CLINICAL STUDY

Expression of betaglycan in pregnant tissues throughout gestation

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Abstract

Background: Betaglycan is a membrane-anchored proteoglycan involved in mediating the passage of transforming growth factor- β (TGF- β), inhibin and activin activities into cells. TGF- β and inhibin-related proteins are growth factors that are expressed by several tissues and in pregnancy. They have a function in modulating the growth, differentiation and invasion of the placental trophoblast.

Objective: To evaluate whether betaglycan is expressed by intrauterine tissues throughout gestation.

Design and methods: Expression of betaglycan mRNA and protein was evaluated (by RT-PCR and immunohistochemistry, respectively) in trophoblast, decidua and fetal membranes collected during the first ($n = 6$ elective terminations of pregnancy, between 8 and 12 gestational weeks) and third ($n = 6$ elective caesarean sections, between 39 and 40 weeks) trimesters of pregnancy.

Results: Betaglycan mRNA was expressed by all gestational tissues, independently of gestational age. Immunoreactive protein was found in decidual cells and in some chorionic, but not epithelial, amniotic cells. With respect to the placental localization, syncytiotrophoblast, but not cytotrophoblast, cells were intensively stained both in the placental bed and in the villous trophoblast, and in some cells within the stroma of terminal villi, of the first and third trimesters of pregnancy. Immunoreactive betaglycan was demonstrated in the endothelial cells of decidual vessels in both the first and third trimesters of pregnancy, whereas endothelial cells of fetal blood vessels in the villous were clearly represented only in first trimester samples, not in those of term placenta.

Conclusions: Betaglycan mRNA and peptide are expressed by the trophoblast, the decidua and the fetal membranes, but the localization of the peptide in vessel walls is dependent on gestational age.

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Introduction

Betaglycan is a membrane-anchored proteoglycan that is involved in mediating the passage of transforming growth factor- β (TGF- β), inhibin A and activin A activities into cells (1) and therefore has an essential role in their signalling. In fact, betaglycan works as TGF- β receptor type III (TGF- β RIII), as it binds TGF- β ; depending on the cell type, it prevents or stimulates the association of TGF- β R type II (TGF- β RII) with type I (TGF- β RI) (2). Betaglycan is also involved in the inhibin A pathway, as it facilitates the binding of inhibin A to the activin receptor type II (ActRII), allowing the formation of a ternary complex including inhibin A, betaglycan and ActRII that is involved in the transduction of the inhibin A pathway into the cells. Finally, betaglycan also interacts with activin A,

preventing the binding of activin A to ActRII and thereby antagonizing the activin signal (1, 3).

TGF- β , inhibin A and activin A are growth factors that are expressed by the placenta (4), where they have essential functions in modulating the growth, differentiation and invasion of the placental trophoblast, and placental hormonogenesis (4–6). In the light of all this evidence, the present study was undertaken to evaluate the expression of betaglycan in first and third trimester placenta, decidua and fetal membranes.

Materials and methods

Specimens of human placenta, maternal decidua and fetal membranes were collected from pregnant women who underwent elective termination of pregnancy

($n = 6$; gestational age between 8 and 12 weeks) and elective caesarean section ($n = 6$; between 38 and 40 gestational weeks). Gestational age was calculated on the basis of the last menstrual period and confirmed by ultrasound evaluation. Informed written consent was obtained from all patients before their inclusion in the study, for which approval was obtained from the local Human Investigation Committee.

The specimens collected were subdivided into aliquots for RT-PCR (samples were immediately frozen in liquid nitrogen for extraction of total RNA) and immunohistochemistry (samples were fixed by immersion in 10% buffered formalin).

RT-PCR

Total RNA was extracted according to the method of Chomczynski and Sacchi (7). Samples were homogenized in Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) and RNA was extracted according to the manufacturer's instructions. RNA was quantified by UV absorption and 1 μ g was reverse-transcribed to prepare complementary DNA (cDNA). Reaction conditions for reverse transcription were: 10 mM Tris-Cl, 50 mM KCl, 5 mM $MgCl_2$ (pH 8.3), 50 U Moloney murine leukaemia virus reverse transcriptase, 20 U RNase inhibitor, 1 mM of each dNTP, 2.5 μ M oligo d(T) primers (Invitrogen) in a volume of 20 μ l. The reaction was run at 42 °C for 15 min, 99 °C for 5 min, and 5 °C for 5 min, and 2 μ l of the product was used for the PCR.

PCR conditions were: 300 mM Tris-Cl, 75 mM $(NH_4)_2SO_4$, 7.5 mM $MgCl_2$ (pH 8.5), 0.25 mM dNTPs, 1 U *Thermus aquaticus* DNA polymerase (Life Technologies), and 0.4 μ M (final concentration) betaglycan primers to a total volume of 50 μ l.

Amplifications were carried out for 1 min at 94 °C, 1 min at 53 °C and 1 min at 72 °C for 35 cycles, followed by a final step of 10 min at 72 °C. Amplification products were visualized on 2% agarose gel and stained with ethidium bromide.

Betaglycan-specific primers used for amplification by PCR were: 5'-CTGTTACCCGACCTGAAAT-3' (sense) and 5'-CGTCAGGAGGCACACTTA-3' (antisense). Computer analysis performed to compare the synthesized oligomers with the human sequences in the gene database of the National Center for Biotechnology Information, using BLAST (8), revealed no significant homology among all other genes. Sequence homology among the different oligomers used in the present study was also avoided, thus excluding possible cross-reactions. The expected size of the amplified fragment was 502 bp; the size of this fragment is specific for RNA, because the primers were localized on different exons. The blank for each reaction consisted of amplifications performed in the absence of reverse transcriptase enzyme.

PCR product identity was confirmed by restriction analysis. The amplified fragment was digested with

Van 91 I (Roche Molecular Biochemicals, Monza, Italy), under the conditions suggested by the manufacturer. The products were separated on 2% agarose gel and stained with ethidium bromide.

Immunohistochemistry

Immunohistochemistry was carried out on sections (5 μ m thick) cut from paraffin-embedded samples, mounted on electrostatically charged slides, and dried overnight at 37 °C. Sections were dewaxed, rehydrated and washed in Tris-buffered saline [20 mM Tris-HCl, 150 mM NaCl (pH 7.6)]. Tissue sections were heated in a microwave oven twice for 5 min at 750 W (EDTA, pH 8) and rinsed in 3% hydrogen peroxide to block endogenous peroxidase. Slides were incubated overnight at room temperature with the primary antibody, goat anti-human TGF- β RIII (R&D Systems, Abingdon, Oxon, UK) diluted 1:1000, and then incubated with secondary antibody (Dako, Glostrup, Denmark) for 30 min at room temperature. Harris haematoxylin was used for nuclear counterstaining. A positive reaction was characterized by the presence of granular brown staining in the cytoplasm.

Negative controls consisted of sections in which the primary antibody was replaced by non-immune serum and by using the antibody preadsorbed with the blocking peptide.

Results

Expression of betaglycan mRNA

Total RNA extracted from trophoblast, decidua and fetal membranes in the first trimester and at term pregnancy was analysed by RT-PCR. As shown in Fig. 1A, a band corresponding in size to product (502 bp) was obtained from the cDNA of all the samples examined. No amplified fragment caused by DNA contamination was detected in any blank experiment (Fig. 1A). As the 502 bp PCR fragment contained a Van 91 I site, PCR products were submitted to the restriction analysis with Van 91 I, yielding products of the expected size (336 and 166 bp) (Fig. 1B).

Distribution of betaglycan peptide

Strong and consistent immunostaining of betaglycan was found in decidual cells obtained from either the first or the third trimester of pregnancy. In decidual vessel walls, the positive stain was restricted to endothelial cells, whereas the other vascular layers (intima and media) were unstained (Fig. 2A,B).

In the placental bed, syncytiotrophoblast, but not cytotrophoblast, cells were intensively stained (Fig. 2C). This was also observed in the terminal villi: syncytiotrophoblast cells were strongly and consistently

Figure 1 (A) Representative results of RT-PCR analysis of betaglycan mRNA during the first trimester (lane 1: trophoblast; lane 3: decidua; lane 4: fetal membranes) and third trimester (lane 5: trophoblast; lane 6: decidua; lane 7: fetal membranes) of pregnancy. M, molecular mass marker (marker VI, Roche Molecular Biochemicals). Lane 2: blank, consisting of amplifications performed in absence of reverse transcriptase enzyme. (B) Verification of the identity of the product from placental betaglycan PCR (restriction analysis). Fragment undigested and digested using *Van 91 I* of decidua, trophoblast and fetal membranes. The sizes of the fragments are indicated. The molecular mass marker (M) is marker VI (Roche Molecular Biochemicals). Troph., trophoblast; Dec., decidua; Memb., fetal membranes.

stained and some stromal cells were positive, but cytotrophoblast cells were completely devoid of stain (Fig. 2D–F). In the villi, endothelial cells were clearly immunostained by betaglycan only in samples collected during the first trimester of pregnancy (Fig. 2D), and not in term placenta (Fig. 2E,F).

Fetal membranes at term showed positivity only in the chorionic connective tissue cells; epithelial amniotic cells were unstained (Fig. 2G).

Discussion

Betaglycan is a membrane-anchored proteoglycan that has a role in TGF- β signaling, functioning as its type III receptor. In the present study, we found that the immunolocalization of betaglycan in gestational tissues (Table 1) overlapped with that of TGF- β , TGF- β RI and TGF- β RII, which have been demonstrated in the decidua, the syncytiotrophoblast and the chorionic plate, but not in the cytotrophoblast (4, 9).

However, betaglycan is also involved in the inhibin A and activin A pathways, as it mediates the passage of inhibin A activity into cells and prevents activin A binding to ActRI, thus antagonizing activin A. Activin A and inhibin A are localized in maternal decidua, amniotic and chorionic cells and in the syncytial layer of placental villi (4, 6, 10–12), the cytotrophoblast remaining unstained. In the light of these findings, we can conclude that the localization of betaglycan overlaps with that of the TGF- β and related receptors, with activin/inhibin β A and α subunits, and with activin receptors (13, 14).

With respect to placental functions, activin A and inhibin A are differently involved in the control of placental hormonogenesis and differentiation. The addition of activin A to primary cultures of human placental cells increases the production of gonadotropin-releasing hormone (GnRH), progesterone, human chorionic gonadotrophin (hCG) (4, 6, 15) and oxytocin (4, 6, 16). In addition, activin A increases the release of prostaglandin E_2 in human amnion-derived cell cultures (12), and has autocrine/paracrine roles in the differentiation of cytotrophoblasts within chorionic villi, by stimulating the outgrowth of cytotrophoblast cells (5). In contrast, inhibin A is not able to affect directly either the secretion of GnRH, progesterone, hCG and prostaglandin E_2 (4, 6, 15), or placental growth and differentiation (5). Thus the lack of inhibin A activity may be attributable to the fact that betaglycan is not expressed by cytotrophoblast and amniotic cells whereas, in contrast, activin exerts an effect because its receptors are localized on the cytotrophoblast and syncytiotrophoblast, and amniotic cells (14).

TGF- β also influences placental functions, inhibiting the outgrowth, differentiation (5) and hormonogenesis (4, 17) of the extravillous trophoblast. Thus, as betaglycan is expressed by syncytiotrophoblasts but not by cytotrophoblast cells (9), we can hypothesize that the effects of TGF- β on the extravillous trophoblast are mediated on the syncytiotrophoblast or, alternatively, through a receptor that has yet to be discovered.

The present study demonstrated immunolocalized betaglycan in endothelial cells of human decidua in the first and third trimesters, but in endothelial cells of trophoblast villi only in the first trimester. Activin/inhibin receptors (such as ActRI and ActRII) have been immunolocalized in placental endothelial cells mainly at term (14), whereas betaglycan staining has been demonstrated only in tissues of early pregnancy. Because activin A is involved in endothelial proliferation and angiogenesis (18, 19), the localization of betaglycan and ActRs in vascular endothelial cells of decidua (13) and villous (14) blood vessels suggests a role for betaglycan in modulating the effect of activin A on vascular adaptations to pregnancy in concert with TGF- β . Furthermore, although inhibin A is

Figure 2 Immunohistochemical staining of betaglycan in the first and third trimesters of pregnancy. (A) First trimester: decidual cells (d) and the thin endothelial layer (e) cells of decidual uteroplacental veins are intensely stained. (B) Term pregnancy: decidual cells (d) and the thin endothelial layer cells (e) of a decidual vein are intensely stained. (C) First trimester of gestation: syncytiotrophoblast (s) but not cytotrophoblast (c) cells are intensely stained. (D) First trimester of pregnancy: syncytiotrophoblast (s) cells around the villus are intensely stained. In addition, inside the villous, the cytoplasm of stromal and endothelial (e) cells of two large vessels [one of which one was full of nucleated red blood cells (r)] are intensely stained. (E) Stem villous at term: stromal (sc) and syncytiotrophoblast (s) cells, but not the wall (intima and media) of either arteries (a) or veins (v), are immunostained. (F) Third trimester: intense betaglycan immunostaining was present in syncytiotrophoblast (s) cells of the terminal villi, but vessel walls (e) and cytotrophoblast cells (indicated by the arrow) are unstained. (G) Fetal membranes: chorionic connective tissue (c) but not amniotic epithelial (a) cells are weakly stained. (H) Negative control (villi at term).

without effect on vascular endothelial cell growth (19), TGF- β , in contrast, is an important modulator of endothelial functions (20). Therefore, betaglycan may serve as mediator of the actions of TGF- β and activin A on placental angiogenesis, inhibiting excessive endothelial cell proliferation in human placenta in early pregnancy or modulating the placental vascular permeability

that is necessary for implantation of the embryo and placentation.

In conclusion, the present study showed that the expression of betaglycan mRNA and protein in placental vessel walls appears to be dependent on gestational age, thus suggesting different roles of TGF- β and activin A during the period of gestation.

Table 1 Summary of the distribution of betaglycan immunostaining in gestational tissues collected from first and third trimesters of pregnancy.

Tissue	Cell type	Staining
Decidua	Stromal cells	+
	Vessels	
	Endothelium	+
	Intima	–
	Media	–
Placental bed	Syncytiotrophoblast	+
Trophoblast villi	Cytotrophoblast	–
	Syncytiotrophoblast	+
	Cytotrophoblast	+
	Stroma	–
	Endothelium	+
	Intima	+ (only at first trimester of pregnancy)
	Media	–
	Vessels	–
Fetal membranes	Epithelial amniotic cells	–
	Chorionic cells	+

+, presence of staining; –, absence of staining.

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