Expression of Transforming Growth Factor-β_2 in Vitreous Body and Adjacent Tissues during Prenatal Development of Human Eye

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Expression of transforming growth factor-β_2 was detected by PCR in the vitreous body, lens, retina, and ciliary-iris complex of human eye at early stages of fetal development. Immunochemical assay of the corresponding protein in eye tissues revealed a correlation between the localization of transforming growth factor-β_2 and the development of intraocular hyaloid vascular network, its regression, formation of the vitreous body, and development of definite retinal vessels.

Key Words: *vitreous body, lens; retina; vasculogenesis and angiogenesis; transforming growth factor-β_2 (TGF-β_2)*

Transforming growth factor-β_2 (TGF-β_2) belongs to the family of multifunctional regulatory cytokine proteins. Three proteins of this family found in mammalian tissues, TGF-β_1, TGF-β_2, TGF-β_3, are presented by dimer polypeptides with a molecular weight about 25-28 kDa. These proteins are synthesized by different cell types and are abundantly present in tissues in both active and latent forms. Being multifunctional signal molecules, TGF-β proteins participate in the regulation of cell proliferation, growth, differentiation, and migration and in the formation and renewal of the extracellular matrix. They play an important role in embryonic development, immune cell responses, regulation of vessel growth, and wound healing [15,17,26].

In the eye of mammals [6,12,18] and humans [14, 16,18], all three proteins of this family are present in both the anterior and posterior eye tissues.

In adult human retina, TGF-β_1 and TGF-β_2 were detected in the inner and outer segments of photoreceptors, ganglionic cells, and cells with dendrite morphology, in the nerve fiber layer, inner plexiform layer and microglia, in retinal smooth-muscle cells, pericytes, and vascular endothelium; TGF-β_2 was found in outer segments of photoreceptors, microglia, and vascular smooth-muscle cells; TGF-β_3 was detected in microglia. In pigmented retinal epithelium, TGF-β_1 is located in the basal part of some cells. The choroid and vitreous hyalocytes contain all three TGF-β proteins [14]. In adult human eye, immunospecific reaction to TGF-β_2 and TGF-β_3 [18] and to TGF-β_1 [16] was shown in pigmented and nonpigmented epithelium of the ciliary body.

TGF-β_2 and TGF-β_3 proteins were detected in cells of pigmented and nonpigmented ciliary epithelium in rabbits [18]. Expression of the gene encoding TGF-β_2 was found in the ciliary epithelium and iris as well as in the equatorial area of the lens in rats [10]. In rat cornea, low levels of matrix RNA (mRNA) for TGF-β_2 and TGF-β_3 were detected [6].

Proteins of the TGF-β family are important signal molecules essential for normal eye morphogenesis

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[3,13,23]. The role of TGF-β signals in eye development is studied on transgenic mice [22,24]. For instance, TGF-β knockout mice have some eye abnormalities [22] similar to those observed in humans with TGF-β gene mutations [2,7]. These abnormalities are a result of disturbed epithelial-mesenchymal relationships [8].

In human eye, all three TGF-β proteins were detected during the prenatal period, TGF-β_2 being a predominant form. It was shown that TGF-β_2 plays the key role in the development of retinal vessels and formation of vessel-free macula in human fetuses on gestation weeks 11-31 [1].

In situ hybridization analysis revealed TGF-β_2 mRNA in all cells of the lens vesicle in 6-week gestation human fetuses and in the anterior epithelium of the lens in 8-week gestation fetuses. At these terms, TGF-β_2 mRNA was also present in the anterior retina and along the edge of the eye cup [9].

Normal growth of retinal vessels and their functional state depends on balanced interaction between angiogenic and antiangiogenic factors [11,24]. Imbalance between these factors leads to various diseases such as prematurity retinopathy, diabetic retinopathy, and macular degeneration of the retina. All these pathologies are associated with the formation of new vessels (neovascularization) capable of invading the vitreous body and inducing its detachment [4]. The central part in the regulation of normal growth of retinal vessels and in angiogenesis control is played by TGF-β_2 protein. This determined the aim of this study: to evaluate the expression of TGF-β_2 gene and localization of the corresponding protein in human eye tissues on gestation weeks 8-31 of prenatal development. Particular attention in this study was given to the inner retinal layers, inner epithelium of the ciliary body, lens, and vitreous body. These tissues are closely related to the growth and regression of the hyaloid vascular system and the formation of definitive blood supply to the retina.

MATERIALS AND METHODS

We examined eye tissues from the abortion material (fetuses on gestation weeks 8-12, 13-14, and 16-20) and material obtained during autopsy of premature nonviable fetuses. The biomaterial was obtained from licensed clinics of the Ministry of Health of the Russian Federation working within the limits of Federal law on health protection and in accordance with the approved list of medical indications. The age of fetuses corresponded to the terms determined by the obstetrician.

The expression of *TGF-β_2* in the retina, lens, ciliary-iris complex, and vitreous body at gestation weeks 9.5-31 was evaluated by PCR. Total RNA was isolated using TRI® Reagent. mRNA fraction was obtained with magnetic particles (Sileks) according to manufacturer's instructions. The first cDNA chain was synthesized on mRNA template using reverse transcriptase SuperScript (GIBCO BRL) and a hexanucleotide random primer or oligo$(dT)_{12\text{-}18}$. PCR on a cDNA template was carried out on an Eppendorf amplifier using Taq polymerase (Sileks). Amplification conditions were as follows: 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C (30-40 cycles). For cDNA standardization, RLP19, a ribosomal protein with a molecular weight of 19 kDa, was used; the content of this protein is similar in different cell types. PCR with forward primer 5'-agggtacagccaatgcccga-3' (exon 4) and reverse primer 5'-ccttggataaatcttgatgatc-3' (exon 6) yielded a PCR product with a length of 326 nucleotides. PCR with TGF-β_2-specific primers forward 5'-gcaggataattgctgcctacg-3' (exon 7) and reverse 5'-ctgcatttgcaagactttacaatc-3' (exon 8) yielded a 302-b.p. PCR product.

Localization of TGF-β_2 protein in eye tissues was studied by a standard immunochemical method on cryostat sections of human fetal eye (gestation weeks 8-31). Mouse monoclonal antibodies to TGF-β_2 (Abcam; 1:20) and secondary antibodies ALEXA 488 or ALEXA 586 (MolecularProbs; 1:1000) were used. Nuclei were stained with Hoechst 42333 (Leica; 1:1000). The results of immunochemical reaction were analyzed under a Leica DM RXA2 fluorescent microscope equipped with a filter set and an Olimpus DP70 camera. Immunochemical localization of the protein was analyzed in the inner retinal layers, inner epithelium of the ciliary body, and in the lens. Other tissues were not analyzed in this study. Reaction performed in the absence of primary antibodies served as the negative control.

RESULTS

PCR analysis revealed expression of *TGF-β_2* gene in the vitreous body of human fetal eye (Fig. 1, *a*). It is known that human vitreous body during the embryonic and early fetal periods is characterized by the presence of cells of mesenchymal origin, *e.g.* cells forming hyaloid vessels, hyalocytes, and macrophages. After regression of hyaloid vessels, hyalocytes become resident cells in the vitreous body. All cells of the vitreous body can express TGF-β_2. These data agree with the results of a previous study where localization of TGF-β_2 protein in vitreous body hyalocytes of adult human eye was immunochemically demonstrated [14].

PCR-analysis of the retina, lens, and ciliary-iris complex showed expression of *TGF-β_2* gene in all studied eye tissues at the specified developmental stages (Fig. 1, *b*).

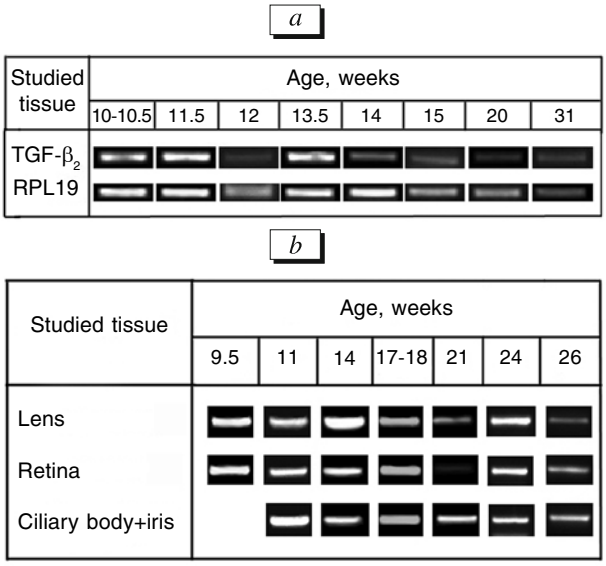

Fig. 1. Expression of *TGF*-β_2 gene in human fetal eye tissues at different stages of development. *a*) vitreous body; *b*) lens, retina, and ciliary-iris complex.

The location of TGF-β_2 protein in the studied tissues was evaluated using immunospecific antibodies.

In the retina of 8-week fetuses, a clear-cut immunochemical signal was seen along the full thickness from the central layer to the periphery. The intensity of immunochemical staining was maximum in the narrow line in the inner limiting membrane at the boundary with the vitreous body. This area of the retina corresponds to inner terminals (footplates) of Muller glial cells (Fig. 2, *a*, *b*) The edge of the eye cap corresponds to presumptive ciliary-iris primordium and is also characterized by intensive immunochemical staining (Fig. 3, *a*, *b*). Our findings on TGF-β_2 localization in 8-week embryos agree with published data on mRNA localization determined in *in situ* experiments [9].

Immunopositive staining in 10-12-week human embryos is seen across the entire thickness of the retina (from the inner to the outer membranes) with predominant localization in the inner layers: in the forming ganglion cells and nerve fiber layers and (as in 8-week embryos) along the inner limiting membrane of the retina (Fig. 2, *c, d*). At latter stages (on gestation weeks 15, 16, 17, 24, and 31), the immunopositive staining was localized in the ganglion cell and nerve fiber layers (Fig. 2, *e-h*).

In the lens of 10-week fetuses, uniform staining of the lenticular epithelium was observed (Fig. 3, *g*). In 12-week fetuses, the immunochemical staining was also present in the lenticular epithelium and involved the equatorial area epithelium. At later stages (gestation weeks 17, 24, and 31), the immunopositive staining was localized primarily in the equatorial area epithelium (proliferative and transitory zones) and in forming lens fibers. However, no immunopositive staining was detected in the anterior lenticular epithelium (Fig. 3, *h*).

In 12-week human fetuses, the first folds of the ciliary epithelium appeared. The immunopositive signal was seen in the inner nonpigmented epithelium of these folds (Fig. 3, *c*, *d*). At all later terms (gestation weeks 17, 24, and 31) the folds become deeper. Immunospecific staining attesting to the presence of

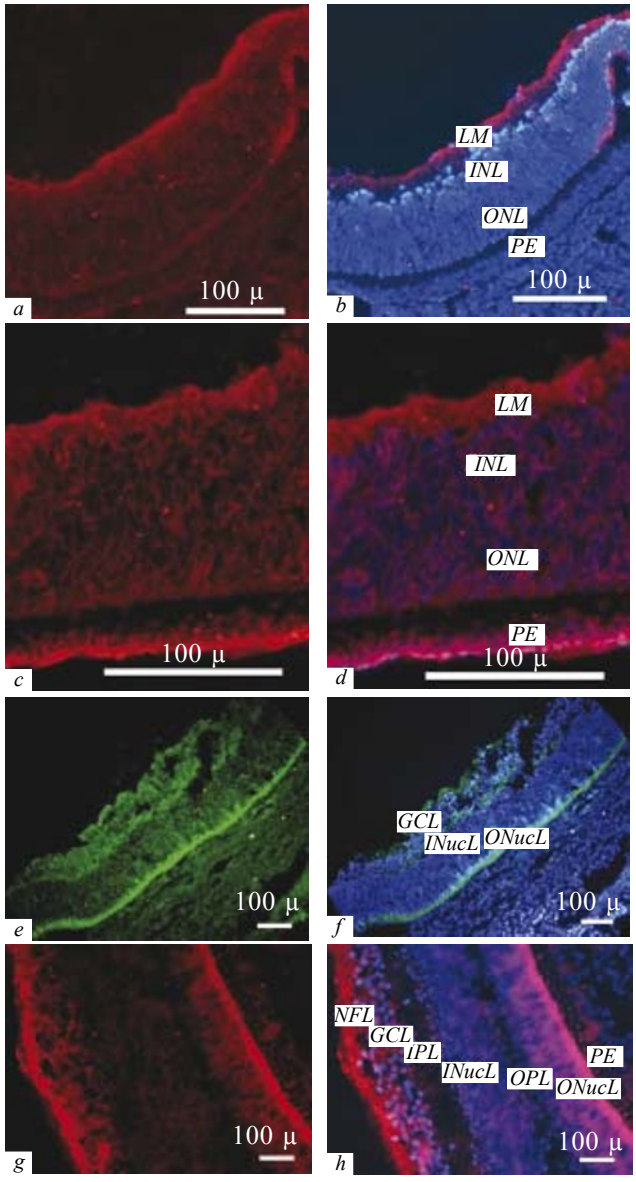

Fig. 2. Immunochemical localization of TGF-β_2 protein in human retina at different stages of prenatal development. *a*, *b)* 8 weeks; *c*, *d*) 10 weeks; *e*, *f*) 15/16 weeks; *g*, *h*) 31 weeks. *LM*: inner limiting membrane, *INL*: inner neuroblast layer, *ONL*: outer neuroblast layer, *PE*: pigmented epithelium, *GCL*: ganglion cell layer, *INucL*: inner nuclear layer, *ONucL*: outer nuclear layer, *NFL*: nerve fiber layer, *IPL*: inner plexiform layer, *OPL*: outer plexiform layer. *a*, *c*, *g*: Antimouse Aleksa 586; *b*, *d*, *h*: Antimouse Aleksa 586+Hoechst 42333; *e*: Antimouse Aleksa 488; *f*: Antimouse Aleksa 488+Hoechst 42333.

TGF-β_2 was also observed in nonpigmented epithelium of ciliary folds (Fig. 3, *e*, *f*).

Thus, our results of PCR analysis on the expression of *TGF-β_2* gene agree with immunochemical data on localization of TGF-β_2 protein in the studied tissues and are mutually complementary.

The epithelial-mesenchymal interactions play an important role in embryonic development of the eye. Differentiation of cells derived from the nerve crest and migrating between the lens and corneal epithelium and the development of primary vitreous body are regulated via the TGF-β-signal pathway and the lens plays a central part in this process [8]. Our studies demonstrated the expression of TGF-β_2 in human fetal lens throughout the studied period.

An important functional characteristic of TGF-β_2 is its participation in the regulation of vasculogenesis and control of angiogenesis, as well as in the formation and renewal of extracellular matrix [5,24]. A characteristic phenomenon of the early development of human eye is the formation of transitory hyaloid vascular network consisting the major hyaloid artery, its branches in the cavity of the primary vitreous body, and vessels of the posterior and anterior tunica vasculosa lentis communicating via lateral anastomoses. These vessels are essential for the growth, nutrition, metabolism, and maturation of internal structures of the developing eye (lens, retina, and vitreous body) and then underwent regression. Regression of the hyaloid vessels starts from gestation week 10 and is completed in the third trimester [25]. Disturbances in mechanisms controlling the development and regression of hyaloid vessels leads to congenital eye abnormalities, *e.g.* persisting hyaloid artery, persisting

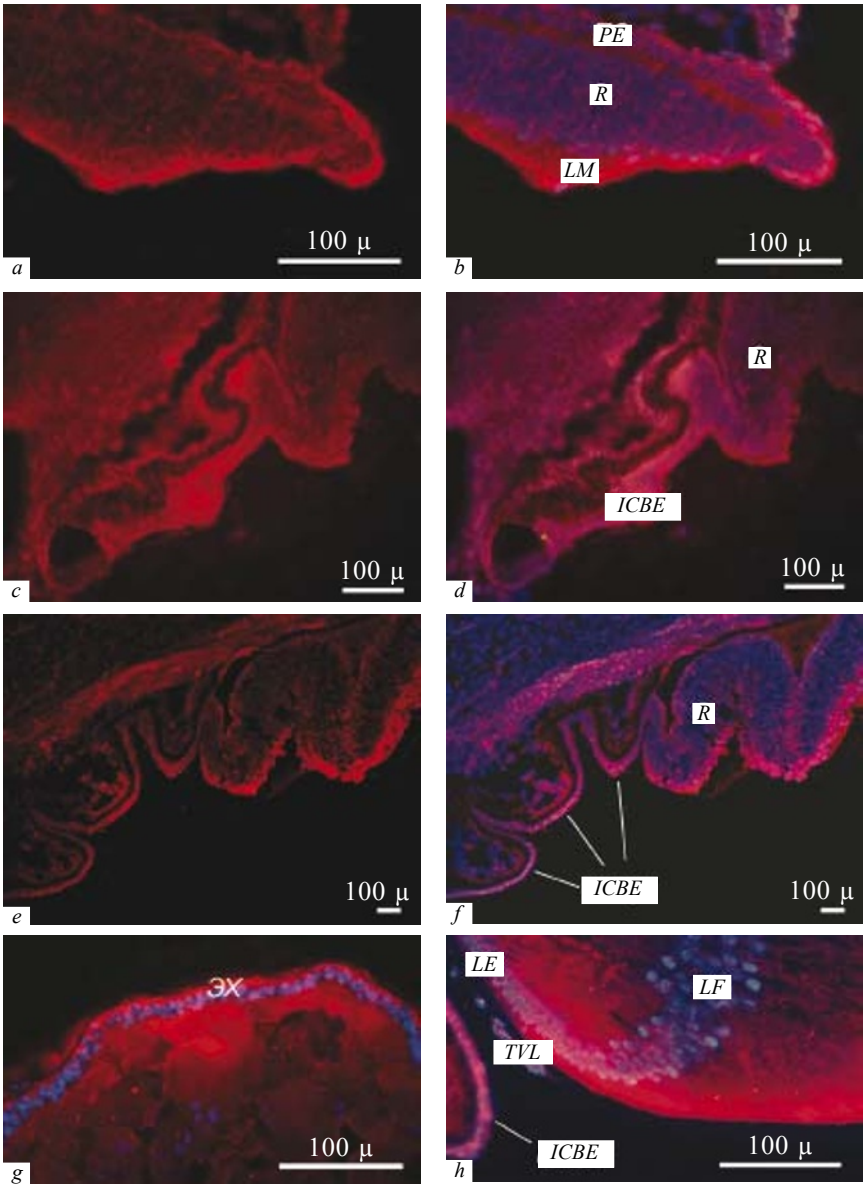

Fig. 3. Immunochemical localization of TGF-β_2 protein in inner ciliary epithelium and lens of human eye at different stages of prenatal development. *a*, *b*: eye cup edge of a 8-week fetus; *c*, *d*: ciliary-iris edge of a 12-week fetus; *e*, *f*: ciliary folds of a 17-week fetus; *g*: lens of a 10-week fetus; *h*: lens of a 17-week fetus. *LM*: inner limiting membrane, *R*: retina, *ICBE*: inner ciliary body epithelium, *LE*: lens epithelium, *LF*: lens fibers, *TVL*: tunica vasculosa lentis. *a*, *c*, *e*: Antimouse Aleksa 586; *b*, *d*, *f-h*: Antimouse Aleksa 586+Hoechst 42333.

hyperplastic primary vitreous, congenital cataract, and prematurity retinopathy [20]. The formation of retinal vessels starts after gestation week 14 (before this term the retina is completely avascular) and actively progresses by week 24 of prenatal development [21].

All processes related to the growth and regression of blood vessels are regulated by angiogenic (vascular endothelial growth factor VEGF and fibroblast growth factor FGF) and antiangiogenic (TGF-β_2, platelet-derived growth factor PDGF, and thrombospondin-1 TSP-1) factors, TGF-β_2 being the major regulator of these processes [11]. TGF-β_2-null mice are characterized by the presence of developmental abnormalities of the eye, including disturbances in vitreous body development and growth and regression of blood vessels [19].

Our experiments demonstrated the expression of TGF-β_2 in the inner retinal layers, nonpignemted epithelium of the ciliary body, lens, and vitreous body cells of human eye at early stages of fetal development. These tissues are related to the growth and regression of vessels and formation and renewal of extracellular matrix in the vitreous body. The results attest to the involvement of TGF-β_2 in the regulation of these processes during the studied period of eye development.

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