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Dexamethasone modifies mitomycin C-triggered interleukin-8 secretion in isolated human Tenon's capsule fibroblasts

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ABSTRACT

Intraoperative mitomycin C (MMC) is widely used to prevent pterygium recurrence and glaucoma filtering bleb failure, but it has been shown to induce corneal inflammation and cell death. Postoperative dexamethasone (DEX) is advocated to reduce MMC-related inflammation and cell death in corneal fibroblasts. Nevertheless, its long-term regulation mechanism in Tenon's capsule remains to be explored. The purpose of this study was to investigate how DEX modifies MMC's effects in human Tenon's capsule fibroblasts (HTFs). HTFs isolated from the pterygium surgical patients ($n = 6$) were treated with MMC at 0, 0.1, 0.2, and 0.4 mg/ml for 5 min and incubated in DEX at 10 μM for 0, 1, 2, and 3 days. Recombinant interleukin-8 (IL-8) was used to verify the effect of MMC-related IL-8 secretion. Cell proliferation of all the treated cells was analyzed by WST-1 assay. The amount of IL-8 secretion in HTFs was determined by enzyme-linked immunosorbent assay. Immunoblotting assay was used to analyze the expression of peroxisome-proliferator-activated receptor gamma (PPARγ) and B-cell lymphoma-extra large (Bcl-xL). Our results revealed that MMC significantly reduced the HTF cell proliferation rate. Additionally, MMC significantly upregulated IL-8 secretion in HTFs concentration-dependently. At 3 days post treatment (dpt), 5-min exposures to 0.1, 0.2, and 0.4 mg/ml MMC resulted in 1.4-fold ($p = 0.012$), 1.6-fold ($p = 0.012$), and 2.5-fold ($p = 0.001$) increases of IL-8 secretion. In contrast, DEX reversed the MMC-retarded cell proliferation rate ($p = 0.036$) and repressed MMC-related IL-8 secretion by 33.5% at 3 dpt ($p = 0.003$). Addition of recombinant IL-8 noticeably suppressed HTF cell proliferation in a concentration-dependent manner. DEX stimulated upregulation of both PPARγ and Bcl-xL at 1 dpt in normal HTFs and at 2 dpt in MMC-treated HTFs. PPARγ silencing reduced expression of PPARγ and Bcl-xL, but enhanced IL-8 secretion ($p < 0.001$). On the other hand, Bcl-xL silencing enhanced IL-8 secretion ($p < 0.001$), but did not affect PPARγ expression. These revealed that IL-8 secretion in HTFs is modulated by PPARγ-dependent Bcl-xL signaling. We conclude that DEX reversed the MMC-inhibited HTF cell proliferation via diminishing the MMC-induced IL-8 secretion, which resulted from a late-phase upregulation of the PPARγ and Bcl-xL. These long-term effects suggest a beneficial postoperative DEX treatment following intraoperative MMC application.

1. Introduction

Mitomycin C (MMC) (Kaufman et al., 2013), bevacizumab (Ozgurhan et al., 2013), and 5-fluorouracil (Prabhasawat et al., 2006) are used as adjuvant anti-fibrotic agents to prevent the pterygium recurrence as well as glaucoma filtering bleb failure (Abraham et al., 2007; Gutierrez-Ortiz et al., 2006). Intraoperative MMC application has a profound anti-scarring activity, but it halts cell growth in Tenon's capsule, resulting in postoperative complications such as a bleb leak, chronic hypotony, and endophthalmitis following trabeculectomy (Bindlish et al., 2002), and scleral melting

Abbreviations: Bcl-2, B-cell lymphoma-2; Bcl-xL, B-cell lymphoma-extra large; DEX, dexamethasone; HTFs, human Tenon's capsule fibroblasts; dpt, days post treatment; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; MMC, mitomycin C; PPARβ, peroxisome proliferator-activated receptor beta; PPARγ, peroxisome proliferator-activated receptor gamma.

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after pterygium excision (Tsai et al., 2002). In addition, a higher concentration of MMC at 0.4 mg/ml is often used in glaucoma surgery (Gedde et al., 2012), ocular surface squamous neoplasia (Besley et al., 2014), and pterygium excision (Koranyi et al., 2012). This approach is also used in the *in vitro* experiments to investigate how MMC affects corneal fibroblasts (Chang et al., 2010; Chen et al., 2012) and corneal epithelial cells (Chen and Chang, 2010). However, local inflammation following MMC application and conjunctival inflammation around the surgical sites may lead to surgical failure and recurrence (Kheirkhah et al., 2008). The mechanism of how MMC triggers inflammatory cytokines, such as interleukin-8 (IL-8) and MCP-1, has been illustrated in corneal fibroblasts (Chou et al., 2007). Nevertheless, whether MMC contributes to the postoperative inflammation in Tenon's capsule is not verified.

Dexamethasone (DEX), a synthetic glucocorticoid, is a commonly used anti-inflammatory agent for ocular inflammatory diseases or after ophthalmic surgery. DEX inhibits interleukin-1β (IL-1β) and interleukin-6 (IL-6) in macrophages (Wang et al., 2012), IL-6 and IL-8 in human corneal fibroblasts (Chang et al., 2010) and primary lung fibroblasts (Van Ly et al., 2011), and TNF-α in primary airway smooth muscle cells (Fujita et al., 2011). Of the inflammatory chemokines, IL-8 is mainly produced by macrophages and plays a critical role in inflammation response. IL-8 is also secreted from non-inflammation tissues by DEX stimulation, such as human corneal fibroblasts (Chang et al., 2010). In addition to the inflammatory response, IL-8 inhibits proliferation of the lung fibroblasts (Zhang et al., 2011) and the ovarian cancer cells (Wang et al., 2011). However, effect of IL-8 secretion on self-regulation of Tenon's capsule remains unclear.

Peroxisome proliferator-activated receptor-gamma (PPARγ) is a nuclear hormone receptor for gene transcription in lipoprotein metabolism (Wang et al., 2013), glucose homeostasis (Sarruf et al., 2009), inflammation (Kersten et al., 2000), and atherosclerosis (Zhang and Chawla, 2004). PPARγ inhibits atherosclerosis by reducing IL-6 and IL-8 expressions (Chinetti et al., 2000). Furthermore, IL-8 synthesis in human lung epithelial cells (Neri et al., 2011) and human gastric epithelial cells (Park et al., 2012b) are negatively regulated by PPARγ dependent pathway. In addition, PPARγ prevents cell apoptosis in the mouse neuroblasts by upregulating expression of B-cell lymphoma-extra large (Bcl-xL), an anti-apoptotic protein (Wu et al., 2009). Therefore, we considered whether PPARγ modulates IL-8 secretion and cell growth of Tenon's capsule after intraoperative MMC application.

Noted the postoperative conjunctival inflammation around the surgical sites after MMC application, including pterygium excision (Kheirkhah et al., 2008) and glaucoma filtering surgery (Lockwood et al., 2013), we investigated herein the conceivable molecular mechanism of how DEX modulates the IL-8 secretion and the cell proliferation of the Tenon's capsule fibroblasts after intraoperative MMC application. We illustrate the potential benefit of postoperative DEX treatment to limit the MMC-triggered cell death in ophthalmic surgery.

2. Materials and methods

2.1. Human Tenon's capsule fibroblasts (HTFs)

HTFs were established from the excised Tenon's capsule of six pterygium surgical patients in compliance with informed consents approved by the hospital's institutional review board. The Tenon's capsule was directly placed at 37 °C with 5% CO_2 in DMEM (Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel), penicillin G (100 IU/ml), and streptomycin (100 μg/ml) until HTFs were isolated. The isolated HTFs in this study were used between passages 2 and 5.

2.2. Treatment of dexamethasone and mitomycin C

Intraoperative MMC at 0.1–0.4 mg/ml is commonly applied to the Tenon's capsule in pterygium excision and filtering surgery (Besley et al., 2014; Koranyi et al., 2012). In addition, an *in vitro* concentration of DEX at 10 μM (4 μg/ml, 0.0004%) is also commonly used for the experiments in our previous study (Chang et al., 2010) as in other series (Hatou et al., 2009). As the tissue concentration after topical application of medication usually drops far below the original concentration and DEX is often applied four times a day for several weeks postoperatively, we applied MMC at 0.1, 0.2, and 0.4 mg/ml MMC for 5 min to the cultured cells followed by incubation of DEX at lower concentration (10 μM) for incubation for up to 3 days to mimic the clinical scenario.

To analyze the effect of DEX on MMC-treated HTFs, the cells were seeded separately in a 6-cm dish or each well of a 96-well plate. After cell attachment overnight, the cells were directly treated with MMC (KYOWA, Tokyo, Japan) at 0, 0.1, 0.2, and 0.4 mg/ml in phenol red–free DMEM (Sigma Aldrich, St. Louis, MO). After 5-min incubation of MMC, the treated cells were quickly washed three times with phenol red–free DMEM and then incubated in DMEM with 10% FBS in the absence or presence of DEX at 10 μM until harvest time points.

2.3. Enzyme-linked immunosorbent assay (ELISA)

To determine the IL-8 secretion in HTFs, the supernatants were collected at 0, 1, 2, and 3 days post treatment (dpt) with MMC. The amount of IL-8 secretion was determined by DuoSet ELISA Development System (Cat. DY208; R&D Systems, Minneapolis, MN) according to the instructions of the manufacturer. Briefly, a 96-well plate was coated with mouse anti-human IL-8 capture antibody at 2 μg/ml overnight at room temperature. Nonspecific binding sites were blocked with 1% bovine serum albumin (BSA). Two hundred microliters of the collected supernatants and seven standard solutions of recombinant human IL-8 ranging from 2000–31.3 pg/ml by two-fold dilution were incubated for 2 h. The plates were incubated with biotinylated anti-human IL-8 detection antibody for 2 h followed by streptavidin-conjugated horseradish-peroxidase for 20 min. The plates were incubated in peroxidase substrate (SureBlue Reserve ELISA substrate; Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 20 min and terminated with 1 N HCl. Finally, absorbance value of all the samples was determined at 450 nm against a reference at 570 nm with a spectrophotometer and calculated by comparison to the standard curve.

2.4. Cell proliferation

To analyze the viability of HTFs, the cells were evaluated with the formazan-based cell proliferation reagent WST-1 (Roche Applied Science, Mannheim, Germany), according to manufacturer's instructions. Briefly, none (as a background control) or 5.0×10^3 cells of HTFs were cultured separately in each well of a 96-well plate and the volume in each well was adjusted to 100 μl under each treatment condition. At 0, 1, 2, and 3 dpt following mock or MMC treatment, 10 μl of reagent WST-1 was directly added into each well and incubated at 37 °C for 2 h. Absorbance of the samples against the background control (medium alone) was measured at 450 nm against a reference at 690 nm as the absorbance values ($A_{450} - A_{690}$) with a spectrophotometer.

2.5. Immunoblotting

To analyze the protein expressions in HTFs, all the cells in a 6-cm dish were disrupted in RIPA lysis buffer (150 mM NaCl; 1% NP-40;