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Expression of Transforming Growth Factor-β Receptors in Normal Rat Retina and Experimental Choroidal Neovascularization

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Purpose: Transforming growth factor-β (TGF-β) plays an important role in the development of choroidal neovascularization. TGF-β transduces signals through the mediation of type I and type II receptors. We investigated the expression of TGF-β receptors in a normal rat retina and a model of experimentally induced choroidal neovascularization.

Methods: Choroidal neovascularization was induced by laser photocoagulation in rat eyes. The expression of TGF-β receptors was determined using immunohistochemical and in situ hybridization methods.

Results: In normal adult rat retinas, immunoreactivity and mRNA expression of TGF-β receptor type I (TβRI) and TGF-β receptor type II (TβRII) were found in the ganglion cells. During the process of neovascularization, immunoreactivity and mRNA expression of TβRI and TβRII were widely distributed in laser lesions soon after photocoagulation; thereafter, these receptors were specifically detected in the endothelial cells of choroidal neovascularization.

Conclusions: The expression of TGF-β receptors in normal rat retinas suggests that TGF-β plays an important role in the homeostasis of normal retina. The upregulation of TGF-β receptors in choroidal neovascularization strongly suggests that TGF-β is most likely transduced through specific receptors and plays an important role in the development of choroidal neovascularization. **Jpn J Ophthalmol 2002;46:525–532**

Key Words: Choroidal neovascularization, neovascularization, retina, transforming growth factor β, transforming growth factor β receptors.

Introduction

Choroidal neovascularization is one of the ocular neovascular diseases that can lead to severe visual impairment and even blindness.[1,2] Choroidal neovascularization involves the formation of neovascularization from the choriocapillaris into the subretinal space. Pathological and morphological studies have indicated the sequential steps of its formation.[3–5]

Various growth factors, including basic fibroblast growth factor[6–8] and vascular endothelial growth factor (VEGF),[9–12] have been suggested as promoters of the development of choroidal neovascularization. The long-term goal of these studies is to use growth factors to treat choroidal neovascularization to arrest its development or progression.

Transforming growth factor-β (TGF-β) has been shown to be a multifunctional cytokine that modulates biological events as diverse as wound healing, angiogenesis, differentiation, chemotaxis, and extracellular matrix deposition and dissolution.[13–15] Since its initial discovery, a myriad of biological effects,

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both paracrine and autocrine, have been attributed to TGF-β, qualifying it as a fundamental regulatory cytokine.[16] All these functions are transduced through specific receptors.[17–21] Two major types of receptors, TGF-β receptor type I (TβRI) and TGF-β receptor type II (TβRII), are widely expressed and are believed to be the signaling molecules.[17–21] TGF-β binds to TβRII first; thereafter, complexes of TGF-β and TβRII recruit TβRI.[22] In these complexes, TβRI is phosphorylated by TβRII and is activated to phosphorylate intracellular signal transducers, including those in the Smad family.[23,24]

In an earlier study, we determined that TGF-β1 and TGF-β2 were normally transcribed in the ganglion cell,[25] and that the mRNA expression of TGF-β1 and TGF-β2 was upregulated during the development of experimentally induced choroidal neovascularization. This suggested that TGF-β acts as a mediator of the neovascular process. There is, however, little information on the process of TGF-β signal induction in a normal retina and in one undergoing neovascularization.

The purpose of this study was to investigate the expression of TβRI and TβRII in normal adult rat retinas and also in retinas undergoing choroidal neovascularization induced by laser.

Materials and Methods

Experimental Choroidal Neovascularization Model

A total of 32 adult pigmented rats (Brown Norway strain) weighing 200–300 g and 10–14 weeks of age were used. After induction of anesthesia (intraperitoneal, 30 mg/kg pentobarbital), the pupils were dilated with phenylephrine and tropicamide. Approximately 10 burns were delivered around the disk of the right eyes by krypton laser. The left eyes were not treated and served as normal controls. The burns were placed separately using a setting of 100 μm diameter, 0.1 second duration, and approximately 100 mW intensity as previously described.[7,8,10,12,25] All procedures were conducted in accordance with the Association for Research in Vision and Ophthalmology statement on the use of animals. The development of choroidal neovascularization in the laser lesions was confirmed by fluorescein angiography.

Tissue Preparation

The animals were sacrificed on days 3, 7, 14, and 28 after the photocoagulation with an overdose of intraperitoneal sodium pentobarbital. Eight rats were used at each time point. The eyes were removed and immediately immersed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 20 minutes at 4°C, embedded in OCT Tissue Tech (Miles, Elkhart, IN, USA), frozen and stored at −80°C. Sections of 7 μm thickness were cut on a cryostat and were used for immunohistochemical examination and for in situ hybridization.

Immunohistochemical Study

Immunoperoxidase analysis was carried out with an LSAB kit (DAKO, Glostrup, Denmark) according to the manufacturer's protocol. Briefly, cryosections were fixed in cold acetone and then treated with 3% hydrogen peroxide to eliminate endogenous peroxidase activity. After blocking with 5% nonfat milk and 10% normal goat serum, the primary antibody (rabbit polyclonal antibody raised against human TβRI or TβRII) (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:100 in dilution) was applied to the sections for 60 minutes. The sections were incubated with a biotinylated goat anti-rabbit IgG antibody (LSAB Kit; DAKO, Carpinteria, CA, USA) followed by horseradish peroxidase-conjugated avidin. 3,3′-Diaminobenzidine (DAKO, Denmark) was used as a chromogen and the slides were counterstained with methyl green. For control staining, preimmune rabbit IgG was used instead of the primary antibody. Sections were observed under a light microscope to detect the localization of immunoreactivity for TβRI and TβRII.

Probes

Human TβRI[19] (provided by Prof. K. Miyazono, National Cancer Institute, Tokyo) and TβRII cDNA[17] (provided by Prof. R. A. Weinberg, Massachusetts Institute of Technology, Cambridge, MA, USA) were used. The *Hind*III fragment (350 base pairs, nucleotides 102–451) of TβRI cDNA was subcloned into the pBluescript II KS+ (Stratagene, La Jolla, CA, USA), then linearized with *Eco*RI and used as a template for the synthesis of an antisense riboprobe or was linearized with *Kpn*I for the synthesis of a sense riboprobe. Human TβRII cDNA (610 base pairs, encoding nucleotides 1–609) was subcloned into the pBluescript II KS+, then linearized with *Bam*HI and used as a template for the synthesis of an antisense riboprobe, or was linearized with *Eco*RI for the synthesis of a sense riboprobe. In vitro transcription was performed with T3 polymerase or T7 polymerase to produce digoxigenin-11-uridine triphosphate (DIG-UTP)-labeled single-strand antisense or sense RNA probes using the DIG RNA Labeling Kit

(Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions.

In Situ Hybridization

To localize TβRI and TβRII mRNAs in the retina, in situ hybridization was conducted according to the manufacturer's recommendations (Roche Molecular Biochemicals). Briefly, the sections were refixed in 4% paraformaldehyde, treated with proteinase K (20 μg/mL), incubated in 0.1 M HCl for 10 minutes to inhibit endogenous alkaline phosphatase activity, then rinsed and dehydrated through a graded ethanol series.

An appropriate amount of DIG-UTP-labeled antisense RNA probe (or sense probe as a control) was denatured and hybridized to pretreated sections overnight at 50°C in a 50% formamide saturated humidified chamber.

After hybridization, the sections were washed with 50% formamide/2× SSC (SSC: 0.3 M NaCl and 0.03 M sodium citrate) at 50°C for 30 minutes, then rinsed in 2× SSC.

Detection of the probe was performed according to the instructions supplied with the DIG Nucleic Acid Detection Kit (Roche Molecular Biochemicals). Briefly, the slides were incubated with alkaline phosphatase-labeled anti-digoxigenin antibody, then washed and treated in a color solution containing nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate for 12 hours. The mRNA signals were detected as dark blue or purple precipitates. Hybridization with sense strand riboprobes as negative control was also performed. Finally, sections were counterstained with methyl green to reveal the nuclei as light blue-green in color and were observed under a light microscope to detect the localization of mRNAs.

Results

Expression of TGF-β Receptors in Normal Retina

Immunoreactivity for TGF-β receptors. Immunoreactivity for TβRI and TβRII was observed in cells of the ganglion cell layer as brown in color. These cells were identified as ganglion cells on the basis of their location in the retina. Immunoreactivity for TβRI and TβRII was also detected in the retinal pigment epithelial (RPE) cell layer (Figures 1A and 1B). However, the retinal endothelial cells and choroidal endothelial cells did not show the immunoreactivity for TβRI and TβRII. Negative control slides showed no immunoreactivity for TβRI (Figure 1C) or for TβRII (data not shown).

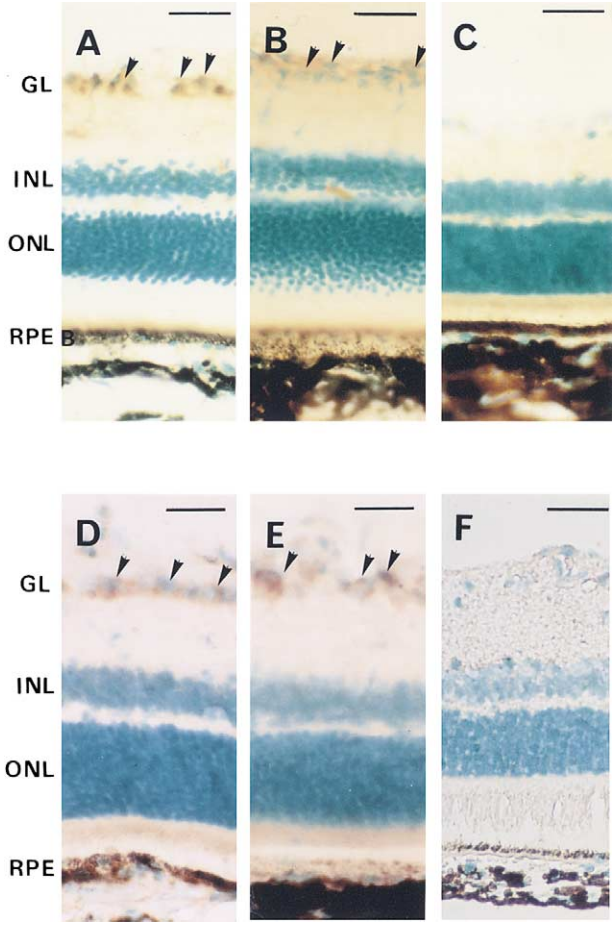

Figure 1. Expression of transforming growth factor-β (TGF-β) receptors in a normal retina. Sections were counterstained with methyl green. Nuclei of the cells are stained light blue-green by methyl green. GL: ganglion cell layer, INL: inner nuclear layer, ONL: outer nuclear layer, RPE: retinal pigment epithelial cell layer. Bars = 50 μm. (**A**,**B**,**C**) Immunoreactivity for TGF-β receptors. Immunoreactivity for TGF-β receptors appears brown in color. (**A**) Immunoreactivity for TGF-β receptor type I (TβRI). Immunoreactivity for TβRI is observed in cells of the ganglion cell layer (arrowheads) and is also detected in the RPE cell layer. (**B**) Immunoreactivity for TGF-β receptor type II (TβRII). Immunoreactivity for TβRII is observed in cells of the ganglion cell layer (arrowheads) and is also observed in the RPE cell layer. (**C**) Control. Control slides showed no immunoreactivity for TβRI. (**D**,**E**,**F**) mRNA expression of TGF-β receptors. Signals are observed as purple precipitates. (**D**) mRNA expression of TβRI. Section was hybridized with antisense riboprobe to detect the expression of TβRI mRNA. Signals are observed in the cells of the ganglion cell layer (arrowheads). (**E**) mRNA expression of TβRII. Section was hybridized with antisense riboprobe to detect the expression of TβRII mRNA. Signals are observed in cells of the ganglion cell layer (arrowheads) but are not apparent in the retinal pigment epithelial cell layer because of the melanin pigments. (**F**) Control. Sections were hybridized with sense probe for TβRI mRNA as controls. No blue precipitate is observed.

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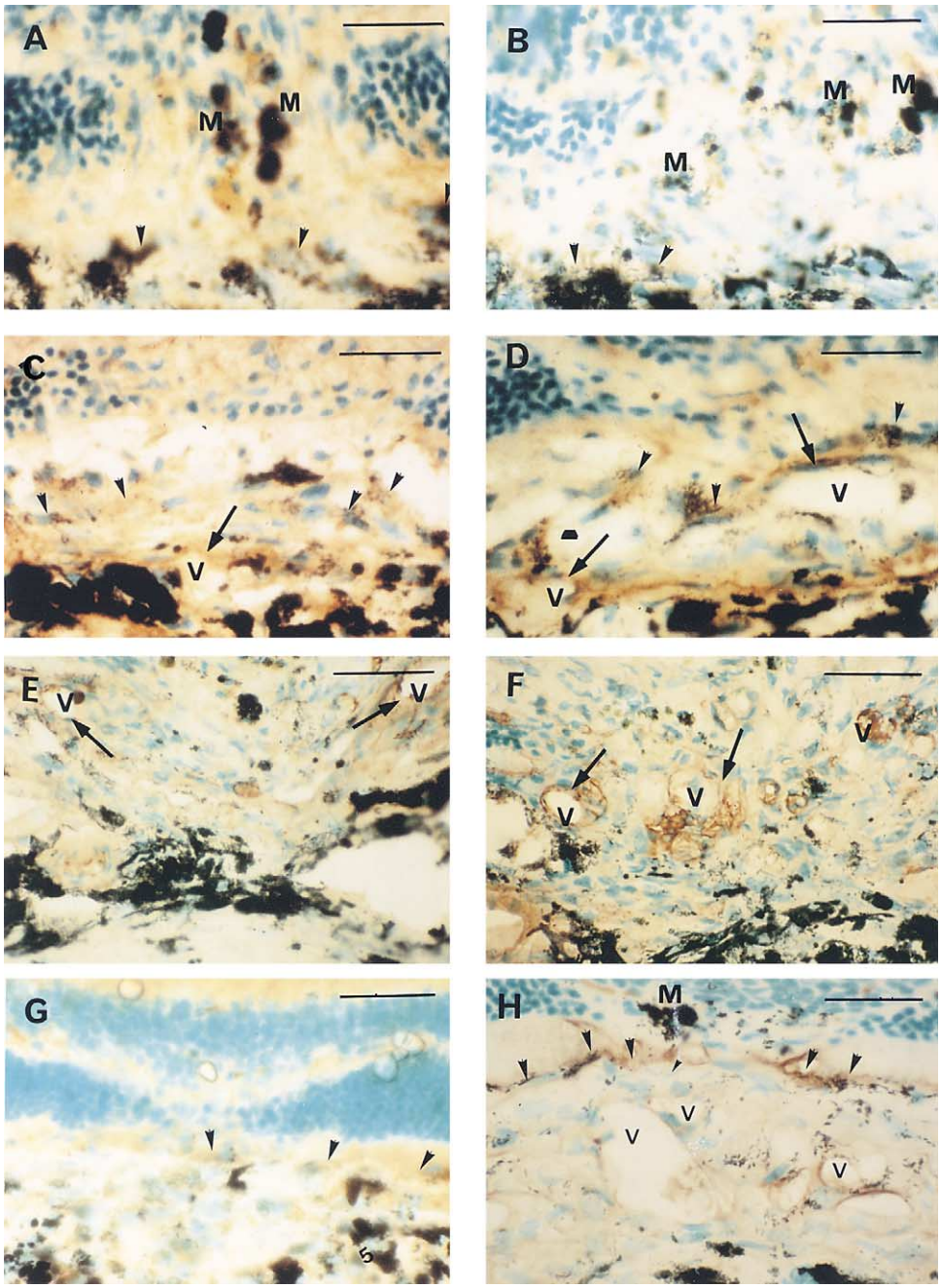

Figure 2. Immunoreactivity for transforming growth factor-β (TGF-β) receptors in the choroidal neovascularization. Immunoreactivity for TGF-β receptors appears brown in color. Nuclei of the cells are stained light blue-green by methyl green. M: macrophages, V: neovascularization. Bars = 50 μm. (**A**,**C**,**E**,**G**) Immunoreactivity for TGF-β receptor type I (TβRI). (**B**,**D**,**F**,**H**) Immunoreactivity for TGF-β receptor type II (TβRII). (**A**,**B**) Three days after laser photocoagulation. Immunoreactivity for TβRI and TβRII is seen in the macrophages (M) and in many cells within the laser lesions (arrowheads). (**C**,**D**) Seven days after laser photocoagulation. Immunoreactivity for TβRI and TβRII is seen in the retinal pigment epithelial (RPE) cells (arrowheads), fibroblasts and endothelial cells (arrows) in the laser lesions. (**E**,**F**) Fourteen days after laser photocoagulation. Immunoreactivity for TβRI and TβRII is specifically observed in endothelial cells (arrows) within the choroidal neovascularization. (**G**,**H**) Four weeks after laser photocoagulation. Immunoreactivity for TβRI and TβRII is decreased in photocoagulated lesions, but still detectable in the RPE cells (arrowheads) and endothelial cells (arrowheads) of the neovascularization.

mRNA expression of TGF-β receptors. Signals for the mRNA of TβRI and TβRII were observed in cells of the ganglion cell layer as purple to blue precipitates. TβRI or TβRII mRNA was barely detectable in the retinal pigment epithelial (RPE) cell layer because of masking by melanin pigments in the RPE cells (Figures 1D and 1E). Signals for the mRNA of TβRI and TβRII were not detectable in the retinal endothelial cells and choroidal endothelial cells. When sense probes for TβRI (Figure 1F) or TβRII were used (data not shown), no blue precipitate was observed in the retina.

Expression of TGF-β Receptors in Choroidal Neovascularization

Immunoreactivity for TGF-β receptors. Three days after photocoagulation, immunoreactivity for TβRI and TβRII was detected in many cells in the laser lesions. These cells appeared similar to macrophages, fibroblasts, and RPE cells by histopathological study and immunohistochemical study as reported previously[7,8,10,12,25] (Figures 2A and 2B). Seven days after photocoagulation, immunoreactivity for TβRI and TβRII was also found in many cells in the laser lesions. These cells expressing TGF-β receptors in the choroidal neovascular tissue were most likely fibroblasts, RPE cells, and endothelial cells (arrows) of the neovascularization (Figures 2C and 2D). After 2 weeks, immunoreactivity for TβRI and TβRII was specifically located in the endothelial cells of the choroidal neovascularization (Figures 2E and 2F). Thereafter, the immunoreactivity for TβRI and TβRII was decreased but still detectable in RPE cells and the endothelial cells of the choroidal neovascularization at 4 weeks (Figures 2G and 2H). Negative controls for TβRI or for TβRII showed no reaction products (data not shown). The data obtained from the immunohistochemical study are summarized in Table 1.

mRNA expression of TGF-β receptors. Three days after photocoagulation, expression of TβRI and TβRII mRNA was detected in the laser lesions. The cells expressing TβRI or TβRII mRNA were macrophages, RPE cells, and fibroblasts as previously reported[7,8,10,12,25] (Figures 3A and 3B). Seven days after photocoagulation, TβRI and TβRII mRNAs were also detected in the RPE cells and endothelial cells in the choroidal neovascularization (Figures 3C and 3D). However after 2 weeks, expression of TβRI mRNA and TβRII mRNA was detected only in endothelial cells of the choroidal neovascularization and RPE cells (Figures 3E and 3F). Four weeks after photocoagulation, expression of TβRI mRNA and TβRII mRNA was barely detectable in choroidal neovascular tissues (data not shown). Sections hybridized with the sense TβRI probes or sense TβRII probes showed no precipitate (data not shown). The data obtained from in situ hybridization are summarized in Table 1.

Discussion

Expression of TGF-β Receptors in Normal Rat Retina

The signal transduction pathway of TGF-β was indicated to be a cooperation between type I and type II receptors in the binding of the ligand and the initiation of signaling.[18–21] Earlier studies demonstrated that the TGF-β binding to receptor I requires the presence of receptor II,[22] and that both receptors are required for the signaling of any response.[19–21,23,24]

In this study, we first demonstrated the expression of both protein and the mRNA of TGF-β receptors in normal rat retinas. TβRI and TβRII immunoreactivity was detected in the ganglion cells and the RPE cells, and TβRI and TβRII mRNAs were also expressed in the ganglion cells. These findings complement our earlier observations of the expression of TGF-β1 and TGF-β2 mRNA in a normal rat retina.[25] Previously, the expression of TβRI and TβRII was investigated in developing rat eyes.[26] It was reported that immunoreactivity for TβRI and TβRII was detected in the corneal and conjunctival epithelial cells, ciliary epithelial cells, lens epithelial cells, RPE cells, and choroids, but was not detected in the neural retina. The reason for this discrepancy is unknown but may be due to a difference in the specificity of the antibody.

We previously demonstrated that TGF-β mRNA was present in ganglion cells and RPE cells and that ganglion cells were the major source of TGF-β.[25] Because both the mRNA and protein localization of TGF-β receptors agree with the localization of ligands, it is highly likely that the TGF-β, which is normally transcribed in ganglion cells or RPE cells, is transduced by TβRI and TβRII cooperatively through autocrine mechanisms in the normal retina. The immunohistochemical localization of TGF-β in photoreceptors has also been reported.[27,28] Because RPE cells are responsible for transporting nutrients from the choriocapillaris to the photoreceptors through the extracellular space, a paracrine pathway would also be a plausible mechanism for the signal induction of TGF-β in the normal retina. These findings support a suggestion that there may be an unrecog-

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Table 1. Expression of Transforming Growth Factor-β (TGF-β) Receptors During the Development of Choroidal Neovascularization*

Days Postphotocoagulation	Protein Expression of TGF-β Receptors								mRNA of TGF-β Receptors							
	TβRI				TβRII				TβRI				TβRII			
	M	RPE	FC	EC	M	RPE	FC	EC	M	RPE	FC	EC	M	RPE	FC	EC
3	+++	++	+		+++	++	+		++	++	+		+	+	+	
7	+	+++	++	+	+	+++	++	++	+	++	+	+	+	++	+	++
14	±	++	+	++	±	++	+	+++	–	+	±	±	–	+	±	+
28	–	+	–	±	–	+	±	+	–	+	–	–	–	+	–	–

*TβRI: TGF-β receptor type I, TβRII: TGF-β receptor type II, M: macrophages, RPE: retinal pigment epithelial cells, FC: fibroblast-like cells, EC: endothelial cells of choroidal neovascularization.

nized in vivo role for TGF-β in maintaining retinal homeostasis.

Expression of TGF-β Receptors in Choroidal Neovascularization

TGF-β can either stimulate or inhibit cellular proliferation depending on cell types and the culture conditions.[13] TGF-β can induce chemotactic migration,[29] affect many aspects of wound healing,[30] and inhibit endothelial cell proliferation that is induced by either acidic or basic fibroblast growth factors (FGF).[31,32] Furthermore, TGF-β can inhibit FGF-stimulated chemotaxis of endothelial cells in vitro.[33] Despite studies indicating that TGF-β inhibits angiogenesis in vitro, TGF-β is thought to be an angiogenic factor in vivo.[34] It has been assumed that the angiogenic response during wound healing is due to the chemoattractant effect of TGF-β on macrophages,[35] ie, TGF-β affects angiogenesis indirectly through the macrophages that secrete angiogenic factors such as tumor necrosis factor α, granulocyte- and macrophage-colony stimulating factor, and interleukin-8.[36–38]

In this study, we demonstrated the expression of TGF-β receptors in laser-induced choroidal neovascularization. Soon after the photocoagulation, we found that many macrophages were observed at the site of the lesion, as reported previously[7,10,12] and these macrophages expressed TGF-β receptors. Because macrophages produce various angiogenic cytokines, it has been supposed that macrophages play a key role in choroidal neovascularization.[39] Previously, we observed an upregulation of TGF-β mRNA during the development of choroidal neovascularization and suggested that TGF-β would play an important role in the process of neovascularization.[25] Our results in this study would confirm the hypothesis that TGF-β affects angiogenesis indirectly through the chemoattractant effect on the macrophages. Seven days after photocoagulation, expression of TβRI or TβRII was found in fibroblasts, RPE cells, and endothelial cells. Because we found earlier that these cells were expressing TGF-β mRNA,[25] we suggest that TGF-β is operating through TβRI and TβRII as an autocrine and/or paracrine mechanism.

Recently, we reported that the expression of VEGF mRNA and its receptor KDR mRNA was also upregulated during the development of choroidal neovascularization.[12] It has been suggested that VEGF is induced in response to TGF-β in fibroblastic and epithelial cells.[40] Thus, similar mechanisms may be operating in the development of choroidal neovascularization. TGF-β, by signaling through TβRI and TβRII, would induce an upregulation of VEGF to promote choroidal neovascularization in the early stage of this model.

Interestingly, 2 weeks after photocoagulation, expression of TβRI and TβRII was observed specifically in endothelial cells of the choroidal neovascularization and not in macrophages. We previously demonstrated that strong TGF-β mRNA signals were located in the endothelial cells of choroidal neovascularization 2 weeks after photocoagulation.[25] TGF-β is known to be a strong inhibitor of the proliferation of endothelial cells[33]; thus TGF-β secreted from endothelial cells would be transduced through receptors specifically expressed in the endothelial cells of the choroidal neovascularization by autocrine and/or paracrine manner and would inhibit neovascularization.

TGF-β is also known to stimulate the production of fibronectin and collagen by fibroblasts.[34] Therefore, it is thought that TGF-β produced by these cells promotes the development of fibrous tissues in the photocoagulated lesion, leading to collagen-rich scar formation and the regression of choroidal neovascularization at 4 weeks.

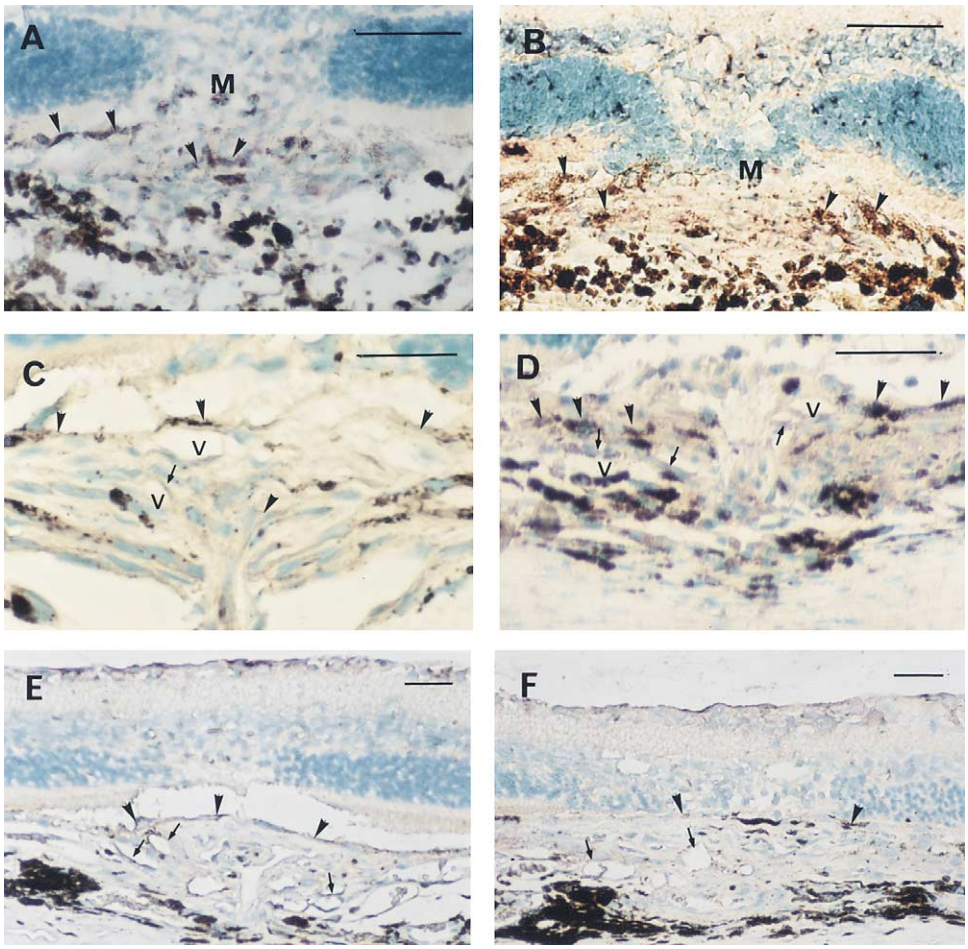

Figure 3. mRNA expression of transforming growth factor-β (TGF-β) receptors in the choroidal neovascularization. Signals are observed as purple precipitates. Sections were counterstained with methyl green. Nuclei of the cells are stained a light blue-green by methyl green. M: macrophages, V: neovascularization. Bars = 50 μm. (**A**,**C**,**E**) mRNA expression of TGF-β receptor type I (TβRI). (**B**,**D**,**F**) mRNA expression of TGF-β receptor type II (TβRII). (**A**,**B**) Three days after laser photocoagulation. Expression of TβRI and TβRII mRNA is observed in macrophages (M) and in many cells in the laser lesion (arrowheads). (**C**,**D**) Seven days after laser photocoagulation. TβRI and TβRII mRNA are present in the retinal pigment epithelial (RPE) cells (arrowheads), fibroblasts and endothelial cells (arrows) in the choroidal neovascularization. (**E**,**F**) Fourteen days after laser photocoagulation. Expression of TβRI and TβRII mRNA is weakly seen in the RPE cells (arrowheads) and endothelial cells (arrows) of the choroidal neovascularization.

We conclude that TGF-β would play an important role in the development of choroidal neovascularization through specific receptors, ie, soon after photocoagulation, TGF-β promotes neovascularization indirectly through the chemoattractant effect on macrophages. Later after photocoagulation, TGF-β inhibits neovascularization through receptors specifically expressed in endothelial cells by autocrine and/or paracrine mechanisms and promotes fibrosis.

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