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Connexin expression and gap junctional intercellular communication in human first trimester trophoblast

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Connexin (Cx) expression and gap junctional intercellular communication (GJIC) are involved in development and differentiation processes, and recently mutation of connexin genes has been implicated in pathologies. In the human placenta, two distinct differentiation pathways of cytotrophoblastic cells coexist and lead to a fusion phenotype (villous trophoblast) and a proliferative/invasive phenotype (extravillous trophoblast). Here we characterized *in situ* and *in vitro* the expression of Cx transcripts and proteins in the villous and extravillous trophoblast of first trimester placenta. In addition, the GJIC functionality was investigated using the gap–fluorescence recovery after photobleaching (gap-FRAP) method. We demonstrated in the villous trophoblast the presence of Cx43 mRNA and of Cx43 protein localized between cytotrophoblastic cells and between cytotrophoblastic cells and syncytiotrophoblast. *In vitro*, a transient functional gap junctional intertrophoblastic communication was demonstrated during the trophoblast fusion leading to the multinucleated syncytiotrophoblast. During the proliferative process of the extravillous trophoblast, Cx40 is expressed in the proximal part of the cell columns. When cytotrophoblastic cells were cultured on Matrigel® for 2 days, α5β1 integrin expression was observed concomitant with the presence of Cx40 mRNA and of Cx40 protein between the cells. No evidence for a GJIC was detected in this induced extravillous phenotype. In addition, Cx32 was detected between some aggregated cells after 72 h of culture. Our data show that the presence of Cx43 allows an inter-trophoblastic GJIC and is associated with the fusion process leading to the villous syncytiotrophoblast and that the presence of Cx40 does not allow GJIC and is associated with the extravillous phenotype.

Key words: connexin/extravillous trophoblast/gap junction/placenta/villous trophoblast

Introduction

In mammals, embryonic development requires the formation of a specific organ, the placenta, which is responsible for physiological exchanges between the developing fetus and its mother *in utero*. In the human, the placenta is characterized by an extensive invasion of the trophoblast into the maternal uterus allowing direct contact of the trophoblast with the maternal blood (haemomonochorial placentation) (Aplin *et al*., 2000; Benirschke and Kaufmann, 2000). After the initial phase of nidation, the human trophoblast proliferates and differentiates along either the villous or the extravillous phenotype (Kaufmann and Castellucci, 1997).

In the villous phenotype, the cytotrophoblastic cells of the floating villi (in the intervillous space) remain attached to the villous basement membrane, and form a monolayer of epithelial cells, which proliferate and differentiate by fusion to form a syncytiotrophoblast covering the entire surface of the villi. The syncytiotrophoblast is primarily engaged in absorptive, exchange and specific endocrine functions.

In the extravillous phenotype, the cytotrophoblastic cells of the anchoring villi proliferate, detach from the basement membrane and aggregate into cell columns to attach to the uterine wall. From there, individual cells migrate into the decidua and the myometrium, remodelling the pregnant endometrium and its vasculature. Alternatively, many of the extravillous trophoblasts (EVT) scattered through the decidua and the myometrium differentiate into multinucleated placental cells. EVT cells share many characteristics with tumour cells, including similar mechanisms underlying their invasive ability (Vicovac and Aplin, 1996; Kaufmann and Castellucci, 1997; Aplin *et al*., 2000; Fisher, 2000). However, unlike tumour cells, proliferation, migration and invasiveness of EVT cells are exquisitely regulated *in situ*, both temporally and spatially, in order to maintain a healthy uteroplacental homeostasis.

Gap junctions are clusters of transmembraneous channels composed of hexameres of connexin (Cx) and provide a pathway for the diffusion of ions and small molecules (for example, cAMP, cGMP, inositol triphosphate, Ca^{2+}). Connexins represent a family of closely related membrane proteins, which are encoded by a multigene family (Willecke *et al*., 1991; Kumar and Gilula, 1996) of at least 21 members in mammals. However, besides common topological features, these junctional channels present a diversity in biophysical properties (such as voltage-dependence and conductance) and some functional or regulation characteristics are determined by the connexin type(s). It is important to note that the presence of most connexins is not restricted to one tissue and that most if not all tissues produce more than one connexin (Bruzzone *et al*., 1996). Furthermore, differentiation

and physiological processes may induce spatiotemporal patterns of connexin synthesis. The exchange of molecules through gap junctions is involved in the control of cell proliferation, in the control of cell and tissue differentiation, in metabolic cooperation and in spatial compartmentalization during embryonic development. Many studies strongly support the hypothesis that the loss of gap junctions and of gap junctional intercellular communication (GJIC) plays an important role in tumorigenesis, and that restoring gap junctional communication in tumour cell lines can normalize the cell phenotype (Tanaka *et al*., 2001).

Little is known about cell–cell communication in the trophoblast during placental development. Earlier, ultrastructural studies have reported the presence of gap junctions between trophoblastic layers of haemochorial placentae (Cronier *et al*., 2001) and between cytotrophoblasts and syncytiotrophoblast of endotheliochorial placentae (Malassiné and Leiser, 1984). In rat placenta, immunolocalization studies have indicated a switch in connexin gene expression (Cx31/Cx26/Cx43) directly correlated to development (Reuss *et al*., 1996). In addition, the importance of connexin expression and of GJIC has been recently illustrated with transgenic mice. In Cx26-deficient mice, transplacental uptake of glucose is decreased leading to embryonic death (Gabriel *et al*., 1998) and Cx31 deficiency causes transient placental dysmorphogenesis (Plum *et al*., 2001).

We have previously demonstrated *in vitro* that a GJIC and Cx43 expression are involved in the differentiation of term villous trophoblast (Cronier *et al*., 1997). Cx40 expression has also been detected in the EVT of first trimester placenta (Winterhager *et al*., 1999, 2000), and Cx32 and Cx43 in the multinucleated cells of the placental bed (Al-Lamki *et al*., 1999). Nevertheless, the functionality of these trophoblastic transmembraneous channels during first trimester placental development remains unknown.

Therefore, the aim of the present study was to characterize *in situ* and *in vitro* the expression of connexin transcripts and proteins in the villous and extravillous phenotypes. In addition, the GJIC functionality was investigated using the fluorescence recovery after photobleaching (FRAP) method. We demonstrated, in villous trophoblast, the presence of Cx43 transcripts and proteins allowing a functional inter-trophoblastic communication. On the contrary, the Cx40 expression in the extravillous phenotype was not associated with a functional GJIC.

Materials and methods

Tissues

Placental tissues (n = 15) (chorionic villi with attached decidua) from first trimester (8–12 weeks) were obtained after legal voluntary interruption of pregnancy. The tissue was washed in calcium and magnesium-free Earle's balanced salt solution (EBSS) supplemented with gentamycin (50 μg/ml; Sigma Chemical Co., St Louis, MO, USA) and immediately transferred to the laboratory. Some samples of tissue were either fast-frozen in isopentane cooled in a liquid nitrogen bath and stored at –80°C or fixed for 3 h in 3% paraformaldehyde for in-situ hybridization. In other cases, chorionic villi were dissected, rinsed and minced for cell isolation.

Trophoblastic cell purification and cell culture

Minced tissue was transferred to pre-warmed EBSS, pH 7.4 containing 0.1% trypsin 1-250 (Sigma), 100 Kunitz units/ml DNase I (Sigma), 1 mmol/l $CaCl_2$, 1 mmol/l $MgSO_4$, 50 μg/ml gentamycin and subjected to two sequential digestions (10 min) at 37°C under gentle agitation. Cells were purified by Percoll gradient centrifugation (Kliman *et al*., 1986). For studies on the villous phenotype, a negative selection procedure was used to obtain a trophoblast preparation without contamination of other cells, by using a monoclonal anti-HLA A, B and C antibody (W6-32HL; Sera Lab, Crawley Down, UK), according to a published method (Schmon *et al*., 1991). This antibody reacts with most cell types (e.g. macrophages, fibroblasts, EVT) but not with villous cyto- or syncytiotrophoblast. Briefly, the isolated cells were transferred to culture dishes coated with the monoclonal antibody. After 15 min at 37°C, non-adherent cells were recovered by gently rocking the dishes and removing them with a pipette. Cells were then diluted to a final concentration of 0.5×10^6/ml in minimum essential medium (MEM) containing 10% fetal calf serum (FCS; Biowest, Nuaillé, France), 25 mmol/l glucose and 50 μg/ml gentamycin and plated on 35 mm plastic dishes (Nunclon; Nunc, Roskilde, Denmark). After a 4 h incubation at 37°C in 5% CO_2, non-adherent cells were removed by three washes with EBSS. To induce the extravillous phenotype, negative selection by anti-HLA A, B and C antibody was omitted and cells were plated on Matrigel-coated dishes (5 mg/ml; Becton Dickinson, le Pont de Claix, France). After a 4 h incubation at 37°C in 5% CO_2, non-adherent cells were removed by three washes with EBSS.

The cells were cultured for 24, 48 and 72 h, and the medium was changed daily. Cytokeratin 07 immunocytochemistry was performed to confirm the cytotrophoblastic nature of the attached cells. The cells were positively stained at 95 and 85% after purification procedure for villous and extravillous trophoblast respectively.

Table I. Antibodies used for immunohistochemistry

Antigens	mAb	Species	Isotypes	Dilution	Source
Cytokeratin 07	OV.TL12/30	Mouse	IgG1	1:200	Dako, Denmark
α5β1 integrin	SAM1	Mouse	IgG2A	1:200	Immunotech, France
Ki67	MIB1	Mouse	IgG1	1:50	Immunotech, France
Cx43	2	Mouse	IgG1	1:200	Transduction Labs, USA
		Rabbit		1:50	Dr Gros, Marseille, France
Cx40		Rabbit		1:50	Dr Gros, Marseille, France
Cx33		Rabbit		1:50	Dr Pointis, Nice, France
Cx32	CX-2C2	Mouse	IgG1	1:200	Zymed, USA
Cx26	CX-12-H10	Mouse	IgG1	1:200	Zymed, USA
Mouse IgG	715-095-150 (FITC)	Donkey		1:100	Jackson Immunoresearch Laboratories, USA
Rabbit IgG	111–025–003 (TRITC)	Goat		1:100	Jackson Immunoresearch Laboratories, USA
Isotypic control	679.1Mc7	Mouse	IgG1	1:50 or 1:200	Immunotech, France
Isotypic control	U7.27	Mouse	IgG2A	1:200	Immunotech, France
Preimmune serum		Rabbit	IgG	1:5	Dr Gros, Marseille, France
Preimmune serum		Rabbit	IgG	1:5	Dr Pointis, Nice, France

mAb = monoclonal antibody; FITC = fluorescein isothiocyanate; TRITC = tetramethylrhodamine B isothiocyanate.

Immunolocalization

For connexins and cytokeratin 07 detection, cultured cells and frozen sections were fixed for 10 min in methanol at –20°C, and for integrin detection, the specimens were fixed for 30 min in 3% (wt/vol) paraformaldehyde. They were then washed three times in PBS and processed using a method similar to that detailed elsewhere (Tabb *et al*., 1992). After incubation in a blocking solution consisting of 2% bovine serum albumin (BSA) and 1% Triton X-100 in PBS for 30 min at room temperature, specimens were washed three times in PBS and incubated overnight in an appropriate dilution of primary antibodies in PBS 1% BSA (Table I) for indirect immunofluorescence. After five further washes in PBS, the second antibodies conjugated to tetramethylrhodamine isothiocyanate (TRITC) and/or to fluorescein isothiocyanate (FITC) (Jackson Immunoresearch Laboratories, Wet Grove, PA, USA) (1/50 and 1/100) were applied for 45 min at room temperature (Table I). After washing, samples were mounted in mounting medium with or without DAPI for nuclear staining (Vector Laboratories, Burlingame CA, USA) and examined on an Olympus Bx60 epifluorescence microscope. All controls, performed by omitting the primary antibody or by non-specific IgG of the same isotype, were negative.

Cx43 in-situ hybridization

Tissue sections (5 µm thick) were collected on 3-aminopropyltriethoxysilane-coated slides and the in-situ hybridization procedure was performed as previously described (Sibony *et al*., 1995). The human 1 kb Cx43 cDNA (gift from Dr M. Mesnil, IARC, Lyon, France) was subcloned into the

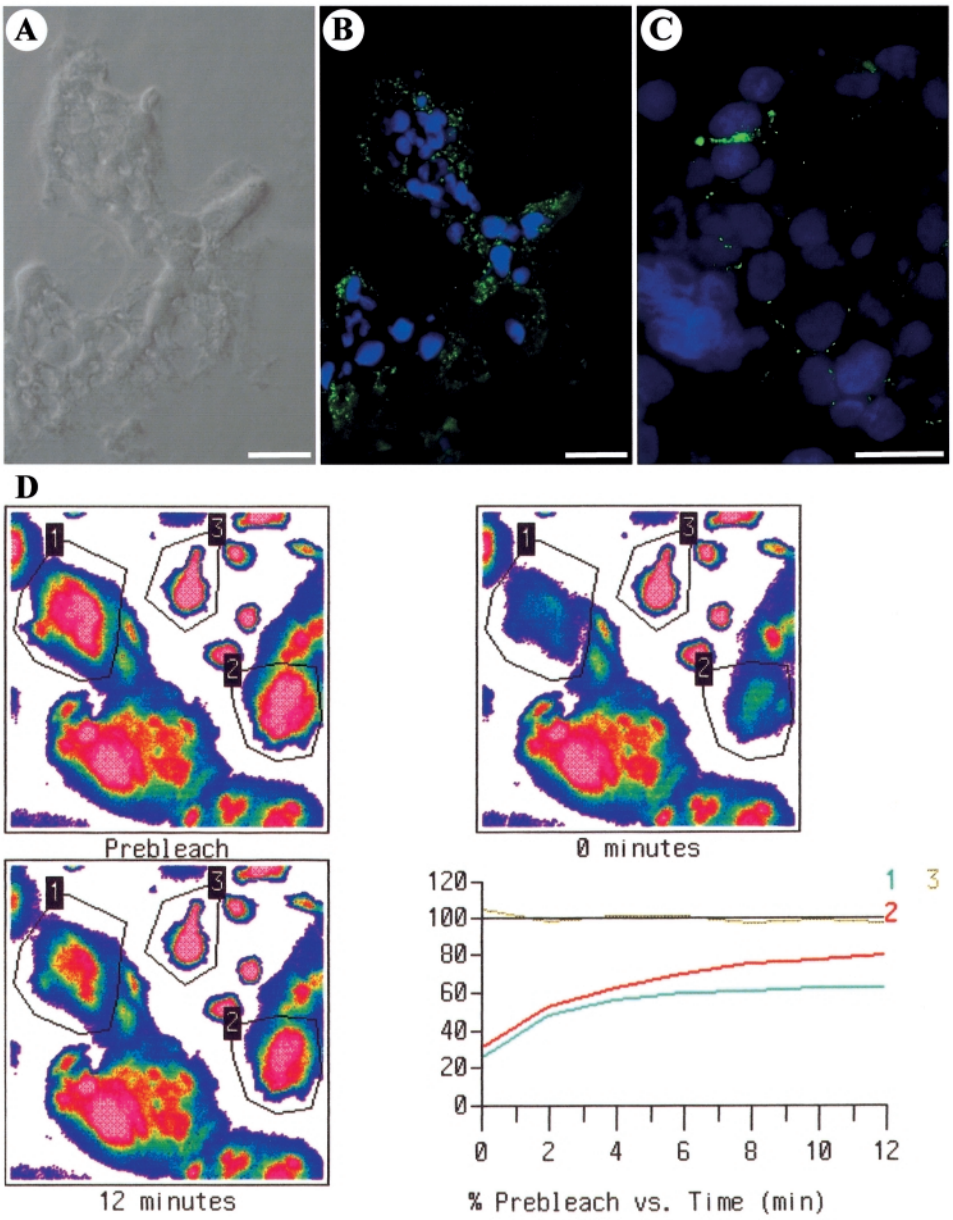

Figure 1. (**A**) Brightfield image of first trimester floating villous section immunostained for Cx43 and DAPI in (**B**). (**B**) Cx43 (green) immunostaining is observed in the trophoblastic layer between contacting cells and within the cytoplasm. (**C**) Transversal section of another floating villous at higher magnification immunostained for Cx43. Cx43 punctate immunofluorescence is observed between two contiguous nuclei presumably of cytotrophoblastic cells and between other contacting nuclei (presumably cyto- and syncytiotrophoblast). Scale bars = 20 µm. (**D**) Gap-FRAP experimentation. Typical computer-generated images of fluorescence distribution in villous trophoblastic cells cultured for 3 days measured during a gap-FRAP experiment. After a pre-bleach scan, the fluorescent dye was photobleached in some selected cells (polygons 1 and 2) by means of a strong laser illumination. Isolated cells (polygon 3) kept unbleached served as a control for the spontaneous fading of fluorescent emission. The evolution of fluorescence intensities was measured starting just after photobleaching for 12 min with a scanning period of 2 min. Corresponding curves of fluorescence evolution in selected cells: fluorescence recoveries in cells 1 and 2 follow a closely exponential time-course, reflecting the presence of open gap junctional channels. Note the low decrease of fluorescence intensity in the control unbleached cell (3) due to repeated scanning.

EcoRI-restriction site of Bluescript KS+ vector. Cx43 antisense and Cx43 sense probes were obtained from linearized vectors with Hind III and Bam HI (Roche, Meylan, France). The radiolabelled probes were generated by in-vitro transcription and incorporation of [^{35}S]uridine triphosphate (specific radioactivity, 1000 Ci/mmol; Amersham, UK). Two serial placental sections were mounted on the same histological slide. One received the antisense RNA probe, and the other received the RNA sense probe as negative control. Heart tissue sections were used as a positive control. Tissue sections were covered with 30–100 µl hybridization buffer (50% formamide, 10% dextran sulphate, 1 mg/ml salmon sperm DNA, 70 mmol/l dithiothreitol and 20×standard saline citrate) containing the radiolabelled probe at a concentration of 10–15×10^3 c.p.m./µl. After the post-hybridization washings, the slides were dipped in NTB2 emulsion (Kodak, Rochester, NY, USA) and exposed at 4°C in dark boxes for 15 days. Slides were developed, fixed and counterstained with Toluidine Blue before examination.

RT–PCR

Total RNA was isolated by the RNeasy method (Qiagen, Hilden, Germany) from isolated highly purified cytotrophoblastic cells cultured on plastic and from isolated cytotrophoblastic cells cultured on Matrigel®. The total RNA was treated with RNase-free DNase I (Life Technologies, France) at 22°C for 5 min.

For Cx43 transcripts, total RNA (5 µg) was transcribed into cDNA using oligo(dT)$_{12–18}$ as primer and superScriptII reverse transcriptase (Roche). One-fifth of the reaction mixture was amplified with *Taq* polymerase (Life Technologies) in a final volume of 50 µl. For the semi-quantitative PCR, each cycle consisted of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min and primer extension at 72°C for 1 min. In order to quantify amplification in exponential phase, primers of GAPDH were added at cycle 8 and PCR products (10 µl) were appropriated at cycles 23, 24, 25, 26 and 27. Primers were designed from previously published sequence data: the human GAPDH 5′ sense CTGCACCACCAACTGCTTAG-3′ and 5′ antisense AGGTCCACCACTGACACGTT-3′; human Cx43 5′ sense AGTCTATC-TTTGAGGTGGCC-3′ and 5′ antisense GGCTGTAATTCATGTCCAGC-3′. Reaction products were resolved on an agarose gel (1.5%) and stained with ethidium bromide. Sizes of the expected amplification products were 1148 base pairs (bp) for Cx43 and 275 bp for GAPDH.

For Cx40 transcripts, RNA samples (350 ng per assay) were reverse-transcribed using the Superscript II reverse transcriptase and oligo(dT)$_{12–18}$ primer (Gibco–BRL, Cergy Pontoise, France). One-third of the cDNA was then used in each PCR reaction. For amplification of the endogenous Cx40 messenger, two primers were chosen in the coding region of the Cx40 human gene to amplify a 500 bp fragment: forward primer S40H, 5′-GCCACGCCATGCACACTGTG-3′, and reverse primer AS40H, 5′-GCTC-TGGACTATGCCCACAGAG-3′. Control PCR were performed on total RNA in order to exclude any genomic DNA contamination. Each PCR reaction was performed with 2.5 IU *Taq* DNA polymerase (Promega, Charbonnières, France) in a 50 µl final volume in the presence of an appropriate 1× buffer plus 25 mmol/l $MgCl_2$, 50 ng of each primer and 20 mmol/l of each dNTP. Amplification was done in a UNOII Biometra thermal–cycler and consisted of 95°C for 3 min, 30×(94°C for 15 s, 60°C for 30 s, 72°C for 40 s) and 72°C for 5 min. After amplification, RT–PCR products were run on an ethidium bromide-stained 1.2% agarose gel and photographed.

Gap-FRAP method

The cell-to-cell diffusion of a fluorescent dye was measured by the gap-FRAP method (Wade *et al*., 1986), using an interactive laser cytometer (ACAS 570; Meridian Instruments, Okemos, MI, USA), which allows for convenient digital video imaging and analysis. After washing, cultured trophoblastic cells were loaded for 10 min at room temperature in saline solution containing the membrane-permeant molecule 6-carboxyfluorescein diacetate (Sigma: 7 µg/ml in 0.25% dimethylsulphoxide). This lipophilic compound is hydrolysed by cytoplasmic esterases to 6-carboxyfluorescein, a hydrophilic derivative which accumulates inside the cells. After washing off the excess extracellular fluorogenic ester to avoid further loading, the fluorescence of some selected cells adjacent to others cells was photobleached by applying strong light pulses from an argon laser set at 488 nm. The fluorescence intensity was recorded in the bleached cells before and after photobleaching for 12 min (each time period = 2 min). In each experiment, one labelled isolated cell, left unbleached, served as a reference for the loss of fluorescence due to repeated scanning and dye leakage, and an isolated bleached cell served as a control. When the return of fluorescence followed a fast step-like course, reaching ⩾90% of the final steady state in <30 s after photobleaching, this indicated that the diffusion of dye was neither prevented by the cell membrane nor limited by the presence of gap junctions. It was inferred that fusion of cell membranes had been completed and that the cellular elements were part of a true syncytium. When the bleached cells were interconnected by open gap junctional channels to unbleached contiguous cells, a fluorescence recovery following a slow exponential time course was measured (Figure 1D) (Cronier *et al*., 1994b; Furger *et al*., 1996). Therefore, the analysis of the kinetic of fluorescence recovery allows discrimination between aggregated cytotrophoblastic cells and syncytiotrophoblast. In our experimental conditions, GJIC was investigated (coupled cells or not) in a population of cells in contact, leading to a percentage of coupled cells (Figure 4B).

Table II. First trimester connexin staining patterns at the fetal–maternal interface, as assessed by immunolocalization

	Floating villi		Anchoring villi	
			Cell column CTB	
	VCTB	STB	Proximal	Distal
Cytokeratin 07	++	++	++	++
Ki67	+	−	++	−
Cx43	+	+	−	−
Cx40	−	−	+++	−
Cx33	−	−	−	−
Cx32	−	−	−	−
Cx26	−	−	−	−

The intensity of staining was estimated by two observers and the results averaged.
VCTB = villous cytotrophoblastic cell; STB = syncytiotrophoblast; CTB = cytotrophoblastic cell.

Results

Connexin expression and GJIC in the villous trophoblast

Among all the connexins tested, only Cx43 was detected in the trophoblast of the floating villi (Table II). *In situ*, Cx43 immunostaining was observed on most villi. Punctate Cx43 immunofluorescence was observed between cytotrophoblastic cells, between cyto- and syncytiotrophoblast and in the syncytiotrophoblastic cytoplasm (Figure 1A–C). Cx43 immunofluorescence was also observed in the mesenchymal cells of the villi. In controls performed by omission of the primary antibody or by using non-specific IgG of the same isotype, no immunoreactivity could be detected (data not shown). In-situ hybridization with Cx43 antisense riboprobe revealed a high accumulation of silver grains in the trophoblastic layers (cytotrophoblastic cells and syncytiotrophoblast: 3.428 ± 0.006 grains/10 µm^2) and in the mesenchymal core of the villi (0.563 ± 0.005 grains/10 µm^2) (Figure 2B). Control sections incubated with the sense probe did not reveal any specific hybridization (Figure 2A).

Villous cytotrophoblastic cells were cultured in 10% FCS on plastic dishes. After 12 h, cells began to emit protrusions and pseudopodia making initial contacts with neighbouring cells. Later, groups of cytotrophoblastic cells in close apposition were observed, consistent with an aggregation stage. Finally, after 72 h, large cellular masses with central nuclei mount and expanding cytoplasm were seen, thus confirming earlier studies (Dodeur *et al*., 1990; Tarrade *et al*., 2001). Only Cx43 was detected in trophoblastic cells cultured on plastic dishes. After 24 h, a strong punctate Cx43 immunofluorescence was mainly observed at the intercellular contacts between pseudopodia of

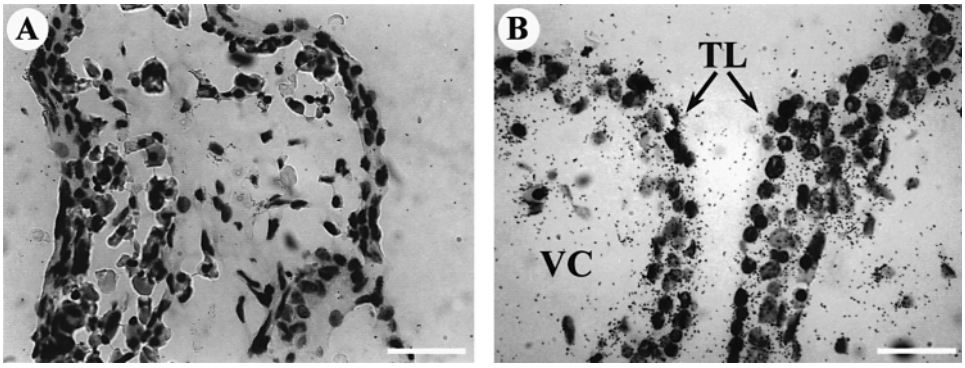

Figure 2. Cx43 in-situ hybridization in first trimester villous section. (**A**) The hybridization signal is absent in control section with the RNA sense probe; (**B**) Localization of Cx43 mRNA in first trimester floating villous section hybridized with ^{35}S-labelled antisense Cx43 riboprobe. The hybridization signal is strong within the trophoblastic layer (TL: 3.428 ± 0.006 grains/10 μm^2) and is also present in the stroma of the villous core (VC: 0.563 ± 0.005 grains/10 μm^2) compared to the intervillous space (0.400 ± 0.002 grains/10 μm^2). Scale bars = 20 μm.

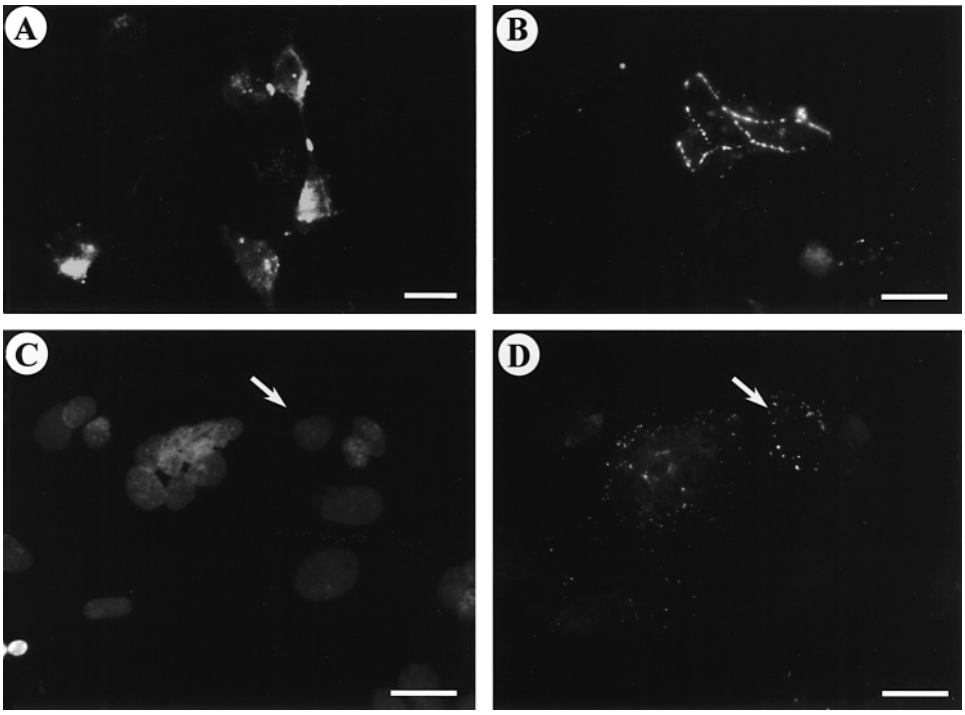

Figure 3. (**A**, **B**) Immunofluorescent localization of Cx43 protein in first trimester trophoblastic cells cultured on plastic dishes. Strong Cx43 spots are observed at the contact between pseudopodia after 24 h (**A**) and at the intercellular boundaries after 48 h (**B**). (**C**, **D**) Double immunofluorescence for Cx43 (**D**) and for nuclear staining with DAPI (**C**) after 72 h of culture. Cx43 punctate immunofluorescence is observed around the cluster of nuclei of a syncytiotrophoblast and at the border of a cytotrophoblastic cell which is still in contact with the syncytiotrophoblast. Scale bars = 10 μm.

neighbouring cells (Figure 3A). After 48 h, Cx43 immunofluorescence was detected at the intercellular boundaries between aggregated cells (Figure 3B). After cellular fusion, the signal disappeared from the cell membrane and appeared in intracytoplasmic spots (Figure 3C, D). No immunoreactivity could be detected in controls. Cx43 expression was confirmed by the presence of Cx43 transcripts in freshly isolated cytotrophoblastic cells and in cytotrophoblastic cells cultured for 72 h (Figure 4A). A single band for the Cx43 PCR product was observed at the expected size (1148 bp) in similar levels in cytotrophoblastic cells and syncytiotrophoblasts. No amplified DNA fragments were identified in the absence of template (data not shown) or in RNA samples incubated without RT (Figure 4A, lanes 1 and 3).

Functionality of the intercellular channels was demonstrated by means of gap-FRAP. Typical changes in the fluorescence intensity of selected cells within a field before and after the photobleaching procedure are shown in Figure 1D, as well as the corresponding fluorescence recovery curves. This slow exponential fluorescence recovery characteristic of GJIC was recorded between cytotrophoblastic cells, between cytotrophoblastic cells and syncytiotrophoblasts and between contiguous syncytiotrophoblasts. Gap junctional coupling was present at all stages of differentiation (Figure 4B) in 5, 14.3 and 15.6% of the trials after 1, 2 and 3 days of culture respectively.

Connexin expression and GJIC in the extravillous trophoblast of the cell column

Due to the use of curettage which disrupts the integrity of the implantation site, the connexin immunostaining was mainly examined in the cell columns of the anchoring villi. *In situ*, a strong punctate Cx40 immunofluorescence was detected between the proliferative cytotrophoblastic cells of the proximal part of the column cells (Figure 5A and B). Expression became weak or absent in the distal part of the column. Other connexins were not detected in the extravillous trophoblasts of the cell columns (Table II).

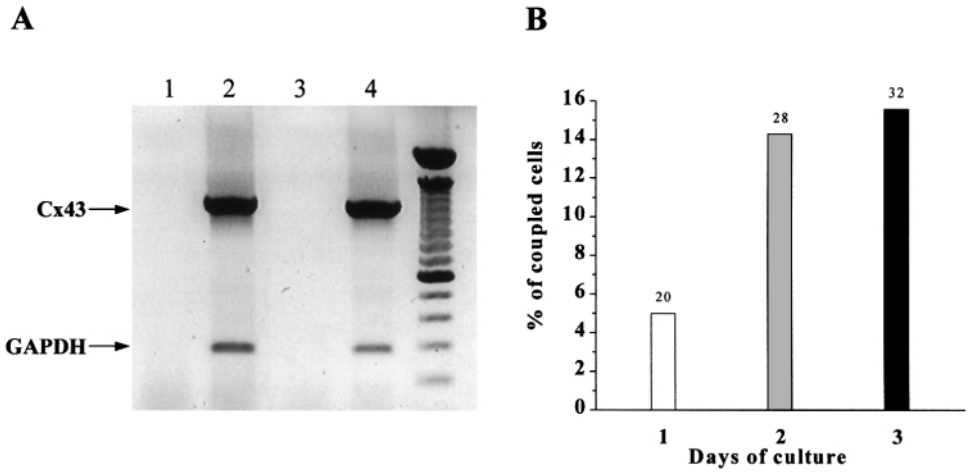

Figure 4. (**A**) Expression of Cx43 mRNA in villous trophoblastic cells as detected by RT–PCR. The expected size of the PCR products is 1148 bp for Cx43. Lane 2: extracts from highly purified cytotrophoblastic cells immediately after cell isolation; lane 4: extracts from trophoblastic cells after 3 days of culture. Lanes 1 and 3 represent respective controls without RT. A 100 bp DNA ladder was used for size analysis. The results shown are representative of three independent experiments. (**B**) Evolution of functional intercellular communication measured by means of fluorescence recovery after the photobleaching method in cultured villous trophoblastic cells. The percentages of coupled cells were measured after 1, 2 or 3 days of culture on plastic dishes. The numbers of intercellular contacts analysed are indicated on top of the bars. GAPDH = glyceraldehyde 6-phosphate dehydrogenase.

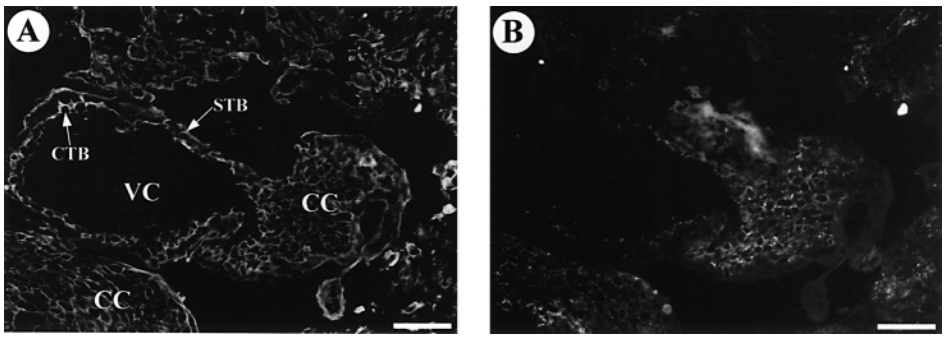

Figure 5. First trimester anchoring villi section double-immunolabelled for cytokeratin 07 (**A**) and Cx40 protein (**B**). Anticytokeratin antibody stained villous cytotrophoblastic cells (CTB), syncytiotrophoblast (STB), cytotrophoblastic cells of the cell columns (CC) and the invasive cytotrophoblastic cells. Punctate immunofluorescence for Cx40 is observed between the cytotrophoblastic cells of the proximal part of the cell column and on the membrane of some invasive cytotrophoblastic cells. Scale bar = 20 μm. VC = villous core.

When first trimester isolated cytotrophoblastic cells were cultured on Matrigel-coated dishes, 4 h after seeding ~60% of cells adhered. They adhered as isolated or aggregated cells but never fused during the first 2 days. During the first 3 days of culture, a cytokeratin 07 immunofluorescence was observed (data not shown). After 2 days of culture, α5β1 integrin immunofluorescence was also observed (Figure 6A, B). Furthermore, a strong punctate Cx40 immunofluorescence was detected between contiguous cells (Figure 6C, D). After 3 days of culture, a Cx32 immunofluorescence was also observed between some aggregated cells (Figure 6F). Cx40 expression was confirmed by the presence of Cx40 transcripts in freshly isolated cytotrophoblastic cells and in cytotrophoblastic cells cultured on Matrigel for 48 h (Figure 6E). A single band for the Cx40 RT–PCR product was observed at the expected size (500 bp) in both specimens (Figure 6E; lanes 1 and 3). No amplified DNA fragments were identified in the absence of template (data not shown) or in RNA samples incubated without RT (Figure 6E, lanes 2 and 4).

To elucidate the presence of a GJIC between cells cultured on Matrigel, the gap-FRAP method was used on cytotrophoblastic cells for 1, 2 or 3 days. GJIC was never detected between contacting cells whatever the period investigated.

Discussion

This study demonstrated the presence of Cx43 (protein and transcript) and of GJIC in first trimester villous trophoblast. Staining with cytokeratin 07, a trophoblast-specific marker, clearly demonstrated the trophoblastic origin of the Cx43 staining. The presence of Cx43 transcripts in villous trophoblast was illustrated by the in-situ hybridization signal and by RT–PCR in isolated and cultured cells. Villous trophoblastic cells in culture are currently used to study certain aspects of the dynamic process occurring during villous differentiation (Kliman *et al*., 1986). The additional step of purification with a monoclonal anti-HLA A, B and C rules out the contamination by other cells containing Cx43 proteins and transcripts such as endothelial and mesenchymal cells. In these conditions, RT–PCR analysis showed that Cx43 transcripts are similarly present in freshly purified cytotrophoblastic cells and in trophoblasts cultured for 3 days (partly syncytiotrophoblast). This result is consistent with Northern blotting analysis showing that the presence of Cx43 transcripts seemed to stay constant during culture of trophoblastic cells (Winterhager *et al*., 1999). It should be noted that numerous studies did not examine functional coupling between the cells, but merely analysed the presence of gap junction RNA/protein, whereas gap junctional

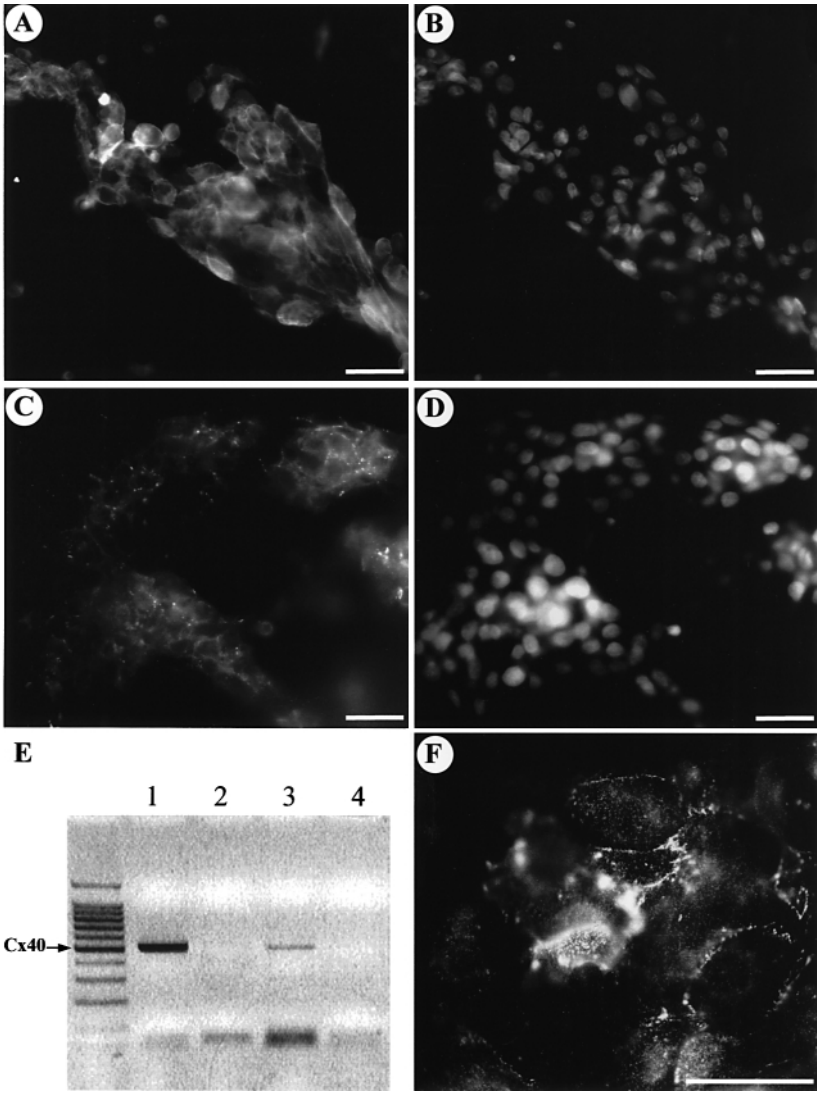

Figure 6. (**A**, **B**) Double immunofluorescence for α5β1 integrin (**A**) and for nuclear staining with DAPI (**B**) in first trimester cytotrophoblastic cells cultured on Matrigel for 48 h. A strong labelling is observed at the periphery of the aggregated cells. Scale bars = 20 μm. (**C**, **D**) Double immunofluorescence for Cx40 (**C**) and for nuclear staining with DAPI (**D**) in first trimester cytotrophoblastic cells cultured on Matrigel for 48 h. Punctate immunofluorescence is observed at the border of aggregated cells. Scale bars = 15 μm. (**E**) Detection of the Cx40 transcript in trophoblastic cells from different stages of development. Lane 1: 500 bp amplified Cx40 cDNA in freshly isolated cytotrophoblastic cells of first trimester placentae. Lane 3: 500 bp amplified Cx40 cDNA in cytotrophoblastic cells of first trimester placentae cultured on Matrigel. Lanes 2 and 4 are corresponding controls of lanes 1 and 3 with the absence of RT. (**F**) Immunofluorescent localization of Cx32 protein in first trimester trophoblastic cells cultured on Matrigel for 3 days. Cx32 spots are observed at the border of some aggregated cells. Scale bar = 20 μm.

communication levels do not necessarily correlate with connexin expression levels (Lo, 1999). Thus, in considering the role of gap junction in differentiation, functional analysis of gap junctional coupling is of critical importance. We have demonstrated for the first time that the presence of Cx43 gap junctional channels allows a functional cell–cell communication between all types of elements in contact during the differentiation of the first trimester villous trophoblastic cells. In culture, the quantitative evolution of GJIC paralleled the morphological differentiation. These data are consistent with our previous study with term trophoblastic cells showing that the ability of villous trophoblastic cells to develop a transient GJIC was a prerequisite for the formation of the syncytiotrophoblast (Cronier *et al*., 1994a). In addition, another role for the presence of gap junction channels between the two trophoblastic layers (cellular and syncytial) of the first trimester villi could be the inter-trophoblastic diffusion and transfer of small molecules (second messengers and nutrients) as suggested for glucose in the labyrinth of the rat placenta (Takata and Hirano, 1997; Gabriel *et al*., 1998).

In the extravillous pathway, the cytotrophoblastic cells proliferate, migrate and invade the decidua and its vasculature. In the proximal part of the cell column, Cx40 expression was demonstrated between cytotrophoblastic cells. This localization correlates with the nuclear expression of the proliferation marker Ki67 (Kaufmann and Castellucci, 1997). To elucidate the functional role of Cx40 expression, isolated cytotrophoblastic cells were cultured on Matrigel. We confirm that in the presence of Matrigel, trophoblastic cells never fused during the first 2 days and that the cells expressed the α5β1 integrin after 2 days of culture (Tarrade *et al*., 2001). This fibronectin receptor immunostaining confirms the extravillous phenotype of these cells (Burrows *et al*., 1993; Damsky *et al*., 1994). A strong Cx40 immunofluorescence was observed between these contiguous cells. This result is substantiated by the presence of Cx40 transcripts. Furthermore, a

recent ultrastructural study demonstrated the presence of gap junctions in the proximal cytotrophoblastic cells of the columns (Enders *et al*., 2001). The functionality of Cx40 expression was therefore investigated by gap-FRAP. In our experimental conditions, no gap junctional communication was demonstrated between cells. Interestingly, in human choriocarcinoma cells expressing Cx40, an extremely low level of electrical coupling has been measured (Hellmann *et al*., 1996). This issue is important because some studies have suggested that electrical coupling through gap junctions may be possible whereas the transfer of fluorescent molecules is restricted (Warner, 1988; Dale *et al*., 1991). It is interesting to note that Cx40 shows a restricted expression pattern in mammals: heart, vascular smooth muscle cells and vascular endothelium (Gros *et al*., 1994). Despite the fact that recent electrophysiological studies support the hypothesis of an ionic coupling in cell lines transfected by Cx40 and Cx43 (Cottrell and Burt, 2001; Valiunas *et al*., 2001), it is currently admitted that Cx40 cannot form functional heterotypic channels with most other connexins (Bruzzone *et al*., 1996). Thus, it could be hypothesized that cells of the proximal part of the column cannot communicate with the villous cytotrophoblastic cells (expressing Cx43), thus preventing them from a villous phenotype differentiation process. A similar model has been proposed in carcinogenesis (Krutovskikh and Yamasaki, 1997) where the absence of communication between tumour cells and surrounding normal cells maintained the dedifferentiated proliferative phenotype of the transformed cell population.

In the distal cell column, when the cytotrophoblastic cells are leaving the column, Cx40 immunostaining disappeared. According to Winterhager *et al*. (1999), Cx40 expression is missing during trophoblast migration and was re-expressed in trophoblastic aggregates within the decidua. Furthermore, in the trophoblastic aggregates in the placental bed, Cx32 and Cx43 immunostaining have been detected (Al-Lamki *et al*., 1999; Cronier *et al*., 2001). These results support the hypothesis of the implication of these connexins in the fusion process leading to the multinucleated cells of the human placental bed (Boyd and Hamilton, 1970).

Recently, principles concerning placental development have emerged using gene knock out approaches in mice (Rossant and Cross, 2001). Due to the striking diversity in placental structure, caution must be exercised in extrapolating findings regarding placental development from one species to another. In rat, a switch in connexin gene expression (Cx31, Cx26, Cx43) was associated with trophoblast development (Reuss *et al*., 1996) and in mice Cx26 and Cx31 deficiencies were shown to cause placental alterations (Gabriel *et al*., 1998; Plum *et al*., 2001). In the study of human placenta, Cx26 and Cx31 was not found to be expressed and a specific connexin expression pattern (Cx43, Cx32, Cx40) was demonstrated during trophoblast differentiation.

In conclusion, this study demonstrated the presence of Cx43 allowing an inter-trophoblastic GJIC and this appeared to be associated with the fusion process leading to the villous syncytiotrophoblast. Moreover, the presence of Cx40 was associated with the extravillous phenotype but not with GJIC.

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