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Distribution of Different Fibronectin Isoforms in the Extracellular Matrices of Human Term Placenta*

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Abstract: Fibronectins are considered to be molecules that play important roles in cell migration and cell differentiation. Fibronectin molecules are thought to influence the differentiation of trophoblasts and the adhesion of these cells to maternal tissues.

The aim of the present study was to analyze the distribution of fibronectin isoforms and to evaluate the differences between matrix-type fibrinoids, and other types of matrices produced by both maternal and fetal tissues.

Tissue samples were collected from different sites of human term placentas. By using immunohistochemistry, the expression of fibronectin isoforms was determined and they were demonstrated by SDS-PAGE and Western blotting methods.

In nearly all tissues reactivities to fibronectin isoforms were determined. The strongest reactivities for cellular and oncofetal fibronectins were found in areas like the basal plate, chorionic plate and chorion laeve, which are occupied by invasive trophoblast cells in high numbers. These results can lead to the conclusion that extravillous trophoblast (EVT) cells emerge from the same differentiation route in different sites of the placenta. Amnion having no EVT cells consisted of all fibronectin isoforms. The presence of oncofetal isoforms especially indicates the high healing activity of this tissue due to the rapidly growing fetus and its membranes.

Key Words: fibronectin, human placenta, extracellular matrix, pregnancy

Introduction

Fibronectins are adhesive mosaic glycoproteins comprised of three general types of homologous iterative modules. These modular units are used as basic building blocks to form domains that develop distinct functions such as the establishment and maintenance of normal cell morphology, cell migration, homeostasis and thrombosis, wound healing, and oncogenic transformation (1).

Although fibronectin is encoded by only one gene, this protein exists in a number of variant isoforms due to alternative splicing and/or post-translational modifications. These comprise modifications such as glycosylations and splicing at three general regions of the precursor mRNA: IIICS, ED-A and ED-B (2). Plasma fibronectin isoforms lack the alternatively spliced domains, whereas cellular fibronectin isoforms contain the ED-A domain in their protein (3).

In transformed cells and malignancies the alternative splicing of the primary transcript of fibronectin is deregulated (4). The fibronectin isoforms containing the domains III-A (ED-A), III-B (ED-B) and III-V (IIICS) are expressed to a higher degree in transformed human cells and in tumor tissues than in their normal counterparts (5,6). Fibronectin containing the III-B sequence are detectable almost exclusively in healing wounds, in fetal tissues and in tumor tissues (7,8).

In the human placenta, the fibronectin molecule is detectable in various sites such as the amnion, basal plate, chorionic plate, decidua, placental fibrinoid, umbilical cord and villi. Although the presence of all fibronectin isoforms is not studied in the extracellular matrices (ECMs) of all these sites, in particular, the special matrix of invasive extravillous trophoblast (EVT) cells, a matrix-type fibrinoid, is evaluated in detail. Huppertz et al. (9)

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showed that the patches of fine fibrillar networks of matrix-type fibrinoids were immunocytochemically reactive with the general (IST-4, IST-6), cellular (IST-9) and oncofetal isoforms (BC-1, FDC-6) of fibronectin. These molecules, especially oncofetal isoforms, are used in the differentiation of trophoblast cells from a proliferative subtype to an invasive subtype.

The aim of this study was to present the differences between matrix-type fibrinoids and the other matrices of fetal or maternal origin. For this purpose, different ECMs were extracted from various regions of human term placentas. Following extractions, the distribution of fibronectin isoforms within these ECMs was evaluated and compared using immunohistochemical and biochemical techniques.

Materials and Methods

Immunohistochemistry

Tissue samples from 7 term human placentas were used. Placental tissue in the form of cubes with a maximal edge length of 20 x 20 x 5 mm^3 were fixed in phosphate-buffered neutral 4% formaldehyde solution for a maximum of 24 h at 4 °C. The specimens were dehydrated in a graded series of ethanol and embedded in paraffin (melting point 52 °C; Merck, Darmstadt, Germany) using xylene as an intermedium, while not exceeding a temperature of 58 °C. Serial sections (3-5 µm) were cut and mounted on glass slides. The sections were then deparaffinized using xylene and a graded series of ethanol (10 min each step).

Immunohistochemical staining was performed according to a standardized sequence as described before (10). In brief, endogenous peroxidases and nonspecific binding were blocked with 3% hydrogen peroxide in methanol and with swine serum (1:20) in 0.05 M Tris-HCl, pH 7.6, 15 mM sodium azide, 6% BSA, respectively. After incubating with primary antibodies (see for details, 11) in 0.05 M Tris-HCl, pH 7.6, 15 mM sodium azide, 12.5% BSA, a biotinylated link antibody (Dako, E453, swine antibody, 1:25) was applied for 30 min. Subsequently, the binding of streptavidin-horseraddish-peroxidase (Dako, P397, 1:400) to the antibody-antigen complexes was detected with an AEC chromogen. Tissue sections were examined and photomicrographs were taken with a Zeiss microscope.

Biochemistry

Tissue preparation

Human term placentas (n = 7) were collected from clinically normal pregnancies terminated by either vaginal deliveries or cesarean sections. After their immediate transfer on ice, samples (5-10 g) from different regions (amnion, basal plate, chorionic plate, decidua, chorion laeve, Nitabuch's fibrinoid, umbilical cord and villi) were cut out as precisely as possible, put into 5% sucrose in PBS, pH 7.4, frozen in liquid nitrogen, and stored at –35 °C until further use.

Extraction procedure and protein determination

The extraction of matrix proteins was performed on ice in the presence of protease inhibitors (Protease inhibitor cocktail, Boehringer) as can be seen in the PhD thesis (see for details, 11).

Before electrophoresis the protein concentration of each aliquot was determined using an Elisa reader (using Microwin or Easywin software program), at either 620 nm with Bradford (Biorad, Munich) or 700 nm with DC-Lowry (Biorad) protein estimation assay.

SDS-polyacrylamide gel electrophoresis and Western blotting

SDS-polyacrylamide gel electrophoresis was carried out under reducing conditions. The 7% separating gel was overlayered with a stacking gel containing 3% polyacrylamide. Samples with the same protein concentration (15-20 µg/per lane), were incubated for 30 min at room temperature in sample buffer made up of 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% sucrose, 0.005% bromphenol blue and 5% 2-mercaptoethanol. Before loading the samples, they were centrifuged for 10 min at 13,500 rpm (Hermle, 220.59 V07). The proteins were separated at 25mA/plate in 25 mM Tris-HCl, pH 6.8, 192 mM Glycine, 0.1% SDS for approximately 1.5 h until the dye front reached the end of the gel. After electrophoresis the gels were either stained with Coomassie blue R-250 or subjected to semi-dry transfer on PVDF membranes (Westran, 0.2 µm, Schleicher & Schuell, Germany) at 200 mA for 20 min. Following the transfer of proteins, the blotted membranes were blocked with 5% nonfat dry milk in 0.05% Tween 20/PBS, pH 7.4 for 2 h and incubated with the primary antibodies (see for details, 11) overnight and developed using gold-labeled goat anti-mouse IgG or IgM for 2 h

and a silver enhancement kit for 40 min (Amersham Life Sciences). The immunoreactivities were evaluated by gel print 2000I (Biophotonics Corporation, MWG-Biotech) and scananalytics (Billerica) (see for details, 11).

Results

Immunohistochemistry

Fibronectins in general (IST-4, IST-6, 3E1)

IST-4 (III-5 domain of all fibronectin isoforms)

Amniotic basal lamina was very faintly immunoreactive with this antibody, whereas the amniotic epithelium and mesoderm showed no immunoreactivity. The chorionic mesoderm was also faintly immunoreactive. The EVT cells in the chorionic plate, appearing like cell islands between the villi and cell columns and in the basal plate, showed quite remarkable, patchy like distribution in their matrices (Figs. 1A-C). In the cell columns immunoreactivity increased from the proximal to distal parts. ECMs of decidual cells in the basal plate were also immunoreactive. The stroma of large stem villi showed immunopositivity, whilst trophoblastic cover remained immunonegative. The walls of large arteries in these stem villi also showed no immunoreactivity. Other types of villi of a smaller caliber did not show immunopositivity in the stroma or in the trophoblastic cover (not shown in data).

IST-6 (III-7/8 domain of fibronectin lacking III-B domain)

The amnion, amniotic epithelium, basal lamina and mesoderm were not immunoreactive to this antibody. The chorionic mesoderm was very faintly immunoreactive. The EVT cells in the chorionic plate, appearing like cell islands between the villi and in the cell columns of the basal plate, showed similar appearances as obtained by IST-4 (Figs. 2A-C). The matrices of decidual cells in the basal plate were also immunoreactive (Fig. 2D). In stem villi, the stroma was immunopositive, the walls of the big arteries gave a very faint reaction and the trophoblastic cover was immunonegative. Other types of villi did not show immunopositivity in the stroma or in the trophoblastic cover.

3E1 (heparin binding domain of all fibronectin isoforms)

Amniotic epithelium showed no immunoreactivities, but basal lamina underlying the epithelium and amniotic mesoderm was immunoreactive to this antibody. The chorionic mesoderm was faintly immunoreactive and EVT cells in the chorionic plate were rarely immunopositive. The stroma of stem villi and anchoring villi were immunoreactive, whereas the stroma of the other types of villi were faintly immunoreactive. The walls of the large arteries were immunopositive. In the basal plate there were spot-like irregular immunoreactivities. The trophoblast cells in cell islands were not immunoreactive. In the basal plate decidua cells were almost immunonegative.

Cellular fibronectins

3E3 (cell binding domain of cellular fibronectins)

This antibody was immunoreactive with amniotic basal lamina and mesoderm, but was not reactive with amniotic epithelium. The chronic mesoderm was immunopositive, but trophoblast cells did not show reactivity. The stroma of villi was immunoreactive, whilst the trophoblastic cover of villi did not show this appearance. The basal plate stroma of anchoring villi and trophoblast cells were very weakly reactive. Decidual ECMs were also very weakly positive.

IST-9 (III-A domain of cellular fibronectins)

IST-9 showed reactivities very similar to those of IST-4 and IST-6. The amnion in all its parts showed no immunoreactivity, whereas the border line of the chorionic mesoderm was very weakly reactive and the patchy distribution of the reactivity in the ECMs of EVT cells was remarkable. The stroma of stem villi were very weakly immunoreactive, but their trophoblastic cover was not reactive. Other types of villi did not show immunopositivity in the stroma or in the trophoblastic cover, but the appearance of cell islands between the villi were immunoreactive. Cell columns in the basal plate containing EVT cells also showed a patchy distribution in their ECMs. The ECMs of decidual cells and EVT cells in the basal plate were also immunoreactive.

Oncofetal fibronectins

FDC-6 (O-glycosylated III-V domain)

FDC-6 was reactive with amniotic basal lamina, whereas it was not reactive with amniotic mesoderm or amniotic epithelium (Fig. 3A). Chorionic mesoderm showed very faint reactivity, while the EVT cells of the chorionic plate had the typical appearance of the patchwork distribution in their ECMs (Fig. 3B). The stroma of the stem villi and of the other types of villi and

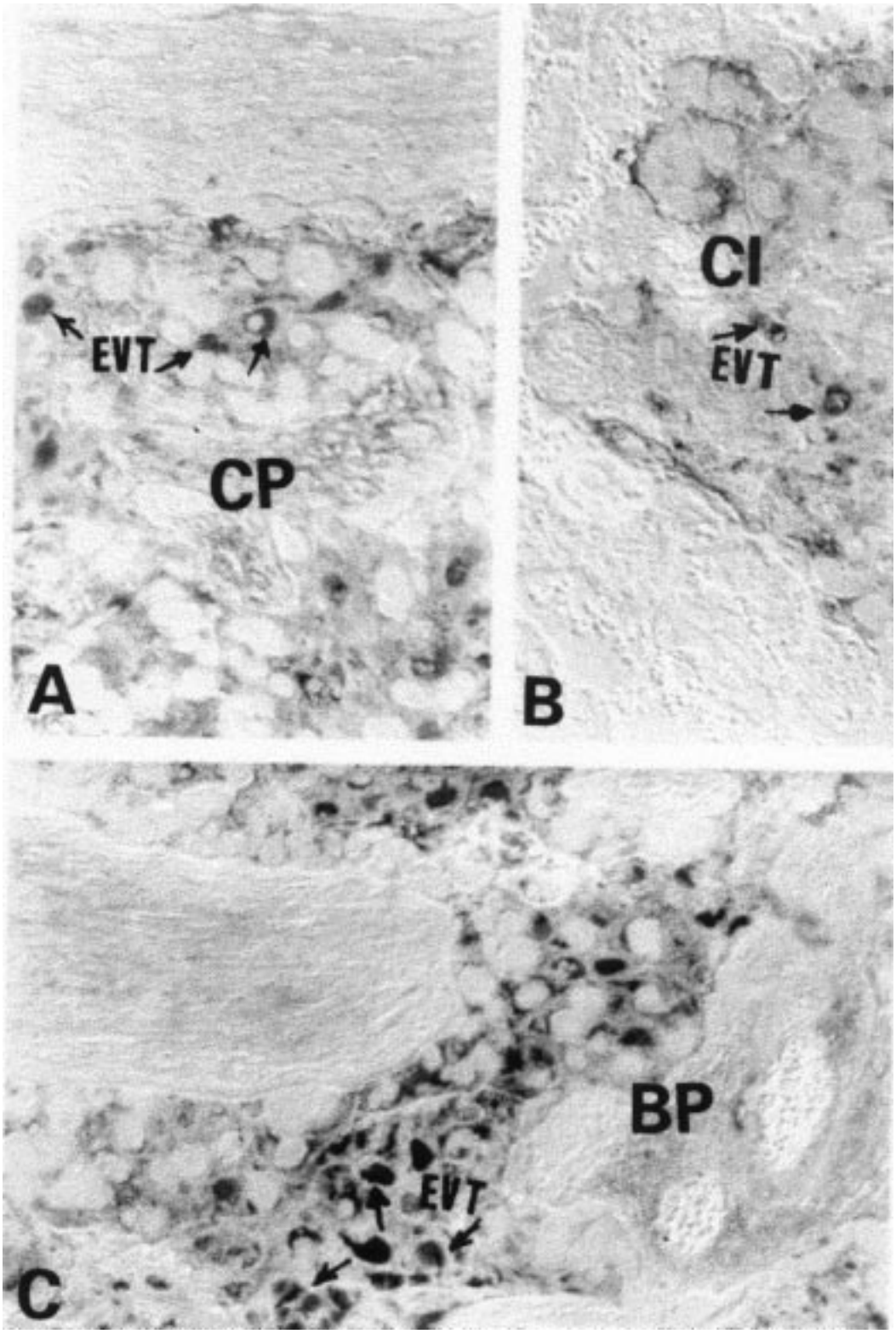

Figure 1. Micrographs showing immunoreactivities with IST-4 in the extracellular matrices of extravillous trophoblast (with EVT) cells in chorionic plate (with CP) (A), in cell islands (with CI) (B) and in basal plate (with BP) (C). Original magnification x25.

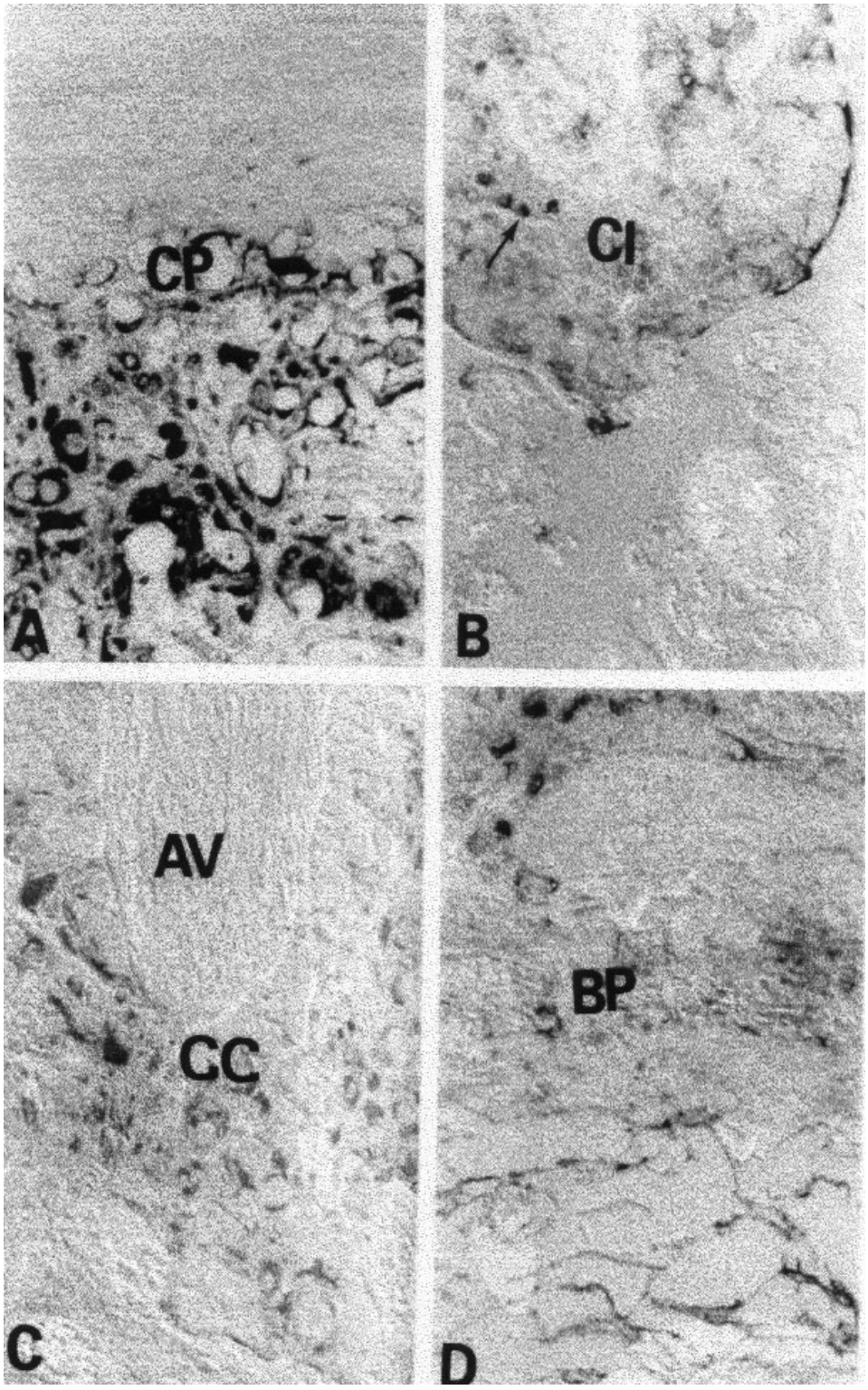

Figure 2. Immunoreactivities with IST-6 in chorionic plate (with CP) (A), in cell islands (with CI) (B), in cell columns (with CC) (C) and in the decidual part of the basal plate (with BP) (D) are seen. A strong immunoreactivity of EVT cells with extracellular matrices in different placental sites is definitely indicated. AV: anchoring villi. Original magnification x25.

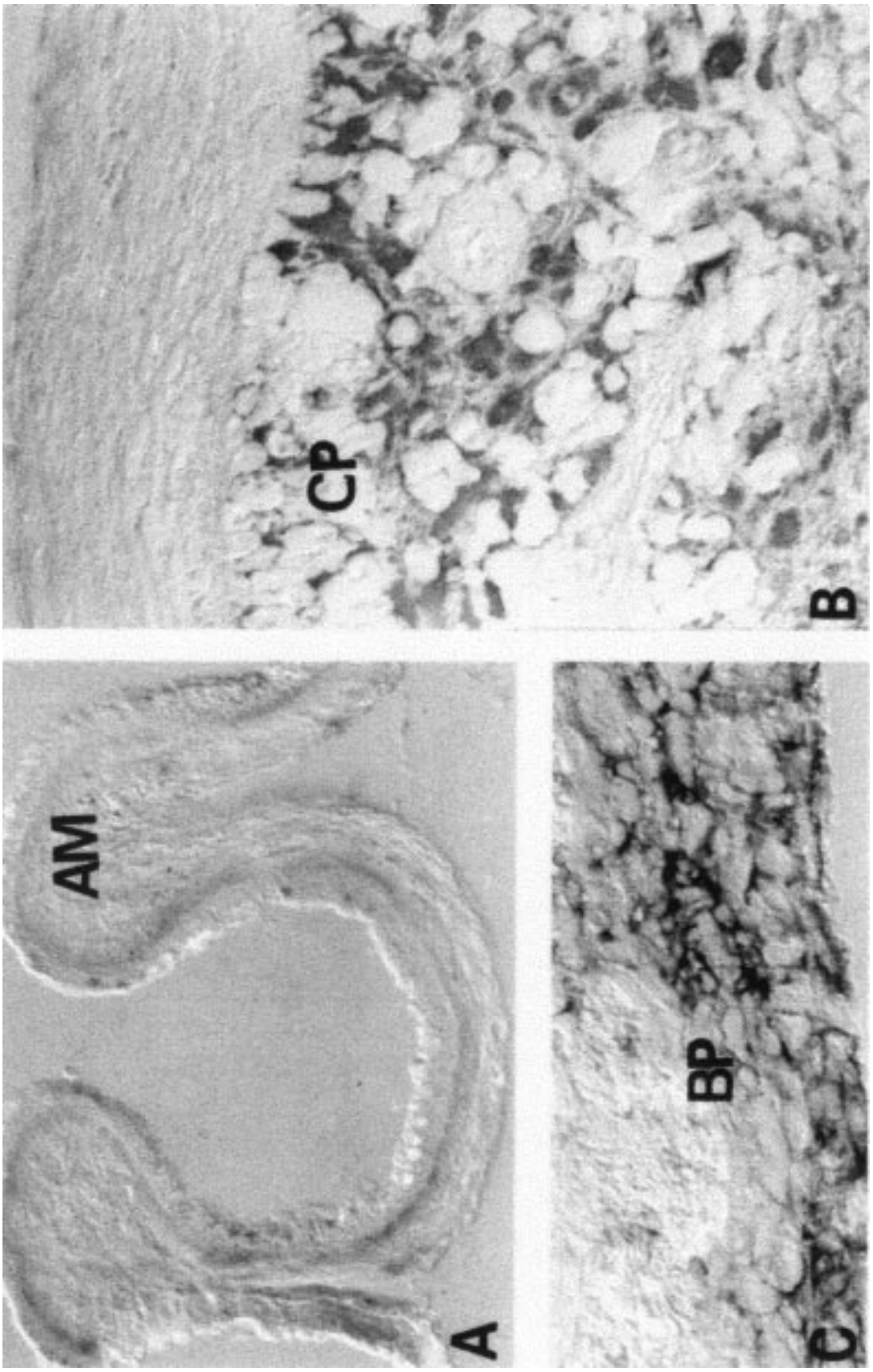

Figure 3. Immunoreactivities with FDC-6 in separated amniotic membrane (with AM) (A), chorionic plate (with CP) (B) and in basal plate (with BP) (C). Amniotic membrane (with AM) and its some components are immunonegative except the basal lamina of amniotic epithelium that is weakly immunopositive, whereas CP with EVT cells and their extracellular matrices and BP give off strong positive immunodetection for the FDC-6 antibody. The chorionic mesenchyme is immunonegative. Original magnification x25.

their trophoblastic cover showed no immunoreactivities. In the cell islands between the chorionic villi tree, EVT cells in the basal plate and also the decidual cells showed reactivities in their ECMs (Fig. 3C).

Biochemistry

Fibronectins in extracellular matrices of human term placenta were solubilized by the application of extraction buffer (for details, 11) and the supernatants that were obtained in this last step were applied on SDS-PAGE (Fig. 4) followed by the quantification of fibronectin isoforms on Western blots. The signal of each fibronectin isoform was analyzed and compared by the integration of detected optical densities (for details, 11).

Fibronectins in general

IST-4 (III-5 domain of all fibronectin isoforms)

The immunoreactivities of this antibody gave the strongest signal with the chorion laeve extract, which was then followed by the preparations from the amnion and chorionic plate. Extracts from the basal plate, villi, umbilical cord and decidua were reactive with IST-4 less intensely, but had densities very similar to each other. The weakest signal was detected in the preparations of Nitabuch's fibrinoid (Figs. 5A-F and 6A-G).

IST-6 (III-7/8 domain of fibronectin lacking III-B domain)

The most intense immunoreactivities to this antibody were detected in the amnion and chorion laeve preparations. The density of chorionic plate extract was very close to the densities detected in the amnion and chorion laeve extracts. The basal plate extract preparation had an intenser immunoreactivity than the preparations from the decidua, umbilical cord and Nitabuch's fibrinoid. While these last three extracts had very similar reactivities, the villi preparation showed the weakest immunoreactivity (Figs. 5A-F and 6A-G).

3E1 (heparin binding domain of all fibronectin isoform)

The immunoreactions of this antibody showed similar intensities with different extracts. Among them, chorion laeve and amnion extracts were the leading dense signals. Chorionic plate and Nitabuch's fibrinoid extracts had the same level of detected density. The umbilical cord followed the last two with very close intensity. The rest of the sequel was formed by the preparations from the basal plate, decidua and villi (Figs. 5A-F and 6A-G).

In addition, another immunoreactive band around the 41-43kD region was detected.

4B2 (gelatin binding domain of all fibronectin isoforms)

This antibody was intensely immunoreactive with the preparation from the chorion laeve, which was then followed by the amnion, chorionic plate, villi, umbilical cord, basal plate and Nitabuch's fibrinoid (Figs. 5A-F and 6A-G).

Cellular fibronectins

3E3 (cell binding domain of cellular fibronectins)

This antibody, similar to the other fibronectin antibodies, was strongly immunoreactive with the extract from the chorion laeve, which was followed by amnion and chorionic plate extracts. Intensity signals from the preparations of the decidua and Nitabuch's fibrinoid were close to each other and were denser than the basal plate and villi (Figs. 5A-F and 6A-G).

IST-9 (III-A domain of cellular fibronectins)

IST-9 showed the strongest immunoreactivity to the chorion laeve extract of, which was followed by signals from chorionic plate and Nitabuch's fibrinoid preparations. The densities of the last two extracts were close to each other and were higher than the signals of amnion and umbilical cord, which were also at the same intensity level. The densities detected from villi, decidua and basal plate extracts completed the sequel with their lower intensities (Figs. 5 A-F and 6A-G).

Oncofetal fibronectins

FDC-6 (O-glycosylated III-V domain)

FDC-6 was significantly immunoreactive with the chorion laeve extract. Amnion and chorionic plate preparations had close intensities and followed the signal of the chorion laeve. The decidua, Nitabuch's fibrinoid, basal plate, villi and umbilical cord extracts followed the first three signals from high to low intensities (Figs. 5A-F and 6A-G).

Discussion

Immunohistochemistry and biochemistry results have revealed that the amniotic basal lamina is mainly formed by fibronectins having III-5, heparin binding, cell binding and O-glycosylated III-V domains. The amniotic

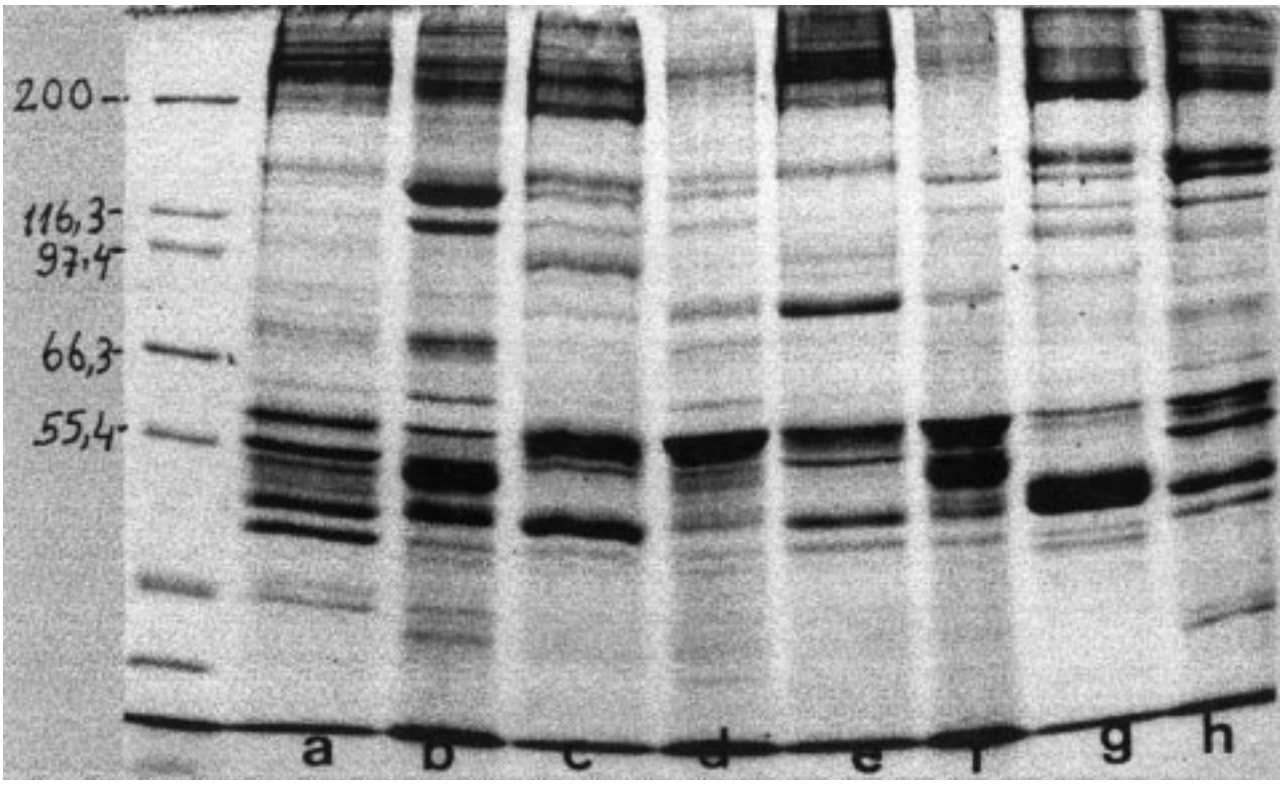

Figure 4. A representative SDS-PAGE with the extracts from different sites in the human term placenta. This application design is also used for Western blots. From left to right: molecular weight markers, extractions from amnion (a), basal plate (b), chorionic plate (c), decidua (d), chorion laeve (e), Nitabuch's fibrinoid (f), umbilical cord (g) and chorionic villi (h). The signal of each fibronectin isoform is detectable for analysis and cooperation by the integration of detected optical densities are seen.

mesoderm is formed by fibronectins having heparin and cellular binding domains. Western blots demonstrated that amnion is also composed of fibronectins having III-7/8 and III-A domains. In previous studies, the presence of fibronectin in amnion was reported (12), while human amnion epithelial cells were shown to secrete cellular (III-A domain containing) and oncofetal (III-B containing) fibronectin isoforms that are assembled in the ECMs (13,14). The expression of oncofetal fibronectin in the amniotic basal lamina can be a part of the healing process due to the rapidly growing fetus and its membranes partly causing shears in the amnion.

In the basal and chorionic plates there are three isoforms of fibronectin (familiar, cellular and oncofetal) present both in imminohistochemistry and in Western blots. Especially in distal parts of the cell columns and in the trophoblastic layer of the chorionic mesenchyme, these isoforms were distributed in a spot-like manner between the EVT cells in agreement with previous reports (9,15). On the other hand, although immunohistochemistry results were not available from the chorion laeve, the trophoblastic layer of the chorionic plate can also be representative of this placental site. In Western blots chorion laeve extracts gave the highest signals with general, cellular and oncofetal fibronectins. This might be due to the concentrated presence of EVT cells and their matrices in this region. The presence of oncofetal fibronectins in the regions where EVT cells are in great numbers especially suggest the importance of the chorion-decidual interface together with the other fibronectin isoforms (16). This would probably influence the differentiation of proliferative trophoblast cells into an invasive type. It would also enhance trophoblast adhesion to maternal tissues and facilitate trophoblast migration.

Decidual tissue was taken from the basal plate for immunohistochemistry, while decidual tissue for Western blots was derived from fetal membranes. In both techniques the results were parallel to each other revealing the presence of fibronectin isoforms (general,

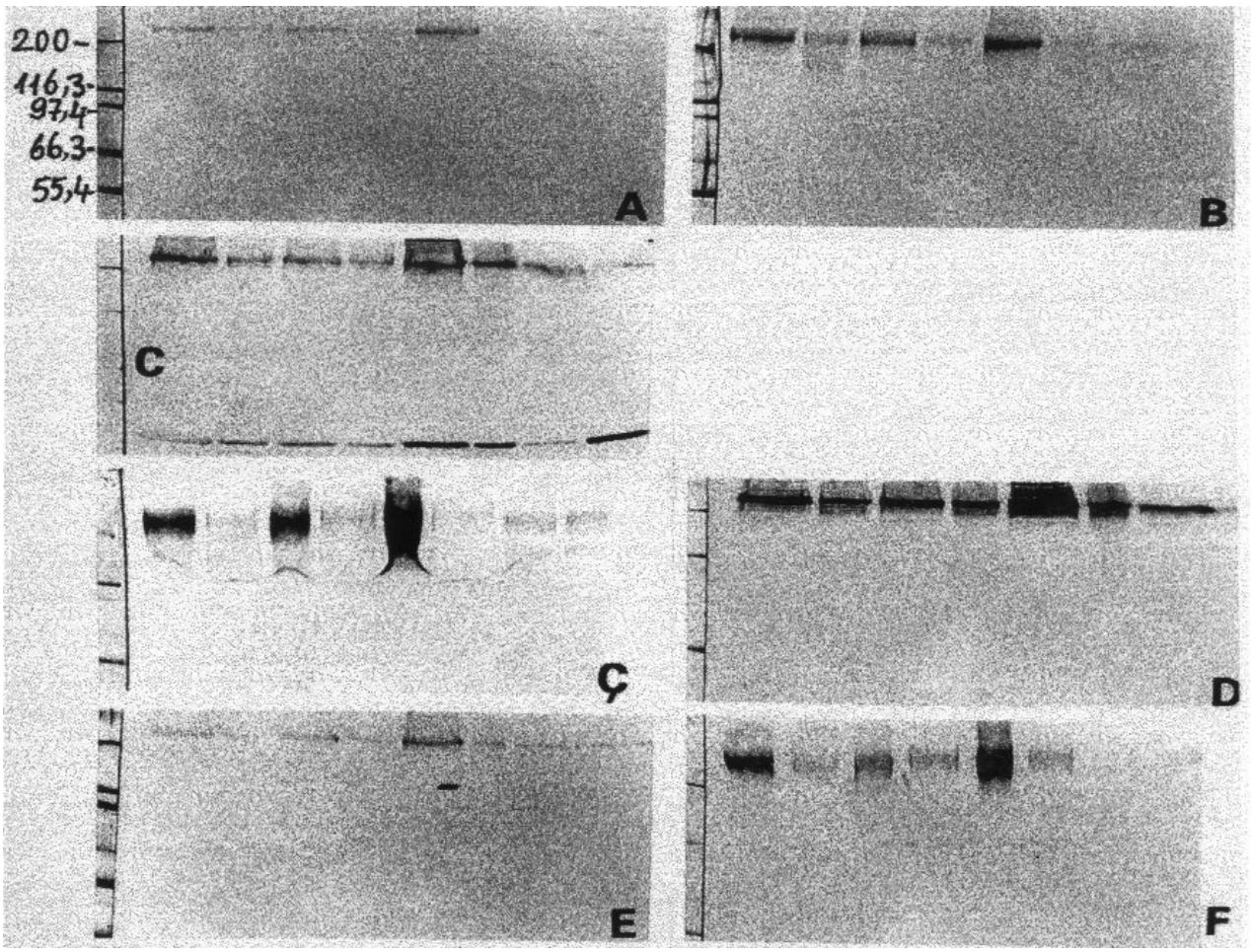

Figure 5. Representative Western blots from the extractions of different placental sites with antibodies against different fibronectin isoforms. The order of blots is similar to that in Figure 4. The immunoreactivity of different levels is seen with IST-4 (A), IST-6 (B), 3E1 (C), 4B2 (Ç), 3E3 (D), IST-9 (E), and with FDC-6 (F).

cellular and oncofetal) in decidual tissue. In a previous study, the presence of fibronectin in the pericellular basement membrane (17), in the pericellular matrix (18) and in cell cytoplasm in fibrillar and punctuate patterns (19) of decidual cells was shown. Interestingly, oncofetal fibronectins having III-B or O-glycosylated III-V domains have been detected only in decidua that has been invaded by the trophoblast (18). The results of this study suggest that the deposition of specific fibronectin isoforms in the decidual matrix may result in a response to EVT cell migration and invasion. Our recent study (20) indicated that there is also a relationship between fibronectin isoforms and pH environment for trophoblastic invasion. We concluded the pH played an important role as a regulator of human trophoblast differentiation.

Besides β chain lacking fibrinopeptide, a variety of polypeptides having molecular masses over 105kD, have been reported in Nitabuch's fibrinoid (21). Among these polypeptides, basement membrane proteins (collagen type IV and laminin) have been shown to be present in this region. In the present study, Western blots also reveal the presence of different fibronectin isoforms; general and cellular fibronectins are the most prominent ones, while oncofetal isoforms and gelatin domain containing isoforms are at very low amounts and almost undetectable.

The synthesis of fibronectin by the venous and arterial endothelia of the human umbilical cord has been previously reported (22) but their types and molecular events were not indicated. In this study, the results obtained from Western blots show the presence of

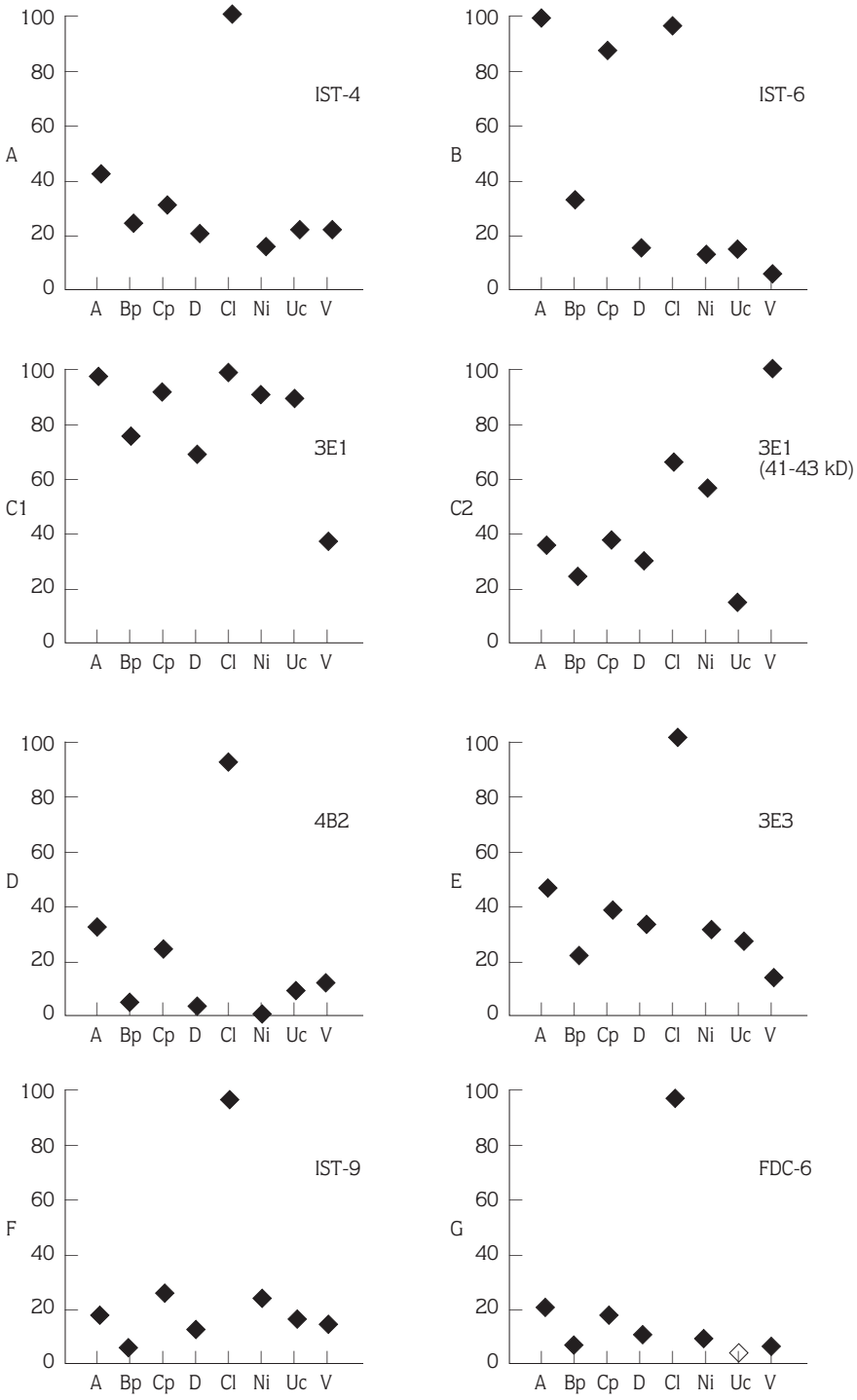

Figure 6. The integrated values of optical densities (y-ordinate) are compared with the extracted tissues (x-ordinate). The strongest reaction is considered to equal 100% and the other reactions were carried out as <100%. The order of blots are similar to that in Figure 5 except for the second graphic of the 3E1 antibody indicated above. The intensity of immunoreaction with IST-4 (A), IST-6 (B), 3E1 (C1 and C2), 4B2 (D), 3E3 (E), IST-9 (F), and with FDC-6 (G) is indicated for comparison.

familiar and cellular fibronectin isoforms in umbilical cord extracts. In addition, the oncofetal isoforms are not detectable.

According to previous studies, in the term placenta, the stroma of the chorionic villi (23,24) and surrounding of the fetal vessels (25) have shown immunoreactivities with fibronectin, while the trophoblastic basement membrane has shown no immunoreactivity (26). In parallel to these reports, this study shows that the stroma of large stem villi (27) and the surroundings of fetal vessels are composed of general and cellular fibronectin isoforms, whereas the presence of these isoforms was not detectable in the trophoblastic cover and in the other types of villi. In addition, the villous stroma did not consist of oncofetal fibronectin isoform that has an O-glycosylated domain in addition to the findings of previous reports (oncofetal fibronectin that has III-B domain) (28).

Finally, we conclude that fibronectin and its isoforms are distributed in different human placental sites with varying intensity due to features of the fetoplacental membrane and decidual components. Fibronectin isoforms are highly detectable immunoreactively in these sites due to the presence of EVT, but in amnionic components having no EVT cells the lack of any immunoreaction of all fibronectin isoforms, except the oncofetal isoform, indicates a high activity of the rapid growth of the fetus and its closed membranes. In conclusion, fibronectin isoforms play an important role in trophoblastic invasion and placental development during pregnancy.

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