Role of eIF5A in TNF-α–Mediated Apoptosis of Lamina Cribrosa Cells

Catherine A. Taylor,[1] *Michelle Senchyna,*[2] *John Flanagan,*[2,3] *Elizabeth M. Joyce,*[2] *Dominic O. Cliche,*[1] *Adrienne N. Boone,*[1] *Shelley Culp-Stewart,*[2] *and John E. Thompson*[1]

Purpose. To determine the role of eukaryotic translation initiation factor 5A (eIF5A) in TNF-α–induced apoptosis of lamina cribrosa (LC) cells.

Methods. LC cells were isolated from optic nerve heads of eyes of two human donors. The cells were treated with TNF-α and camptothecin, a TNF synergist, and the incidence of apoptosis was scored by Hoechst staining. Expression of eIF5A protein in response to camptothecin or a combination of camptothecin and TNF-α was determined by Western blot analysis. The ability of small inhibitory (si)RNAs directed against eIF5A to protect LC cells from TNF-α–induced apoptosis was determined by Hoechst and TUNEL staining of transfected LC cells.

Results. TNF-α and camptothecin synergized to induce greater than two times more apoptosis in LC cells than when the cells were treated with TNF-α or camptothecin separately. Expression of eIF5A protein increased significantly after 8 hours of exposure to TNF-α and camptothecin, but not in response to camptothecin alone. siRNAs directed against eIF5A reduced apoptosis of LC cells in response to TNF-α and camptothecin by between 35% and 69%, as determined by Hoechst staining. An siRNA against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) also reduced apoptosis of LC cells by 42%. TUNEL of transfected LC cells treated with TNF-α and camptothecin revealed an 80% reduction in apoptosis with siRNA against eIF5A.

Conclusions. TNF-α, in synergy with camptothecin, induces apoptosis in human LC cells. eIF5A is upregulated by LC cells in response to TNF-α, and siRNAs against eIF5A protect LC cells from apoptosis. Thus, eIF5A appears to be a novel pro-apoptotic protein in the TNF pathway and a possible target for treatment of glaucoma. (*Invest Ophthalmol Vis Sci.* 2004;45: 3568–3576) DOI:10.1167/iovs.03-1367

Glaucoma describes a group of potentially blinding ocular disorders that involve progressive optic neuropathy of unknown etiology, frequently associated with elevated intraocular pressure (IOP).[1] The major site of injury appears to be at the level of the lamina cribrosa (LC).[2] Two common glial cell types have been identified within the LC: optic nerve head (ONH) astrocytes (type 1β) and LC cells.[3–6] They play an essential role in the mechanical and metabolic support of the optic nerve axons and are capable of responding to local mechanical and ischemic insult. In glaucoma, these activated cells are thought to lead to death of the retinal ganglion cells (RGCs) by apoptosis[3,4,7–16] and to remodeling of the LC.

The involvement of neurotoxic substances, particularly tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and nitric oxide (NO), in glaucoma is also becoming increasingly clear. TNF-α is a proinflammatory cytokine that mediates the response of the immune system to infection and trauma and is capable of inducing apoptosis in susceptible cells through activation of caspases.[17,18] TNF-α is dramatically upregulated by reactive astrocytes and microglia cells after ischemic and excitotoxic brain injury and contributes to neuronal loss in injured brain.[19–22] Similarly, TNF-α and its receptor have been found to be upregulated in the glaucomatous eye.[23] As well, it has been demonstrated that TNF-α and NO are secreted by glial cells in response to simulated ischemia and elevated hydrostatic pressure and induce apoptosis in cocultured retinal ganglion cells.[24] A recent study has also shown that a polymorphism of TNF-α, which results in increased TNF-α expression, is associated with primary open-angle glaucoma (POAG) in Chinese subjects.[25] It appears, therefore, that TNF-α may be an important factor in the neurodegenerative process of the glaucomatous eye.

Eukaryotic translation initiation factor 5A (eIF5A) was originally isolated from immature red blood cells and identified as a translation initiation factor on the basis of its ability to stimulate the formation of methionyl-puromycin, a dipeptide analogue, under in vitro conditions.[26,27] However, this view is no longer popular, because these experiments have never been replicated in situ and because deletion of eIF5A in yeast leads to only marginal decreases in total protein synthesis.[28] eIF5A protein is posttranslationally activated by conversion of a conserved lysine in the N terminus to the unusual amino acid, hypusine. It has been proposed that hypusination of eIF5A is necessary for survival, because inactivation of both eIF5A isoforms in yeast blocks cell division.[28–31] Several studies have suggested that eIF5A functions as a nucleocytoplasmic shuttle for specific subsets of mRNAs involved in cell division rather than as a factor in global protein synthesis.[28–37] However, the possibility that eIF5A also has a function during cell death has recently been suggested.[38,39]

Elevated levels of TNF-α are known to be present in the LC of glaucomatous eyes.[23–25,40,41] We describe evidence indicating that TNF-α in conjunction with camptothecin, a known TNF synergist, induces apoptosis in LC cells, and that this effect is inhibited by small inhibitory (si)RNAs directed against eIF5A.

Materials and Methods

Human LC Cell Culture

Paired human eyes of healthy donors were obtained within 48 hours after death, from the Eye Bank of Canada, Ontario Division, and were

From the [1]Department of Biology and the [2]School of Optometry, University of Waterloo, Waterloo, Ontario, Canada; and the [3]Department of Ophthalmology and Vision Sciences, University of Toronto, Toronto, Ontario, Canada.

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Corresponding author: John E. Thompson, Department of Biology, University of Waterloo, Waterloo, Ontario, N2L 3G1 Canada; jet@sciborg.uwaterloo.ca.

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used to initiate primary cell cultures. LC cell lines 506 and 517 were established from the ONHs of eyes of an 83-year-old male and a 17-year-old male, respectively. ONHs (with attached pole) were removed and placed in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotic/antimycotic, glutamine, and 10% fetal bovine serum (FBS) for 3 hours. The ONH button was retrieved from each tissue sample and cut into four small pieces with fine dissecting scissors. Explants were cultured in 12.5-cm^2 plastic culture flasks in DMEM. Cell growth was observed within 1 month in viable explants. Once the cells reached 90% confluence, they were trypsinized and subjected to differential subculturing to produce LC and astrocyte cell populations. LC cells were enriched by subculture in 25-cm^2 flasks in DMEM supplemented with gentamicin, glutamine, and 10% FBS. Cells were subcultured and maintained on an ongoing basis, according to this protocol.

The identity and purity of cell populations obtained by differential subculturing were determined with fluorescent antibody staining on eight-well culture slides. Cells were fixed in 10% formalin solution, washed three times with Dulbecco's phosphate-buffered saline (DPBS), permeabilized for 5 minutes in 0.5% Triton X-100, and washed three times with DPBS. After the fixed cells were blocked with 1% BSA in DPBS, antibodies were diluted in 0.1% bovine serum albumin (BSA) in DPBS and applied to the cells in six of the wells. The remaining two wells were treated with only 0.1% BSA solution or secondary antibody and thus served as the negative control. Cells were incubated with the primary antibodies for 1 hour and washed three times with DPBS. Secondary antibodies were diluted in DPBS, added to each well, and incubated for 1 hour at room temperature. After washing with DPBS, the slide was washed in water, air-dried, stained with Hoechst 33258 as described later, and overlaid with McIlvaine's buffer (0.021 M citric acid and 0.058 M $Na_2HPO_4.7H_2O$ [pH 5.6]). Immunofluorescence was viewed under a fluorescence microscope with appropriate filters and compared with control wells that were not treated with primary antibody. All primary antibodies were obtained from Sigma-Aldrich (Oakville, Ontario, CA). All secondary antibodies were purchased from Molecular Probes (Eugene, OR) and included Alexa 488 goat anti-mouse IgG (1:100), Alexa 568 goat anti-mouse IgM (1:85), and Alexa 488 goat anti-rabbit IgG (H+L; 1:100). Primary antibodies used to identify LC cells were anti-collagen I (mouse monoclonal IgG; 1:400), anti-collagen IV (mouse monoclonal IgG; 1:400), anti-laminin (rabbit polyclonal IgG; 1:20), anti-cellular fibronectin (mouse monoclonal IgM; 1:400), anti-glial fibrillary acidic protein (GFAP; mouse monoclonal IgG; 1:400), and anti-α-smooth muscle actin (mouse monoclonal IgG; 1:400). Cell populations were deemed to comprise LC cells if they stained positively for collagens I and IV, laminin, cellular fibronectin, and α-smooth muscle actin and negatively for GFAP. Both LC cell lines were fully characterized and found to contain more than 90% LC cells.

Construction of siRNAs and Transfection

siRNAs directed against human eIF5A were used specifically to suppress expression of eIF5A in LC cells. Target sequences for siRNA in the human eIF5A transcript (GenBank accession number NM_001970; http://ncbi.nlm.nih.gov/GenBank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) were identified using the design guidelines suggested by Ambion, Inc. (Austin, TX; http://www.ambion.com/techlib/tb/tb_506.html). The sequence was scanned for the pattern $AA(N_{19})$ with a G/C content of 35% to 50%, avoiding regions within 50 to100 nucleotides of the start and termination codons and regions containing four or more G's in a row. A BLAST search was performed on potential siRNA target sequences to ensure specificity. Six siRNAs were generated by in vitro transcription (Silencer siRNA Construction Kit; Ambion, Inc.). Four siRNAs were generated against human eIF5A (siRNAs 1–4). Two siRNAs were used as the control: an siRNA directed against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) provided in the kit and an siRNA (siRNA-5) with the reverse sequence of eIF5A-specific siRNA-1, which does not target eIF5A or any other known human gene product. The siRNAs were generated according to the manufacturer's protocol. In brief, for each siRNA constructed, two DNA oligonucleotides were synthesized (Sigma-Aldrich), encoding the desired siRNA strands and an 8-base sequence complementary to the 5′ end of a T7 promoter primer. The oligonucleotides were annealed to the T7 promoter primer, filled-in with Klenow fragment, and used in an in vitro transcription reaction with T7 RNA polymerase. The reactions from both strands of the desired siRNA were then combined to permit annealing of the two siRNA strands and treated with DNase and RNase to remove the template and any single-stranded RNA. Column purification was then used to purify the resultant double-stranded siRNA complexes. The target sequences for the eIF5A and control siRNAs are siRNA-1, 5′-AAAGGAATGACTTCCAGCTGA-3′; siRNA-2, 5′-AAGATCGTCGAGATGTCTACT-3′; siRNA-3, 5′-AAGGTCCATCTGGTTGGTATT-3′; siRNA-4, 5′-AAGCTGGACTCCTCCTACACA-3′; and siRNA-5, 5′-AAAGTCGACCTTCAGTAAGGA-3′.

LC cells were transfected with siRNA (Lipofectamine 2000; Invitrogen, San Diego, CA). Cells were seeded onto eight-well culture slides at 7500 cells per well and allowed to reach 40% to 70% confluence before transfection was initiated. Transfection medium sufficient for one well of an eight-well culture slide was prepared by diluting 25.5 picomoles of siRNA to a final volume of 21.2 μL in serum-free medium (Opti-Mem; Sigma-Aldrich). Transfection agent (0.425 μL Lipofectamine 2000; Invitrogen) was diluted to a final volume of 21.2 μL in the serum-free medium and incubated for 7 to 10 minutes at room temperature. The diluted transfection reagent mixture was then added to the diluted siRNA mixture, and the mixture was incubated at room temperature for 20 to 30 minutes. To initiate transfection, the cells were washed once with DMEM, and 135 μL of DMEM was then added to the well and overlaid with 42.4 μL transfection medium. The cells were incubated in the culture chamber for 4 hours. After this incubation, 65 μL of medium containing 30% FBS was added to the well, and the cells were incubated for 72 hours before treatment with 50 μM camptothecin (Sigma-Aldrich) and 10 ng/mL of TNF-α (Leinco Technologies, St. Louis, MO) to induce apoptosis.

For Western blot analysis, transfection of cells with siRNA was performed in 24-well plates in the same conditions, except that the volumes were increased by 2.3-fold. At the end of the 72-hour incubation, cells were treated with camptothecin and TNF-α, and cell lysates were collected for Western blot analysis.

Detection of Apoptotic Cells

Transfected cells that had been treated with TNF-α and camptothecin were stained with Hoechst 33258 (Sigma-Aldrich) to determine the percentage of cells undergoing apoptosis. Briefly, cells were fixed with a 3:1 mixture of absolute methanol and glacial acetic acid and then incubated with Hoechst stain (0.5 μg/mL Hoechst 33258 in PBS). After a 10-minute incubation in the dark, the staining solution was discarded, the chambers separating the wells of the culture slide were removed, and the slide was washed three times for 1 minute each with deionized water. After the slides were washed, a few drops of McIlvaine's buffer was added, and the cells were overlaid with a coverslip. The stained cells were viewed under a fluorescence microscope equipped with a UV filter (UV-G 365, filter set 487902) to identify Hoechst-stained nuclei. Cells with brightly stained or fragmented nuclei were scored as apoptotic. A minimum of 200 cells was counted per well.

Apoptotic cells were also identified with TUNEL (DeadEnd Fluorometric TUNEL; Promega, Madison, WI), which detects the DNA fragmentation that is a characteristic feature of apoptotic cells. After the cells were Hoechst stained and the apoptotic cells counted, the mounting medium was removed from the slide with deionized water, and the slide was labeled by TUNEL according to the manufacturer's protocol. The cells were viewed under a fluorescence microscope with a UV filter (UV-G 365, filter set 487902; Carl Zeiss Meditec, Oberkochen, Germany) to count the total number of nuclei in the field. Using the same field of view, the cells were then viewed using a fluorescein filter (Green H546, filter set 48915), and any nuclei fluorescing bright green were scored as apoptotic. The percentage of apoptotic cells in the field of view was calculated by dividing the number of bright green nuclei

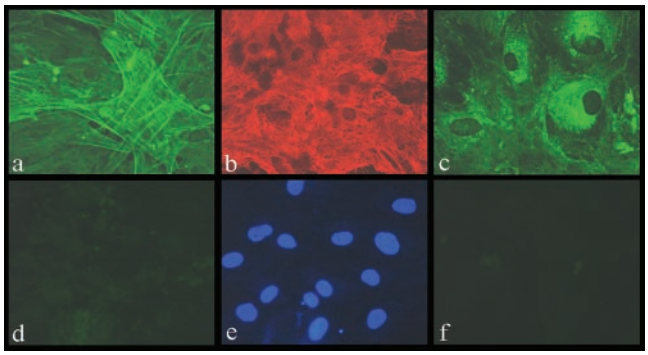

FIGURE 1. Characterization of LC cells by immunofluorescence. Human LC cells (line 506) isolated from the ONH of the eye of an 83-year-old male donor were characterized by immunofluorescence. Primary antibodies or staining were (**a**) actin; (**b**) fibronectin; (**c**) laminin; (**d**) GFAP; (**e**) Hoechst-stained nuclei (same field as in **d**); (**f**) control with Alexa 488 goat anti-mouse IgG alone (secondary antibody for actin and GFAP, without primary antibody). Magnification, ×400.

counted using the fluorescein filter by the total number of nuclei counted under the UV filter. A minimum of 200 cells was counted per well.

Protein Extraction and Western Blot Analysis

Protein was isolated for Western blot analysis from LC cells growing on 24-well plates by washing the cells twice in PBS (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4) and then adding 50 μL of boiling lysis buffer (2% SDS, 50 mM Tris-HCl [pH 7.4]). The cell lysate was collected in a microcentrifuge tube, boiled for 5 minutes, and stored at −20°C. Protein concentrations were determined by protein assay (Bicinchoninic Acid Kit; BCA; Sigma-Aldrich). For Western blot analysis, 5 μg of total protein was fractionated on a 12% SDS-polyacrylamide gel. The separated proteins were transferred to a polyvinylidene difluoride membrane. The membrane was blocked for 30 seconds with polyvinyl alcohol (30 seconds; 1 μg/mL), washed well with water, and then incubated for 1 hour in blocking solution of 5% skim milk in PBS-T. The primary antibody was diluted in a solution of 5% milk in PBS and incubated with the membrane for 1 hour. The primary antibodies used were anti-eIF5A (BD Transduction Laboratories, Lexington, KY; mouse IgG) and anti-β-actin (Oncogene Science, Manhasset, NY; mouse IgM) each at a dilution of 1:20,000 in 5% milk. The membranes were washed three times in PBS-T and incubated for 1 hour with the appropriate HRP-conjugated secondary antibodies diluted 1:5000 in 1% milk in PBS. The blot was washed, and a Western blot analysis detection kit (ECL Plus; Amersham Pharmacia Biotech, Piscataway, NJ) was used to detect antibody-bound proteins. After detection for eIF5A, the blots were stripped according to the protocol provided by the chemiluminescence detection system and reprobed with the actin antibody to confirm equal loading.

RESULTS

Characterization of Cultured LC Cells

Two LC cell lines were established from human ONHs obtained from male donors 83 (506) and 17 (517) years of age. The cells isolated from the human LC had the same broad, flat morphology with prominent nuclei observed for LC cells in other studies.[5,6] In addition, consistent with previous characterizations of LC cells,[5,6,42–44] the human LC cells showed immunoreactivity to α-smooth muscle actin (Fig. 1a) as well as to the extracellular matrix proteins, cellular fibronectin (Fig. 1b), laminin (Fig. 1c), collagen I, and collagen IV (data not shown). Consistent with previous findings,[6] negative immunoreactivity of the human LC cells to GFAP was also observed (Fig. 1d). Positive immunoreactivity to GFAP would have resulted in green fluorescence. The presence of cells in the GFAP-negative field (Fig. 1d) was confirmed by the presence of Hoechst-stained nuclei in the same field (Fig. 1e). The control treated with secondary antibody for actin and GFAP, but no primary antibody, is shown for comparison (Fig. 1f). The data shown in Figure 1 are for LC cell line 506, and comparable data were obtained for LC cell line 517. These observations collectively indicate that the cell lines comprised primarily (>90%) LC cells.

TNF-α–Induced Apoptosis

Because TNF-α is believed to play an important role during the glaucomatous process, the susceptibility of LC cells to the cytotoxic effects of this cytokine was examined. Many normal cells are known to be resistant to TNF-α cytotoxicity and hence camptothecin, a topoisomerase I inhibitor that has been shown to synergize with TNF-α and other members of the TNF family to induce death in a variety of cell types,[45–47] was used to sensitize LC cells to TNF-α cytotoxicity. Confluent LC cells were exposed to either TNF-α, camptothecin, or a combination of camptothecin and TNF-α for 48 hours. Hoechst staining revealed that TNF-α alone was not cytotoxic to LC cells (Fig. 2). Camptothecin was able to induce apoptosis in LC cells. However, the combination of TNF-α and camptothecin treatment more than doubled the number of cells undergoing apoptosis compared with camptothecin alone (Fig. 2). Thus, camptothecin clearly increases the ability of LC cells to initiate apoptosis in response to TNF-α.

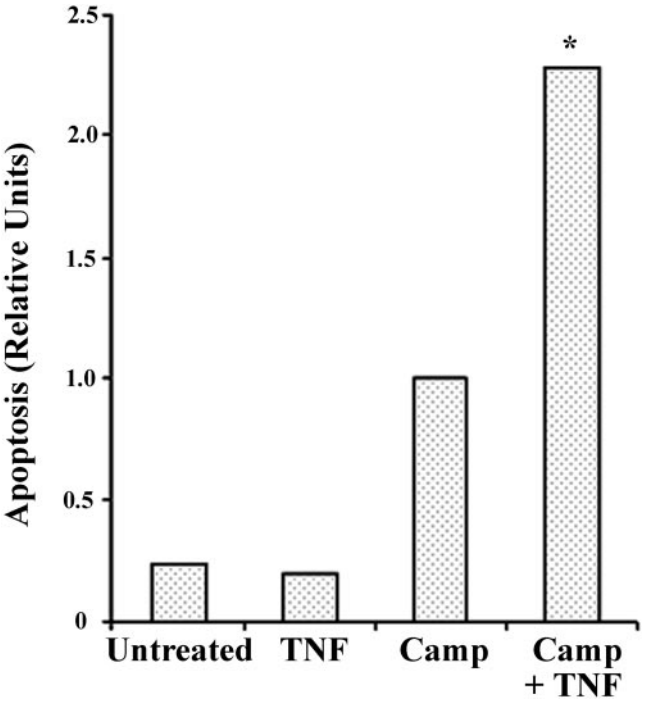

FIGURE 2. Apoptosis of LC cells in response to treatment with camptothecin and TNF-α. LC cells (line 506) were seeded onto an eight-well culture slide. Three days later, the confluent cells were treated with either 10 ng/mL TNF-α, 50 μM camptothecin, or 10 ng/mL TNF-α+50μM camptothecin. An equivalent volume of DMSO, a vehicle control for camptothecin, was added to the untreated control cells. The cells were stained with Hoechst 33258 48 hours after treatment and viewed by fluorescence microscopy. Cells with brightly stained condensed or fragmented nuclei were scored as apoptotic. The percentage of apoptotic cells was normalized to the value for camptothecin-treatment, which was set at 1. *Significantly different from the camptothecin-treated sample by paired Student's *t*-test ($P < 0.05$; $n = 2$).

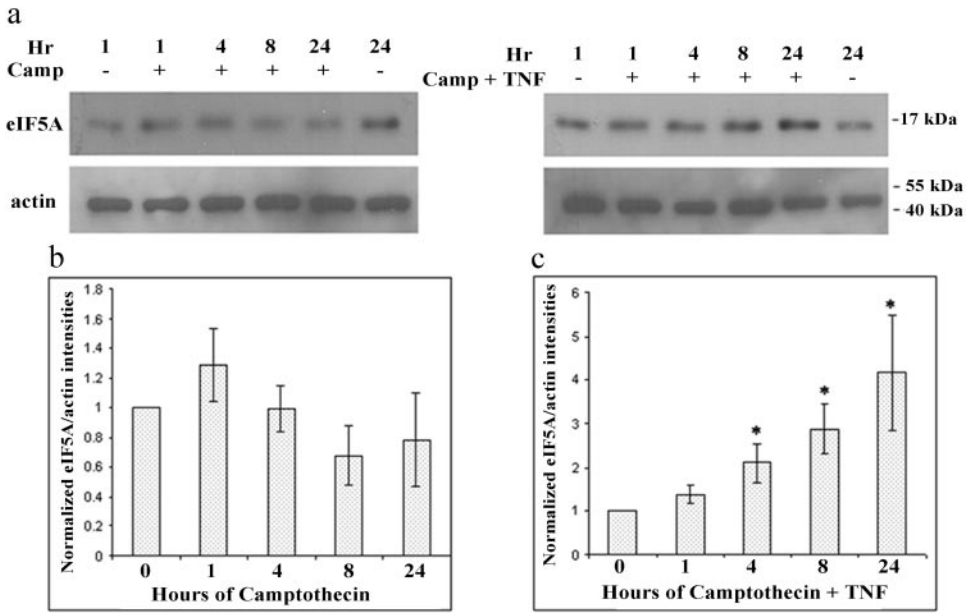

FIGURE 3. Expression of eIF5A during camptothecin or TNF-α plus camptothecin treatment of LC cells. LC cells (line 506) were seeded onto a 24-well plate. Three days later, the LC cells were treated with either 50 μM camptothecin or 10 ng/mL TNF-α+50 μM camptothecin, and cell lysate was isolated 1, 4, 8, and 24 hours later. An equivalent volume of DMSO was added to control cells as a vehicle control, and cell lysate was harvested 1 and 24 hours later. (**a**) Western blot analysis of cell lysate from LC cells treated with camptothecin or combined camptothecin and TNF-α; (**b**, **c**) plot of relative intensities of eIF5A bands normalized to actin in (**a**). *Significantly different from the control sample by paired Student's *t*-test ($P < 0.05$; $n = 3$).

Eukaryotic Translation Initiation Factor 5A

eIF5A is a nucleocytoplasmic shuttle protein known to be required for cell division and recently also suggested to be involved in apoptosis.[38,39] In light of its proposed involvement in apoptosis, we examined the expression of eIF5A protein in LC cells being induced to undergo apoptosis by treatment with camptothecin plus TNF-α. Basal levels of eIF5A protein were detectable in LC cells, and its expression did not change significantly on treatment with camptothecin alone (Figs. 3a, 3b). By contrast, a significant upregulation of eIF5A protein was observed after 4, 8, and 24 hours of camptothecin/TNF-α treatment (Figs. 3a, 3c). The finding that eIF5A expression did not increase in the presence of camptothecin alone indicates that its strong upregulation in the presence of combined camptothecin and TNF-α is a result of the cytokine (Fig. 3). The upregulated expression of eIF5A also preceded the induction of apoptosis (Figs. 2, 3), suggesting a possible involvement of eIF5A in the apoptotic pathway downstream of TNF-α receptor binding.

To examine further the involvement of eIF5A in TNF-α–induced apoptosis in LC cells, a series of four siRNAs (siRNAs 1–4) targeting eIF5A were designed and synthesized by in vitro

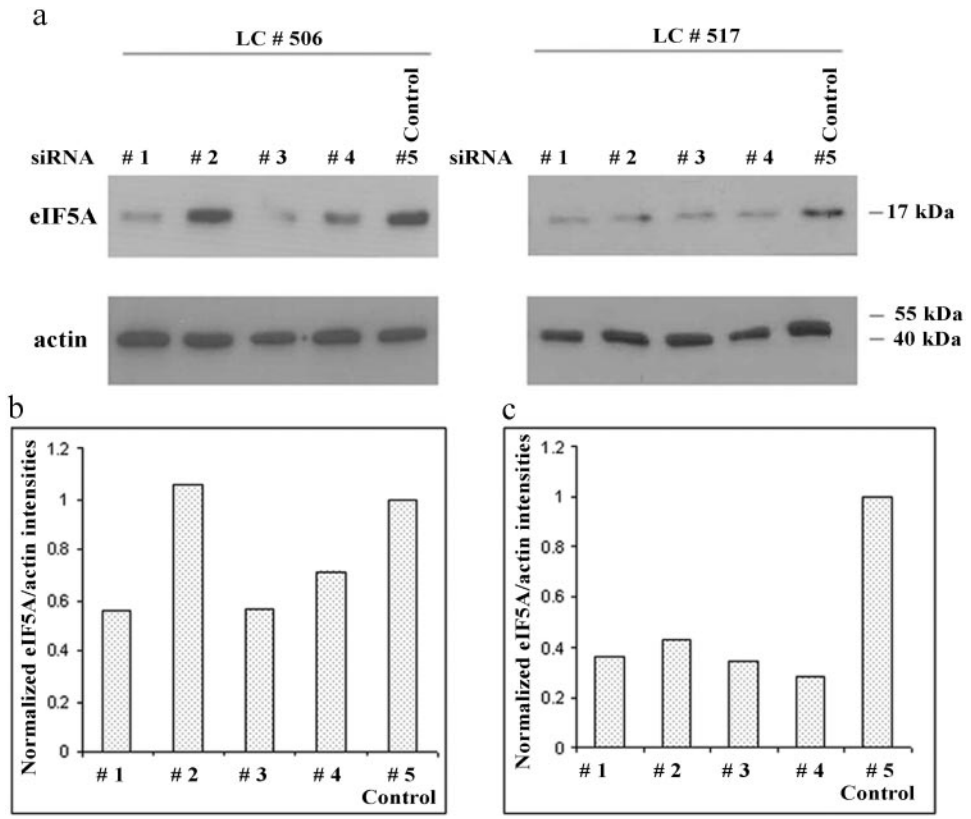

FIGURE 4. Expression of eIF5A in LC cells after transfection with siRNAs. LC cells (lines 506 and 517) were seeded onto a 24-well plate. Three days later, the LC cells were transfected with eIF5A siRNAs 1 to 4, or control siRNA-5. Three days after transfection, the cell lysate was Western blotted with anti-eIF5A antibody. The bound antibody was detected by chemiluminescence and exposed to x-ray film. The membrane was then stripped and reblotted with anti-β-actin as an internal loading control. (**a**) Western blot analysis; (**b**, **c**) plots of relative intensities of eIF5A bands from (**a**) normalized to actin for cell lines 506 and 517, respectively.

transcription. To determine the effectiveness of the siRNAs in suppressing eIF5A protein expression, LC cells (lines 506 and 517) were transfected with each of the siRNAs, and expression of eIF5A protein in the cell lysates was examined 72 hours later (Fig. 4). For comparison, cells were also transfected with a control siRNA (siRNA-5), which has the same nucleotide composition as siRNA-1 but is not able to recognize eIF5A. siRNA-1, -3, and -4, significantly suppressed eIF5A expression in LC cells in both cell lines (Fig. 4). siRNA-2 suppressed eIF5A expression in LC cell line 517 but not in line 506 (Fig. 4).

All four siRNAs against eIF5A were also capable of protecting transfected LC cells (line 506) from apoptosis induced by a 24-hour treatment with TNF-α and camptothecin (Fig. 5). Using Hoechst staining to detect apoptotic cell death, the siRNAs (siRNAs-1–4) were found to reduce apoptosis of LC cells by 59% (siRNA-1), 35% (siRNA 2), 50% (siRNA-3), and 69% (siRNA-4). An siRNA against GAPDH also reduced apoptosis of LC cells by 42% (Fig. 5). GAPDH is known to have cellular functions independent of its role as a glycolytic enzyme, including a proposed involvement in apoptosis of cerebellar neurons.[48–50] In a similar experiment, we also demonstrated that siRNA 1 was able to reduce TNF-α+camptothecin–induced apoptosis in LC line 517 cells by 53%, indicating that eIF5A siRNAs are protective for LC cells isolated from different ONHs (Fig. 6). These observations support the contention that eIF5A is involved in apoptosis and suggest that it may be an important intermediary in the pathway leading to TNF-α–induced apoptosis in LC cells.

To confirm that LC cells exposed to TNF-α and camptothecin were dying by classic apoptosis, DNA fragmentation was evaluated in situ with the TUNEL method. LC cells (line 506) were transfected with eIF5A siRNA (siRNA-1) or control siRNA (siRNA-5) treated 3 days later with TNF-α and camptothecin for 24 hours, and scored for apoptosis by TUNEL. The cells were also stained with Hoechst to facilitate visualization of the nuclei. As illustrated in Figure 7, 46% of LC cells transfected with the control siRNA were positive for TUNEL staining while only 8% of LC cells transfected with eIF5A siRNA-1 were positively labeled, indicating that the eIF5A siRNA provided greater than 80% protection from apoptosis. TUNEL detects DNA strand breaks which occur before the condensation of chromatin and fragmentation of nuclei discerned by Hoechst staining. In keeping with this, it is apparent from Figure 7 that although not all TUNEL-stained cells are staining brightly with Hoechst, all the brightly stained Hoechst nuclei are staining with TUNEL. Similar results were obtained with eIF5A siRNA-4, which provided greater than 60% protection from apoptosis relative to the control siRNA (data not shown).

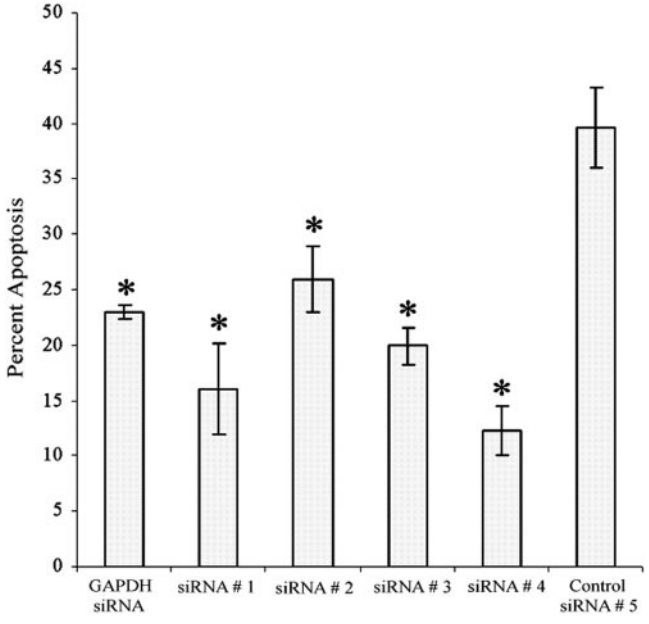

FIGURE 5. Apoptosis of LC cells transfected with eIF5A siRNAs and treated with TNF-α and camptothecin. LC cells (line 506) were seeded onto an eight-well culture slide. Three days later, the LC cells were transfected with GAPDH siRNA, eIF5A siRNAs 1 to 4, or control siRNA-5. Seventy-two hours after transfection, the transfected cells were treated with 10 ng/mL TNF-α+50 μM camptothecin. Twenty-four hours later, the cells were stained with Hoechst 33258 and viewed by fluorescence microscopy. Cells with brightly stained, condensed, or fragmented nuclei were scored as apoptotic. Means ± SE are indicated for results of four independent experiments. *Significantly different from the control sample (siRNA-5) by paired Student's *t*-test ($P < 0.05$; $n = 4$).

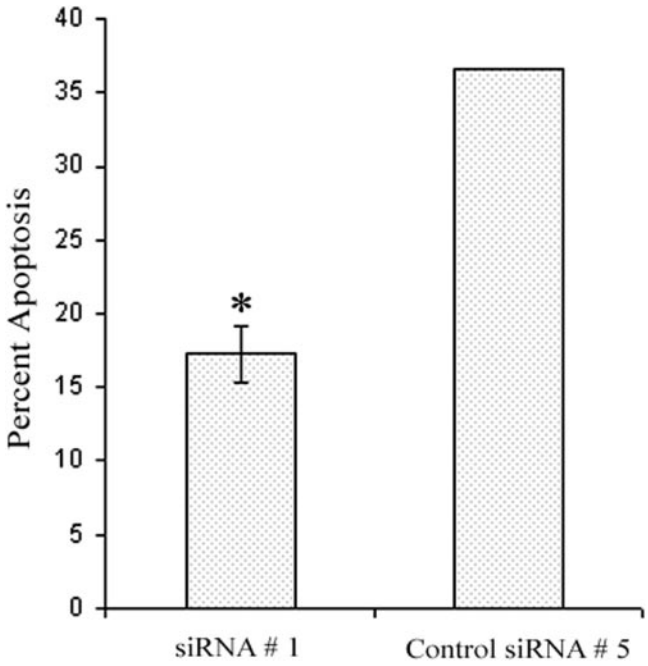

FIGURE 6. Apoptosis of LC cells transfected with eIF5A siRNA-1 and treated with TNF-α and camptothecin. LC cells (line 517) were seeded onto an eight-well culture slide. Three days later, the cells were transfected with either eIF5A siRNA-1 or control siRNA-5. Seventy-two hours after transfection, the transfected cells were treated with 10 ng/mL TNF-α+50 μM camptothecin. Twenty-four hours later the cells were stained with Hoechst 33258 and viewed by fluorescence microscopy. Cells with brightly stained, condensed, or fragmented nuclei were scored as apoptotic. *Significantly different from the control sample by paired Student's *t*-test ($P < 0.05$; $n = 2$).

DISCUSSION

TNF-α is known to be produced in glaucomatous eyes[23–25,40,41] and appears to contribute to the apoptotic cascade leading to the death of retinal ganglion cells.[24] A number of other studies have also helped to highlight the important role that TNF-α plays in neurodegenerative processes associated with glaucoma. For instance, optic nerve degeneration has been observed in eyes injected intravitreally with TNF-α.[51] Also, a genetic polymorphism in the human TNF-α promoter that is associated with elevated TNF-α levels has been found more frequently in Chinese patients with POAG than in the control group,[25] suggesting a role for TNF-α in the glaucomatous process.

It is apparent from the present study that TNF-α, in the presence of additional stressors, can induce apoptosis in LC cells. The LC consists of perforated cribriform plates that form

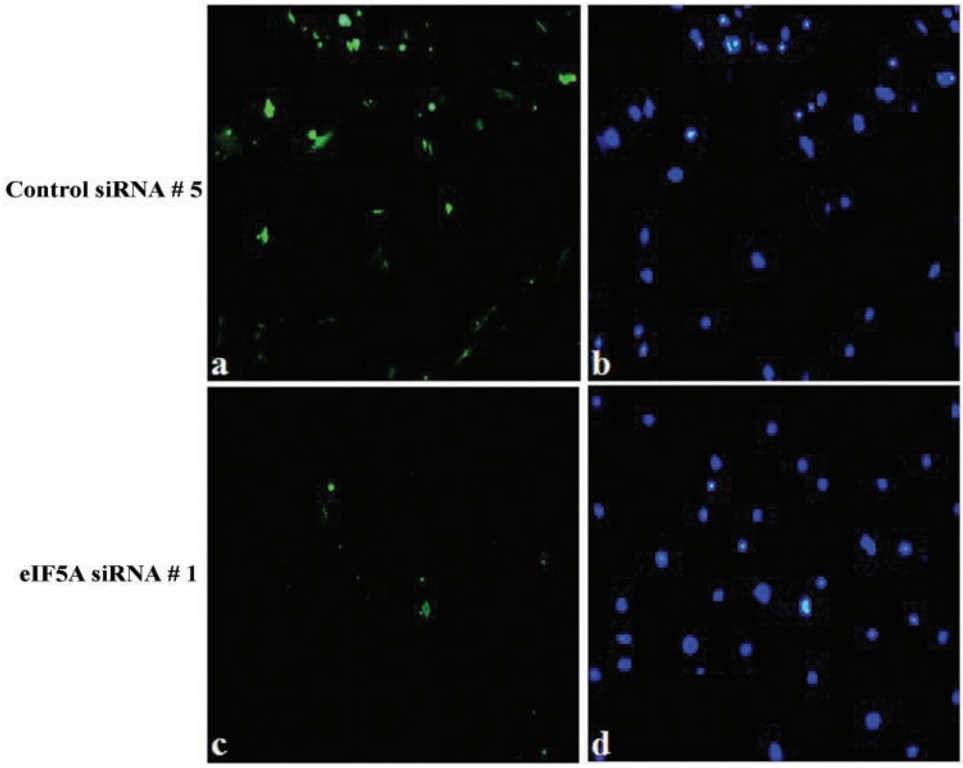

FIGURE 7. TUNEL-labeling of LC cells transfected with eIF5A siRNA-1 and treated with TNF-α and camptothecin. LC cells (line 506) were seeded onto an eight-well culture slide. Three days later, the cells were transfected with either eIF5A siRNA-1 or control siRNA-5. Seventy-two hours after transfection, the cells were treated with 10 ng/mL TNF-α+50 μM camptothecin. Twenty-four hours later, the cells were stained with Hoechst 33258, and DNA fragmentation was evaluated in situ by the TUNEL method. Control siRNA-5: (**a**) Fluorescence microscopic image with a fluorescein filter used to visualize TUNEL of the fragmented DNA of apoptotic cells; (**b**) same field as in (**a**) observed through a UV filter to visualize Hoechst-stained nuclei. eIF5A1 siRNA-1: (**c**) fluorescence microscopic image with a fluorescein filter used to visualize TUNEL of the fragmented DNA of apoptotic cells; (**d**) same field as in (**c**) observed through a UV filter to visualize Hoechst-stained nuclei. A minimum of 200 cells per treatment were counted. The results are representative of two independent experiments. Magnification, ×400.

a latticelike structure across the optic nerve canal, and the axons of the retinal ganglion cells pass through this lattice as they exit the eye.[52] Two common glial cell types have been identified within the LC: the LC cells and the ONH astrocytes (type 1β).[3–6,42,43] LC cells are by far the most common cell type in the LC and have a distinctive morphology, being large and flat, with a prominent nucleus.[3,6,42,43] Both cell types express neural cell adhesion molecule (NCAM) but only the type 1β astrocytes express GFAP.[6] As such the LC cells have been identified as a distinct LC cell type. Both LC cells and type 1β astrocytes are capable of responding to local mechanical and ischemic insult and have been shown to express TGF-β,[53] neurotrophins and their receptors,[6] bone morphogenetic proteins (BMPs),[54] and glial cell line-derived neurotrophic factor (GDNF) and its receptors.[5] LC cells, but not the type-1β astrocytes, were also found to proliferate in response to exogenous GDNF.[5] In the present study, LC cells were isolated from the ONHs of eyes from 83- and 17-year-old male human donors with no known history of eye disease. The morphology of the isolated LC cells and their immunoreactivity to actin and the extracellular matrix proteins, collagen, laminin, and cellular fibronectin, proved to be the same as that described previously for cultured LC cells.[5,6,42–44] The isolated LC cells were also distinguishable from ONH astrocytes on the basis of their morphology and negative immunoreactivity to GFAP, recognized criteria for discriminating between these two cell types.[5,6,42–44]

When LC cells were exposed to TNF-α alone, apoptosis was not observed. This finding is consistent with previous reports to the effect that, although TNF-α is cytotoxic to a wide variety of tumor cells, it does not induce apoptosis in many normal cells in the absence of a secondary signal such as DNA damage or other types of cellular stress.[55–57] In the present study, primary cultures of LC cells were rendered responsive to TNF-α cytotoxicity by treatment with camptothecin, a topoisomerase-I inhibitor which is known to synergize with TNF-α and other members of the TNF family to induce death in a variety of cell types.[45–47] Treatment with camptothecin alone induced apoptosis in LC cells, but the levels of apoptosis were more than two times higher in the combined treatment of camptothecin and TNF-α than with camptothecin alone, in keeping with observations in other types of cells.[45–47] This indicates that LC cells are clearly susceptible to TNF-α–induced apoptosis, once they have been sensitized by treatment with camptothecin.

Although the process is not fully understood, TNF-α activates both apoptotic and antiapoptotic signaling pathways simultaneously in the same cell.[56] The mechanism underlying its ability to promote survival in some cells and induce apoptosis in others is also not clearly understood, but the transcription factor, NF-κB, probably plays a central role. NF-κB is activated by TNF-α receptor–binding and has been implicated in cell survival, proliferation, suppression of apoptosis, and inflammation. The ability of topoisomerase inhibitors such as camptothecin to potentiate TNF-α cell killing is thought to be due principally to inhibition of TNF-α–induced activation of NF-κB, which reduces antiapoptotic signaling and allows apoptosis to occur.[58,59] Although camptothecin does not replicate the ischemic environment secondary to elevated IOP in a glaucomatous ONH, there are two significant similarities between the effects of camptothecin and ischemia on TNF-α function. First, increased production of TNF-α is well documented in experimental models of ischemia,[60] and ischemia, like camptothecin,[45–47] potentiates TNF-α cytotoxicity. For example, a synergistic effect on loss of neuronal viability has been observed in response to the combination of ischemia/reperfusion and TNF-α treatment.[61] Second, both camptothecin treatment and ischemia have been associated with a decrease in NF-κB,[62,63] which is significant, because TNF-α cytotoxicity is thought to be limited by NF-κB activation. This suggests that camptothecin and glaucoma-related ischemia potentiate TNF-α cytotoxicity through similar mechanisms and raises the possibility that TNF-α may be cytotoxic to LC cells in the glaucomatous ONH. This is further supported by the observation that the TNF-α receptor, TNFR1, appears to be constitutively expressed in cells of the ONH, particularly in the glaucomatous ONH.[41] Thus, TNF-α–induced apoptosis of LC cells could play a role in the development or progression of glaucoma.

The elevated IOP often associated with glaucoma is thought to cause remodeling of the ONH, including stretching and compression of the LC cribriform plates, resulting in blockage of axoplasmic flow and reduced neurotrophin transport to the retinal ganglion cells.[52,64] Neurotrophins are a family of growth factors that are essential for neuron survival, and hence their depletion would ultimately result in the death of retinal ganglion cells.[6] It has also been proposed that ischemia secondary to elevated IOP contributes to retinal ganglion cell death in the glaucomatous eye, possibly through upregulation of TNF-α.[24,65–67] Specifically, retinal glial cells secrete TNF-α and NO when subjected to conditions that simulate ischemia or elevated IOP, and when cocultures of glial cells and retinal ganglion cells were exposed to these conditions, apoptosis was observed only in the retinal ganglion cells.[24] Moreover, inhibitors of TNF-α and, to some degree, inhibitors of nitric oxide synthase (iNOS) were able to block the apoptotic death of retinal ganglion cells in these cocultures, suggesting a crucial role for TNF-α in neurotoxicity.[24] Reactive astrocytes in the ONH of glaucomatous eyes are also known to produce increased amounts of NOS and TNF-α.[23–25,40,41,68–70] Moreover, in light of the recent finding that ONH LC cells may serve as an alternate source of neurotrophins for retinal ganglion support, especially during POAG where the primary source of neurotrophins, retrograde transport, is blocked,[6] it is possible that if loss of LC cells due to TNF-α cytotoxicity occurred, it could contribute indirectly to retinal ganglion cell death.

Of particular interest is the finding in the present study that expression of the unique hypusine-containing protein eIF5A is upregulated during the apoptotic response of LC cells to TNF-α. eIF5A is an unusual translation initiation factor, in that it is believed to function as a nucleocytoplasmic shuttle for specific subsets of mRNAs rather than as an initiation factor in global protein synthesis.[28] This view is supported by the observations that ligands that bind eIF5A share highly conserved motifs,[37] and depletion of active eIF5A causes the disappearance of only specific mRNA species from polysomes.[34] The ability of inhibitors of the hypusination modification to block cell proliferation has prompted the view that eIF5A facilitates the translation of mRNAs involved in cell division.[32,35,36] However, a possible role for eIF5A in cell death has also been suggested, since there is evidence that eIF5A may regulate programmed cell death in plant tissues.[38] As well, an inhibitor of the hypusine modification was found to protect human umbilical vein endothelial cells (HUVECs) from serum starvation–induced apoptosis.[39] These observations raise the possibility that eIF5A also facilitates the translation of proteins involved in the apoptotic process. This contention is reinforced by the finding in the present study that eIF5A is upregulated coincident with the induction of apoptosis in LC cells treated with TNF-α/camptothecin. Moreover, no significant change in eIF5A expression was observed in response to camptothecin alone, indicating that the upregulation of eIF5A after treatment with TNF-α/camptothecin represents a direct response to TNF-α stimulation. This observation is in keeping with a previous report of interferon (IFN)-α induced upregulation of eIF5A.[71] IFN-α, like TNF-α, is capable of inducing apoptotic cell death in responsive cells.[72–74]

The contention that eIF5A is involved in TNF-α–induced apoptosis of LC cells is further supported by the finding that eIF5A expression and TNF-α cytotoxicity were both inhibited by eIF5A siRNA. Four eIF5A siRNAs were tested, and they all proved capable of significantly suppressing eIF5A expression and inhibiting TNF-α–induced apoptosis of LC cells measured by Hoechst staining as well as TUNEL. Indeed, siRNA-1 inhibited TNF-α–induced apoptosis by up to 80%. Moreover, similar results were obtained when cells of two separate lines of LC cells, lines 517 and 506, were treated with eIF5A siRNA, indicating that the ability of eIF5A siRNA to block TNF-α–induced apoptosis is common to LC cells in general and not an aberrant trait of a particular LC line.

An siRNA against GAPDH also proved capable of protecting LC cells from TNF-α–induced apoptosis, although not as effectively as most of the eIF5A siRNAs. GAPDH is a glycolytic enzyme that has been shown to have roles in other cellular functions, including transcription,[75] DNA replication and repair,[76] and apoptosis.[77] Alterations in GAPDH function have been implicated in neurodegenerative diseases,[78–80] and GAPDH has been implicated in apoptosis of rat cerebellar neurons.[49] Moreover, antisense oligonucleotides against GAPDH have been shown to protect cerebellar neuron cells from apoptosis.[48–50]

It is well established that TNF-α production by reactive astrocytes and microglia after ischemic brain injury contributes to neuronal loss and that inhibition of TNF-α production is protective against brain damage.[20–22,81] The similar nature of neuronal damage in ischemic brain and in glaucomatous optic neuropathy suggests that inhibition of TNF-α and/or TNF-α–induced apoptosis in the glaucomatous eye could have potential neuroprotective effects. The present study indicates that TNF-α produced in the glaucomatous eye could have detrimental effects on LC cells and that the nucleocytoplasmic shuttle protein, eIF5A, is involved in this process. In particular, siRNA-mediated inhibition of eIF5A expression provided strong protection against TNF-α induction of apoptosis in LC cells. Thus, eIF5A may be a target for the development of therapeutic agents for glaucoma, although it remains to be established whether the protective function of eIF5A inhibitors extends to nonmitotic cells, such as retinal ganglion cells.

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