Mini Review

Epithelial-mesenchymal transition in the pathogenesis of pterygium

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A pterygium is a wing-shaped, fibrovascular conjunctival outgrowth, invading centripetally into the clear cornea, and its incidence is associated with sun exposure (ultraviolet radiation). We investigated whether epithelial-mesenchymal transition (EMT) is involved in the pathogenesis of pterygium. Histopathology showed aberrant fibrotic proliferation beneath the pterygium epithelium, with epithelial processes extending into the stroma. Transmission electron microscopy revealed the dissociation of epithelial cells, which were surrounded by activated fibroblast-like cells. Characteristic downregulation of E-cadherin and intranuclear accumulation of β-catenin and lymphoid-enhancer-factor-1 in pterygial epithelium were also observed by immunohistology. Interestingly, epithelial cells extending into the stroma were positive for both α-SMA/Vimentin and cytokeratin 14. Snail and Slug were immunopositive in the nuclei of pterygial epithelial cells, but not in normal corneal epithelial cells. These results suggested that EMT of basal epithelial cells may play a key role in the pathogenesis of pterygium.

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Pterygium

A pterygium is a fibrovascular conjunctival outgrowth that centripetally invades from the corneal limbus into the central cornea (Fig.1A). Epidemiologically, the incidence of pterygium is known to be associated with sun exposure (ultraviolet radiation), and therefore the disease is sometimes regarded as a benign tumor[1-3]. A large pterygium approaching the central cornea can cause visual loss due to irregular astigmatism, requiring surgical excision. As bare sclera excision of pterygium is associated with a high recurrence rate, pterygium excision is often combined with conjunctival autograft, mitomycin C, beta-irradiation or other adjunctive therapies to reduce recurrence rates[4,5].

Although the pathogenesis of pterygium remains unknown, pterygial epithelial cells (PECs) are believed to have acquired

an altered balance between proliferation and apoptosis[6-11]. The stromal portion of a pterygium shows aberrant accumulation of extracellular matrix molecules such as collagen fibers, with pathological features characteristic of elastotic degeneration[12-15]. These facts indicate that pterygial fibroblasts and PECs are phenotypically altered from resident cells of the ocular surface, contributing to the fibrotic changes observed in pterygia.

Epithelial-mesenchymal transition and the eye

Fibrosis is a common pathological event observed in various organs of the body. Recent studies have demonstrated that the cellular origin of fibroblasts during disease are not only remnants of embryonic development, but may also arise from tissue-specific epithelial cells and circulating pools[16]. In particular, the phenomenon of epithelial cells changing their phenotype to fibroblastic cells following morphogenic pressure from injured tissue is called epithelial-mesenchymal transition (EMT)[17-18]. EMT is a well-recognized mechanism involved in the dispersion of cells during vertebrate embryogenesis, and is also observed in the adult during repair of injured tissue, as well as in the initial stages of cancer metastases[19-21].

The important role of EMT in pathogenesis of several diseases of eye has been also reported. Saika et al reported possible involvement of EMT in cataractogenesis in both human and mouse, and subretinal fibrosis after retinal detachment[22-25]. Kawakita et al. previously reported that corneal limbal epithelial cells (LECs) residing in the peripheral cornea can undergo EMT following air exposure *in vitro*[26]. As a result, LECs were shown to invade the underlying stroma leading to histological findings similar to fibrosis. Since the basal limbal epithelium include putative corneal epithelial/progenitor cells, it is reasonable to hypothesize that altered LECs undergo EMT as one of the mechanisms involved in the pathogenesis of pterygium.

Histopathology of eyebank corneas with pterygium

An intact pterygium attached to an eyebank cornea offers the unique opportunity to observe the ultrastructure of pterygia *in situ*. Gross histological examination of an intact pterygium showed irregular thinning of the epithelium with the appearance of fibrous tissue between the epithelium and Bowman's layer (Fig.1B, C). Pterygial tissue has invaded centripetally from the conjunctiva towards the central cornea. Towards the head of the pterygium, epithelium thickness was irregular and fibrous proliferation was observed between the basal epithelium and Bowman's layer. The high-magnification view (Fig.1B) reveals the dissolution of the Bowman's layer behind the leading head of the lesion, which was replaced by massive fibrovascular proliferation. In the pterygial body, massive basophilic amorphous material was observed in the stroma, which is consistent with previous reports[27].

During EMT, epithelial cells show less intercellular adherence junctions, tight junctions and desmosomes leading to the loss of cellular polarity. Cytokeratin intermediate filaments are also disassembled in order to rearrange their F-actin stress fibers to express filopodia and lamellopodia[16]. Transmission electron microscopy showed newly synthesized collagen fibrils accumulating between the epithelium and Bowman's layer at pterygial head. Basal PECs have irregular cytoplasmic membranes and are attached to the underlying fibrotic tissue. Slightly behind the pterygium head, the Bowman's layer disappeared and basal PECs attached directly onto the fibrous tissue (Fig.2A). Basal PECs had a cuboid, relatively small cell contour and high cytoplasmic electron density with cytoplasmic fibrils. The basement membrane is multilaminated and intermingled with irregular extra cellular collagen fibers (Fig.2B). PECs found invading into the underlying stroma revealed higher cytoplasmic electron density, enlarged intercellular spaces, and irregular basal cytoplasmic membranes, no longer showing adhesion complexes (Fig.2C,D). Subepithelial fibrotic tissue contained numerous enlarged fibroblast-like cells.

EMT markers in pterygium head epithelial cells

Co-expression of epithelial keratins with the mesenchymal marker α-SMA or vimentin is a classical sign of EMT. We examined EMT in pterygial epithelium by immunohistochemistry against the mesenchymal marker α-SMA or vimentin, and the basal epithelial marker cytokeratin 14 (K14). Examination of pterygium on the eyebank cornea revealed that these α-SMA+, K14+ cells/ vimentin+, K14+ cells were localized at the leading edge of epithelial invaginations into the stroma (Fig. 3B). Normal corneal and limbal epithelium did not express α-SMA/ Vimentin (Fig.3C,E).

Since the loss of membranous localization of E-cadherin protein and transcriptional repression of its mRNA are hallmarks of EMT[21], we also examined the staining pattern of E-cadherin in pterygium and normal corneal epithelium. E-cadherin was observed lining the cytoplasmic membrane in the central corneal epithelium as well as in pterygium tissue. However, pterygium samples demonstrated the focal loss of membranous staining of

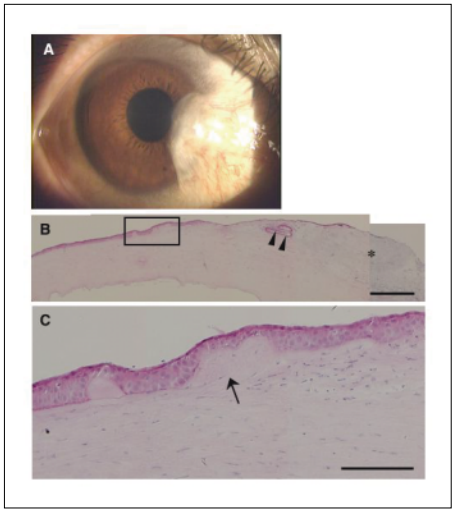

Fig.1 Clinical and histopathology of pterygium. (Partly adapted from Kato et al[39])

A: Clinical appearance of pterygium.

B: Representative pathological appearance of an intact pterygium found in an eyebank cornea (bar = 1 mm). Arrowheads; epithelial cells extending into the superficial stroma. Asterisk; basophilic amorphous material in the stroma

C: High magnification of the open square in A. Arrow; discontinuity of Bowman's membrane (bar = 100 μm).

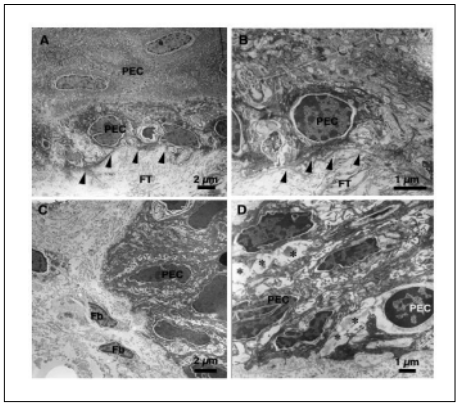

Fig.2 Transmission electron micrographs of pterygial epithelium. (Adapted from Kato et al[39])

A: Pterygial epithelial cells (PEC) slightly posterior to the pterygial head. Arrowheads; indentations associated with the basement membrane.

B: High magnification of basal PECs. Arrowheads; multi-layered basement membrane.

C: Leading head of an epithelial indentation extending into the underlying stroma.

D: Adjacent section of C. Asterisks; longer enlarged intercellular.

PEC, pterygial epithelial cell; Fb, fibroblast-like cells; FT, fibrous tissue.

E-cadherin (Fig.4A). Those results supported our hypothesis that EMT plays a key role in the progression of pterygia.

Downstreaming β-catenin pathway activation and upregulation of MMP-7 in pterygium

Beta-catenin links E-cadherin and α-catenin to the cytoskeleton to form a complex that maintains normal epithelial polarity and intercellular adhesion. When E-cadherin is downregulated during EMT, β-catenin accumulates in the cytoplasm where it binds to cytosolic T cell-factor/lymphoid enhancer factor (LEF) transcription factors. The resulting complex is shuttled into the nucleus and activates the expression of target genes such as cyclin D1, c-myc, MMP-7, and membrane type (MT)1-MMP[28-31]. We demonstrated that PECs focally lost membrane-bound E-cadherin and β-catenin (Fig.4A-D). Examination of surgically excised tissue revealed both the membrane-associated expression and cytoplasmic/nuclear localization of both E-cadherin and β-catenin within the same field of view (Fig.4A,B), suggesting that a discontinuity exists between pterygium and normal epithelial tissue. Furthermore, PECs towards the pterygial head simultaneously had nuclear staining for LEF-1 (Fig.4E). These findings indicate that the β-catenin signaling pathway is activated in this area.

The canonical β-catenin signaling pathway is a necessary component to drive EMT in development and is frequently activated in cancer[32]. Beta-catenin/LEF-1 complexes also play a key role in EMT during the pathogenesis of colon cancer[31,33,34]. mRNA of MMP-7, one of the down-stream genes of β-catenin/LEF-1 complex, was uniquely expressed in pterygial epithelial cells, and not by corneal limbal epithelial cells taken from the limbus of donor corneas (Fig.4G). MMP-7 was previously shown to be present in the leading edge of pterygium tissue[35], and may be involved in the destruction of basement membrane and stromal tissue surrounding the pterygium head.

Expression of the E-cadherin repressor proteins Snail and Slug

Snail-related zinc-finger transcription factors Snail and Slug, are repressors of E-cadherin transcription essential for initiating mesodermal development during gastrulation[21]. Strong expression of Snail and Slug was observed in the nucleolus of pterygial epithelial cells (Fig.5A-C), but not in normal corneal epithelium (Fig.5D) or normal limbal epithelium (Fig.5E).

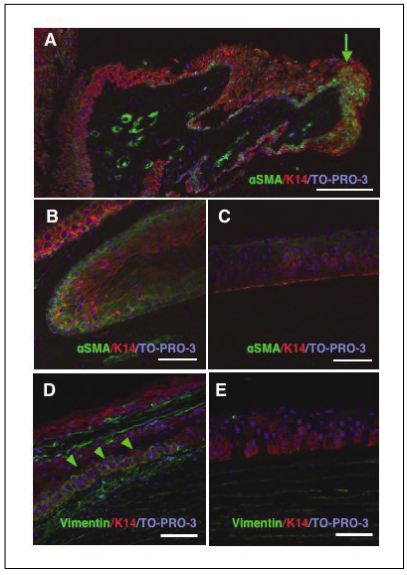

Fig.3 Immunohistochemistry of α-SMA/K14 and vimentin/K14 (Adapted from Kato et al.[39])

A: Double immunohistochemistry for α-SMA (green) and K14 (red) in surgically excised pterygial tissue (bar = 100 μm).

B: Double immunohistochemistry for α-SMA (green) and K14 (red) in pterygial epithelium in eyebank corneas (bar = 50 μm).

D: Double immunohistochemistry for Vimentin (green) and K14 (red) in pterygial epithelium in eyebank cornea (bar = 50 μm).

C,E: Normal corneal epithelium (bar = 50 μm).

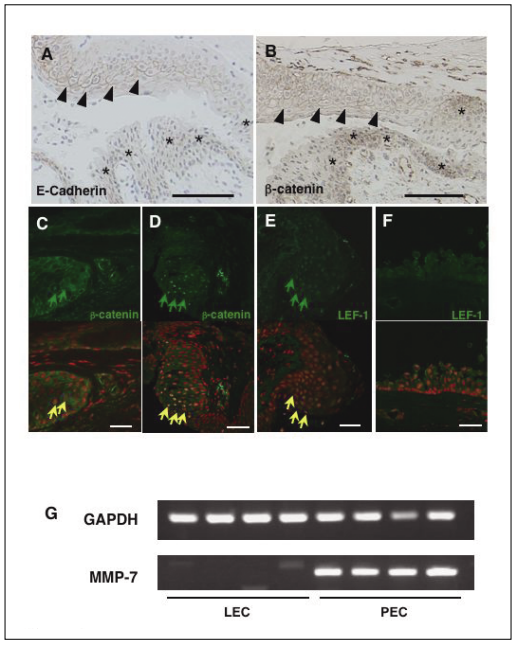

Fig.4 Immunohistochemistry of E-cadherin and β-catenin pathway molecules. (Adapted from Kato et al.[39])

A: E-cadherin in a surgically excised pterygium. Asterisks; focal loss of membrane-associated E-cadherin. Arrowheads; membrane-associated staining for E-cadherin (bar = 100 μm).

B: Immunohistochemistry for β-catenin in surgical excised pterygium. Arrowheads; membrane-bound staining. Asterisks; focal cytoplasmic staining. (bar = 100 μm).

C,D: Nuclear translocation of β-catenin in pterygial epithelium in eyebank cornea (C) and surgically excised pterygium (D) (bars = 50 μm).

E: LEF-1 in the nuclei of pterygial epithelial cells (bar = 50 μm).

F: Normal corneal epithelium (bar = 50 μm).

G: RT-PCR for MMP-7 in pterygial epithelial cells (PEC) and limbal epithelial cells (LEC).

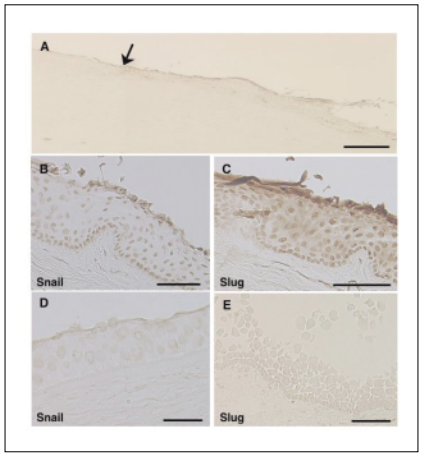

Fig.5 Immunohistochemistry of E-cadherin repressors. (Adapted from Kato et al.[39])

A: Composite micrograph of Snail immunohistochemistry. (bar = 200 μm).

B: Snail expression in the nucleus of pterygial epithelial cells (bar = 50 μm).

C: Slug expression in the nucleus of pterygial epithelial cells (bar = 50 μm).

D,E: Normal corneal (D) and limbal epithelium (E) (bars = 50 μm).

Possible causes of EMT in pterygium

EMT is triggered by several extracellular signals that include both ligand dependent signaling by soluble growth factors such as transforming growth factor (TGF)-β, FGF families and hepatocyte growth factors (HGF)[36], as well as cellular interaction with extracellular matrix proteins such as collagen and hyaluronic acid[37]. While the signaling pathways leading to EMT following ligand activation are complex, the downregulation of E-cadherin is a major final common pathway. Repression of E-cadherin is driven by the transcriptional regulators Snail and Slug, which translocate into the cell nucleus following phosphorylation of Ser residues[38].

Although our results strongly suggest that EMT is a major factor in the progression of pterygium, specific upstream events triggering Snail activation in PECs are yet to be elucidated. The cellular changes observed might be the result of an aberrant wound healing response. Mutations of the genome may be involved, and there is also the possibility that persistent inflammation causes the upregulation of MMP-7, as well as other effectors. Further investigations are needed to link ultraviolet exposure and/or inflammation to the induction of EMT in the pathogenesis of pterygium.

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