ARTICLE

Regulation of TIGR/ MYOC gene expression in human trabecular meshwork cells

JON R. POLANSKY, DONALD J. FAUSS, CARIN C. ZIMMERMAN

Abstract

Glucocorticoid (GC) treatment of human trabecular meshwork (HTM) cells produces delayed, progressive cellular and extracellular protein/glycoprotein inductions with characteristics matching those for intraocular pressure elevation with corticosteroid eyedrops. The cloning of the Trabecular Meshwork Inducible Glucocorticoid Response (TIGR) gene from this system has suggested possible environmental and genetic influences in relation to glaucoma mechanisms. As reported here, the major GC-induced increase of TIGR expression in HTM cells is reduced approximately 4-fold by basic fibroblast growth factor (bFGF, 100–1000 pM), with a somewhat smaller inhibition noted with the thyroid hormone triiodothyronine (T_3, 100 nM). Such endogenous 'protective' factors could help balance stimulatory effects on TIGR gene expression from 'stress' and/or mechanical perturbations in the trabecular meshwork. TIGR coding region mutations affecting the gene's olfactomedin (OLF) homology domain may also perturb biosynthetic pathways and cellular homeostatic functions. Our recent studies have shown the OLF domain corresponds to a major translocational 'pause', an area where critical processes for normal TIGR biogenesis are expected to take place. Observations that Glu323Lys (and other mutations early in the OLF domain) altered the pattern of paused protein intermediates provide possible clues to previously unexplained pathogenetic mechanisms. HTM cell transfection studies using TIGR–green fluorescent protein (GFP) fusions showed increased and altered distribution of the expressed protein with constructs missing the OLF domain, an effect also found with the Pro370 Leu mutation for early-onset glaucoma. The data suggest an activation of stress/apoptotic pathways in HTM cells as a potential mechanism for environmental/genetic interactions in glaucoma pathogenesis.

Key words Basic fibroblast growth factor, Cytokines, Endoplasmic reticulum, Gene expression, Genetics, Glaucoma, Glucocorticoids, MYOC, Oxidative stress, Protein biogenesis, Thyroid hormones, TIGR, Trabecular meshwork

The human trabecular meshwork (HTM) is the major pathway for the outflow of aqueous humour. Limitations on the study of the HTM cell type were overcome by methods developed by our laboratories several years ago for growth and propagation of HTM cells as stable, morphologically differentiated monolayers.[1–3] The HTM cell culture system permitted reproducible studies of the structural and functional properties of this unique endothelial-like cell which performs a number of functions required for preservation of the normal outflow pathway.[3,4] Alterations involving HTM cells have been of primary interest in attempts at understanding the increased resistance to outflow that results in elevation of intraocular pressure (IOP) as a major risk factor in glaucoma.

Our investigations of the temporal and dose-response effects of environmental and pharmacological effects in the HTM cell culture system permitted a definition of the 'homeostatic' responses of this cell type compared with those of neighbouring fibroblasts and a variety of non-ocular cell types. Studies of brief versus prolonged effects of glucocorticoids (GCs) in the HTM system provided the key experimental data leading to the cloning of TIGR as a putative gene for glaucoma.[5–11] The major progressive GC induction of proteins at 55–57 kDa (termed the 55 kDa induction) was not found in other cell types examined, and its migration on two-dimensional gels (SDS/isoelectric focusing) did not match a known protein. This fact, and the finding that a large 55 kDa induction was also found with GC treatment of trabecular meshwork tissues in organ culture, suggested that efforts should be made to identify the gene(s) responsible.

Differential library screening of HTM cells treated with GC for 10 days (vs control and 1 day treatments) led to the cloning and characterisation of a gene with the anticipated

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GC induction properties. It had the appropriate predicted molecular weight, and expression of the recombinant protein showed species that matched the position of the 55 kDa proteins on two-dimensional gels. The induction was named 'TIGR' for the Trabecular Meshwork Inducible Glucocorticoid Response gene. Structural evaluations of its cDNA showed features expected for a secretory molecule capable of several types of interactions with itself and other molecules. This included the presence of a putative signal peptide and a motif for N-glycosylation. A role for TIGR–TIGR and TIGR–protein interactions was proposed to involve its leucine zipper region (where some have seen myosin homology). Glycosaminoglycan (GAG) and glycoprotein interactions also appeared reasonable to consider based on consensus motifs for hyaluronate binding and GAG initiation.[11]

The observation that the proteins rapidly appeared in the medium after pulse-labelling in healthy HTM cells with no signs of cellular injury, suggested a process by which proteins (and/or glycoproteins) secreted by the cells *in situ* could contribute to an increase in outflow resistance, potentially involving interactions with other extracellular molecules.[10,11] Tunicamycin experiments[8] showed elimination of the N-glycosylated form(s), and signal sequence cleavage of TIGR was readily demonstrated using microsomal preparations.[12] These data indicate that TIGR biogenesis involves entering the secretory pathway and modification by the endoplasmic reticulum (ER). The extracellular movement of TIGR from the ER appears to involve Golgi functions that can be inhibited by Brefeldin A, based on recent studies conducted in GC-treated HTM cells (Fauss and Polansky, unpublished data 1999).

The possibility that the induced TIGR species could be contributing to primary open-angle glaucoma (POAG) as well as steroid glaucoma was supported by the finding that different 'stress' treatments (including oxidative injury and phorbol ester exposure) substantially increase TIGR mRNA levels in HTM cells.[9,10] This agreed with prior findings of 55 kDa cellular inductions observed 1–3 days after oxidative injury produced by exposure to hydrogen peroxide.[10] Studies of meshwork tissues showed increased TIGR immunofluorescence was associated with cases of adult POAG and exfoliation glaucoma in addition to GC treatments.[10,13] The reversibility of outflow resistance changes observed with corticosteroid-induced IOP elevations was readily explained by the reversibility of the GC induction of TIGR in HTM cells and proposed degradative pathways. The putative role(s) of TIGR in POAG and certain secondary glaucomas might be less readily reversible due to oxidative cross-linking or other secondary changes following interactions with other extracellular matrix (ECM) molecules.

Concurrent with these cellular and molecular approaches, other laboratories were using classical linkage analyses to define the locations of 'glaucoma genes'. The *GLC1A* locus for a juvenile form of POAG described by Sheffield *et al.*,[14] in particular, became a focus for research by many laboratories attempting to identify the unknown gene. There were suggestions that the unknown *GLC1A* gene could also be involved in adult POAG cases,[15] but this was not readily accepted. We had found evidence of alterations involving the TIGR gene in an 'intermediate'-age POAG family, but were surprised by fluorescent *in situ* hybridisation[16] results that placed the gene within a narrowed *GLC1A* inclusion interval. Potentially related was the observation of E. Lutjen-Drecoll (personal communication 1997) that certain connective tissue changes in the cribriform region of the meshwork appeared to link juvenile glaucoma and steroid glaucoma. It appeared possible that studies of the regulation of TIGR gene could provide information to help explain the increased responsiveness of patients with POAG to raise IOP during corticosteroid treatment protocols, as observed in the earlier glaucoma literature.[17]

Widespread interest in TIGR followed the report by Stone *et al.*[18] that demonstrated single base coding region defects in the gene accounted for the linkage of juvenile glaucoma to the *GLC1A* locus, with certain defects also showing a relationship to adult cases of POAG. Subsequent confirmation and extensions of this idea have come from the efforts of several major laboratories and clinicians internationally. Currently, there is general agreement that a number of point mutations in the third exon of TIGR (known variously as *TIGR/MYOC*, *MYOC/TIGR* or *MYOC*) can be regarded as causally related to glaucoma over a spectrum of ages.[19–33] Interestingly, certain TIGR coding region defects (e.g. Pro370Leu and Tyr437His) appear to be associated with an earlier onset and more rapidly progressive juvenile glaucoma phenotype, while others (Gln368Stop) are associated with either a milder or adult phenotype. Observations within families with the same mutations are showing patterns of different ages of onset, with implications for discovering new gene products that directly or indirectly influence TIGR gene expression, including protective or susceptibility factors that could be involved in glaucoma pathogenesis. The variations in phenotype seen with the same TIGR mutations have also suggested a role for individual differences in the expression of TIGR protein(s)/glycoprotein(s), as well as potentially interacting molecules.

Here we report results from our continuing studies of the regulation of TIGR expression in HTM cells, including comparisons with other cell types that express TIGR but which do not show the same responsiveness to 'environmental' influences. Effects of growth factors and thyroid hormones to counterbalance GC effects are presented as representing potentially relevant regulators of TIGR gene expression in the HTM cell type. Studies of TIGR mutations in the gene's olfactomedin (OLF) homology domain are also considered, with data from transfection studies in which TIGR–GFP constructs containing the Pro370Leu mutation or deletion of the OLF domain are compared with results from the normal sequence. The importance of the OLF domain suggested by these studies complements our current cell-free

evaluations of TIGR biogenesis in which translocon functions of the ER appear to be playing an important role.[12]

Methods

Cell culture and experimental treatments

HTM cells previously established and characterised in our laboratories were used for the current experiments.[1–4] Third to fifth passage HTM cultures were removed from cryopreserved stocks, plated at approximately 10 000 cells/cm^2, and grown 7–10 days post-confluency in Dulbecco's modified Eagle's (DME) medium with initially 15% fetal calf serum (FCS) and 100 nM basic fibroblast growth factor (bFGF; FGF-2, Intergen, human recombinant) used during active growth. At confluence the serum concentration was reduced to 10% FCS and bFGF treatment discontinued. Cultures were maintained another 10–14 days to obtain stable endothelial-like monolayers, before using them for experimental studies.

The effects of TIGR gene expression and protein synthesis were tested on these stable HTM monolayer cultures using 100–500 nM dexamethasone (DEX), as being in the range achieved in the aqueous humour by topical corticosteroid eyedrops. The influence of varying doses of bFGF from 10 to 3000 pM (0.17 to 50 ng/ml) on the GC induction of TIGR was evaluated by addition of the growth factor along with DEX (100 nM) every 2 days in DME (10% FCS) medium; the influence of 100 nM triiodothyronine was evaluated by addition of the hormone with DEX (100 nM) every 2 days in DME 10% hyothyroid (charcoal-treated) FCS after maintaining the cells in this medium for 4 days prior to the treatments. The effects of sublethal oxidative stress were evaluated using 0.1–0.6 mM hydrogen peroxide treatments to HTM cells in phosphate-buffered saline (PBS) for 30 min, a concentration that stimulated the cultures to produce an increased amount of the 55 kDa induction over a 24–72 h period, as described previously.[10]

Fourth passage human non-pigmented ciliary epithelial (NPE) cells and retinal pigment epithelial (RPE) cells grown in our laboratories were taken from frozen stocks and grown as differentiated monolayers as previously described.[34–36] Schlemm's canal epithelial (SCE) cells obtained in collaborative studies[37] were grown using the same conditions we used for HTM cells. The cultures were grown to stable, confluent monolayers. Comparisons with HTM cells involved treatments with 500 nM DEX added with medium changes every 2 days. The TIGR mRNA levels in the cells were evaluated by semi-quantitative RT-PCR, and the induction of proteins/glycoproteins studied as described below.

Glucocorticoid effects on HTM cell protein/glycoprotein synthesis

GC effects on specific protein/glycoprotein synthesis were determined by addition of [^{35}S]methionine to label newly synthesised proteins in HTM cells. Confluent cultures which had been exposed to DEX for varying times (medium changes 24 h prior to labelling) were placed into methionine-free medium with 300–500 μCi [^{35}S]methionine (New England Nuclear; specific activity 1100 Ci/mmol), 10% FCS and the appropriate concentration of DEX. GC inductions in the cytosol were evaluated 15–60 min after addition of [^{35}S]methionine (short labelling times providing an estimate of protein synthetic rates). Proteins/glycoproteins present in the HTM tissue culture medium were from 2 h serum-free DME collections after a 3 h initial cell labelling. Pulse-chase experiments, which were performed with and without serum, had been used initially in the selection of these conditions. Immediately after collection, each sample was exposed to lysis buffer (20 mM Tris-HCl, pH 7.6; 10 mM $MgSO_4$; 0.1% TX100; chymostatin, 2 μg/ml; leupeptin, 4 μg/ml; bacitracin, 25 μg/ml; PMSF, 1 mM) at 4°C. The trichloracetic acid (TCA) precipitable counts of the cell lysates were used to normalise the amounts of sample added to the gel electrophoresis lanes. The samples were diluted according to TCA assay results and gel buffer was added to a final concentration of 10% glycerol, 2% SDS, 5% beta-mercaptoethanol, and 62.5 mM Tris, pH 6.8. Samples were then boiled for 2 min, cooled, spun and loaded onto gels.

Previously published methods involving computerised spot matching (PDQuest) programs on three different autoradiogram exposures of large-format two-dimensional gels[10] were used to quantitate DEX effects on specific protein synthesis in the HTM cultures for the time course experiment comparing different HTM cell lines. Phosphorimager quantitations were also performed as a more efficient approach to quantify the inductions in the bFGF dose-response studies. Phosphorimager screens were scanned using a Phosphorimager model 400E, with gel files stored on a magneto-optical disc. Analyses were performed using the Image Quant v. 3.22 software package, which allowed the quantitation of radiolabelled protein bands over a four log-unit range of intensity.

Transfection studies of primary HTM cell cultures

TIGR cDNA–green fluorescent protein (GFP) cDNA fusions, with the GFP cDNA fused to the 3′ end of the TIGR cDNA, were synthesised for (i) wild-type TIGR, (ii) a truncation at the *Avr*II site at the beginning of the OLF homology domain, and (iii) the Pro370Leu mutation associated with a severe juvenile glaucoma phenotype. The constructs were made from the TIGR cDNA cut with *Eco*RI + *Bam*HI inserted into the plasmid vector pEGFP-N3 (Clontech), made especially for expression in human cell lines. The expression of the fusion protein is driven by the CMV promoter. The Quik-Change Site-directed Mutagenesis Kit (Stratagene) was used to create the Pro370Leu mutation in the TIGR–GFP cDNA expression construct. The truncated TIGR–GFP construct (lacking the OLF domain) was made by converting the *Avr*II site to a *Bam*HI site and inserting into the same pEGFP

vector. All constructs were verified by sequencing. High-quality DNA for transfection was obtained using the Qiagen Midi-Plasmid Prep kit.

Transient transfections were performed in primary HTM cells grown to stable, confluent monolayers on tissue culture plastic microscope slides, in which the lipid reagents Superfect (Qiagen) and LipofectAMINE (BRL) were employed according to the manufacturers' protocols. Various concentrations of DNA:LipofectAMINE were used to optimise the transfection using the 57-year-old HTM cell line. A 12 well plate was used, and the ratio is listed as μg DNA:μl LipfectAMINE reagent for each well: (1) 1:5, (2) 1:10, (3) 1:20, (4) 2.5, (5) 2:10, (6) 2:20. Wells 7–12 used the same concentrations, but a different DNA construct (mutant) was used. For Superfect, a 6 well plate was used, and the ratio was listed as above: (1) 1:30, (2) 2.5:30, (3) 5:30, (4) 10:15, (5) 2:15, (6) 5:15. Expression of the GFP–cDNA constructs was evaluated over a 10 day period using a fluorescence microscope. The LipofectAMINE reagent, which showed more GFP fluorescence, was preferred over the Superfect reagent for the experimental comparisons, with a 2:10 concentration showing 5–10% of the HTM cells being fluorescent. The period 3–4 days following transfection showed maximal fluorescence using the 2:10 ratio with LipofectAMINE. TIGR mRNA expression in the transfected cells was examined by RT-PCR in which the wild-type and Leu370 GFP fusion constructs were evaluated using a restriction site introduced with the Pro370Leu mutation.

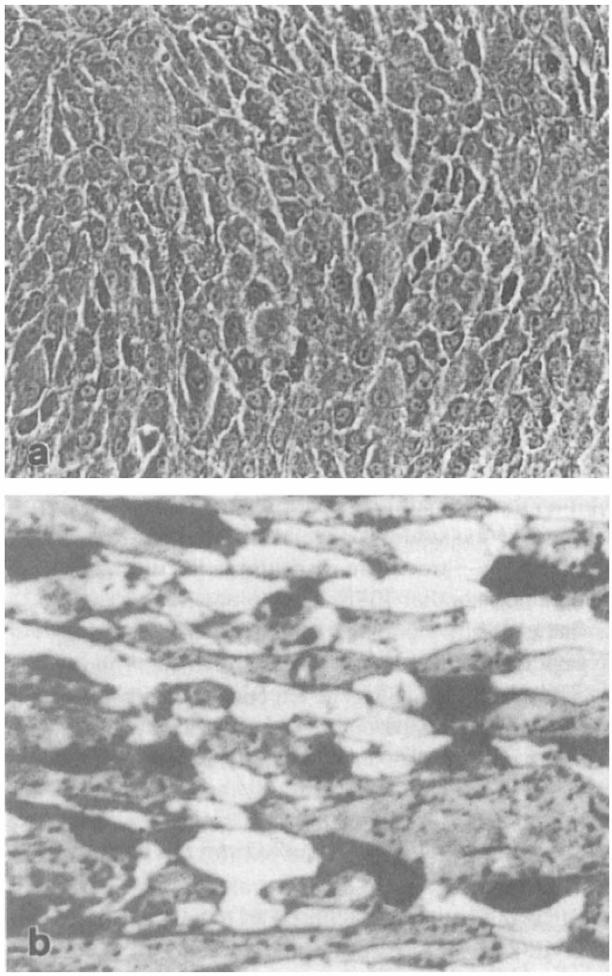

Fig. 1. *Human trabecular meshwork (HTM) cells, showing comparison of the typical endothelial-like morphology of stable HTM monolayer cultures (a) compared with a cross-section of the corneoscleral meshwork from a young individual (b). Beginning with cryopreserved stocks of cells stored in liquid nitrogen, we obtain rapidly dividing cultures of HTM cells with 1/8 to 1/12 split ratios as described in Methods. If maintained 7–10 days post-confluency, the HTM culture adopts the stable morphological appearance shown.*

RT-PCR

Semi-quantitative RT-PCR for determining regulation of TIGR gene expression, including the studies performed as part of the TIGR–GFP transfection experiments, employed the following primer sets: 5′ EX1 CCA GTA TAC CTT CAG TGT GGC or 5′ GFPRI CTC AAG CTT CGA ATT CGG CAC GAG; 3′ EX 3 TGG CCT AGG CAG TAT GTG AAC; 5′ AVR 2 TGC CTA GGC CAC TGG AAA GCA; 3′ GFP#1 CTG CAC GCC GTA GGT CAG; 5′ G3PDH TGA AGG TCG GAG TCA ACG GAT TTF GT; 3′ G3PDH CAT GTG GGC CAT GAG GTC CAC CAC; 5′ EX2 GAG CTA ACT GAA GTT CCT G; 3′477 CAG GGG GTT GTA GTC AAT CAT GCT. The first primer set (EX1 + EX3) that was used as a control amplifies both the construct mRNA and the endogenous TIGR mRNA; the expected product size is 821 bp. There are two sets of primers that both amplify only the mRNA from the construct. One set (GFPRI5′ + EX3′) utilises vector sequences at the 5′ end of the construct message, which verifies that full-length cDNAs have been obtained; the expected product size is 1030 bp. The other set (GFP#1 + AVR II) uses one primer sequence unique to the GFP cDNA combined with a TIGR primer; the expected product size is 781 bp. A primer set for G3PDH (Clontech), a housekeeping gene, was also used to verify that the amounts of cDNA were equivalent among all the samples; the expected product size is 983 bp. Primer sets were designed to span introns to avoid amplifying genomic DNA potentially present in small amounts. Additional verification of the mRNA isolations involved treating with DNase (RNase free), and a no RT control. The RT-PCR reaction was performed using 3 μl of cDNA product, with the following conditions: 94 °C 3 min, 94 °C 45 s, 72 °C 45 s, 60 °C 1 min, 30 s for 30 or 35 cycles as indicated, 72 °C, 7 min, 4 °C, soak. To prevent contamination, Ready-To-Go PCR amplification beads (Pharmacia) were used. Each individual tube contained *Taq* polymerase, buffer, and dNTPs necessary for PCR. Water, primers and cDNA were added to individual tubes separately.

The evaluations of GC effects on TIGR mRNA, including the ability of bFGF and triiodothyronine to influence GC-induced TIGR, were conducted using 1 μg of total RNA to generate cDNA and the 5′ EX1 + 3′ EX3 primer set. Earlier semi-quantitative RT-PCR evaluations of GC (and hydrogen peroxide) effects on HTM and other ocular cell types employed TIGR primer sets and methods published previously.[11]

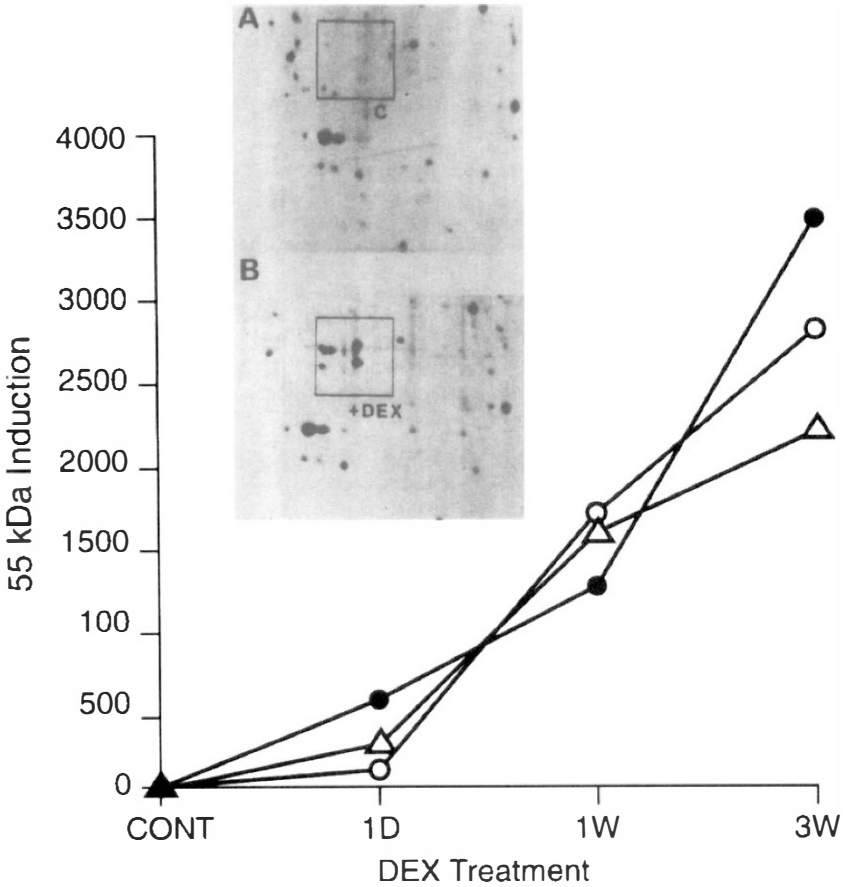

Fig. 2. *Quantitative evaluations of the 55 kDa TIGR induction(s) by glucocorticoids in confluent HTM cells from three different donors. The amount of labelled product found in the boxed area of two-dimensional gels was evaluated according to Methods in control and dexamethosone (DEX) (100 nM)-treated HTM cultures, at 1 day, 1 week and 3 week time points. One hour labelling was used for these evaluations, since earlier pulse-chase versus equilibrium (24 h) labelling experiments indicated that longer labelling periods would decrease our ability to detect the '55 kDa' induction(s). The induced proteins/glycoproteins appeared to be rapidly secreted extracellularly.*

For the transfection experiments, total mRNA was isolated using the RNAeasy kit from Qiagen. Three to four days after transfection, the cells were rinsed three times in cold PBS, then lysed by the addition of RNA lysis buffer + BME (Buffer RLT). The lysed cells were then homogenised, and the resulting lysate bound to a spin column, washed, and the RNA eluted as directed by the RNAeasy manual. The RNA was quantified by spec readings at OD 260 nm. The average yield was 12 μg per well (from a 6 well plate). For cDNA synthesis, 2 μg of total RNA was used. The RNA was denatured by heating to 75 °C for 3 min, then put on ice. The cDNAs were synthesised by adding oligo-dT primer, dNTPs, buffer, and MMLV reverse transcriptase to a volume of 30 μl, incubating at 42 °C for 1 h, then stopping the reaction by heating to 75 °C for 10 min.

Results

Using the growth conditions described in Methods, confluent HTM cultures with the appearance shown in Fig. 1a are obtained as a model system for evaluating the properties and responses of this unique cell type. A cross-section of the corneoscleral meshwork showing the HTM cells *in situ* lining the trabecular beams is shown in Fig. 1b.

Induction of TIGR protein(s) and mRNA in HTM cells

Fig. 2 presents a quantitative comparison of the 55 kDa (TIGR) GC induction in confluent HTM cells from three different donors. Stable, morphologically differentiated HTM cultures were required for reproducibly large inductions to be seen, with dividing 'undifferentiated' HTM cultures showing variable and markedly reduced inductions. Use of two-dimensional gel (SDS/isoelectric focusing) minimised interference from other proteins of the same molecular weight (with different isoelectric points), facilitating comparisons of the time course effects of 100 nM DEX treatments on the '55 kDa' induction(s) from the different donors. The HTM cells from the donors all showed progressively larger inductions of the proteins at 55–57 kDa (pI 5.4–5.8) with sustained hormone exposures. Quantitative measurements of the boxed region over time demonstrated all three HTM 'lines' had an increase from barely detectable levels in control, to noticeable increase at 1 day for one subject, with all lines progressively increasing their expression levels after 1 and 3 weeks of hormone treatment. The final levels of induction were very high, reaching approximately 3% of the labelled cellular proteins visualised on two-dimensional gels. The donor differences in induction times appear reasonable based

Table 1. *Glucocorticoid induction of TIGR expression*

Cell type	mRNA			55 kDa extracellular induction
	Control	1 day	4 days	
HTM	+/−	1+	4+	4+
SCE	0	½+	1+	½+
NPE	½+	½+	1+	½+
RPE	½+	½+	½+	0

Cell types: HTM, human trabecular meshwork; SCE, Schlemm's canal epithelial; NPE, non-pigmented ciliary epithelial; RPE, retinal pigment epithelial.

Confluent, stable cultures of HTM, SCE, NPE and RPE cells are compared for the observed glucocorticoid induction of TIGR expression. Relative inductions of TIGR mRNA standardised from the RT-PCR and dot blot comparisons are shown in the three left-hand columns, for control, 1 day and 4 day dexamethasone (DEX) (500 nM)-treated conditions. A relative scale is used to facilitate comparisons. Estimated amounts based on a dot blot standardisation are: 0, <.1% total mRNA; +/−, near 0.1%; ½+, approximately 0.3%; 1+, approximately 0.5%. A 4+ increase is 2% or greater of the total mRNA. A separate relative scale is used to indicate the amount of '55 kDa' extracellular protein/glycoprotein induction seen after 4 day DEX treatments (right-hand column), with '½+' being barely detectable, and '4+' representing approximately 5% of the total radiolabelled media proteins on SDS gels obtained after 2 h serum-free collections, according to Methods.

on variations observed in the IOP elevations observed when human subjects are exposed to topical corticosteroid eyedrops over the course of several weeks.

Table 1 presents data obtained from experiments in which the effects of 500 nM DEX on TIGR gene expression in HTM are compared with those observed in studies of other important ocular cell types. Confluent, stable cultures of HTM, SCE, NPE and RPE cells were studied using a relative induction scale (as explained in the notes to Table 1) for effects on TIGR mRNA using semi-quantitative RT-PCR, and for TIGR protein expression using biochemical labelling. The baseline (untreated) levels of TIGR mRNA appeared higher in NPE and RPE. Higher baseline levels in NPE compared with HTM had previously been noted in hybridisation experiments using the full-length TIGR cDNA. DEX treatments resulted in slight inductions of TIGR mRNA for SCE and NPE cells, and no change in RPE cells. Both NPE and SCE showed some 55 kDa protein in the medium after biochemical labelling, with none for the RPE. The 4+ mRNA and extracellular 55 kDa inductions seen with HTM cells were qualitatively different from the responses seen with the other ocular cell types.

Semi-quantitative RT-PCR was used in an earlier study to examine a number of stimuli of TIGR mRNA in the HTM cell type.[10] Fig. 3 presents a representative experiment showing markedly higher levels of TIGR mRNA after 10 days versus 1 day of DEX treatment to the HTM cultures. The figure also shows that oxidative stress induces TIGR within 1 day after injury, agreeing with effects seen by biochemical labelling. Both the GC effects and oxidative injury responses were confirmed by quantitative dot blot dilutions. Additional RT-PCR and dot blot experiments showed results compatible with the delayed time course and dose-response characteristics on

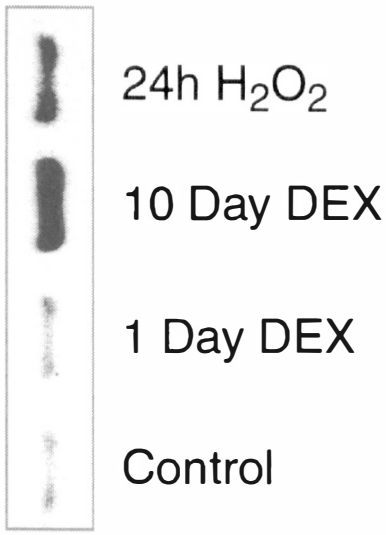

Fig. 3. *Semi-quantitative RT-PCR for TIGR mRNA expression, comparing control (no dexamethasone (DEX)), 1 day DEX (100 nM) treatment, and 10 day DEX (100 nM) treatments, with 24 h post-injury with hydrogen peroxide (H_2O_2). The samples were normalised using primers for the relatively stable 'housekeeping' G3PDH mRNA as described in Methods (data not shown).*

the different HTM lines determined by the biochemical labelling studies of the GC-induced proteins/glycoproteins in the cell and media.

bFGF and thyroid effects on the GC induction(s)

Fig. 4 presents the initial RT-PCR experiments that showed bFGF could markedly reduce the induction of TIGR mRNA seen with GC treatments to HTM cultures. Similar effects have been found in experiments using two different HTM cell lines. As shown here, 1000 pM (17 ng/ml) bFGF markedly reduced the TIGR mRNA levels (approximately 80%) compared with that observed with DEX (100 nM) exposure for the 6 day experimental study. Two-dimensional gel studies of the cellular 55 kDa

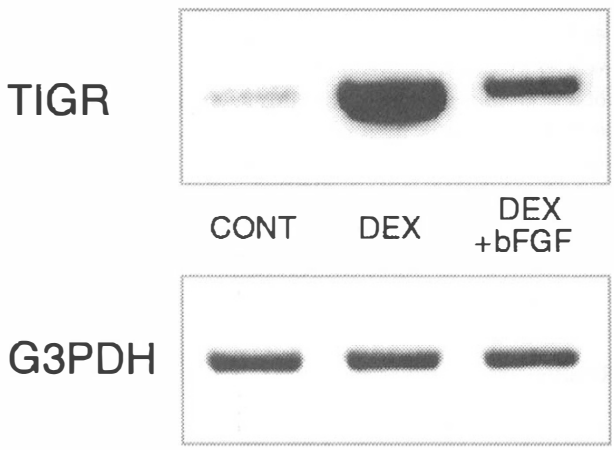

Fig. 4. *Semi-quantitative RT-PCR for TIGR mRNA expression, comparing control (no DEX), DEX (100 nM) treatment, and DEX (100 nM) + basic fibroblast growth factor (bFGF) (1000 pM) treatment after 6 days, according to Methods. The G3PDH standard for each sample is shown.*

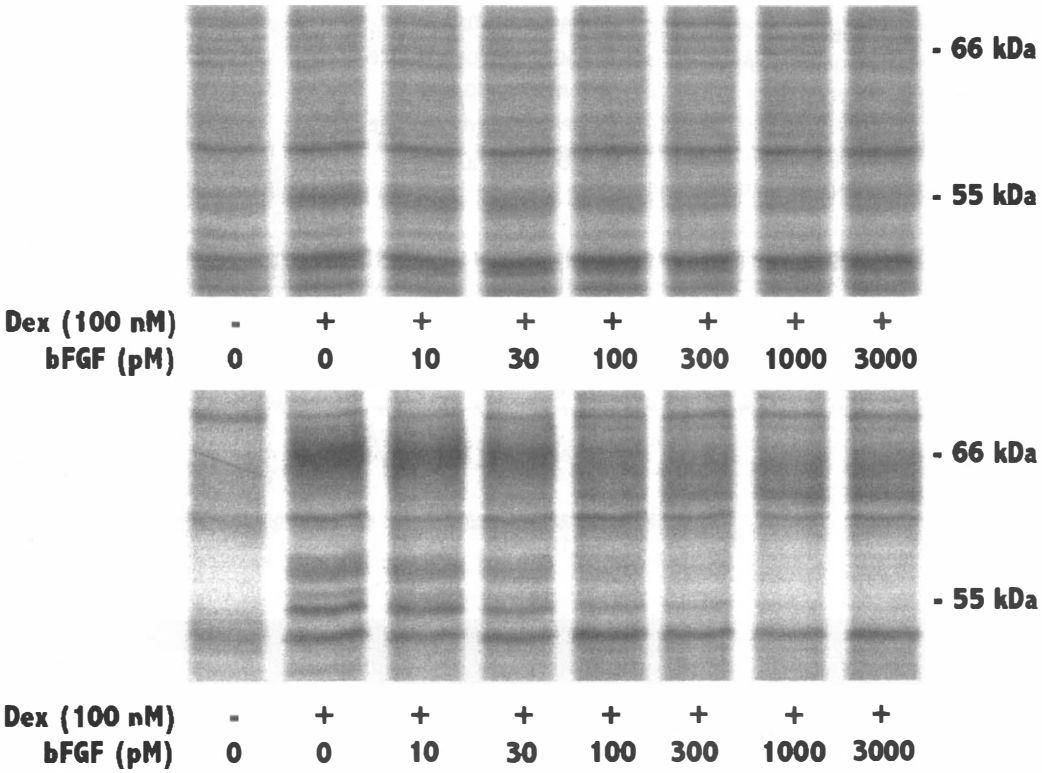

Fig. 5. *SDS polyacrylamide gel electrophoresis of the GC inductions found in HTM media using stable, confluent cultures from a 30-year-old donor. On the left is shown: –DEX (control) versus +DEX (100 nM) media inductions, with prominent 55–57 kDa bands and a broad band near 66 kDa seen. On the right, the effect of varying concentrations of bFGF to lessen the DEX inductions is shown, with noticeable reductions beginning at approximately 100 pM (1.7 μ/ml). Phosphorimager analyses were used for quantitative comparisons.*

induction(s) by biochemical labelling showed a similar magnitude for the effect using this concentration of bFGF (data not shown).

Fig. 5 shows a bFGF dose-response study for its ability to block the inductions seen with 100 nM DEX in the HTM cell layer (top) and media (bottom) evaluated by one-dimensional SDS gels. A similar analysis proved useful for defining DEX dose-responses in earlier studies, in which 100 nM DEX showed slightly less than maximal inductions.[16] As presented in Fig. 5, the 55 kDa cell, and 55 and 66 kDa protein(s) were substantially induced in the '+DEX, 0 bFGF' condition compared with the '–DEX, 0 FGF' control, in both the HTM cell and media evaluations. The inductions were somewhat reduced with bFGF concentrations of 10–30 pM, with a clear effect at 100 pM (1.7 ng/ml). The 100 pM concentration showed a larger effect on the media induction(s) than those in the cell layer, suggesting an effect on TIGR secretion as well as mRNA that merits further investigation. The maximal effect of bFGF in blocking the DEX inductions was achieved with 300–1000 pM concentrations.

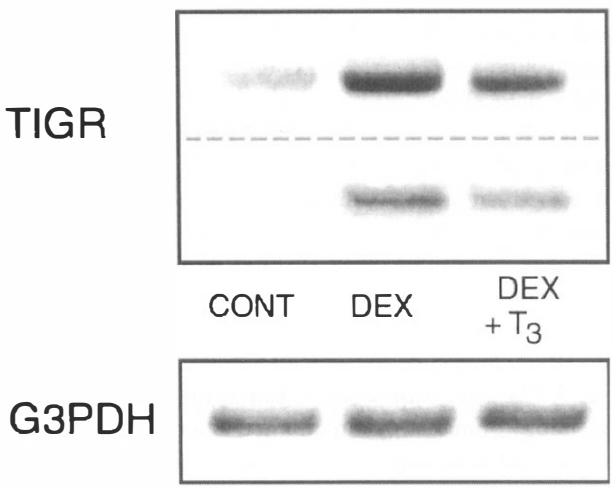

Fig. 6. *Semi-quantitative RT-PCR for TIGR mRNA expression, comparing control (no DEX), DEX (100 nM) treatment, and DEX (100 nM) + triiodothyronine (T_3, 100 nM) treatment. Assays for the RT-PCR at 23 cycles (upper) and 21 cycles (lower) are shown. The G3PDH standard for each sample is shown.*

Fig. 6 provides data for TIGR mRNA expression, comparing control (no DEX), DEX (100 nM) and DEX (100 nM) + triiodothyronine (100 nM) treatments. Since the effect was somewhat less dramatic than that observed with bFGF, assays using 23 and 21 RT-PCR cycles are shown. The T_3 effect was also observed in one- and two-dimensional gel studies of labelled 55 and 66 kDa inductions in different HTM cell lines, although no dose-response data for the effect have yet been obtained. Following these determinations, it will also be of interest to look for interactions between bFGF, other growth factors and thyroid hormones on TIGR gene regulation.

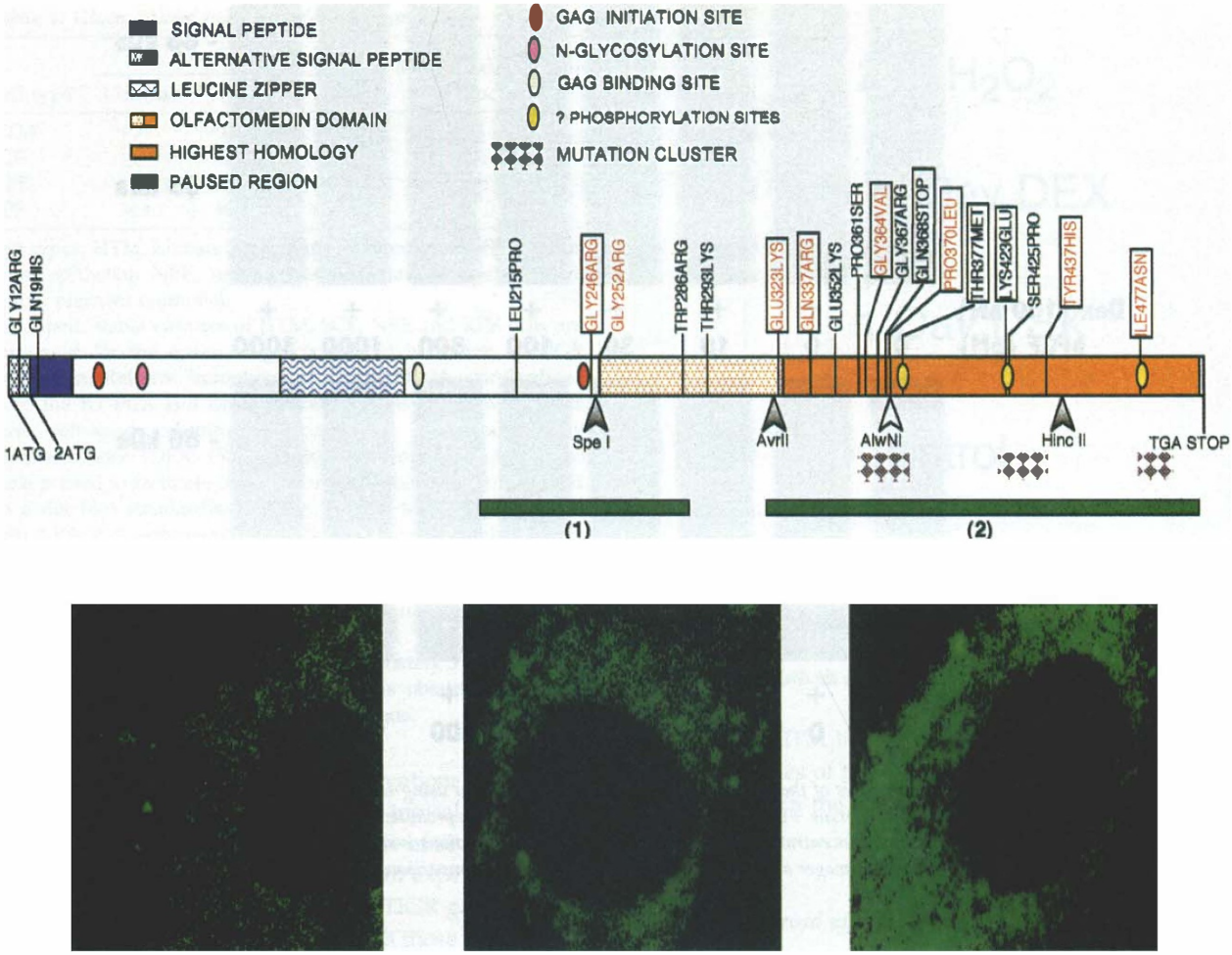

Fig. 7. *TIGR coding region, structural map (top); and evaluations of the TIGR-GFP fusions transfected into confluent HTM cells. Important features of the structural map are explained in the legend and text. The structural map show sites for key restriction enzymes (arrows), including the* AvrII *site used to produce the construct without the OLF domain. Among the sequence variants made by site-directed mutagenesis is also the Pro370Leu mutation used in the transfection studies. Regions for translocational pausing in cell-free microsomal systems are shown underneath the diagram by two green lines: as noted, the major translocational pause (#2) coincides with the gene's OLF homology domain;*[12] *a shorter pause (#1) has recently been discovered and needs further characterisation. HTM cells used in transfection experiments with TIGR-GFP constructs are shown from left to right as: wild-type TIGR, TIGR containing the Pro370Leu mutation, and TIGR truncated at the* AvrII *restriction site. The experiment presented was 4 days after transfection with a ratio of 2 μg DNA/10 μl LipofectAMINE reagent, according to Methods. Confocal microscopy with ×60 objective at ×2 –×4 magnification was used to record the findings.*

TIGR gene coding region structure and possible effects of sequence changes

Fig. 7 (top) presents a schematic map of the TIGR coding region, and an initial group of sequence variants (made by site-directed mutagenesis) being evaluated in our laboratories. Indicated on the figure are the location of predicted motifs for the normal TIGR sequence, including the proposed signal peptide, an N-glycosylation site, a leucine zipper (coil-coil) region, a hyaluronate or other GAG binding site, and potential GAG initiation sites in the N-terminal part of the molecule, with the region of highest OLF homology shown in the darker orange. Most disease-associated mutations occur in this OLF homology domain, with an interesting clustering of mutations occurring in three regions around putative phosphorylation sites.[32] The regions with a high frequency of mutations within the OLF domain are indicated below this structure with the purple diamonds.

Among the sequence variants made by site-directed mutagenesis is also the Pro370Leu mutation used in the transfection studies, and the Glu323Lys and Gln337Arg which have shown effects in translocational processing assays. Regions for translocational pausing as determined by cell-free microsomal systems are shown by two green lines. The major translocational pause (#2) coincides with the gene's OLF homology domain as we reported;[12] a shorter pause (#1) has recently been discovered and needs further characterisation.

Results of TIGR-GFP transfections using the HTM cell model are presented in the photographs at the bottom of Fig. 7. The data support an important role for the OLF homology domain in maintaining the normal appearance of the wild-type sequence of TIGR. TIGR constructs containing the Pro370Leu mutation, and TIGR truncated at the *Avr*II restriction site (which deletes the region of highest OLF homology) resulted in substantially increased fluorescence as well as possible differences in localisation, compared with that seen with the wild-type

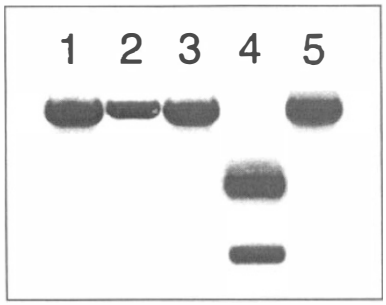

Fig. 8. *Evaluation of TIGR mRNA expression in transfected HTM cells by RT-PCR, according to Methods. The lanes shown are RT-PCR products for: (1) DEX (500 nM) treatment (non-transfected) for 5 days, (2) transfected wild-type TIGR-GFP, (3) transfected Prp370Leu TIGR-GFP, (4) transfected Pro370Leu TIGR-GFP cut with* AlwNI, *and (5) transfected wild-type TIGR-GFP cut with* AlwNI. *For the PCR/restriction enzyme digest assay, a different primer set (5′EX2 + 3′477) which encompasses the mutation and also spans introns was used; the expected band size is 665 bp. When cDNAs were amplified with 5′EX2 + 3′477, PCR product was cut with the restriction enzyme* AlwNI. *This site is created with the Leu370 mutation, and will not cut a PCR product generated from the endogenous TIGR gene. This results in two bands: a 327 bp band and a 458 bp band. In this manner, the relative amount of mRNA derived from the Leu370GFP construct can be compared with that derived from the endogenous TIGR gene.*

TIGR-GFP construct. Evaluations of TIGR mRNA expression in transfected HTM cells by RT-PCR, shown in Fig. 8, permitted a validation that both the wild-type TIGR-GFP and the Leu370TIGR-GFP constructs were expressed at high levels.

Discussion

The studies presented here support and extend several observations made in our prior papers concerned with TIGR gene regulation and expression in HTM cells. HTM cell type specificity was emphasised by the finding that GC treatment of HTM cells stimulates TIGR mRNA and protein/glycoprotein expression in qualitatively different ways compared with the other ocular cells types studied, including minor effects in Schlemm's canal epithelium (SCE) and non-pigmented epithelium (NPE), and lack of a response in retinal pigment epithelium (RPE). This further supports the importance of focusing attention on HTM cells for studies of TIGR gene regulation beyond our prior work with scleral and corneal fibroblasts, and a few non-ocular cell types.[5,10] The observation that stable HTM monolayers are necessary to see reproducibly large inductions of TIGR mRNA and the appearance of the characteristic pattern of extracellular protein(s)/glycoprotein(s) suggests that 'differentiation' factors as well as cellular specificity may be required for experimental evaluations of certain aspects of TIGR gene expression and its regulation.

The effects of bFGF and triiodothyronine in reducing the GC stimulation of TIGR have substantial conceptual implications. The findings suggest that these and other 'counterbalancing' regulators might function to 'protect' against stimulation of TIGR production by a variety of stimulators. The stimulatory effects include TGFβ and mechanical perturbations as described by Tamm *et al.*[38] in addition to GCs and oxidative injury. Conceptually, one needs to consider whether a decrease in counterbalancing growth factors, thyroid hormones and/or other factors could contribute to outflow obstruction in POAG by an abnormally increased TIGR production. Members of the FGF family of growth factors are being investigated as protective factors in other cell types, with impressive effects of bFGF described for reducing damage in light-induced retinal degeneration models.[39] Potentially important effects of bFGF in protecting against 'apoptotic' stimuli have been described in lens epithelium[40] and vascular endothelium.[41] The ability of relatively low doses of bFGF to reduce the GC induction of TIGR gene expression in HTM cells could be related to these observations. The triiodothyronine effect is of additional interest because of the earlier proposals of an association between thyroid hormone deficiency and glaucoma, an idea that has received renewed attention in recent clinical and basic science studies.[42,43] Growth factors such as bFGF act through a variety of possible regulatory steps to influence gene expression and cellular functions; often these steps are believed to involve activation of receptor kinases as part of a regulatory cascade of events that could also influence gene expression. Thyroid hormones act to regulate gene transcription directly, with interactions known for a variety of hormonal and pharmacological stimuli.

The sequence information obtained for the putative TIGR promoter[11] revealed consensus motifs for a variety of hormonal response elements, including possible GC response elements and a possible thyroid response element. A number of motifs potentially involved in stress responses are also found, including possible AP-1, AP-2, NFκB, and a shear stress response element. We have previously discussed AP-1 sites in relation to the induction of TIGR mRNA found with phorbol ester treatments.[10] The oxidative stress effects may involve NFκB, AP-1 and other motifs, but such effects could also be substantially more complicated. Likewise, growth factor and thyroid hormone effects modifying GC responses could be due to influences on the TIGR promoter, but the wide variety of influences of these factors opens the possibility of several levels of regulation. Booth *et al.*[44] recently suggested that the shear stress response motif could be involved in sensing fluid flow across the endothelial cells and possibly helps to explain the induction of TIGR mRNA by mechanical stretch in HTM cells.[38,45] Continued study of the effects of various modulators in HTM cells compared with other cell types could provide information regarding further aspects of TIGR gene expression, and potential interactions of the pathways involved.

The successful transfection studies of TIGR–GFP constructs into confluent HTM monolayers could provide a useful step in considering questions of gene regulation and potential effects of TIGR gene mutations. The observed differences in the fluorescence patterns of TIGR–GFP constructs containing a deletion in the OLF domain are of interest because of the importance of this

domain both for glaucoma genetics and for understanding pathways involved in TIGR protein biogenesis. The increased staining and different localisation patterns observed with the Pro370Leu construct that appear similar to the construct missing the OLF-homology domain, could involve a functionally relevant change, especially since the Pro370Leu mutation was selected because it has shown a consistently early and severe juvenile POAG phenotype. Since our TIGR–GFP transfections are transient, however, methods to achieve prolonged expression beyond 10 days are desirable for understanding chronic effects. The TIGR–GFP transfection studies showing increased fluorescence with the Pro370Leu mutation might involve a variety of possible processes, including altered targeting or binding to proteins due to the ER sorting function(s) that might change its kinetics within the secretory pathway. Future studies will involve the use of smaller epitope tags placed in different locations in assessing potential mutation-induced alterations in different secretory and cellular compartments, since the relatively large GFP addition to the TIGR molecule may influence the results observed.

The OLF domain was found in our prior studies to coincide with a major translocational pause. This is a crucial region for complex secretory proteins, in which the kinetics of the growing nascent chain slow down, permitting exposure of the polypeptide to the cytosol. This could be important for modifications by cytosolic enzymes (e.g. kinases) as well as proper subunit formation and folding.[46] Effects of TIGR gene mutations, and also possibly certain environmental influences and cell-specific factors that could influence the modifications taking place during biogenesis, could involve effects during this early phase of translocation and synthesis. Such mechanisms may be crucial for understanding the pattern of TIGR mutations associated with glaucoma in this region, including some of the effects of mutations described by Rozsa *et al*.[32] as affecting putative phosphorylation sites. Studies of mutations with only minor predicted structural alterations (e.g. Glu323Lys) occurring at the beginning of the OLF domain generate abnormal forms in assays of translocational processing.[12]

Defects occurring during translocation and/or translation may invoke ER sorting functions, degradative pathways and chaperone functions, some of which may activate stress and/or apoptotic pathways. A report by Zhou and Vollrath[47] that used transfections of tagged TIGR constructs into human kidney (HK) cells may be related to the processes being considered. The study involved evaluations of several TIGR mutations in which an increased ratio of the insoluble/soluble TIGR protein in detergent buffer was able to distinguish mutations from polymorphisms. Further studies may provide information regarding specific mechanisms in this interesting model system, some of which may be relevant to effects in HTM cells. The possibility that abnormal forms of a protein could stimulate cell signalling pathways and the role for chaperone functions in the HTM appear to have some precedent in the findings being obtained in studies of the 'unfolded protein response' (UPR) in the secretory pathway.[48] The UPR response involves the induction of cellular signalling pathways through an ER transmembrane protein with structural similarities to mammalian growth factor receptor kinases, along with the up-regulation of the transcription of ER resident genes involved in protein folding. This or a related cell signalling response to TIGR mutations could provide a mechanism for our earlier proposal that some TIGR mutations produce a stress induction of TIGR proteins/glycoproteins in which both affected and normal genes could be influenced.[16] Interesting studies by B. Yue (personal communication, 2000) have recently found evidence for a mitochondrial form of TIGR that also could be related to the proposed stress-induced 'apoptotic' pathway(s). Her findings suggest that accumulation of the mitochondrial TIGR may be a result of the marked GC-induced increase in the expression of TIGR shown in HTM cultures. The ER pathway for TIGR secretion could also be involved in this process, since she called attention to a precedent for mitochondrial glycoproteins having a potential origin in the ER,[49] and her data show apparent N-glycosylation of the TIGR molecule. Regarding the idea that stimulation of apoptotic pathways could take place with GC treatments of HTM cells, Nguyen observed a noticeable elevation of bcl-2 in our 10 day, 500 nM DEX treated HTM cultures by RT-PCR (unpublished observations). If HTM cells respond to abnormal forms of TIGR with an activation of 'stress' pathways, this would provide a means for alterations in the TIGR protein biogenic pathways to intersect with the proposed GC and oxidative stress pathways for glaucoma pathogenesis. Of course, effects at a later stage involving abnormal protein targeting or alterations influencing intracellular or extracellular activities of the TIGR species produced may play roles in certain of the disease-associated TIGR mutations.

Transfections of TIGR constructs are also being explored by other groups in standard cell lines to provide information on the steps involved in TIGR biogenesis. A recent report by Tomarev's laboratory[50] evaluated TIGR–GFP constructs with deletions in exon 1, in which the authors showed a diminution of the association of GFP–TIGR constructs with microtubules. They interpret the data as being consistent with a secretory biosynthesis pathway which could involve a membrane-related function of cellular TIGR in egress and/or intracellular trafficking pathways; they also called attention to other OLF-related molecules that have proposed ER or membrane functions. McKay and Stamer (personal communication) have also used TIGR–GFP constructs transfected into established cell lines, with an initial emphasis on exon 1 in relation to effects on targeting pathways. They have suggested a role for TIGR as a cytosolic protein that would not have its signal peptide cleaved and would not enter the ER secretory pathway. Nguyen *et al*.[11] from our laboratory also considered that such a form of TIGR might exist in HTM cells, but this was based only on protein sequence data

from the baculovirus Sf9 insect cell system (in which the ER pathway could be saturated through the high levels produced in this system used to produce rTIGR). Our more recent studies of GC-treated HTM cells have found only the cleaved form of TIGR by N-terminal sequencing of media proteins, with the upper band in the 55–57 kDa region due entirely to N-glycosylation (Fauss and Polansky, unpublished observations). The possibility remains, however, that a cytosolic pathway for TIGR could exist as proposed by McKay and Stamer, and may have a major role in other cell types. Also, it is not possible to exclude the possibility that different forms of TIGR could predominate in HTM cells under certain treatment conditions.

The development of improved methods of performing TIGR gene transfections in HTM cells and other cell types should help in addressing a number of questions concerned with TIGR gene mutations, including potential environmental/genetic interactions and effects due to other gene products. Combined with information from other model systems, such evaluations should offer exciting opportunities to explore glaucoma mechanisms (and possible interventions) of clinical significance. Investigations into the effects of different stimuli producing potentially different cellular and extracellular forms of the TIGR protein(s)/glycoprotein(s) need to be conducted in HTM cells as compared with other potential target cells. In this regard, current research efforts by other groups characterising TIGR gene regulation and potential effects of mutations in cells in the back of the eye, such as astrocytes and lamina cribrosa cells, may help to explain additional effects of the TIGR gene with regard to visual field loss.

Continued efforts to define the pathways involved in normal and mutated forms of TIGR could provide important clues into physiological/pathogenic mechanisms. In the coming years it will be of interest to determine the spectrum of effects produced by agents that both stimulate and reduce TIGR mRNA. These studies are likely to be important in addition to direct measurement of effects on gene expression. Influences of chronic TIGR stimulation by GCs and other agents, in addition to the effects of TIGR mutations on cell signalling and apoptotic pathways, need to be explored in much greater detail. Determinations of TIGRs 'life cycle' in different cellular compartments (including synthetic and degradative pathways produced by alterations in the molecule's biogenic pathways), may also help to explain certain pathological effects.

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