Potentiation of Bortezomib-Induced Apoptosis by TGF-β in Cultured Human Tenon's Fibroblasts: Contribution of the PI3K/Akt Signaling Pathway

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Purpose. To investigate the effects of bortezomib on cell apoptosis and proliferation in human Tenon's capsule fibroblasts (HTFs) after cotreatment with TGF-β.

Methods. The effect of bortezomib on the apoptosis and cell proliferation of cultured HTFs was determined with FACS analysis and 3-[4,5-demethylthiazol-2,5-diphenyl-2H-tetrazolium bromide] (MTT) assay with or without cotreatment of TGF-β. The apoptotic effect of cotreatment of bortezomib and TGF-β through the PI3K/Akt pathway was determined by Western blot analysis. The mRNA level of Bcl-2 and Bax was determined by RT-PCR. p53 expression, DNA-PKcs cleavage, and c-Jun phosphorylation were determined.

Results. Cotreatment with bortezomib (5 μM) and TGF-β (10 μM) increased the proportion of apoptotic cells in HTFs on FACS analysis, whereas either bortezomib or TGF-β treatment alone did not. The MTT assay also showed that when bortezomib was cotreated with TGF-β, the cell proliferation of HTFs induced by TGF-β treatment was significantly decreased at 72-hour incubation. The cotreatment of bortezomib and TGF-β specifically decreased the Akt phosphorylation induced by TGF-β, indicating on Western blot analysis that these changes are mediated by the PI3K/Akt pathway. The mRNA level of an apoptosis-related factor, *Bcl-2*, was significantly reduced, and p53 expression, DNA-PKcs cleavage, and c-Jun phosphorylation were increased after cotreatment.

Conclusions. Bortezomib-induced apoptosis is potentiated by TGF-β cotreatment in cultured HTFs by inhibition of the PI3K/Akt pathway, indicating that the effect of bortezomib may be potentiated when the level of TGF-β is elevated, as is observed in the postoperative period. (*Invest Ophthalmol Vis Sci.* 2010; 51:6232–6237) DOI:10.1167/iovs.10-5747

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Excess scarring of the conjunctiva after glaucoma filtration surgery is a major cause of the failure of surgical treatments.[1,2] Treatment with antimetabolites, such as mitomycin C (MMC) and 5-fluorouracil (5-FU), could decrease the frequency of scar formation and improve the surgical outcome. However, these drugs could be associated with serious complications such as bleb leakage, hypotonus maculopathy, and infective endophthalmitis.[3,4] Moreover, the response of individual patients to these agents can be idiosyncratic, making dose titration difficult.[5] These findings lead us to the need for novel agents that can efficiently increase the surgical outcome with less toxicity and fewer complications.

High levels of transforming growth factor (TGF)-β signaling have been implicated in excessive scarring. The TGF-β family, consisting of three isoforms denoted as β1, β2, and β3, regulates cell functions, such as proliferation, differentiation, apoptosis, and production of extracellular matrix.[6,7] In the eye, aqueous humor contains abundant TGF-β.[8] Importantly, TGF-β is the most potent growth factor in aqueous humor with regard to the stimulation of Tenon's capsule fibroblasts. This molecule is found in significantly higher concentrations in the aqueous humor of patients with glaucoma.[9–11] The wound-healing response after trabeculectomy is unique in that the wound site is bathed in aqueous humor, which surrounds the subconjunctival tissues in a functioning bleb. Thus, TGF-β is not only a key cytokine in the process of tissue repair and the most potent stimulator of scarring in the eye, it also may interact with drugs applied during filtration surgery.[6,8,9] In a previous report,[6] TGF-β was shown to reduce the antiscarring effect of MMC in a rabbit filtration model.

Bortezomib is a dipeptidyl boronic acid proteasome inhibitor that interferes with the function of the 26S proteasome complex. The drug disrupts the degradation or function, or both, of diverse cell cycle-, signaling-, and survival-related proteins, including NF-κB, p53, Bax, and stress-activated kinases, among others.[12–14] For reasons that remain unclear, proteasome inhibitors are lethal to tumor cells but relatively sparing toward their normal counterparts.[15] According to a current report by Sarkozi et al.,[16] bortezomib had cell type-specific effects on apoptosis/survival genes and pathways. In preclinical studies, proteasome inhibitors such as bortezomib disrupt multiple signaling pathways and potently induce apoptosis in tumor cells.[17]

Given that the proteasome inhibitor bortezomib demonstrated antiproliferative and proapoptotic effects, we hypothesized in the present study that it might prevent the proliferation and induce the apoptosis of cultured HTFs. This information could be useful in the consideration of clinical applications of bortezomib in glaucoma filtration surgery and for understanding the mechanism of surgery-induced scar formation.

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Materials and Methods

Cell Culture

In compliance with the provisions of the Declaration of Helsinki, human subconjunctival fibroblasts were obtained from excised Tenon's capsule specimens during strabismus surgery. Written informed consent was obtained before operative excision. Institutional human experimentation committee approval was also granted. Briefly, five 5-mm sections of Tenon's capsule were collected, minced, and placed in a 35-mm culture dish containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 g/mL penicillin, and 50 g/mL streptomycin (DMEM-10). Cells were allowed to migrate from the explanted tissue and were then incubated at 37°C in a 5% CO_2 environment. When a confluent monolayer of the primary culture stage was obtained, the cells were incubated with 0.05% trypsin and 5 mM EDTA at 37°C for 5 minutes and transferred to a 100-mm culture dish containing DMEM-10. Cells that maintained proliferative potential and fibroblast-like elongated morphology between the third and fifth passages were used for this study.

Bortezomib and TGF-β Treatment of Fibroblast Monolayers

The proteasome inhibitor bortezomib (Velcade; Millennium Pharmaceuticals, Cambridge, MA) was dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C. HTFs were seeded in multi-well tissue culture plates (BD Biosciences) and incubated overnight to allow attachment. The fibroblast monolayers were then washed and treated with a single application of TGF (10 ng/mL) and bortezomib (5 μM).

FACS Analysis

The number of apoptotic cells was quantified using propidium iodide (PI) staining and flow cytometry. Cells (3×10^5–5×10^5) were permeabilized and stained using 5 μL annexin V-FITC and propidium iodide (annexin V-FITC Apoptosis Detection Kit; BioVision Inc., Mountain View, CA). DNA staining was analyzed with a FACSCalibur (Becton-Dickinson, San Jose, CA).

MTT Assay

HTFs were seeded in 96-well cell culture plates. Cells were allowed to settle for 1 to 3 days in the presence or absence of TGF-β (10 μM) and bortezomib 0.05, 0.5, 5.0 μM) in a total volume of 200 μL per well. MTT (4, 5-dimethyl-thiazol-2yl)-2, 5-diphenyltetrazolium bromide) stock solution (20 μL) (5 mg/mL in PBS; Sigma-Aldrich) was added to each well, and cells were incubated at 37°C for 3 h. The MTT solution was discarded by gently inverting plates, and wells were filled with 200 μL DMSO. After plates were shaken vigorously for 20 minutes, the absorbance of each well was read with a spectrophotometric plate reader (Quant; BioTEK Instruments, Inc., Seoul, South Korea), which detected emissions at 560 nm.

Reverse Transcriptase–Polymerase Chain Reaction Analysis of *Bcl-2* and *Bax* mRNA Expression

Total cellular RNA was isolated using reagent (Trizol; Invitrogen-Gibco, Carlsbad, CA) according to the manufacturer's instructions. The RNA concentration and purity were determined spectrophotometrically (SmartSpec 3000; Bio-RAD, Hercules, CA). Total RNA was converted to cDNA using a first-strand synthesis kit (Superscript; Invitrogen-Gibco).

The PCR reaction for *Bcl-2* involved denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute and by a final incubation at 72°C for 10 minutes. The reaction mixture contained primer sequences specific to *Bcl-2* (forward, 5′-TTC TTT GAG TTC GGT GGG GTC-3′; reverse, 5′-TGC ATA TTT GTT TGG GGC AGG-3′).

The PCR reaction for *Bax* involved denaturation at 94°C for 5 minutes and then 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, followed by a final incubation at 72°C for 10 minutes. The reaction mixture contained primer sequences specific for *Bax* (forward, 5′-AGA GGA TGA TTG CCG CCG-3′; reverse, 5′-CAA CCA CCC TGG TCT TGG ATC-3′).

The quantity of amplified products was analyzed using an image documentation system (ImageMaster VDS; Pharmacia Biotech Inc., Uppsala, Sweden). Reverse transcription polymerase chain reaction (RT-PCR) products were analyzed by electrophoresis on 1% agarose gels. β-Actin levels were used to normalize mRNA expression levels.

Western Blot Analysis

Total cell lysates were prepared from cultured HTFs using lysis buffer (25 mM HEPES [pH 7.5], 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.05% [vol/vol] Triton X-100, 0.5 mM dithiothreitol [DTT], 0.4 mM phenylmethylsulfonyl fluoride [PMSF]; Sigma-Aldrich, St. Louis, MO), 2 μg/mL leupeptin (Sigma-Aldrich), and 2 μg/mL aprotinin (Sigma-Aldrich). After centrifugation for 10 minutes at 12,000*g*, protein content was determined using a BCA assay (Pierce, Rockford, IL) with bovine serum albumin as the standard.

For Western blot analysis, supernatant proteins (30 μg/lane) were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Hybond; GE Healthcare, Piscataway, NJ). Membranes were blocked with TBST (20 mM Tris, 137 mM NaCl [pH 7.4], and 0.02% [vol/vol] Tween 20) containing 5% (wt/vol) BSA, incubated with primary antibodies diluted in TBST for 24 hours at 4°C, and washed three times with TBST.

Primary antibodies were detected by incubating the membranes with horseradish peroxidase-conjugated anti–mouse or anti–rabbit antibody diluted 1:1000 in TBST for 1 hour, followed by three washes in TBST. Immunoreactive proteins were visualized using chemiluminescence detection reagents (ECL; Pierce) applied to autoradiograph films. Blots were stripped and reprobed according to the manufacturer's (GE Healthcare) instructions. Antibodies used for Western blot analysis included anti–DNA-PKcs, phospho-p53, p53 (Santa Cruz Biotechnology, Santa Cruz, CA), and phospho-c-Jun, phospho-ERK1/2, ERK1/2, phospho-JNK, JNK, phospho-p38, p38 mitogen-activated protein kinase (MAPK), phospho-Akt, and Akt antibodies (Cell Signaling, Danvers, MA).

Statistical Analysis

Data are presented as mean ± SD. Statistical comparisons between groups were performed using one-way ANOVA, followed by the Bonferroni multiple range test of significance. Statistical significance was accepted at $P < 0.05$.

Results

Induction of Fibroblast Apoptosis by Cotreatment with TGF-β and Bortezomib

Cells were incubated with TGF-β (10 μM), bortezomib (5 μM), or both for 72 hours, after which they were stained and underwent FACS analysis to determine the amount of apoptosis and necrosis. Viable cells excluded propidium iodide and did not bind annexin-V. Apoptotic and necrotic cells externalized phosphatidylserine on the plasma membrane and were labeled with annexin-V. Apoptotic cells with intact membranes excluded propidium iodide, whereas necrotic cells took up propidium iodide, which stained nuclear DNA. Cotreatment with TGF-β and bortezomib increased the proportion of apoptotic cells in HTFs, whereas TGF-β or bortezomib treatment alone did not (Fig. 1).

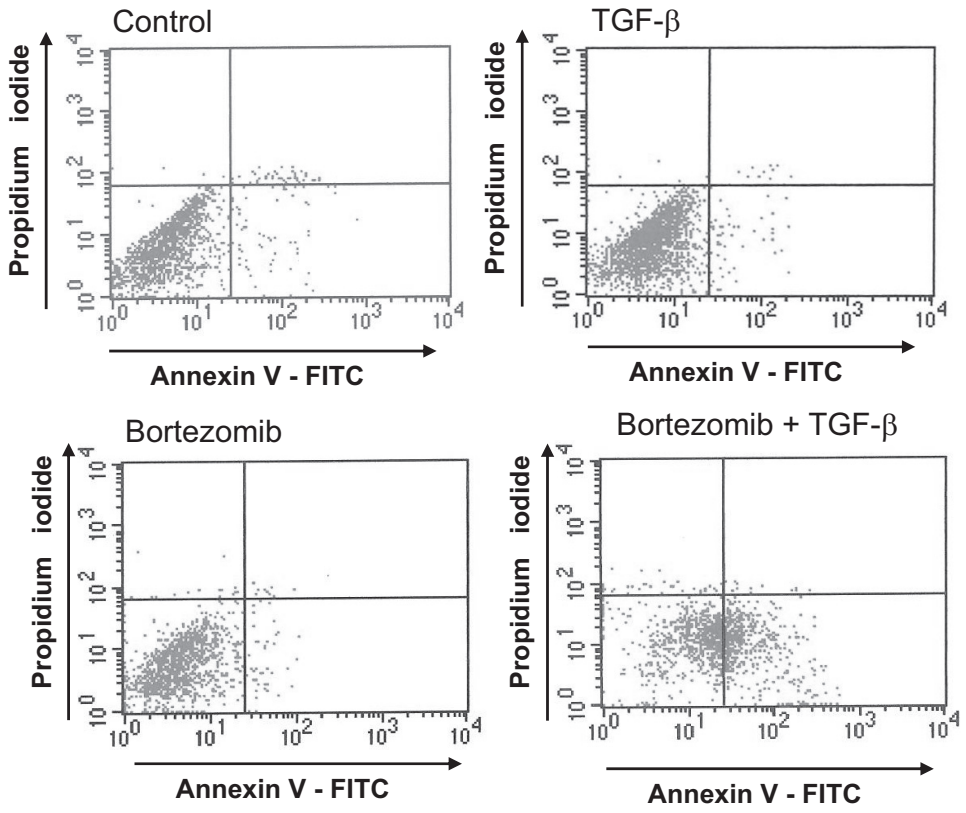

FIGURE 1. The effect of cotreatment with bortezomib and TGF-β on cultured HTFs: FACS analysis. Cells were incubated with bortezomib, TGF-β, or both for 72 hours, after which they were harvested. The DNA was stained with propidium iodide, and the cells were analyzed using FACS. Cotreatment with bortezomib and TGF-β increased the proportion of apoptotic cells. Representative images from three independent experiments are shown.

Time-Dependent and Dose-Dependent Effects of Bortezomib on Cultured Human Tenon's Fibroblasts

There was a significant decrease in the cell proliferation of HTFs compared with control at 72 hours when cells were treated with a 5-μM solution (Fig. 2A). The MTT assay demonstrated that TGF-β (10 μM) treatment significantly increased the proliferation of HTFs at all time points. However, when bortezomib (5 μM) was cotreated with TGF-β (10 μM), the cell proliferation of HTFs induced by TGF-β treatment was significantly decreased at 72-hour incubation (Fig. 2B).

Induction of Akt Dephosphorylation by Cotreatment with TGF-β and Bortezomib

ERK, p38, and PI3K/Akt signaling pathways have been proven to be involved in apoptosis and cell proliferation.[18–21] Thus, we examined whether bortezomib might have an effect on these signaling pathways. In the present experiment, TGF-β treatment strongly induced the phosphorylation of Akt at 24 hours and slightly enhanced the phosphorylation of ERK. However, when bortezomib was cotreated with TGF-β, the phosphorylation of Akt induced by TGF-β treatment was significantly decreased (Fig. 3A). The activation state of other markers of the MAPK signaling pathway (e.g., p38 and ERK) was also examined; no significant changes were observed (Figs. 3B, 3C).

Expression of Bcl-2 and Bax

Cells were incubated with TGF-β, bortezomib, or both for 24 hours. After this the cells were harvested, RNA was isolated, and RT-PCR analysis was performed to determine the expression levels of the apoptosis-related factors *Bcl-2* and *Bax*. Cotreatment with TGF-β and bortezomib significantly reduced *Bcl-2* mRNA expression and slightly increased *Bax* expression in HTFs (Fig. 4).

Upregulation of p53 Expression and Induction of p53 Phosphorylation by Bortezomib Treatment and Cotreatment with TGF-β and Bortezomib

Because p53 is a substrate of the ubiquitin-proteasome pathway, we first examined the effect of bortezomib on p53 protein ex-

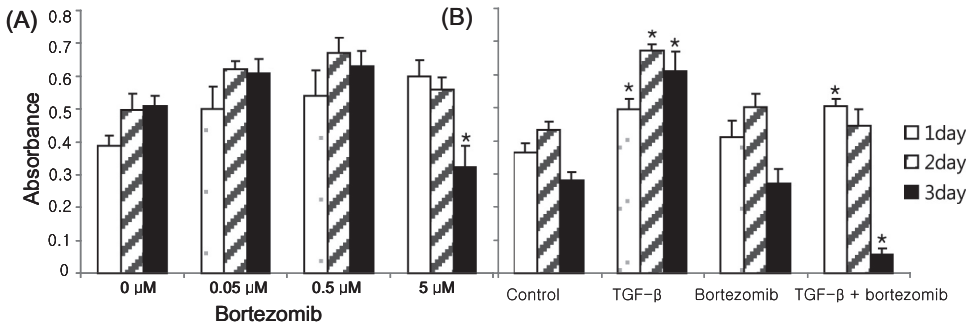

FIGURE 2. **(A)** Time- and dose-dependent effects of bortezomib on HTF proliferation. Cells were incubated with various concentrations of bortezomib for various times, after which cell proliferation was measured using the MTT assay. **(B)** Time-dependent effects of bortezomib, TGF-β, or both. Cotreatment with bortezomib and TGF-β significantly reduced cellular proliferation of HTF. Data are the mean ± SD of results from three independent experiments. $^*P < 0.05$ compared with control.

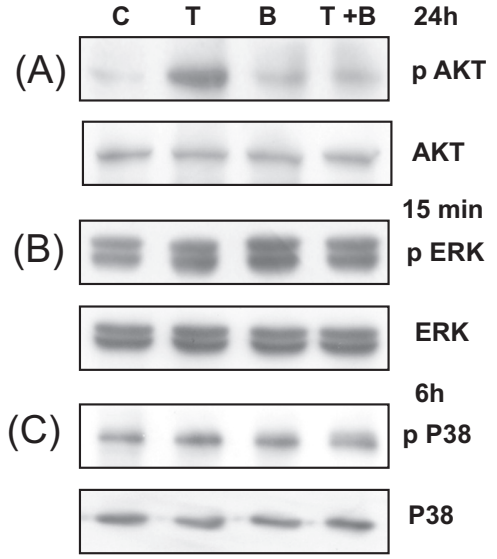

FIGURE 3. The effect of cotreatment with bortezomib and TGF-β on activation of the PI3K/Akt pathway. (**A**) Akt phosphorylation. TGF-β treatment strongly induced phosphorylation of Akt at 24 hours, and cotreatment with TGF-β significantly reduced Akt phosphorylation. (**B**) ERK and (**C**) p38 phosphorylation. The activation state of other markers of MAPK signaling pathway (e.g., p38 and ERK) were also examined, and no significant changes were observed. Representative images from three independent experiments are shown. C, control; T, TGF-β; B, bortezomib; T+B, TGF-β + bortezomib.

pression in HTFs. Cells were incubated with bortezomib for up to 8 hours. As expected, bortezomib treatment induced the phosphorylation of p 53 and upregulated p53 protein expression in a time-dependent manner. Expression was highest at 8 hours (Fig. 5A). We next examined the effect of cotreatment with TGF-β and bortezomib, which further enhanced the phosphorylation and expression of p53 protein (Fig. 5B).

Induction of JNK and c-Jun Phosphorylation by Bortezomib Treatment and Cotreatment with TGF-β and Bortezomib

The stress response in HTFs after bortezomib treatment was evaluated by measuring the expression of phosphorylated c-Jun NH_2-terminal kinase (JNK) and c-Jun. Bortezomib induced the

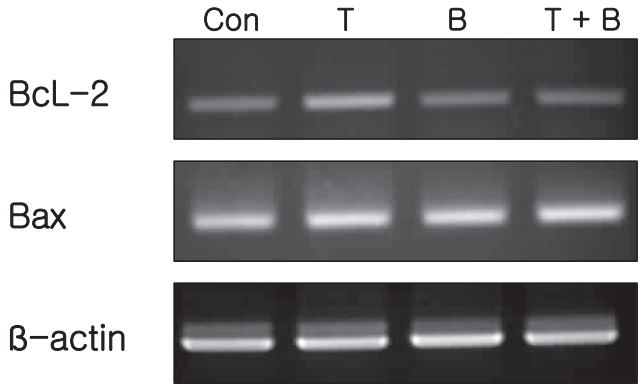

FIGURE 4. The effect of cotreatment with bortezomib and TGF-β on Bcl-2 and Bax mRNA expression in HTFs. Cotreatment with bortezomib and TGF-β markedly reduced Bcl-2 mRNA expression and slightly enhanced Bax expression. Representative images from three independent experiments are shown. Con, control; T, TGF-β; B, bortezomib; T+B, TGF-β + bortezomib.

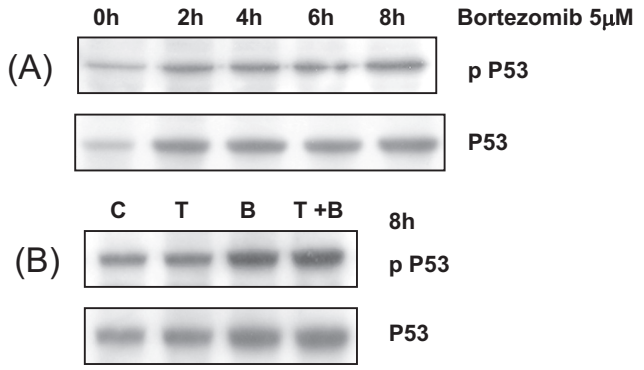

FIGURE 5. (**A**) Time course for the effect of bortezomib on protein expression and phosphorylation of p53. Bortezomib increased protein expression and phosphorylation of p53 in a time-dependent fashion. (**B**) The effect of cotreatment with bortezomib and TGF-β on protein expression of p53. Bortezomib treatment and cotreatment with bortezomib and TGF-β increased the protein expression and phosphorylation of p53. Representative images from three independent experiments are shown. C, control; T, TGF-β; B, bortezomib; T+B, TGF-β + bortezomib.

phosphorylation of JNK and c-Jun proteins in a time-dependent fashion, which peaked at 1 hour and 24 hours, respectively (Figs. 6A, 6B). We next examined the effect of cotreatment with TGF-β and bortezomib. TGF-β treatment weakly induced the phosphorylation of c-Jun, whereas both bortezomib and cotreatment with TGF-β and bortezomib further enhanced the phosphorylation of JNK and c-Jun (Figs. 6C, 6D).

Induction of DNA-PKcs Cleavage in HTF by Bortezomib Treatment and Cotreatment with TGF-β and Bortezomib

Previous reports have shown that DNA-PKcs is a target for IL-1–converting enzyme (ICE)-like and CPP32-like apoptotic protease and that bortezomib induces DNA-PKcs cleavage in multiple myeloma cells.[12,22,23] Thus, we examined whether DNA-PKcs was cleaved by treatment with bortezomib, TGF-β, or both.

Bortezomib treatment alone induced the cleavage of DNA-PKcs (180-kDa protein), and cotreatment with TGF-β potentiated the cleavage of DNA-PKcs (Fig. 7).

DISCUSSION

In the present study, we examined the effects of bortezomib on apoptosis and proliferation in cultured HTFs after cotreatment with TGF-β, which mimics postoperative conditions observed after glaucoma filtration surgery.

FACS analysis revealed that cotreatment with TGF-β and bortezomib strongly potentiated apoptosis, whereas treatment with either bortezomib or TGF-β alone had little effect. In previous reports, proteasome inhibitors including bortezomib induced the apoptosis of various tumor cells in vitro and marked in vivo antitumor activity.[13,24,25]

Cotreatment with bortezomib and TGF-β also significantly inhibited the cellular proliferation of cultured HTFs, as determined by MTT assay. Several previous reports have demonstrated TGF-β–induced proliferation of HTFs, which might play a critical role in scar formation during the postoperative period after filtration surgery.[1,26,27] In the present study, the antiproliferative effects of bortezomib were significantly potentiated when applied with TGF-β. These results indicated that bortezomib might be more effective during the postoperative period, when TGF-β levels are elevated.

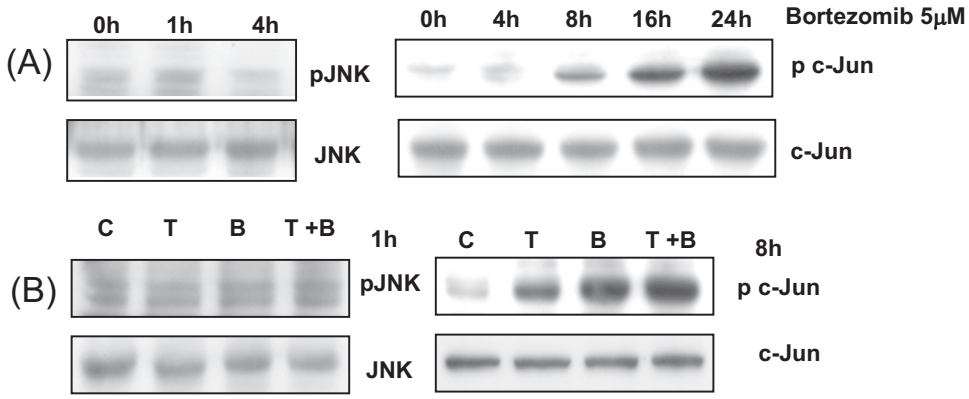

FIGURE 6. (**A**) Time course for the effect of bortezomib on JNK and c-Jun activation. Bortezomib induced the phosphorylation of JNK and downstream target c-Jun. (**B**) The effect of cotreatment with bortezomib and TGF-β on the phosphorylation of JNK and c-Jun. Bortezomib treatment and cotreatment with bortezomib and TGF-β increased the phosphorylation of JNK and c-Jun. Representative images from three independent experiments are shown. C, control; T, TGF-β; B, bortezomib; T+B, TGF-β + bortezomib.

In cultured HTFs, TGF-β treatment induced proliferation, implicating its critical role in scar formation during the postoperative period after filtration surgery.[1,26–28] In the present study, we demonstrated a significant increase in HTF proliferation as determined by MTT assay and Akt phosphorylation after treatment with TGF-β treatment alone.[29] However, when TGF-β and bortezomib were administered simultaneously, the phosphorylation of Akt was significantly reduced; cell proliferation was decreased and bortezomib-induced apoptosis was increased, implicating that HTF proliferation and survival after treatment with TGF-β were mediated by the PI3K/Akt pathway. Bortezomib treatment suppressed the PI3K/Akt signaling pathway and, therefore, HTF proliferation. The present results are in accordance with previous reports showing that TGF-β promoted myofibroblast resistance to apoptosis by activation of the PI3K/Akt signaling pathway.[30–32] PI3K/Akt pathway activation by TGF-β might also promote resistance to apoptosis in HTF, as was observed in myofibroblasts.[30–32] Although the exact mechanisms by which cotreatment with TGF-β–potentiated bortezomib-induced apoptosis should be elucidated in further investigations, the present study demonstrated, for the first time, the effect of bortezomib and TGF-β cotreatment in the inhibition of proliferation and the induction of apoptosis in cultured HTFs.

Recent data also indicate that bortezomib may promote apoptosis, in part by its effects on Bcl-2 family members.[14,33] We found that bortezomib treatment decreased Bcl-2 mRNA levels, and cotreatment with TGF-β further potentiated the effect. Similar results were reported for bortezomib in human glioblastoma multiforme cells and human pancreatic cancer cell lines.[34,35]

In the present study, we demonstrated that treatment with bortezomib alone and cotreatment with TGF-β increased p53 expression and phosphorylation. Bortezomib also induced the expression of p53 and upregulated p53-induced gene expression in the induction of apoptosis in other cancer cell lines.[36–38]

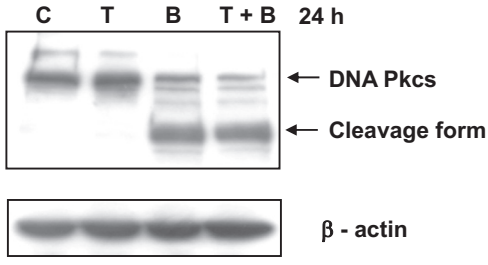

FIGURE 7. The effect of cotreatment with bortezomib and TGF-β on DNA PKcs cleavage. Bortezomib treatment and cotreatment with bortezomib and TGF-β induced DNA-PKcs cleavage in HTFs. Representative images from three independent experiments are shown. C, control; T, TGF-β; B, bortezomib; T+B, TGF-β + bortezomib.

Cotreatment with bortezomib and TGF-β also induced the phosphorylation of JNK and c-Jun. Bortezomib has been shown to induce the transcriptional triggering of apoptotic cascades, the downregulation of growth/survival kinases, and stress kinases. JNK, one of these stress-response proteins, and its downstream protein c-Jun mediated bortezomib-induced apoptosis in multiple myeloma cells and esophageal squamous cell carcinoma cells.[12,39]

Cotreatment with bortezomib and TGF-β increased DNA-PKcs cleavage. DNA-PKcs is a phosphatidylinositol kinase that has a crucial role in the repair of damaged DNA and the phosphorylation of p53.[40,41] In a previous report regarding molecular mechanisms of bortezomib, the drug was found to impair DNA repair by cleavage of DNA-PKcs and to activate p53, resulting in the induction of apoptosis in multiple myeloma cell lines.[12]

In the present study, we used cultured HTFs treated with TGF-β as an in vitro model of fibrosis and scar formation after filtration surgery. The major drawback of this in vitro approach was that this model could not exactly represent the complexity of the physiologic condition after glaucoma filtration surgery because many other growth factors and cellular components are involved in the postoperative scarring process. However, HTFs and TGF-β have been known to be key players in postoperative scarring after glaucoma filtration surgery, and this may be considered a prescreening model to be used to select for substances to treat scarring processes in further in vivo models.[6,8–9] On the other hand, findings obtained from animal models do not necessarily apply to the human in vivo condition, as evidenced by CAT-152, a recombinant human monoclonal antibody to human TGF-β2. The drug has demonstrated some promise in the animal model, but clinical applications remain uncertain.[5,42] Our results should encourage further in vivo studies regarding efficacy, toxicity, and the potential role of bortezomib as a means to prevent bleb failure in glaucoma filtration surgery.

In summary, the present study demonstrated that cotreatment with TGF-β and bortezomib significantly inhibited cell proliferation and induced apoptosis in cultured HTFs through the inhibition of the PI3K/Akt pathway and p53 induction, DNA-PKcs cleavage, and c-Jun phosphorylation. Increased levels of TGF-β were observed in the aqueous humor of glaucoma patients; our in vitro model mimics the pathophysiological changes observed after glaucoma filtration surgery. Bortezomib may thus prove to be therapeutically useful for inhibiting excessive scarring in the wound after glaucoma filtration surgery. Further studies are required to determine the in vivo effects of the drug in animal models and to establish proper dosage and safety.

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