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Galectin-3 enhances extracellular matrix associations and wound healing in monkey corneal epithelium

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ABSTRACT

Poor healing of epithelial wounds in cornea is a major clinical problem, leading to persistent epithelial defects and ulceration. The primary cause is poor cell migration over the wound. Carbohydrate-binding protein galectin-3 binds to extracellular matrixes (ECMs) and promotes lamellipodia formation by cross-linking to α3 integrin. Recombinant galectin-3 also facilitates wound healing in the rodent cornea. The purposes of the present experiments were to: (1) establish epithelial wound healing models in monkey corneal explant culture, the models more relevant to human, (2) evaluate the healing effect of galectin-3 in our models, and (3) determine if galectin-3 enhances cell adhesion by interacting with ECMs on corneal surface and their ligand integrins. Monkey corneas with central wounds produced by sodium hydroxide (NaOH) or n-heptanol were incubated with or without recombinant galectin-3. The defected area was stained with sodium fluorescein. Primary isolated corneal epithelial cells from monkey were cultured with or without galectin-3 on plates coated with ECMs or integrins, and the number of adhering cells was counted. Galectin-3 expression in various eye tissues was visualized by immunoblotting. NaOH caused loss of epithelial cells and basement membrane. n-Heptanol removed epithelial cells, but the basement membrane was retained. These corneal defects spontaneously became smaller in a time-dependent manner. Exogenous galectin-3 enhanced wound healing in both NaOH and n-heptanol models. Galectin-3 also enhanced cell adhesion onto the major ECMs found in the basement and Bowman's membranes and onto integrins. Relatively high levels of galectin-3 were detected in corneal and conjunctival epithelium, but tear fluid contained negligible galactin-3. These results suggested that the enhanced binding of epithelial cells to ECMs and integrins caused by galectin-3 might promote cell migration over wounded corneal surfaces. Since tear fluid contained relatively low levels of galectin-3, exogenous galectin-3 may be a beneficial drug to enhance re-epithelialization in human corneal diseases.

1. Introduction

Following the loss of the corneal epithelium, the remaining epithelial cells attempt to cover the denuded wound. Poor healing of corneal wounds is a major clinical problem, leading to persistent epithelial defects and ulceration. Corneal epithelial defects may be caused by various situations such as refractive surgery, contact lens wear, dry eye, diabetes, and burns from chemical and thermal agents (Ambrósio et al., 2008; Galentine et al., 1984; Schultz et al., 1981; Chahud et al., 2009). Corneal epithelial wound healing is characterized by three phases; cell migration, cell proliferation, and cell differentiation (Suzuki et al., 2003). Inadequate migration of epithelial cells is the primary cause of poor healing. Although the growth factors, neurotrophins, fibronectin and cytokines accelerate corneal re-epithelialization, they have not been developed to clinical drugs. For example, epidermal growth factor accelerates corneal epithelial healing in a clinical trial (Pastor and Calonge, 1992), but corneal neovascularization was a risk factor (Yang

Abbreviations: ECM, extracellular matrix; NaOH, sodium hydroxide; FAK, focal adhesion kinase; HBSS, Hank's balanced salt solution; MEM, Minimum Essential Medium; H & E, hematoxylin and eosin; DMEM/F12, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12; DPBS, Dulbecco's phosphate buffer saline; BSA, bovine serum albumin; TTBS, Tris-buffered saline with 0.05% Tween 20; MMP, matrix metalloproteinase.

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et al., 2013; Burling et al., 2000).

Galectins are β-galactoside-binding animal lectins, and are expressed in human cornea (Hrdlicková-Cela et al., 2001). The gene homology for galectin-3 between human and mouse/rat is 87% and 83%, respectively. This is nearly the same as the 95% homology between man and monkey (*M. mulatta*). Re-epithelialization of corneal wounds is significantly slower in galectin-3 knock-out mice, and exogenous galectin-3 accelerates epithelial wound healing in rodent cornea (Cao et al., 2002; Yabuta et al., 2014). Potentially, galectin-3 ligand binding may modulate cell-to-cell and cell-to-extracellular matrix (ECM) interactions (Pricci et al., 2000). Galectin-3 cross-links N-glycans of integrin α3β1 (a ligand to laminin-5), induces lamellipodia formation, and promotes migration of corneal epithelial cells (Saravanan et al., 2009). In tumor cells, interaction of galectin-3 with integrin α5β1 modulates cell adhesion and motility through fibronectin remodeling (Lagana et al., 2006).

The corneal epithelial basement membrane plays important roles in maintaining a healthy epithelium and healing wounds (Torricelli et al., 2013). Galectin-3 enhances corneal wound healing by promoting adherence of epithelial cells onto collagen IV in the basement membrane (Yabuta et al., 2014). Other components of the basement membrane, such as fibronectin and laminin, are also involved in process of wound healing. Fibronectin increases adhesion and motility of corneal epithelial cells through activating Rac1-p21-activating kinase pathway (Kimura et al., 2006). Laminin-5 binding to α3β1 integrin accelerates keratinocytes migration by activation of focal adhesion kinase (FAK)-Rac1 pathway and formation of lamellipodia (Choma et al., 2007, 2004). These previous reports indicated that exogenous galectin-3 might be a good candidate drug to promote corneal wound healing. However, most studies used rodents, where Bowman's membrane is thinner and forms a fuzzy border with the stroma, compared to the abundant Bowman's membrane in human (Merindano et al., 2002; Hayashi et al., 2002). Thus, the purposes of the present study were to (1) establish a more relevant epithelial wound healing models using cultured explanted monkey corneas, (2) evaluate the efficacy of galectin-3 on corneal wound healing in our monkey model, and (3) measure the influence of galectin-3 on the adherence of monkey epithelial cells onto ECMs and integrins.

2. Materials and methods

2.1. Experimental animals

Sixty eyes, two tear samples, and a blood sample from 36 rhesus monkeys (*M. mulatta*) ranging in age from 1 to 12 years were obtained at necropsy from the Oregon National Primate Research Center (Beaverton, OR). Acceptable variability (see below) in tissue sampling was unavoidable, because, for ethical reasons, monkey tissues could only be obtained when they became available from experiments unrelated to the present studies. The excised eyes were soaked in ice-cold Hank's balanced salt solution (HBSS) (Life Technologies, Carlsbad, CA), and the average time between death and use of eyes was less than 2 h. Experimental animals were handled in accordance with the ARVO Statement for the use of Animals in Ophthalmic and Vision Research and the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23).

2.2. Culture of corneal explants

Corneal wounds were produced in the central cornea of dissected globes by applying a 7.5 mm diameter filter paper disk soaked in 1 N sodium hydroxide (NaOH) (Sigma–Aldrich, St. Louis, MO) for 60 s or n-heptanol (Alfa Aesar, Ward Hill, MA) for 90 s. The cornea was then rinsed with HBSS, and damaged epithelium was gently removed with a surgical blade. The cornea was then excised along with a narrow rim of sclera, and the conjunctiva was trimmed off as much as possible. The corneal sizes were similar between young and adult monkeys and were nearly equal between right and left eyes (e.g., 3 yrs = 13 mm diameter; 11 yrs = 14 mm diameter). The corneal explants were then incubated in Minimum Essential Medium (MEM) (Life Technologies) containing 1% (vol/vol) MEM non-essential amino acids (Life Technologies), 2 mM L-glutamine (Life Technologies) and 100 unit penicillin/mL and 100 μg streptomycin/mL (Life Technologies) at 37 °C. Human recombinant galectin-3 (BioVision, Inc., Milpitas, CA) was added at the level of 20 μg/mL because galectin-3 was previously shown to enhance corneal wound healing in a mouse organ culture in a dose dependent manner to at least 20 μg/mL (Cao et al., 2002).

The corneal wounds were stained with 1% (wt/vol) fluorescein sodium (Sigma–Aldrich), and excess fluorescein was gently wiped from the area. Images were captured with a FluorChem FC2 imager (Alpha Innotech Corp., San Leandro, CA) and complied in image analysis software (ImageJ Launcher 1.4.3.67). Percent healing was calculated as: 100 − (stained area at each point/stained area at 0 h) × 100. To avoid artifacts due to repeated fluorescein staining and wiping, all corneas were measured at one time point, so that 21 eyes were used in an experiment for spontaneous wound healing. Thus, twenty eyes from 10 monkeys were used to assess the effect of galectin-3: 5 eyes each for NaOH or n-heptanol treatment alone for controls, and the contralateral corneas were treated 5 eyes each with galectin-3 plus NaOH or galectin-3 plus n-heptanol.

One cornea from each time point in each group (9 eyes) was subjected to histological observations. The corneal explants were fixed in buffered formalin more than 1 day and were processed for paraffin-embedded sections. Four-micrometer of sections were stained with hematoxylin and eosin (H & E).

One cornea from each group (3 total eyes) was subjected to transmission electron microscopy. Monkey corneas were immediately fixed after wounding in 0.1 M sodium cacodylate buffer (pH7.4) containing 1.5% (wt/vol) glutaraldehyde and 1.5% (wt/vol) paraformaldehyde, then postfixed in 1% (wt/vol) osmium tetroxide, and embedded in Epon epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed with an electron microscope FEI Tecnai 12 TEM (FEI, Hillsboro, OR).

2.3. Isolation of monkey corneal epithelial cells

Monkey corneal epithelial cells were isolated following a protocol previously reported, with some modifications (Kawakita et al., 2004). Enucleated corneas were incubated overnight at 4 °C in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (Life Technologies) with Corneal Epithelial Cell Growth Kit (American Type Culture Collection, Manassas, VA) containing 15 mg dispase II/mL (Roche Diagnostics Corp., Indianapolis, IN). Corneal epithelial sheets were peeled off in HBSS and were then digested with 0.05% trypsin-EDTA solution (Life Technologies) at 37 °C. After 3 min incubation, the reaction was stopped by addition of 1.3% (wt/vol) soybean trypsin inhibitor (Sigma–Aldrich). Corneal epithelial cells were collected after dissociation by pipetting, filtration through a 100 μm cell strainer (BD Biosciences, Franklin Lakes, NJ), and washing with DMEM/F12.

2.4. Adhesion of monkey corneal epithelial cells to ECMs and integrins

Ninety six well polystyrene culture plates (Greiner Bio-One International GmbH, Kremsmünster, Austria) were coated for 1 h at