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TNF-α upregulates HIF-1α expression in pterygium fibroblasts and enhances their susceptibility to VEGF independent of hypoxia

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ABSTRACT

The clinical manifestations of pterygium are characterized by rapid growth and postoperative recurrences. We had previously proposed that hypoxia-inducible factor (HIF)-1α recruits progenitor cells during the development and progression of pterygia. Recently, it was reported that various stimuli, including inflammation, could activate HIF-1α even under normoxic conditions. The ocular surface directly faces external environments, and is thus frequently exposed to inflammatory insults. First, we examined the gene expression of HIF-1α, its downstream molecule, vascular endothelial growth factor (VEGF)-A, and VEGF receptor (VEGFR)-2 in corneal and conjunctival cells compared with cultured human umbilical vein endothelial cells. Corneal fibroblasts had high expression of VEGFR-2 in the presence of TNF-α, and HIF-1α was activated by TNF-α in diverse ocular surface cells. The HIF-1α/VEGF/VEGFR signaling pathway in response to TNF-α was evaluated in cultured human pterygium fibroblasts (HPFs) at the gene and protein levels and was compared to treatment with cobalt chloride ($CoCl_2$), a hypoxic mimetic, to exclude the effect of hypoxia. Although VEGF-A expression was not changed by TNF-α, expression of HIF-1α and VEGFR-2 was enhanced in HPFs treated with TNF-α, independent of hypoxia conditioning. In addition, VEGF-C gene expression was activated solely by TNF-α in HPF, but VEGF-B levels were not significantly affected. These results may provide mechanistic explanations for the uniquely vigorous proliferation of pterygium fibrovascular tissue during TNF-α-induced ocular surface inflammation.

1. Introduction

The ocular surface area, including the cornea and conjunctiva, directly faces external environments and is thus frequently exposed to various chronic inflammatory insults such as ultraviolet (UV) radiation, infection, allergy-inducing dust, and dryness. Many proinflammatory cytokines are activated accordingly; of these, tumor necrosis factor (TNF)-alpha (α) plays a major role in orchestrating inflammatory effects in a broad range of cell types (Aggarwal, 2003). TNF-α activates the canonical nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway as part of the acute inflammatory response, and thus controls cell proliferation, differentiation, migration, and apoptosis (Pahl, 1999). For example, at the ocular surface, UV irradiation triggers secretion of TNF-α from corneal and conjunctival epithelial cells (Gamache et al., 1997; Kennedy et al., 1997) and TNF-α may stimulate proliferation of Tenon's capsule fibroblasts (Cunliffe et al., 1995).

Pterygium is a triangular ocular surface fibrovascular tissue that develops and proliferates in response to stress due to UV light exposure, inflammation, and/or ocular irritation. Capillaries ingrown from the stroma into the pterygium epithelium were suggested to be a reaction to hypoxia (Seifert and Sekundo, 1998). Interestingly, the relatively low vascular density of the nasal bulbar conjunctiva is essential, and increased vulnerability to tissue hypoxia is correlated with intrinsic susceptibility to pterygium formation at the nasal conjunctival area (Ha and Kim, 2006). This concept was supported by studies that verified increased expression of hypoxia-inducible factor (HIF)-1α, a central component of the oxygen sensor system, in pterygium tissues compared to normal conjunctiva (Lee et al., 2007; Pagoulatos et al., 2014). Mechanistically, it was suggested that accumulated HIF-1α induced by chronic hypoxic insult at the pterygium epithelium might elicit

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downstream stromal cell-derived factor (SDF)-1 expression to recruit circulating progenitor cells and contribute to copious vascularization and fibrosis at the pterygium stroma (Kim et al., 2013b, 2015).

Among five members of the VEGF family, VEGF-A is best known for angiogenesis. HIF-1α and vascular endothelial growth factor (VEGF)-A, one of its downstream molecules, are generally activated by hypoxia. However, recent studies have shown that HIF-1α signaling can be unexpectedly activated by inflammatory cytokines, growth factors, oncogenes, reactive oxygen species, bacterial lipopolysaccharide, and hormones even under normoxic conditions (Dery et al., 2005; Zhou and Brune, 2006). For example, TNF-α induced accumulation of HIF-1α through a NF-κB-dependent pathway under normoxic conditions in human embryonic kidney cells (Zhou et al., 2003). However, the relevant studies have mostly focused on cancers as ischemic and hypoxic diseases for the purpose of supportive therapeutic strategies (Frede et al., 2007). Given that the suggested pathogenesis of pterygium proposes a link to chronic inflammation with involvement of TNF-α and interleukin (IL)-1beta (β) (Solomon et al., 2000; Siak et al., 2011), it is plausible that TNF-α induced inflammation may also upregulate HIF-1α production in pterygial cells and further induce proteins involved in angiogenesis and vascular remodeling, such as VEGF-A.

It has been reported that pterygium fibroblasts might play a pivotal role in the pathogenesis of pterygia. In addition, pterygium stromal fibroblasts are largely responsible for fibrovascular tissue proliferation (Chen et al., 1994; Kim et al., 2016a). However, there are no previously published reports on the HIF-1α and VEGF-A axis in pterygium fibroblasts during TNF-α-induced inflammatory stimulation independent of hypoxia. In general, pterygia reveal very variable and changeable patterns of clinical progression and recurrence. Therefore, identifying pterygial cell-specific signaling induced by ocular surface inflammation is of special interest. Accordingly, we first screened for changes in HIF-1α, VEGF-A, and VEGF receptor (VEGFR) gene expression with TNF-α or a hypoxic mimetic in various cell types present at the ocular surface. Furthermore, we investigated the effect of TNF-α-induced NF-κB activation on HIF-1α/VEGF-A/VEGFR signal activation under normoxic conditions in human pterygium fibroblasts (HPFs) to elucidate the mechanism underlying the exacerbation of vascularization by ocular inflammation in pterygia.

2. Materials and methods

2.1. Isolation and culture of corneal cells

Human corneal tissues without previous ocular diseases were obtained for corneal transplantation and stored at 4 °C in storage medium (Optisol-GS, Bausch & Lomb, Rochester, NY, USA). After excision of the central 8-mm round cornea for transplantation, the peripheral corneal tissues were used for culture of human corneal epithelial cells (HCEpCs), human corneal endothelial cells (HCEnCs), and human corneal stromal fibroblasts (HCFs). Corneal tissues were washed 6 times with phosphate-buffered saline (PBS) containing 5 × antibiotic (penicillin/streptomycin) solution.

To isolate HCEpCs, corneal button tissues were cut into 6 to 8 pieces after stripping the endothelium and incubated in Dulbecco's Modified Eagle Medium and Ham's Nutrient Mixture F-12 (DMEM/F12) containing 2.4 U dispase II (Sigma-Aldrich, St. Louis, MO, USA) for 2 h at 37 °C. Loosened corneal epithelial sheets were peeled off with forceps and incubated in 0.25% trypsin/EDTA for 10 min at 37 °C. The sheets were separated into single cells by pipetting. The cells were collected after centrifugation at 1200 rpm for 5 min and transferred to tissue culture dishes for attachment. Primary HCEpCs were maintained in medium (EpiLife®, Cascade Biologics, Portland, OR, USA) containing human corneal growth supplement (HCGS, Cascade Biologics).

For the isolation of HCFs, cut pieces of corneal button tissues were placed in 6-well plates. After adhesion for 10 min, each explant was covered with alpha-minimum essential medium (MEM) (Invitrogen-Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 100 units/mL penicillin/streptomycin.

The isolation and culture of HCEnCs was performed as described previously (Kim et al., 2016b). Briefly, cells on the corneal endothelial layer were isolated using a peel-and-digest approach. Detached corneal endothelial cell clusters were rinsed once in EGM-2MV BulletKit™ (Lonza, Walkersville, MD, USA) and further dissociated to obtain smaller cell clumps, which were washed and collected after centrifugation at 1200 rpm for 5 min, and plated on FNC-coated tissue culture dishes for attachment. Isolated cells were cultured in EGM-2MV BulletKit™.

Cultured corneal cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. When the cells reached 80–90% confluency, they were subcultured using 0.25% trypsin/EDTA. Experiments were performed using cells at the third passage.

2.2. Isolation and culture of stromal fibroblasts from pterygia and normal conjunctiva

Specimen collection and culture of stromal fibroblasts from pterygium and normal conjunctiva were performed as described previously (Kim et al., 2013a). Normal conjunctival tissue including the underlying Tenon's capsule and pterygium tissue were obtained during cataract surgery and pterygium excisional surgery, respectively. Pterygium specimens were obtained from the central portion of the pterygium body from four patients: (1) a 48 year-old (y/o) female with grade T3 pterygium based on the T grading system (Tan et al., 1997); (2) a 71 y/o male with grade T3; (3) a 35 y/o female with grade T3; and (4) a 35 y/o female with grade T3. These tissue samples were used for explant cultures to generate human conjunctival stromal fibroblasts (HCJFs) and HPFs. HCJFs and HPFs were cultured in α-MEM containing 10% FBS and 1% penicillin/streptomycin. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and used in experiments at passages three to five.

2.3. Culture of human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (Manassas, VA, USA) and used in experiments at passages two to five. HUVECs were grown in EGM-2 (Lonza), 2% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin and maintained at 37 °C in a humidified atmosphere with 5% CO_2. Cells were starved in EBM-2 with 1% FBS for 6 h before TNF-α treatment.

2.4. Drug treatment

Cultured HUVECs, HCEpCs, HCFs, HCEnCs, HCJFs, and HPFs were treated with TNF-α (300-01, PeproTech, Rocky Hill, NJ, USA) at a concentration of 20 ng/mL for 24 h to induce NF-κB activation for inflammation. As for inducing hypoxia conditioning, the cells were treated with cobalt (II) chloride ($CoCl_2$; 15862, Sigma-Aldrich), which is a well-known hypoxia mimetic and a strong inducer of HIF-1α, at a concentration of 100 μM for 12 or 24 h. Before experiments, we confirmed that treatment with TNF-α or $CoCl_2$ under the above conditions did not reveal cytotoxicity decreasing the viability of HPFs (Supplementary Fig. 1).