogen) supplemented with 25 μg/ml bovine pituitary extract, 0.2 ng/ml EGF and 0.3 mM $CaCl_2$. Stimulations were performed after incubating the cells overnight in the same medium without the added EGF and pituitary extract. Cells were stimulated for the indicated times at 37 °C, rinsed quickly in ice-cold phosphate-buffered saline (171 mM NaCl, 10.1 mM Na_2HPO_4, 3.35 mM KCl, 1.84 mM KH_2PO_4, pH 7.2) and reactions were terminated by addition of SDS-containing sample buffer. Samples were normalized for protein using the BCA reagent (Pierce) and run on 10% polyacrylamide gels in Mini-Protean 3 apparatus (Bio-Rad). Western blotting was performed according to standard procedures, and blots were developed using the SuperSignal® Dura detection kit (Pierce). Equal loading in lanes was verified by staining blots with Ponceau S red (Block et al., 2004). All experiments were performed at least three times with triplicate determinations. To stratify the cells, they were transferred to Dulbecco's Modified Eagles Medium:F12 1:1, with 10% new-born calf serum and 10 ng/ml EGF, which contains high levels of calcium for 3 days (Gipson et al., 2003). Stimulations under the stratified conditions were done after starvation overnight with no EGF and 2% new-born calf serum in the same medium.

2.3. Wounding in organ culture

To inflict wounds in rabbit eyes in organ culture, a 7.5 mm diameter mark was made with a trephine, and the epithelium was removed by means of an Algerbrush II (Alger Equipment Co.) and a sharp forceps. The wounded corneas were excised with a 2–3 mm scleral rim and placed on hemispheric supports made from the round end of transfer pipets (Samco Scientific Corporation) in 12-well dishes. The wound was briefly stained with 0.1% fluorescein and photographed. They were subsequently incubated submerged at 37 °C in Ham's F12 Medium:Dulbecco's Modified Eagle's medium (1:1). Initial time-course experiments determined the optimal incubation time of the organ cultures to 60 h. The time-course analysis demonstrated that healing takes place during the whole incubation period, i.e., that the cells are actively covering the wound even at the end of the incubation period. The corneas were then stained with 1% alizarin red in phosphate-buffered saline and photographed again. The percentage of healing was calculated using MetaMorph software (Molecular Devices) based on the areas of the wounds before and after incubation for each cornea.

3. Results

3.1. Activation of the EGF receptor by hydrogels

HCLE is a human corneal limbal epithelial cell-line that has been immortalized by a three-step process involving abrogation of $p16^{INK4A/Rb}$ and p53 functions, and overexpression of the catalytic subunit of the telomerase holoenzyme. When grown in low Ca^{2+} concentrations, they grow as monolayers, but stratify upon transfer to high Ca^{2+}. They express a range of keratins and mucins similar to that expressed in native corneal epithelium (Gipson et al., 2003).

Unstratified HCLE cells were incubated with hydrogels that are commonly used in commercially available artificial tears, using concentrations in the ranges of the formulations. To test for activation of the EGF receptor, we immunoblotted extracts with an antibody that recognizes the EGF receptor phosphorylated on tyrosine 1173, which is the major binding site for the adaptor protein shc (Batzer et al., 1994). Of the seven polymers that were tested, five activated the EGF receptor (Fig. 1). Methyl cellulose and povidone were most potent, but significant activation was also seen with hydroxypropylmethyl cellulose (also known as hypromellose), carboxymethyl cellulose, and polyvinyl alcohol. Dextran, polyethylene glycol, glycerol, and propylene glycol 400, were inactive. The activating ability of the compounds is not dependent on charge, as most of the compounds are uncharged. Three of the active compounds have a backbone of cellulose, whereas the synthetic compounds polyvinyl alcohol and povidone do not, so there is no obvious structural requirement for the ability to activate the EGF receptor. Other components in artificial tears, which are occasionally listed as ''active'' ingredients, such as glycerol and propylene glycol did not activate the EGF receptor (Fig. 1). Methyl cellulose, hydroxypropylmethyl cellulose and carboxymethyl cellulose were also tested on stratified HCLE cells and found to activate the EGF receptor (data not shown). The level of activation was lower than that seen in stratified HCLE cells, but it should be noted that the cells are grown under very different conditions in the two states (see methods), and that a direct comparison is therefore not possible.

Time-course studies showed that activation by the hydrogels was rapid, and a peak of activity was detected starting at 15 s. For illustration, the time-course of activation by methyl cellulose is presented in Fig. 2A. The receptor activation was accompanied by a peak of ERK1/2 activation after a lag phase of 3.5–5 min. This is qualitatively similar to that seen after stimulation with ligands of the EGF receptor, which also results in a peak of activation. For comparison purposes, cells were stimulated with HB-EGF for 10 min, which shows the maximal level of stimulation that can be archived in the HCLE cells with exogenous ligand.

The EGF receptor has been reported to be activated by changes in tonicity of the medium (Lezama et al., 2005),