Expression of αB-Crystallin and Vascular Endothelial Growth Factor in Conjunctival Squamous Cell Carcinoma

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Abstract. *Aim: To examine the expression of αB-crystallin and vascular endothelial growth factor (VEGF) in conjunctival squamous cell carcinoma (CSCC). Materials and Methods: Seven CSCCs and three normal conjunctivas that were surgically excised were studied. Paraformaldehyde-fixed, paraffin-embedded tissue sections were processed for immunohistochemistry with antibodies against αB-crystallin, its phosphorylated forms, and VEGF. In vitro experiments were conducted to investigate the effects of mitomycin C (MMC) treatment on the expression of αB-crystallin and VEGF secretion. Results: αB-Crystallin and VEGF were strongly expressed in CSCCs compared to normal conjunctivas. αB-Crystallin immunoreactivity was co-localized with that for VEGF in CSCCs, whereas these signals were reduced in CSCC tissues treated with MMC before excision. MMC treatment suppressed the αB-crystallin expression and VEGF secretion in cultured conjunctival cells in a dose-dependent manner. Conclusion: This study demonstrated αB-crystallin and VEGF expressions in human CSCCs, which may play a role in the pathogenesis. αB-Crystallin expression, and VEGF secretion were reduced by MMC, indicating a novel therapeutic mechanism in MMC treatment for human CSCC.*

Conjunctival squamous cell carcinoma (CSCC) is one of the most common malignant tumors seen by ophthalmologists, accounting for 4-29% of all ocular adnexal tumors (1). The carcinoma originates from the squamous epithelial cells of the conjunctiva, characterized by extensive feeding vessels, and basement membrane invasion. It tends to occur in elderly men, but can also develop in young immunosuppressed patients. Although surgical excision/cryosurgery is conducted as a therapeutic option, enucleation or orbital exenteration may eventually be required in selected cases (2-4). This disease exhibits an aggressive and invasive nature, which subsequently leads to distant metastasis (5-7). Recently, in addition to conventional surgical excision, topical mitomycin C (MMC) eye drops were proven to be effective in reducing the tumor volume and risk of recurrence, as a first-line treatment (8, 9).

Vascular endothelial growth factor A (VEGF) is a well-known, potent angiogenic factor. Numerous studies have demonstrated that VEGF is overexpressed in solid tumor cells, and plays a critical role in tumor growth and metastasis through pathological angiogenesis (10, 11). Therefore, VEGF has been considered to be an interesting therapeutic target in the treatment of cancer for a long time. Indeed, the combination of anti-VEGF therapy and chemotherapy prolongs the life expectancy of patients with metastatic lung, colon, and breast cancer (12). Since anti-VEGF therapy was found to contribute to tumor regression in patients with CSCC (13, 14), VEGF may participate in its pathogenesis and tumor progression.

αB-Crystallin, a predominant protein of the ocular lens (15), belongs to the small heat shock-protein family. In addition to being a structural protein, recent studies reported that αB-crystallin was also expressed in various non-lenticular tissues, which contributes to the protection of cells from stress-induced damage by acting as a molecular chaperone and anti-apoptotic regulator (16-22). Indeed, marked expression of αB-crystallin has also been demonstrated in some tumors, predicting a poor clinical outcome (23-25). Furthermore, in response to various stresses, αB-crystallin is known to be phosphorylated at three serine (Ser) residues, Ser19, 45, and 59 (26, 27), with phosphorylation on Ser59 conferring maximal cytoprotection (28, 29).

Recently, Ghosh et al. demonstrated that αB-crystallin strongly interacted with VEGF based on molecular structural

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analysis (30). Furthermore, we showed that αB-crystallin played a critical role in the promotion of angiogenesis as a molecular chaperone of VEGF, and regulated pathological angiogenesis together with VEGF (28, 31). However, to the best of our knowledge, the involvement of αB-crystallin and VEGF has yet to be elucidated in CSCC.

In this study, we examined αB-crystallin expression in the normal conjunctiva, as well as CSCC, humans, where the co-localization of αB-crystallin and VEGF was analyzed by double-staining immunohistochemistry. Furthermore, we examined the effect of MMC on the expression of αB-crystallin and VEGF, both in vivo and *in vitro*.

Materials and Methods

Preparation of human tissues. Seven CSCC tissues were obtained from patients who underwent local surgical excision or orbital exenteration at the Department of Ophthalmology, Hokkaido University Hospital, Sapporo, Japan from 2005 and 2009. Two patients underwent orbital exenteration due to intraorbital tumor involvement. One patient was treated with MMC (0.04%, four times daily for three weeks) before surgical treatment. Three normal conjunctivas obtained during cataract surgery were also examined. The clinical characteristics of all patients are summarized in Table I. This study adhered to the tenets of the Declaration of Helsinki, and was approved by the Ethics Committee of Hokkaido University (approval number: 010-0172), and written informed consent was obtained from each patient. All CSCCs and normal conjunctivas were fixed in 4% paraformaldehyde soon after the tissues were removed in an operating room, and were submitted for hematoxylin and eosin (H & E) staining and immunohistochemistry. Two of the CSCC tissues were further used for western blot analysis.

Immunofluorescent staining. The slides were dewaxed in xylene overnight, dehydrated in a series of ethanol, and rinsed in phosphate-buffered saline for 10 min. As pre-treatment, microwave-based antigen retrieval was performed in 100 mM citrate buffer (pH 6.0). These slides were incubated with 0.1% bovine serum albumin (BSA) for 30 min. Sections were incubated with rabbit polyclonal antibody against human αB-crystallin (1:100 dilution; Stressgen, Ann Arbor, MI, USA) and mouse monoclonal antibody against human VEGF (1:50; Abcam, Tokyo, Japan) at room temperature overnight. Binding of the primary antibody was localized with Alexa Fluor® 546 goat anti-rabbit secondary antibody (1:200 dilution; Life Technologies, Eugene, Oregon, USA) and Alexa Fluor® 488 goat anti-mouse antibody (1:100 dilution; Life Technologies) for 30 min, respectively.

Double-immunofluorescent staining of phosphorylated serine forms of αB-crystallin and VEGF. To investigate the immunoreactivity of phosphorylated forms of αB-crystallin and VEGF in CSCC, we also performed double immunostaining in serial slides of the same patient, using rabbit αB-crystallin phosphorylated (pSer)19 (1:100 dilution; Novus Biologicals, Littleton, CO, USA), pSer45 (1:100 dilution; Stressgen), and pSer59 (1:100 dilution; Abcam) polyclonal antibody, respectively, and mouse monoclonal antibody against human VEGF (1:50; Abcam) as the primary antibody. Finally, all sections were mounted with mounting media with 4', 6-diamino-2-phenylindole (DAPI) (SlowFade® Gold antifade reagent with DAPI, Invitrogen). All slides were examined using a Keyenece BZ-9000 (Keyence, Osaka, Japan) microscope.

Chemicals and cells. Cultured human conjunctival epithelial cells were purchased from the European Collection of Cell Cultures (Clone 1-5c-4; ECACC, Manassas, VA, USA) and maintained in complete medium 199 (Sigma-Aldrich, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) under a humidified atmosphere containing 5% CO_2 at 37˚C. After growing to complete confluency, the culture medium was replaced with serum-free medium or serum-free medium with MMC (Sigma-Aldrich, St. Louis, MO, USA) at the following concentrations: 0.1% (1 mg/ml), 0.01% (0.1 mg/ml) and 0.001% (0.01 mg/ml), respectively. Sixteen hours later, the supernatants and cells were collected for further analysis.

Enzyme-linked immunosorbent assay (ELISA). Protein levels of VEGF in supernatants of cultured human conjunctival cells treated as above were measured using human VEGF ELISA kits (R&D Systems, Minneapolis, MN, USA) and normalized to total protein (BCA Protein Assay Kit; Thermo Scientific, Rockford, IL, USA), according to the manufacturers' protocols.

Western blot analysis. Harvested human conjunctival cells were sonicated in lysis buffer (1× RIPA buffer; Cell Signaling Technology, Danvers, MA, USA) with protease inhibitor (Roche, Basel, Switzerland) on ice, centrifuged at 1,3850 ×g for 20 min at 4˚C, and supernatants were collected for western blot analysis. Proteins were also extracted from two CSCCs (patient 6 and 7) and total protein was measured as described above. Equal amounts of each sample of cell lysates (8 μg) or CSCC (14 μg) were electrophoresed on 15% sodium dodecylsulfaet-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corporation, Billerica, MA, USA). After blocking with 5% skim milk in tris-buffered saline with tween 20 (TBST) for 2 h, the membranes were incubated with the rabbit αB-crystallin polyclonal antibody (1:1,000 dilution; Stressgen) overnight at 4˚C. Next, the membrane was washed with TBST and incubated with a 1:5,000 dilution of horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG for 60 min at room temperature. Immunoreactive bands were visualized using chemiluminescent reagents (ECL Plus; GE Healthcare, Little Chalfont, Buckinghamshire, UK) and measured using a LAS-4000 mini camera system (Fujifilm, Tokyo, Japan).

Statistical analysis. Data are presented as the mean±SEM. Statistical evaluations were performed using Student's *t*-test. The accepted level of significance for all tests was $p<0.05$.

Results

Expression and co-localization of αB-crystallin and VEGF in CSCC. CSCC exhibited a diffuse cellular population with atypical cells containing prominent and hyperchromatic nuclei, as compared with normal conjunctival squamous epithelia (Figure 1A and F). There was no sebaceous differentiation in any of the CSCC cases examined in this study. In CSCCs, αB-crystallin was strongly expressed in the cytoplasm of carcinoma cells, as compared to marginal

expression in normal conjunctival epithelia (Figure 1B and G). Similarly, carcinoma cells were strongly positive for VEGF, which was homogeneously expressed in the cytoplasm of neoplastic cells, whereas normal conjunctival squamous epithelia showed moderate immunoreactivity for VEGF (Figure 1C and H). Furthermore, double-staining immunohistochemistry revealed the co-localization of αB-crystallin with VEGF in CSCC (Figure 1E and J). To further confirm the existence of αB-crystallin protein in CSCC, western blot analysis revealed the expression of αB-crystallin in human CSCC tissues obtained from patient 6 and 7 (Figure 2, Table I).

Differential immunoreactivity of phosphorylated αB-crystallin in CSCC. To investigate the expression of phosphorylated αB-crystallin in CSCC, we performed immunostaining using antibodies against phosphorylated forms of αB-crystallin in serial sections from the same CSCC patients. αB-Crystallin phosphorylated at Ser59 exhibited significantly stronger immunoreactivity than the other phosphorylated forms, suggesting that phosphorylation at Ser59 contributes to the pathology of CSCC. Compared with phosphorylation at Ser59, phosphorylation at Ser19 and Ser45 led to moderate and weak immunoreactivity, respectively (Figure 3).

Decreased immunoreactivity of αB-crystallin and VEGF in CSCC treated with MMC. We were aware of the difference in immunoreactivity of αB-crystallin and VEGF among human CSCCs with and without pre-tment of MMC before tissue removal. In patients with CSCC previously treated with topical 0.04% MMC eye drops for three weeks, marginal immunoreactivity of αB-crystallin and VEGF was detected compared to those without previous MMC treatment (Figure 4). This suggests that treatment with MMC affects the expression of αB-crystallin and VEGF.

MMC treatment decreased αB-crystallin expression and VEGF secretion in cultured human conjunctival cells. To further confirm whether treatment with MMC modulates the expression of αB-crystallin or VEGF in stressed conjunctival cells, we performed an *in vitro* experiment using cultured human conjunctival epithelial cells. Before starting serum starvation (0 hours), the expression of αB-crystallin was barely detectable. However, the expression of αB-crystallin increased 16 hours after starvation by the replacement of serum-free cultured medium, while the expression was reduced following MMC treatment in a dose-dependent manner (Figure 5).

Furthermore, we investigated whether MMC addition reduced VEGF protein levels in the supernatants of cultured human conjunctival cells using ELISA. The VEGF protein was undetectable before starting serum-free starvation, whereas the VEGF protein concentration in the supernatants increased significantly 16 hours after replacement by serum-free culture medium (450.7±64.1 ng/mg, $p<0.001$; Figure 6). Interestingly, the VEGF protein concentration decreased significantly following 0.1 and 0.01% MMC treatment (10.2±4.2 and 113.4±2.4 ng/mg, respectively, $p<0.05$; Figure 6). Treatment with 0.001% MMC also reduced the VEGF protein levels but non-significantly (287.0±70.7 ng/mg, $p>0.1$; Figure 6).

Table I. *Clinical characteristics of patients enrolled in this study*

No.	Age (years)	Gender	CSCC	Pre-operative topical MMC	Orbital exenteration
1	70	M	Y	N	Y
2	69	M	Y	N	N
3	58	M	Y	N	N
4	45	F	Y	Y	Y
5	40	M	Y	N	N
6	41	M	Y	N	N
7	71	M	Y	N	N
8	54	M	N	N	N
9	70	F	N	N	N
10	70	F	N	N	N

M, Male; F, female; MMC, mitomycin C; Y, yes; N, no; CSCC, conjunctival squamous cell carcinoma.

Discussion

In the present study, we found that VEGF was expressed in human CSCCs using immunohistochemistry. The results suggest that VEGF plays an important role in human CSCC growth and angiogenesis similarly to other systemic tumors (32-35). Moreover, this study demonstrated the co-localization of αB-crystallin with VEGF in human CSCC. Immunoreactivity for αB-crystallin was weak in normal human conjunctival epithelial cells, whereas intense immunoreactivity for αB-crystallin was noted together with strong VEGF expression in CSCC, indicating that αB-crystallin was up-regulated during tumorigenesis of CSCC.

Recently, we showed the co-localization of αB-crystallin and VEGF in neovessels of the epiretinal membrane in human diabetic retinopathy (31). In addition, we demonstrated that αB-crystallin interacted with VEGF as a molecular chaperone, protecting VEGF from degeneration, and subsequently stimulating VEGF secretion, and eventually promoting neovascularization (28). We and other colleagues demonstrated that the absence of αB-crystallin attenuated retinal and tumor neovascularization using αB-crystallin knockout mice (28, 36). Considering that VEGF possibly contributed to the development of CSCC as in other solid tumors (13, 14), the co-localization of αB-crystallin and

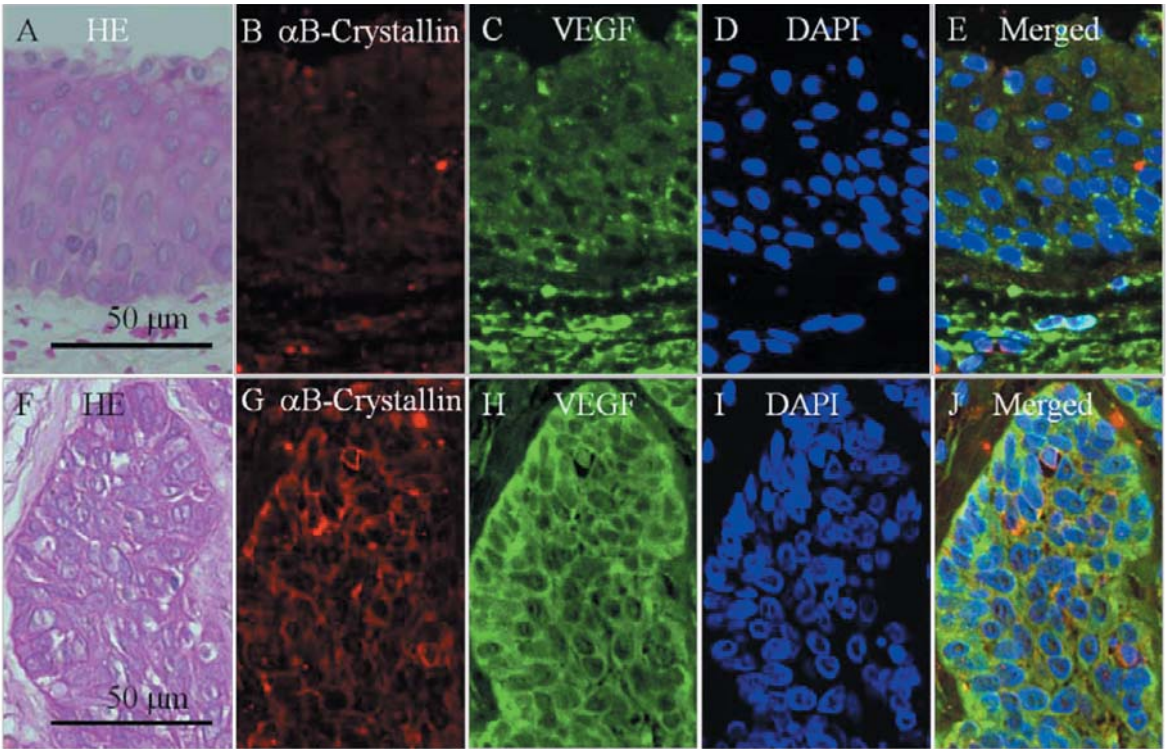

Figure 1. *Hematoxylin-eosin staining and immunohistochemistry for αB-crystallin, as well as vascular endothelial growth factor (VEGF) in normal human conjunctiva (top panel) and conjunctival squamous cell carcinoma (CSCC, bottom panel). A, F: Hematoxylin-eosin staining; B, G: αB-crystallin; C, H: VEGF; D, I: 4', 6-diamidino-2-phenylindole (DAPI) nuclear staining; E, J: merge of B, C, D and G, H, I, respectively. Note that both αB-crystallin and VEGF expression are up-regulated in CSCC, as compared with normal conjunctival epithelium.*

VEGF, and up-regulation of αB-crystallin in CSCC suggest that αB-crystallin expression is of marked importance in the pathogenesis and development of CSCC as a molecular chaperone for VEGF. It is known that αB-crystallin chaperone activity is regulated by its phosphorylation (27). This study also demonstrated that αB-crystallin was markedly phosphorylated at Ser59 in tumor cells, consistent with previous reports on αB-crystallin phosphorylation during intraocular neovascularization (28). Therefore, αB-crystallin phosphorylation might also contribute to the regulation of αB-crystallin activity and VEGF as a molecular chaperone in CSCC.

In this study, we observed reduced expression of αB-crystallin and VEGF in human CSCC tissues pre-treated with topical MMC. Being widely used as topical adjunctive therapy in ocular diseases over the past two decades, MMC is thought to induce cellular apoptosis *via* DNA damage, either by alkylation or free radical injury (37), or to cause cytotoxicity by inhibiting RNA and protein synthesis, and by protein damage following the generation of free radicals (38). Our *in vitro* analysis demonstrated that αB-crystallin expression and VEGF protein secretion decreased on treatment with MMC in cultured human conjunctival cells under serum starvation in a dose-dependent manner.

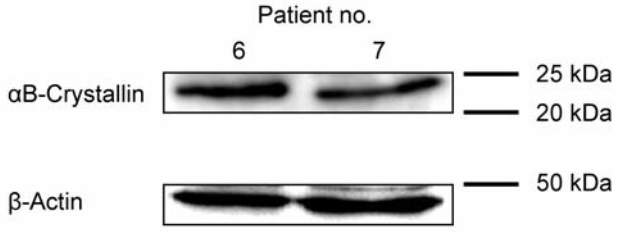

Figure 2. *Western blot analysis using proteins extracted from conjunctival squamous cell carcinomas (CSCCs). Note that the expression of αB-crystallin is clearly detected in CSCC tissues.*

Considering that αB-crystallin is necessary to protect VEGF from degeneration, MMC treatment might lead to a decrease in VEGF protein expression, directly through VEGF protein down-regulation, or indirectly through effects on αB-crystallin protein, and subsequently result in the inhibition of angiogenesis and tumor growth.

It was recently demonstrated that the down-regulation of alphaB-crystallin by siRNA led to the suppression of tumor growth (39, 40). We showed that the down-regulation of αB-crystallin in the siRNA-transfected cells led to a significantly

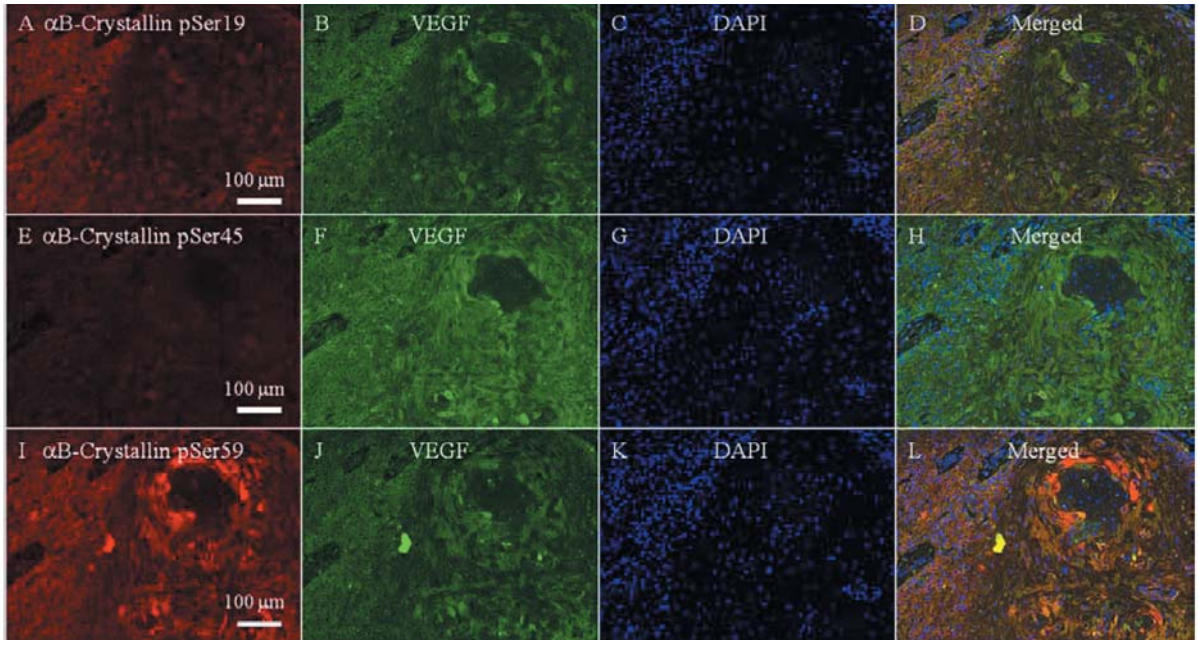

Figure 3. *Immunohistochemistry of phosphorylated serine forms of αB-crystallin and vascular endothelial growth factor (VEGF) in serial sections of conjunctival squamous cell carcinoma (CSCC). A: αB-Crystallin pSer19; D: αB-crystallin pSer45; I: αB-Crystallin pSer59; C, G, K: 4', 6-diamidino-2-phenylindole (DAPI) nuclear staining; B, F, G: VEGF; D, H, L: merged image. Note that alphaB-crystallin pSer59 exhibited the strongest immunoreactivity among these three phosphorylated serine forms of αB-crystallin in CSCC.*

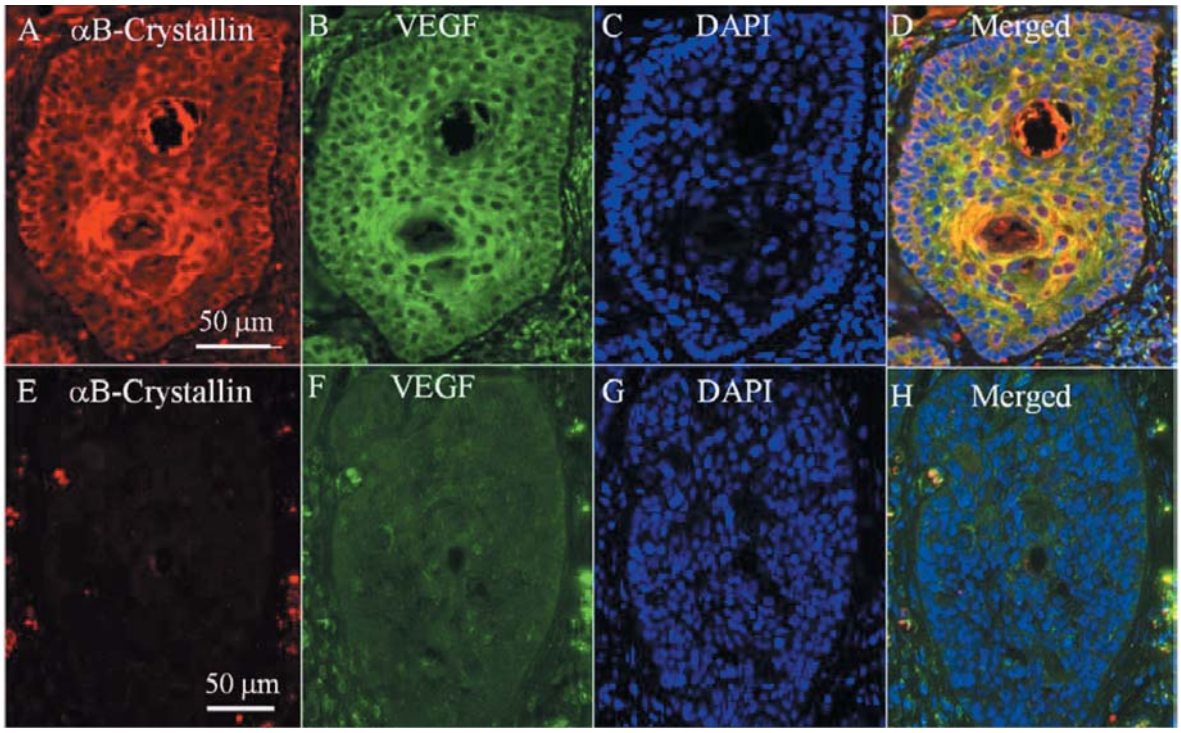

Figure 4. *Deceased αB-crystallin and vascular endothelial growth factor (VEGF) expression in conjunctival squamous cell carcinoma (CSCC) following mitomycin C (MMC) treatment. Top panel: CSCC treated without MMC, bottom panel: CSCC treated with MMC. Note that in CSCC treated with MMC, both αB-crystallin and VEGF expression are weaker than those in CSCCs treated without MMC. A, E: αB-Crystallin; B, F: VEGF; C, G: 4', 6-diamidino-2-phenylindole (DAPI) nuclear staining; D, H: merged image of A, B, C and, E, F, G, respectively.*

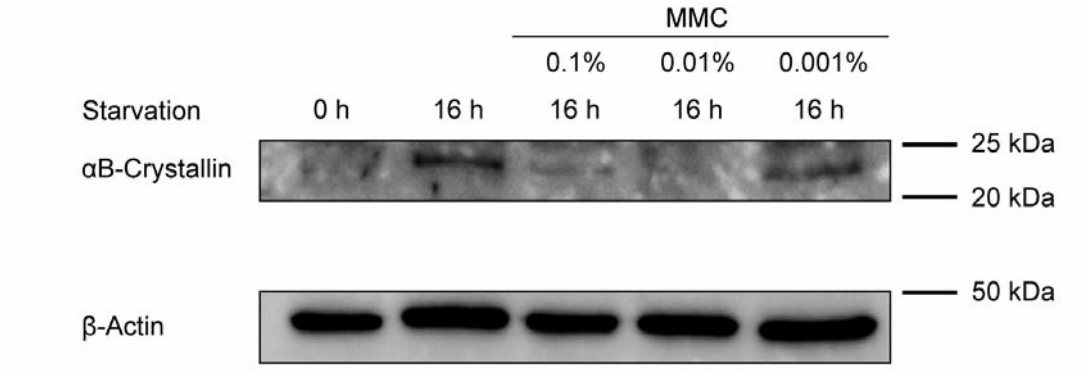

Figure 5. *Expression of αB-crystallin protein in cultured human conjunctival cells treated with mitomycin C (MMC). The αB-crystallin protein level increased 16 hours after starvation, while it decreased following MMC treatment, especially at concentrations of 0.1 and 0.01%.*

lower expression of VEGF *in vitro* (28), suggesting that the down-regulation of αB-crystallin by MMC addition also inhibits tumor growth *via* a decrease in VEGF secretion. Thus, it may be beneficial to reduce αB-crystallin expression for the suppression of CSCC development through VEGF down-regulation. Although further studies are needed to clarify the mechanism underlying the tumorigenesis of CSCC through αB-crystallin together with VEGF, the suppression of αB-crystallin expression may be a novel therapeutic approach for patients with CSCC.

Competing Interests

None.

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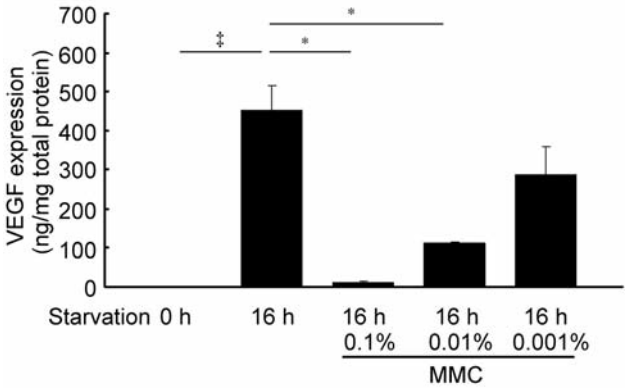

Figure 6. *Inhibition of vascular endothelial growth factor (VEGF) protein in cultured human conjunctival cells treated with mitomycin C (MMC). VEGF secretion was significantly induced 16 hours after starvation, while it decreased in a dose-dependent manner following MMC treatment. Bars show the average VEGF protein concentration in each group. Values are the mean±SEM. *p<0.05, †p<0.01, ‡p<0.001; N=3, Student's t-test.*

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