CURRENT EYE RESEARCH
2016, VOL. 41, NO. 8, 1035–1043
http://dx.doi.org/10.3109/02713683.2015.1088954

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Transforming Growth Factor Beta 1 Modulates the Functional Expression of the Neurokinin-1 Receptor in Human Keratocytes

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ABSTRACT

Purpose: Transforming growth factor beta 1 (TGF-β1) is a cytokine involved in a variety of processes, such as differentiation of fibroblasts into myofibroblasts. TGF-β1 has also been shown to delay the internalization of the neurokinin-1 receptor (NK-1 R) after its activation by its ligand, the neuropeptide substance P (SP). NK-1 R comprises two naturally occurring variants, a full-length and a truncated form, triggering different cellular responses. SP has been shown to affect important events in the cornea – such as stimulating epithelial cell proliferation – processes that are involved in corneal wound healing and thus in maintaining the transparency of the corneal stroma. An impaired signaling through NK-1 R could thus impact the visual quality. We hypothesize that TGF-β1 modulates the expression pattern of NK-1 R in human corneal stroma cells, keratocytes. The purpose of this study was to test that hypothesis.
Methods: Cultures of primary keratocytes were set up with cells derived from healthy human corneas, obtained from donated transplantation graft leftovers, and characterized by immunocytochemistry and Western blot. Immunocytochemistry for TGF-β receptors and NK-1 R was performed. Gene expression was assessed with real-time polymerase chain reaction (qPCR).
Results: Expression of TGF-β receptors was confirmed in keratocytes *in vitro*. Treating the cells with TGF-β1 significantly reduced the gene expression of NK-1 R. Furthermore, immunocytochemistry for NK-1 R demonstrated that it is specifically the expression of the full-length isotype of the receptor that is reduced after treatment with TGF-β1, which was also confirmed with qPCR using a specific probe for the full-length receptor.
Conclusions: TGF-β1 down-regulates the gene expression of the full-length variant of NK-1 R in human keratocytes, which might impact its signaling pathway and thus explain the known delay in internalization after activation by SP seen with TGF-β1 treatment.

ARTICLE HISTORY
Received 18 March 2015
Revised 31 July 2015
Accepted 25 August 2015

KEYWORDS
Cornea; cytokines; neuropeptides; stroma; substance P

Introduction

Eye trauma that results in corneal ulceration is an important cause of visual impairment and is estimated to lead to 1.5–2 million new cases of unilateral blindness every year.[1] Maintaining a transparent cornea is of utmost importance after injury, infection, or penetrating transplant surgery, all of which can otherwise lead to formation of opaque corneal tissue, i.e., scarring. The corneal stromal transparency is based on tightly regulated and highly organized collagen fibers.[2] Certain proteoglycans, such as lumican[3] and keratocan,[4] are responsible for maintaining the highly controlled collagen organization. These proteoglycans are normally produced by keratocytes, the primary resident cells of the stroma, which also produce the extracellular matrix (ECM) surrounding them. After an injury, keratocytes can be activated by cytokines in the tear fluid,[5] or by factors produced by the corneal epithelium,[6] like transforming growth factor Beta (TGF-β).

TGF-β is a cytokine comprising three isoforms in mammals, TGF-β1, TGF-β2, and TGF-β3, displaying different and sometimes opposing effects during corneal wound healing.[7] TGF-β1 and TGF-β3 bind to the high affinity type II receptor TGFBR2. This association subsequently triggers the dimerization of TGFBR2 with the low affinity type I receptor TGFBR1.[8,9] TGF-β2 requires the intervention of the type III receptor betaglycan to present the ligand to TGFBR2.[10,11] TGF-β is a cytokine which is restricted to the epithelium in healthy corneas but which upon injury is released into the stroma,[12] where it is involved in cell proliferation, migration, and differentiation.[13] TGF-β1 triggers transformation of quiescent keratocytes into myofibroblasts, contractile cells expressing alpha smooth muscle actin (αSMA) and producing ECM components including collagen.[14] The newly produced collagen fibrils are disorganized and result in corneal haze which often disappears after myofibroblasts apoptosis.

TGF-β has also been shown to delay internalization of the neurokinin-1 receptor (NK-1 R) after its activation by its ligand, the neuropeptide substance P (SP), in T lymphocytes.[15] NK-1 R is encoded by the TACR1 gene which gives rise to two variants, a full-length variant comprising 407 amino acids, and a truncated form resulting from a splicing, lacking

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the last 96 amino acids of the intracellular C-terminal region.-[16] Contrary to the truncated receptor, the full-length variant has a high affinity for SP and induces a rapid activation of the extracellular-signal-regulated kinases (ERK).[17]

Both SP and NK-1 R are expressed by human corneal epithelial cells and keratocytes in the stroma.[18] SP is an 11 amino acid pro-inflammatory neuropeptide encoded by the preprotachykinin-A (PPT-A) gene and is expressed by neuronal as well as non-neuronal cell.[18–20] It enhances cells proliferation and reduces or delays apoptosis *in vitro* and *in vivo*[21] in an Akt-dependent manner.[22,23] In an alkali burn rabbit model, exogenously administered SP enhanced the healing of the corneal epithelium and reduced the bleeding.[24] Furthermore, SP regulates the expression of some matrix components such as collagen type I and III in explants of ligaments.[25] An impaired signaling through NK-1 R could thus disturb the corneal ECM and impact visual quality.

In the present work, we studied whether TGF-β1 could impact the expression pattern of the NK-1 R in human keratocytes *in vitro*.

Materials and methods

Isolation and primary culture of human keratocytes

Healthy human corneal material was obtained from the corneal biobank at the University Hospital of Umeå, either as whole corneas that were not used for transplantation by lack of matched recipient, or from transplantation graft leftovers from surgery. The tissue, which is tested according to Swedish law for infectious transmitted diseases like HIV etc, is derived from deceased individuals who had chosen, when alive, to donate their corneas post-mortem for transplantation and research. In case of suspicion of disease or abnormal cell count by the biobank, the corneas were discarded. Both central and peripheral cells were used in the experiments. Healthy donors used in this study comprise a male/female ratio of 1/6 with average age of 65.0 and 66.3 (range: 37–90), respectively. The study was vetted by the Regional Ethical Review Board in Umeå (2010-373-31M) without objections. The study was performed according to the principles of the Declaration of Helsinki.

Samples were scraped using a sterile scalpel to remove any remaining epithelial or endothelial cells before being washed in sterile Hanks' Balanced Salt Solution (HBSS; Invitrogen™, CA, USA, # 14170). The remaining stroma was then minced with a scalpel and digested in collagenase (Clostridopeptidase A, Sigma-Aldrich®, Stockholm, Sweden, # C-1030) diluted in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12, Fisher Scientific, Göteborg, Sweden, # 21331-046) to a concentration of 2 mg/ml for 30 min at 37°C. The digestion product was then transferred to a culture dish containing DMEM/F-12 supplemented with 2% fetal bovine serum (FBS; Invitrogen™, CA, USA, # 16000), 1% penicillin-streptomycin (Invitrogen™, CA, USA, # 15410), and 0.2% L-Glutamine (Invitrogen™, CA, USA, # 25030) and cultured at 37°C in a humidified atmosphere supplemented with 5% CO_2. Medium was replaced every second to third day until the cells reached confluence. Confluent cells were detached with 0.05% trypsin–EDTA (Invitrogen™, CA, USA, # 25300) and split in a 1:3 ratio. Cells from passage three to four were used for experiments.

Immunocytochemistry

10^4 cells per well were seeded in eight well chamber slides (BD Falcon™, Bedford, MA, USA, # 354118) overnight before being fixed in 2% paraformaldehyde (PFA) diluted in 0.1 M phosphate buffered saline (PBS) (pH 7.4) for 10 min. Fixed cells were washed repeatedly in PBS, then blocked with 1:20 diluted normal serum (Table 1) corresponding to the host type of the secondary antibody for 15 min. After carefully disposing of the serum, cells were incubated with the primary antibody (Table 2) overnight at 4°C. Washing and blocking were repeated and secondary antibody (Table 3) was added for 30 min at 37°C. Cells were washed and mounted in Vectashield mounting medium for fluorescence (Vector Laboratories, Burlingam, CA, USA, # H-1500). A control well was also prepared for each secondary antibody by replacing the primary antibody with PBS. A Zeiss Axioskop 2 plus microscope equipped with epifluorescence and an Olympus DP70 digital camera were used for analysis.

Stimulation of human keratocytes

2.5×10^5 cells per well in six well plates (Sarstedt, Newton, NC, USA, # 83.1839) were cultivated and kept in serum-reduced medium (containing 0.1% of FBS) for 24 h. Then, cells received either 10 ng/ml of GW 788388 (Tocris bioscience, Bristol, UK, # 3264), a selective inhibitor of TGF-β1 receptor, or the equivalent volume of medium. 20 min later, cells received either 10 ng/ml of

Table 1. Normal serum used for immunocytochemistry.

Serum	Code	Source	Dilution
Donkey	017-000-121	Jackson I.R., West Grove, PA, USA	1:20
Rabbit	X0902	Dako, Glostrup, Denmark	1:20
Swine	014-000-121	Jackson I.R., West Grove, PA, USA	1:20

Table 2. Primary antibodies used for immunocytochemistry.

Antigen	Code	Source	Type	Dilution
CD34	sc-9095	Santa Cruz Biotechnology®, Dallas, TX, USA	Rabbit	1:50
ALDH	ab52492	Abcam®, Cambridge, UK	Rabbit	1:100
Keratocan	sc-66941	Santa Cruz Biotechnology®, Dallas, TX, USA	Rabbit	1:100
Lumican	sc-166871	Santa Cruz Biotechnology®, Dallas, TX, USA	Mouse	1:50
TGFBR1	ab155258	Abcam®, Cambridge, UK	Rabbit	1:100
TGFBR2	ab78419	Abcam®, Cambridge, UK	Mouse	1:50
TGFBR3	ab166705	Abcam®, Cambridge, UK	Mouse	1:100
NK-1R interal loop	sc-5220	Santa Cruz Biotechnology®, Dallas, TX, USA	Goat	1:100
NK-1R C-terminal	s8305	Sigma St Louis, MO, USA	Rabbit	1:100

Table 3. Secondary antibodies used for immunocytochemistry.

Secondary antibody	Code	Source	Dilution
Alexa Fluor 488, donkey anti-goat	A-11055	Invitrogen, Carlsbad, CA, USA	1:300
TRITC-conjugated rabbit anti-mouse	R0270	Dako, Glostrup, Denmark	1:20
TRITC-conjugated swine anti-rabbit	R0156	Dako, Glostrup, Denmark	1:40

human recombinant TGF-β1 (R&D system, Abingdon, UK, # P01137) or the equivalent volume of medium. Cells were then incubated 24, 48, or 72 h at 37°C in a humidified atmosphere supplemented with 5% CO_2 before being lysed.

RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

Isolation of total RNA was performed with an RNeasy kit (Qiagen, Sollentuna, Sweden, # 74106) following the manufacturer's instructions on 2.5×10^5 cells per well. Isolated RNA was converted to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems®, # 4368813) following the manufacturer's instruction. Conversion was performed on an Eppendorf Mastercycler EP Gradient S (VWR International, Spånga, Stockholm, Sweden), using the following protocol: 10 min at 25°C followed by 120 min at 37°C and 5 min at 85°C. Quantitative PCR (qPRC) was performed using TaqMan fast universal PCR master mix (Life technology, Stockholm, Sweden, # 4352042) and NK-1 R probes, of which the first (TACR1, Life technology, Stockholm, Sweden, # Hs00185530-m1) targets both forms of the receptor and the second (TACR1, Life technology, Stockholm, Sweden, # Hs01025732-m1) is specific for the full-length variant, αSMA probe (ACTA2, Life technology, Stockholm, Sweden, # Hs00909449_m1), and housekeeping gene probes, human beta actin (ACTB) (Life technology, Stockholm, Sweden, # 4352935) and 18 s rRNA Oligo Mix (Life technology, Stockholm, Sweden, # 4332641). Samples were run on a ViiA 7 Real-Time PCR System (Life technology, Stockholm, Sweden) using the following settings: 20 s of denaturation at 95°C followed by 40 cycles of 95°C for 1 s and 60°C for 20 s. All samples were run in technical duplicates and the mean value was used for analyses.

Protein extraction and western blot

2.5×10^5 cells per well in six well plates (Sarstedt, Newton, NC, USA, # 83.1839) were cultivated in DMEM/F-12 medium containing either 0.1% or 2% FBS. After 24 h of culture, cells were washed in ice-cold PBS and lysed in Radio-immunoprecipitation assay buffer supplemented with 0.5% of protease inhibitor cocktail (Sigma, St. Louis, MO, USA # P1860). Samples were then diluted in Laemmli Sample buffer supplemented with 2-mercaptoethanol. Proteins were separated by gel electrophoresis under denaturing conditions and transferred to PVDF membranes. Blots were incubated with primary antibodies (Table 4) overnight at 4°C. Blots were then washed and incubated with horseradish peroxydase-conjugated secondary antibodies (Table 5) for 1 h at room temperature, washed again, and incubated with chemiluminescent reagents (Amersham Pharmacia Biotech INC, Piscataway, NJ, USA, # RPN2232). Images were taken by Odyssey® Fc imaging system (LI-COR, Lincoln, NE, USA). Densitometry was performed using Image J analysis software (NIH).

Statistical analysis

Data represent means ± standard deviation (SD) of three replicates. All experiments were performed at least three times with successfully repeated results. GraphPad Prism 5 software was used for data analysis. Statistical significance was determined using one-way ANOVA followed by Bonferroni post-hoc comparison test for the qPCR (more than two groups) or t-test for the Western blot densitometry analysis (two groups). Statistical significance was predetermined at $p < 0.05$.

Table 4. Primary antibodies used for Western blot.

Antigen	Code	Source	Type	Dilution
CD34	sc-9095	Santa Cruz Biotechnology®, Dallas, TX, USA	Rabbit	1:200
ALDH	ab52492	Abcam®, Cambridge, UK	Rabbit	1:500
Keratocan	sc-66941	Santa Cruz Biotechnology®, Dallas, TX, USA	Rabbit	1:200
Lumican	sc-166871	Santa Cruz Biotechnology®, Dallas, TX, USA	Mouse	1:200
Procollagen I	ab155258	Santa Cruz Biotechnology®, Dallas, TX, USA	Goat	1:100

Table 5. Secondary antibodies used for Western blot.

Secondary antibody	Code	Source	Dilution
Anti-rabbit IgG HRP-linked	7074	Cell Signaling, Danvers, MA, USA	1:2000
Anti-mouse IgG HRP-linked	7076	Cell Signaling, Danvers, MA, USA	1:2000
Anti-goat IgG HRP-linked	Sc-2020	Santa Cruz Biotechnology®, Dallas, TX, USA	1:2000

Results

Characterization of isolated cells from donor cornea

In this study, cells were cultured in medium supplemented with 2% FBS, then switched to a medium containing a low amount of FBS, 0.1%, 24 h prior to stimulations. However, keratocytes are considered to be quiescent cells in the cornea and FBS is known to activate them into fibroblasts. Cells were therefore studied for the presence of keratocyte markers after 24 h of culture in medium supplemented with 2% or 0.1% FBS by immunocytochemistry and Western blot. CD34 is now considered as a well-established marker of keratocytes *in vivo* and its expression decreases upon cell activation.[26] Our cells showed a weak but positive staining for CD34, with only a few cells positively stained in 0.1% FBS (Figure 1A), but all cells in the 2% FBS condition (Figure 1B). Western blot analysis confirmed the expression of CD34 in both culture conditions, however densitometry analysis did not show any significant difference between the two conditions (Figure 1I). Aldehyde dehydrogenase (ALDH) is a crystallin expressed by keratocytes to help maintain corneal transparency, and its expression decreases when keratocytes turn into repair keratocytes or fibroblasts.[27] In this study, no ALDH was observed by immunocytochemistry in cells cultured in 0.1% FBS (Figure 1C) and very few cells displayed a weak positive staining for ALDH in the 2% FBS culture condition (Figure 1D). Bands were however visible on Western blot analysis for both conditions, with a significantly higher expression in cells cultured in medium supplemented with 2% FBS (Figure 1J). Keratocan and lumican are the two most

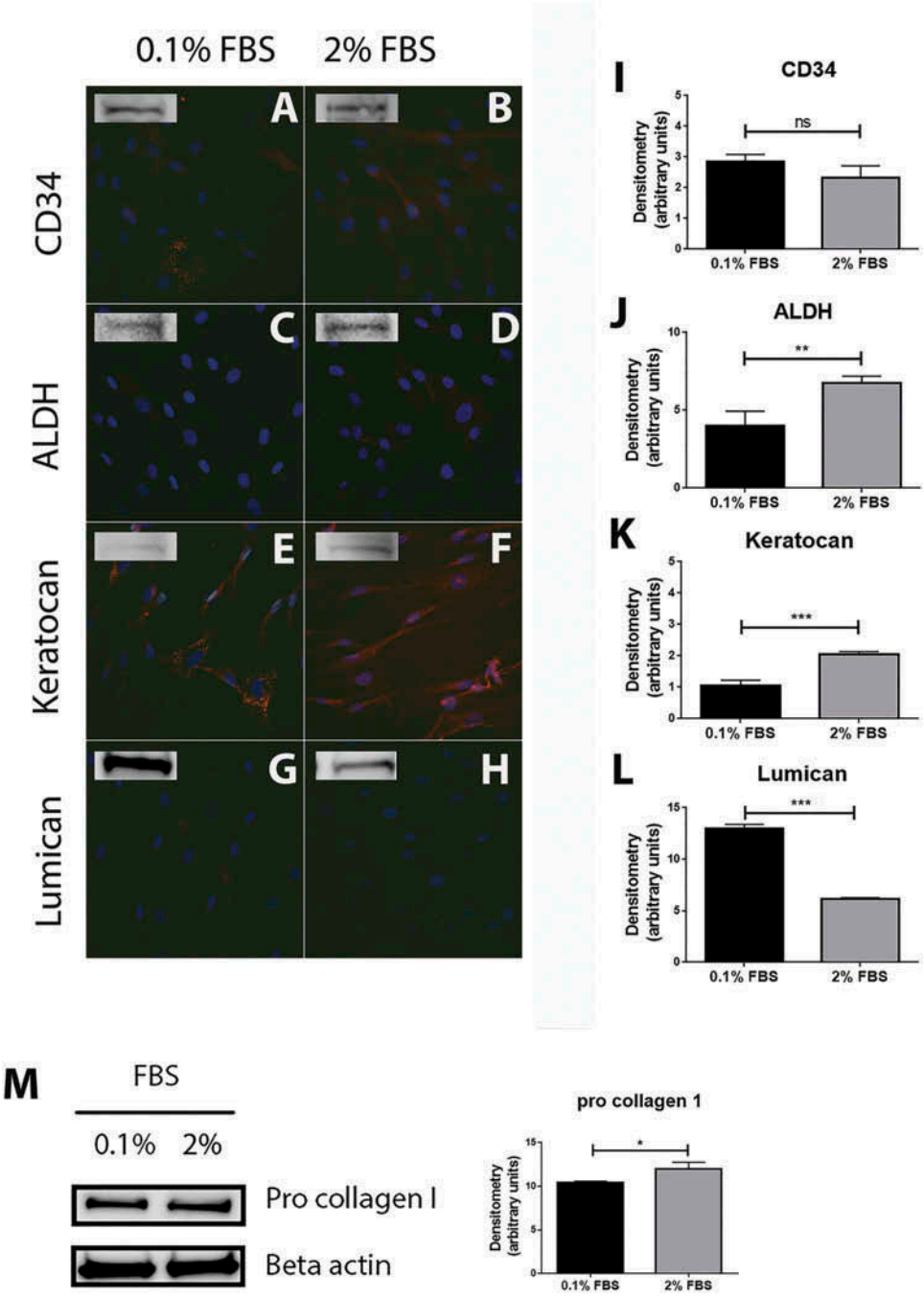

Figure 1. Characterization of cells extracted from healthy human cornea. Immunocytochemistry of the cells extracted from healthy human cornea after 24 h of culture in DMEM medium supplemented with either 0.1% or 2% fetal bovine serum (FBS) for 24 h shows that several cells were weakly stained for CD34 in 0.1% FBS condition (A) whereas the majority of cells showed a weak but positive staining in 2% FBS (B). ALDH (C and D) and lumican (G and H) stainings showed almost no positive reactions in both culture conditions. Keratocan was highly expressed in both 0.1% FBS (E) and 2% FBS (F) culture conditions. Western blot experiments were also performed on the cells and showed that all the markers were expressed by the cells in both culture conditions. Densitometry analysis further revealed no statistically significant (ns) difference in expression of CD34 between the cells cultured in 0.1% and 2% FBS (I). ALDH (J) and keratocan (K) had a significantly higher expression in cells cultured in 2% FBS than in 0.1% FBS (**$p < 0.01$ and ***$p < 0.001$, respectively). On the contrary, cells expressed significantly more lumican (L) in the 0.1% FBS than in 2% FBS culture conditions (***$p < 0.001$). In addition, cells strongly expressed pro collagen I in both culture conditions, with a significantly higher expression in medium supplemented with 2% FBS (*$p < 0.05$) (M). Values are means ± SD.

commonly used markers of keratocytes and their expression decreases as well as the cells get activated.[28,29] All cells stained strongly positive for keratocan in 0.1% (Figure 1E) and 2% (Figure 1F) FBS conditions and Western blot densitometry analysis showed a significantly higher expression of keratocan in cells cultured in 2% FBS than in cells cultured in 0.1% FBS (Figure 1K). The opposite was observed for lumican, for which expression was significantly higher in cells cultured in 0.1% FBS as compared to cells cultured in 2% FBS (Figure 1L). No positive staining for lumican was observed by immunocytochemistry (Figure 1G and H) though strong bands were seen by Western blot. As keratocytes are cells that produce the corneal ECM, cells capacity to produce collagen was assessed by Western blot through expression of pro collagen

I. A strong band could be observed in both cell culture conditions (Figure 1M), confirming that the cells produce ECM. Pro collagen I expression was significantly higher in cells cultured in 2% FBS condition than cells cultured in medium supplemented with 0.1% FBS.

TGF-β1 down-regulates NK-1 R gene expression in keratocytes

Human keratocytes were studied for the presence of TGF-β receptors. Immunocytochemistry showed that TGFBR1, TGFBR2, and TGFBR3 were highly expressed by all cells with a slightly higher expression of TGFBR1 and TGFBR3 in cells cultured in medium supplemented with 2% FBS (Figure 2B and F) as compared to cells cultured in 0.1% FBS medium (Figure 2A and E). Culture condition did not impact TGFBR2 expression (Figure 2C and D). To determine the effect of TFG-β receptor activation on keratocytes, cells were stimulated with a recombinant human TGF-β1 for 24, 48, or 72 h. mRNA levels of αSMA, a marker of corneal fibroblast activation into myofibroblasts,[30] and of NK-1 R were subsequently determined by qPCR. TGF-β1 significantly increased the expression of αSMA (Figure 2G) after 24 h, which was expected, as TGF-β is known to induce myofibroblasts. This verifies the stimulation efficiency. Furthermore, qPCR showed that TGF-β1 stimulation significantly decreased the gene

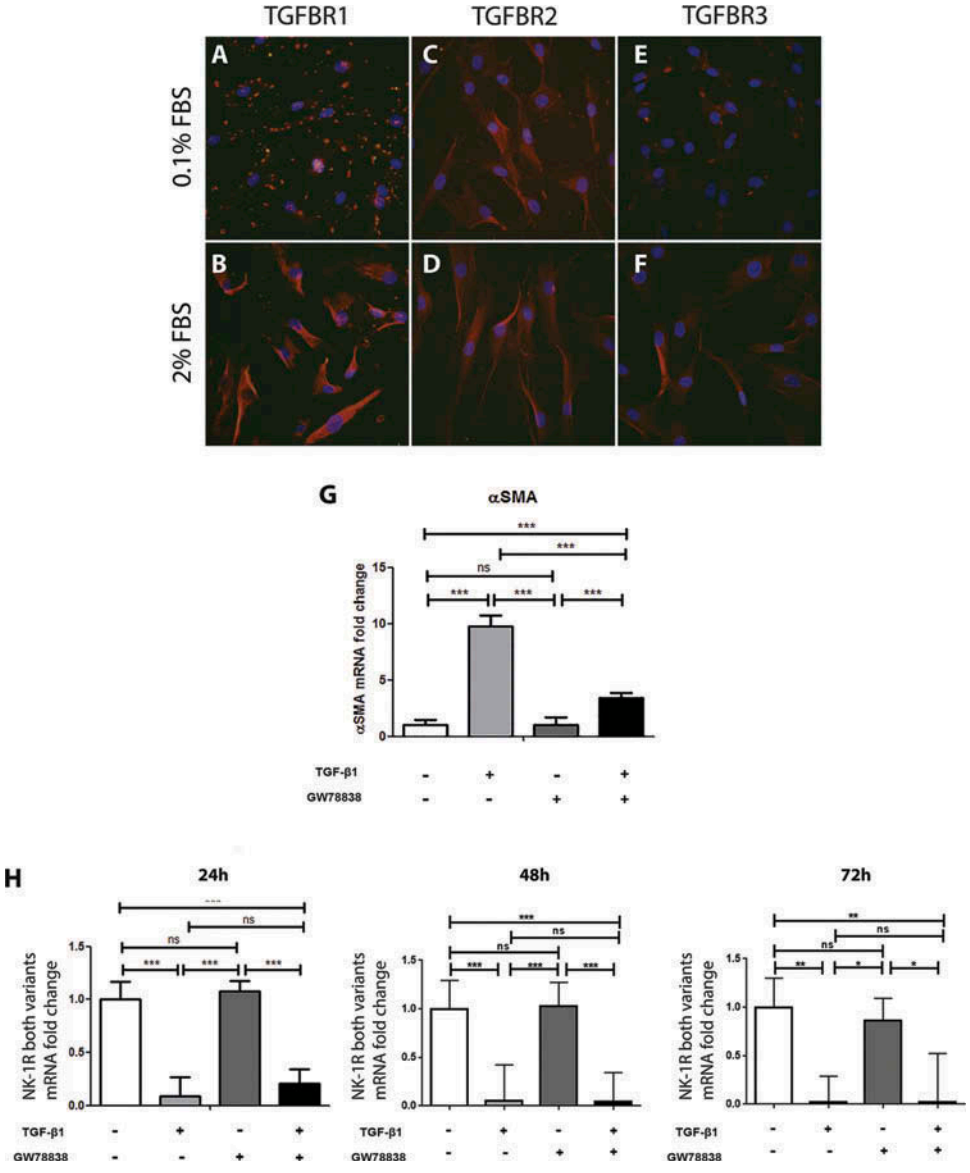

Figure 2. TGF-β stimulation of human keratocytes *in vitro*. Immunocytochemistry of human keratocytes *in vitro* revealed the presence of TGF-β receptor I (TGFBR1; red) in all cells cultured in medium supplemented with 0.1% FBS (A) and 2% FBS (B). TGF-β receptor II (TGFBR2; red) was also strongly positive in all cells in the 0.1% (C) and 2% (D) FBS culture conditions. TGF-β receptor III (TGFBR3) showed a more intense staining in the 2% FBS culture (F) than in the 0.1% FBS condition (E). All slides were double stained with DAPI (blue) to identify cell nuclei. ((G) and (H)) Cells were treated with TGF-β1 (10 ng/ml), the TGF receptor I inhibitor GW788388 (10 ng/ml) or both, 24, 48, or 72 h prior to RNA extraction and conversion to cDNA. qPCR showed an increase of αSMA mRNA levels (G) in TGF-β1 treated cells compared to untreated cells 24 h after stimulation. GW 788388 significantly reduced the effect of TGF-β1. For the NK-1 receptor, mRNA levels decreased significantly in TGF-β1 treated cells as compared to untreated (***$p < 0.001$ at 24 and 48 h, **$p < 0.01$ at 72 h). GW 788388 slightly reduced the effect of TGF-β1 at 24 h but not significantly (ns). Values are means ± SD.

expression of NK-1 R (Figure 2H). This decrease was observed at all three time points studied. However, pre-incubation of the cells with GW 788388, an inhibitor of TGF receptor I, did not significantly attenuate the NK-1 R gene expression decrease induced by TGF-β1 stimulation.

TGF-β1 specifically down-regulates the expression of the full-length isoform of NK-1 R

To further elucidate whether it is one particular or both isoforms of NK-1 R that are down-regulated by TGF-β1, cells were stimulated with TGF-β1 for 24 h, then fixed and stained with two antibodies targeting different regions of the receptor. One antibody (# sc-5220) recognizes an inner-loop, thus identifying both isoforms. The other one (# s8305) targets the C-terminal region and is therefore specific of the full-length NK-1 R. In untreated keratocytes, both the internal loop (Figure 3A) and the C-terminal region (Figure 3C) were immunopositive. Overlap image (Figure 2E) revealed that both the full-length receptor (recognized by both antibodies) and the truncated receptor (recognized by only one of the antibodies) were expressed. On the TGF-β1 treated keratocytes, however, the truncated receptor was still expressed (Figure 3B) but the full-length form showed almost no positive reaction (Figure 3D), which is also seen in the overlap figure (Figure 3F) in which no more structures identified by both antibodies are visible. TGF-β1 thus seems to down-regulate only the full-length receptor, without impacting the truncated isoform of NK-1 R. The specific down-regulation of the full-length receptor was furthermore confirmed by qPCR using a probe (# Hs01025732-m1) that is specific to the mRNA of the full-length isotype (Figure 3G). Results also showed that the full-length isotype decreased expression was maintained 48 h after stimulation, and still showed the same trend, although not statistically significant, at 72 h.

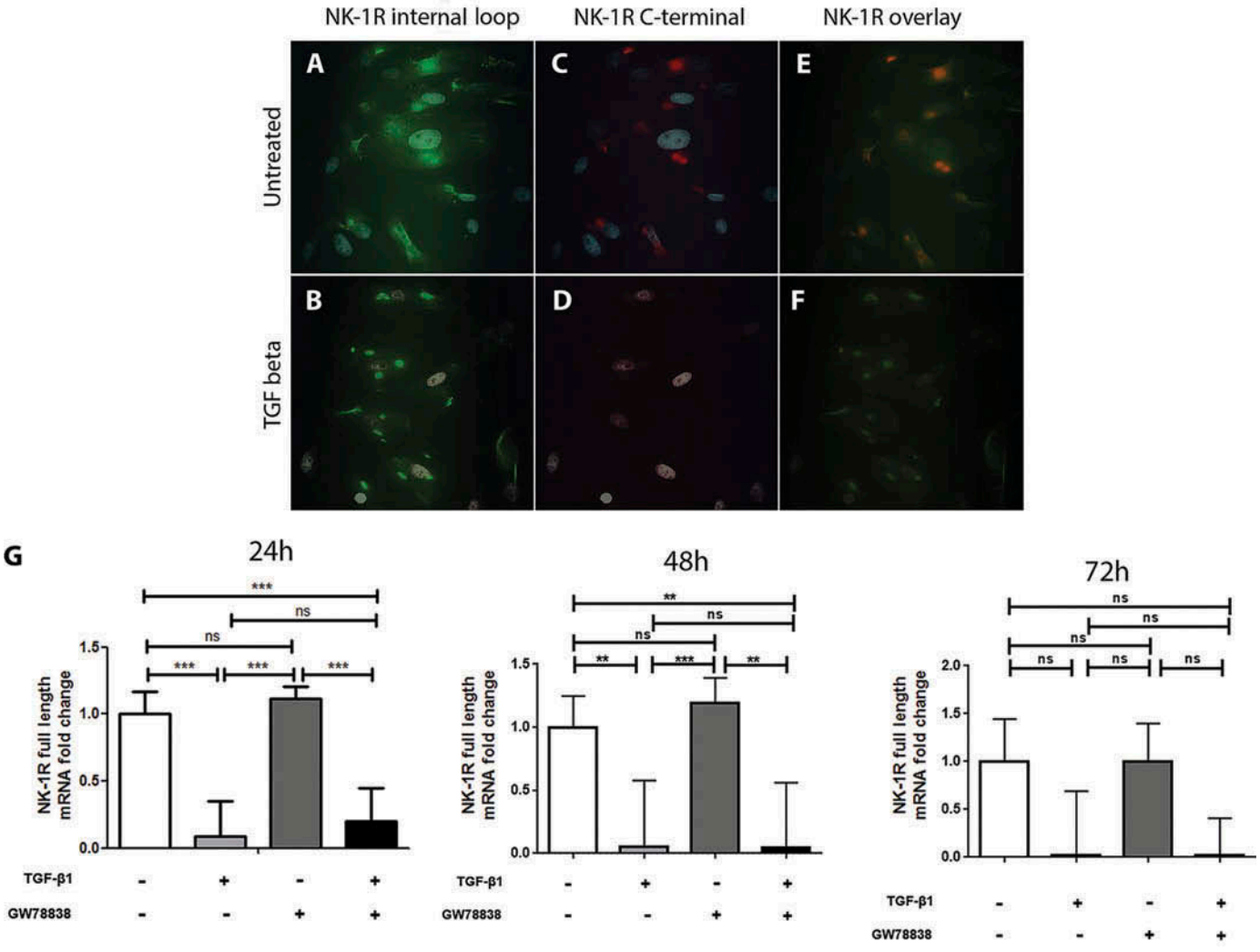

Figure 3. TGF-β1 down-regulates the expression of the full-length but not of the truncated form of NK-1 R in human keratocytes *in vitro*. Immunocytochemistry of the untreated ((A), (C), and, (E)) or TGF-β1 treated ((B), (D), and (F)) human keratocytes *in vitro* shows that untreated cells display positive reactions for the antibody targeting the inner-loop of NK-1 R (green) (A) as well as for the antibody targeting the C-terminal of the receptor (red) (C). Both antibodies overlap (yellow) but some green staining is still visible, indicating that both forms are expressed (E). The reactions of the inner-loop antibody are still present in the TGF-β1 treated cells (B), but the full-length receptor is not expressed anymore (D), as is confirmed by the overlapping image in which no yellow color can be seen (F). DAPI (blue) was applied to identify nuclei. (G) Quantitative real-time PCR, using a probe that specifically targets the full-length form of the NK-1 receptor further confirms the down-regulation of this isoform of the receptor after TGF-β1 treatment (***$p < 0.001$ at 24 h, **$p < 0.01$ at 48 h, not significantly (ns) at 72 h). The TGF receptor I inhibitor GW 788388 reduces the effect of TGF-β1 at 24 h, but this was not significant (ns). Values are means ± SD.

Discussion

Soon after an injury occurs, quiescent keratocytes get activated, stop expressing lumican and keratocan, start expressing stress fibers due to changes in the ECM tension,[31] and finally express αSMA, a marker of myofibroblasts.[32,33] The generated myofibroblasts produce less crystallins, which decrease the transparency of the corneal stroma,[34] and produce high amounts of ECM components,[14] leading to haze in some cases.[35] Key regulators of the wound healing response are likely to be constitutively produced in order to be quickly available,[36] like TGF-β which is produced as a precursor containing the latency-associated protein and the latent TGF-binding protein, anchoring it to the ECM until a proteolytic cleavage converts it into its mature form.[37] TGF-β binds to type IV collagen of the basement membranes of the epithelium with a high affinity[34] and it has been noted that the expression of fibrotic markers is only observed if Bowman's layer is disrupted[37] thereby allowing TGF-β release in the stroma. Therefore, TGF-β plays a central role in corneal wound healing as it is responsible for myofibroblast transformation, and topical application of blocking antibodies to TGF-β prevents corneal fibrosis in a rabbit model.[38]

In our study, we extracted stromal cells from healthy human corneas. As the culture medium used was supplemented with FBS, which is known to change keratocytes phenotype,[39] we investigated the expression of several keratocyte markers by immunocytochemistry and Western blot. CD34, a glycosylated transmembrane protein that is expressed by keratocytes *in vivo*, is lost when cells are cultured *in vitro* on plastic with serum-containing medium.[40] In our experiments, cells cultured in 0.1% FBS showed no positive staining for CD34, and cells in 2% FBS only showed a weak staining but on all cells. However, clear bands were observed in both culture conditions by Western blot. The difference is likely due to a less efficient antibody in immunofluorescence. The same was observed for ALDH and lumican, two other keratocyte markers that are decreased in activated cells,[27,41] with weak or absence of staining by immunofluorescence but bands were seen in Western blot. Keratocan, a crystallin expressed by keratocytes, showed a strong staining in both culture conditions as well as bands on Western blot. Surprisingly, keratocan had a significantly higher expression in cells cultured with more FBS. Cells also strongly expressed pro collagen I, showing their ability to produce ECM components. In summary, the extracted cells showed characteristic markers of keratocytes despite the presence of FBS in the medium, and it seems that switching to a concentration of 0.1% FBS 24 h before stimulation slightly increases the expression of two out of four markers. Most of the studies comparing the effect of FBS on cultured keratocytes used 10% versus 0% FBS,[42–45] five times more FBS than we used in our experiments.[46] This might explain why our cells still express some keratocyte markers despite the presence of serum, even though those levels are low.

In our study, we assessed the presence of TGF-β receptor I, II, and III at the cell surface of human keratocytes *in vitro* by immunocytochemistry. The cells were then stimulated with human recombinant TGF-β1 which resulted in an increase in αSMA mRNA levels. This result showed that stimulation was effective, as it is known that TGF-β activates fibroblasts into myofibroblast.[47,48] Myofibroblasts generation also correlated to a drop in NK-1 R gene expression, and this decrease remained up to 72 h after addition of TGF-β1. Upon TGF-β binding to the high affinity TGFBR2, the receptor transphosphorylates TGFBR1 and associates with it in a complex containing two type II and two type I receptors. TGFBRI subsequently phosphorylates downstream kinases in the cell.[49–51] Addition of TGFBRI blocker significantly attenuated TGF-β1 enhanced α-SMA expression in our study, as compared to TGF-β1 alone. However, it did not significantly prevent this decrease in NK-1 R expression. One explanation could be that the efficient dose of the blocker used to attenuate α-SMA expression is not sufficient to prevent NK-1 R expression decrease.

NK-1 R has two naturally occurring variants, a full-length form comprising 407 amino acid residues and a truncated form lacking 96 amino acids from the C-terminal domain. The full-length receptor is mostly expressed in the human brain whereas the truncated form is found in the central nervous system as well as in peripheral tissues.[52] SP binds to both isoforms, and such binding induces responses by both receptors although through different pathways. The full-length receptor activation leads to a transient increase in intracellular calcium, activation of nuclear factor kappa B (NFκB), production of interleukin 8 (IL-8), phosphorylation of protein kinase C delta (PKCδ), and fast activation of the ERK (1–2 min). Activation of the truncated isoform induces a decrease in IL-8, inhibition of PKCδ phosphorylation, and a later peak in ERK activation, around 20–30 min.[17] It has also previously been shown that exogenously added TGF-β delays NK-1 R internalization after SP binding.[15] Among the demonstrated effects of SP, some could be of importance during corneal wound healing, such as CD29+ cell recruitment,[24] cell proliferation,[53] anti-apoptotic agent,-[22,23] cell migration,[54] and angiogenesis.[55]

In our study, we investigated which forms of NK-1 R are expressed by human keratocytes and which forms are down-regulated following TGF-β1 stimulation. We found that both receptor variants were expressed by the cells, but only the full-length receptor was regulated by TGF-β1. Other studies have also shown differences in the regulation of NK-1 R isoforms during cell differentiation.[56] For example, mRNA expression of NK-1 R truncated form is higher in patients with high grade dysplasia than in patients with carcinoma.[57] Also, undifferentiated monocyte/macrophage THP-1 cells only express NK-1 R truncated form, but start expressing the full-length after phorbol myristate acetate (PMA)-differentiation.[58] These regulations imply that the cells can switch the expression of NK-1 R isoforms when they differentiate. In our experiments, TGF-β stimulated cells differentiated into αSMA-positive myofibroblasts, and then almost exclusively expressed the receptor's truncated variant. The truncated NK-1R has a 10 times lower affinity to SP as compared to the full-length, and it is believed that the lack in the C-terminal domain results in the stimulation of the truncated isoform leading to a prolonged cellular responsiveness and reduced homologous desensitization, as compared to

stimulation of the full-length receptor.[59] Further studies will be necessary to investigate what is the benefit for myofibroblasts to down-regulate the expression of the full-length and keep only the truncated NK-1 R.

To conclude, human keratocytes express both variants of the neurokinin-1 receptor. After a TGF-β1-mediated differentiation to myofibroblasts, the cells keep expressing the truncated variant of the receptor, but the full-length expression is highly decreased.

Acknowlegments

The authors thank Mr. Peter Boman for technical assistance and scientific advice. We also thank Dr. Maria Brohlin and Ms. Randi Elstad for help in providing the donated corneas from the biobank, as well as to all the OR-staff at the Ophthalmic Surgery Clinic, especially Dr. Berit Byström, of the University Hospital of Umeå for deliverance of graft leftovers.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.
Financial support was obtained by P.D. from the national Swedish Research Council (grant no. 521-2013-2612), the J.C. Kempe and Seth M. Kempe Memorial Foundations (for S.L.), the Swedish Society of Medicine, the Cronqvist foundation, the foundation Kronprinsessan Margaretas Arbetsnämnd för synskadade (KMA), the foundation Ögonfonden, and Västerbotten County Council (Spjutspetsmedel). Financial support was furthermore provided to P.D. through a regional agreement between Umeå University and Västerbotten County Council (ALF).

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