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Inhibitory effects of trehalose on fibroblast proliferation and implications for ocular surgery

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ABSTRACT

Trehalose is a disaccharide which plays an important role in preserving cells from completely dehydrated circumstances. In this study, we investigated effects of trehalose on proliferative activity of fibroblasts and epithelial cells both in vitro and in vivo. As in vitro assessment, normal human dermal fibroblasts and normal human epidermal keratinocytes were cultured in media containing various concentrations of trehalose. Growth activities of cells were evaluated with MTT assay and diff-quick™ staining. Expressions of vimentin and α smooth muscle actin (α-SMA) changed by trehalose were semiquantitatively measured by Western blot. As an in vivo study, 5% or 10% trehalose was topically instilled onto rabbit eyes after simple conjunctival incision or trabeculectomy. Condition of the surgical wound was evaluated by morphologically and immunohistochemically using isolectin B4 and antibodies specific for vimentin and α-SMA. Intraocular pressures (IOPs) after trabeculectomy were compared between eyes treated with trehalose and 0.04% mitomycin C (MMC). Results obtained by in vitro experiments showed that growth activities of cultured fibroblasts and keratinocytes were inhibited by trehalose in a dose-dependent manner. Fibroblasts were strongly inhibited by trehalose concentrations ≧ 5% of trehalose, whereas keratinocytes were less inhibited compared to fibroblasts. Expressions of vimentin and α-SMA were reduced by trehalose. With in vivo experiments, postoperative application of trehalose resulted in less firm adhesion between conjunctiva and sclera compared to controls. Immunohistochemical studies showed reduced staining of isolectin B4, vimentin and α-SMA in conjunctival wounds treated by topical trehalose. Also, after trabeculectomy, IOP remained in a low range during instillation of topical trehalose solution. We concluded that trehalose has inhibitory effects on proliferation of fibroblasts and vascular tissues, partially due to inhibition of transformation of fibroblasts into myofibroblasts in wound tissues. The present results imply that trehalose can be a potential agent for preventing postoperative fibrous scar formation after ocular surgery such as glaucoma filtration surgery.

1. Introduction

Postoperative adhesion is one of many serious complications for most surgeries. In particular, ophthalmic surgeons occasionally have a hard time overcoming postoperative scar formation at the time of re-operation for strabismus, retinal detachment or glaucoma, particularly filtering surgery for glaucoma.

When inflammation occurs in the tissues due to external or internal causes, inflammatory cells such as leukocytes, fibroblasts, macrophages, mast cells and platelets gather in and around the affected areas. These cells crosstalk by up- or down-regulating cytokines (interleukin 1, tumor necrosis factor α, etc.), growth factors (epidermal growth factor, transforming growth factor (TGF) α/β, platelet-derived growth factor, basic fibroblast growth factor, vascular endothelial growth factor, etc.), and chemokines among each other (Ooi et al., 2006; Banerjee et al., 2007; Hashida et al., 2005; Li et al., 2007; Nakamura et al., 2004; Singer and Clark, 1999; Wynn, 2008; Lee et al., 2008; Abu EI-Asrar et al., 2008). As a result, the process of inflammation becomes aggravated. Above all, fibroblasts play the most important role in scar formation after wound-healing processes. In the usual process of wound healing, inflammatory cells gather moderately and in a balanced manner, and the wound heals nicely and completely. Conversely, when excessive wound healing occurs with extraordinary and abnormal numbers of fibroblasts gathering around the wound, an abnormal scar forms as a result. As an initial sign of abnormal scar formation,

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fibroblasts are known to transform into myofibroblasts that specifically express α smooth muscle actin (α-SMA) in the inflammatory or wound area (Honjo et al., 2007; Abe et al., 2001; Mori et al., 2005). The greater the number of myofibroblasts transformed in the wound, the firmer and more solid the resulting scar becomes. For example, the main reason for failure of glaucoma filtration surgery is excessive scar formation in subconjunctival or episcleral tissues (Skuta and Parrish, 1987; Mietz et al., 1996; Bruno et al., 2007).

Trehalose, α-D-glucopyranosyl-α-D-glucopyranoside, is a disaccharide isomer of sucrose with numerous interesting properties. This substance is widely present in animals, plants, insects and microorganisms and plays an important role in preserving cells from completely dehydrated circumstances (Crowe et al., 1992; Mayer and Candy, 1969). Trehalose reportedly has an inhibitory effect on denaturation of protein and membranes in bacterial (Leslie et al., 1995) and human cells (Guo et al., 2000), protective effects on cryo-preserved cells under freeze-dried condition (Nakamura et al., 2008) and corneal epithelial cells under dry eye conditions (Matsuo et al., 2002), and possible therapeutic effects against the progression of Huntington's disease (Tanaka et al., 2004). The safety and efficacy of topical instillation of trehalose eye drops have been already confirmed (Matsuo, 2004).

Although the protective effects of trehalose on epithelial cells are well known, effects on fibroblasts, particularly actively proliferating fibroblasts in inflammatory or wound tissues, remain largely unknown. To the best of our knowledge, the control of fibroblasts in inflammation and wound healing with trehalose has not yet been reported. The present study investigated whether trehalose could inhibit or accelerate proliferation of fibroblasts using in vitro and in vivo experiments. If trehalose shows inhibitory effects against fibroblast proliferation, it would offer a potential agent for preventing postoperative fibrous scar formation, particularly after glaucoma filtration surgery, and may help to avoid serious complications such as endophthalmitis and bleb failure after using mitomycin C (MMC) as currently applied during trabeculectomy (Kitazawa et al., 1991, 1993; Greenfield and Parrish, 1996; Sihota et al., 2000; DeBry et al., 2002).

2. Materials and methods

2.1. Cell culture and cell treatment

Cell culture dishes, plates, centrifuge tubes, and other plastic ware were purchased from Nunc™ (Thermo Fisher Scientific, Roskllide, Denmark). Normal human dermal fibroblasts (NHDF), normal human epidermal keratinocytes (NHEK) and Medium 106S were purchased from Kurabo Industries (Osaka, Japan). NHDF and NHEK were inoculated in 15-mm dishes at 1000 cells/dish and cultured with or without trehalose containing medium (40%, 20%, 10%, 5%, 2.5%, 1.25% or 0.65%) for 2–11 days. Culture media were changed at 24 h once into the media containing the same concentration of trehalose (0% to 40%), respectively, and then culture was continued with the same culture media without any change. Cultured cells were stained with diff-quick™ staining (Dade Behring, Deerfield, Illinois) after 3, 6 or 10 days. For these cells, time courses of cell growth and survival were measured colorimetrically using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) test at the time of 2, 5, 9 or 11 days and 5 dishes were analyzed per group at each time point. The MTT-cell growth assay kit was purchased from Chemicon International (Temecula, California). Since the final goal of the present study is to examine the possibility of availability of trehalose in clinical practice, we used human cell lines instead of rabbit ones. In addition, since both human and rabbit conjunctival fibroblasts and keratocytes are not currently commercially available, we used human skin keratinocytes and fibroblasts for them.

2.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

NHDF were cultured with or without 5% trehalose-containing medium for 6 or 9 days. NHDF were dissolved in lysis buffer (1× Roche complete mini protease inhibitor cocktail provided by the manufacturer, Roche Applied Science, Indianapolis, Indiana). The same amount of protein (15 μg) was loaded on each lane of a 12% acrylamide gel, electrophoresed and electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, California) at 18 V overnight. After nonspecific binding was blocked by blocking buffer, 5% bovine serum albumin and 1% normal swine serum in phosphate-buffered saline containing 0.05% of Tween 20 (0.05% TW-PBS) at room temperature for 2 h, the membrane was probed with primary antibody against α-SMA (DAKO, Glostrup, Denmark) diluted to 1:5000 in blocking buffer at room temperature overnight. After washing 3 times with 0.05% TW-PBS, the membrane was incubated in blocking buffer containing horseradish peroxidase (HRP)-labeled rabbit-anti-mouse immunoglobulin (Ig) G (DAKO) diluted to 1:5000 at 4 °C overnight. Specific antigen-antibody binding α-SMA was visualized using an ECL kit® (GE Healthcare Life Sciences, Piscataway, New Jersey). After detection of the band for α-SMA antibody, a Re Blot Western blot Recycling kit® (Chemicon International) was used to remove anti-α-SMA antibody. Density of each band was quantified by a densitometer.

The membrane was then blocked by 5% skim milk, and probed with primary antibody against vimentin (Thermo Scientific, Waltham, Massachusetts) diluted to 1:5000 in blocking buffer at 4 °C overnight. After washing 3 times with TW-PBS, the membrane was incubated with HRP-labeled goat anti rabbit IgG (DAKO) diluted at 1:5000 at 4 °C overnight. Specific antigen-antibody binding for vimentin was visualized using an ECL kit® (GE Healthcare Life Sciences). As an internal protein control, rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Abcam, Cambridge, UK) was re-probed to the membrane and treated similarly.

2.3. Flow cytometry analysis

The rate of apoptosis in cultured cells was determined by an annexin V-affinity assay that detects the surface exposure of phosphatidylserine during apoptosis. After NHDF was cultured until about 80% confluent, medium was exchanged with 106S medium containing 5% trehalose. After 1week, cells were harvested by using cell scraper and were centrifuged and washed twice with PBS at 1000 rpm for 5 min at room temperature. Cells were resuspended in 1× binding buffer (BD Biosciences, Franklin Lakes, New Jersey). Samples (100 μl) were transferred to 5 ml-culture tubes and were added by 5 μl of PE annexin V (BD Biosciences) and 5 μl 7-ADD (BD Biosciences). After samples were vortexed and incubated for 15 min at room temperature in the dark, samples were added by 400 μl of 1x binding buffer to each tube. Annexin V-positive cells were detected by a fluorescence-activated cell sorting (FACS) flow cytometer (FACSCalibur, BD Biosciences).

2.4. Animal experiments

All experimental procedures were designed to ethnically conform to both the Association for Research on Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic Vision Research and the guidelines of our own institution. A total of 48 Japanese white rabbits (body weight, 1 kg; purchased from