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Status of p53 in first-trimester cytotrophoblastic cells

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p53 has been called the cellular gatekeeper of the genome because it can induce cell-cycle arrest in G1, apoptosis or affect DNA replication in response to DNA damage. As p53 has been observed in first-trimester cytotrophoblastic cells (CTB), but its expression in normal cells is generally not detectable because of its short half-life, p53 could play an important role in cellular differentiation and/or in the control of the invasion of trophoblastic cells; therefore, p53 status was investigated in these cells. Using different antibodies recognizing different epitopes of p53 protein, abundant p53 expression was observed both in nuclear and in cytoplasmic compartments of first-trimester CTB. Whereas p53 was detected in the nuclei of few trophoblastic cells with an antibody recognizing the N-terminal epitope of the protein, high expression level of p53 in the cytoplasm of CTB was detected with an antibody recognizing the middle part of p53. The lack of immunoreactivity of p53 with antibodies recognizing the epitopes located at the N-terminus of p53 and the high level of p53 protein observed in the cytoplasm of CTB suggest that the N-terminus of p53 is involved in the formation of complexes. These cytoplasmic complexes were detected under non-reducing conditions in western blot analysis and had apparent molecular weights (MW) of 195, 167 or 125 kDa. These complexes could prolong the half-life of p53 in the cytoplasm of CTBs. By contrast, in the nuclei of CTBs, p53 seems to be present as a tetramer.

Key words: complex/CTB/cytoplasm/nucleus/p53

Introduction

The transcription factor p53 activates the transcription of its target genes by binding to a specific consensus DNA sequence consisting of two copies of a 10-bp DNA motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ separated by 0–13 bp (El-Deiry *et al*., 1992). Wild-type (wt) p53, but not mutants of p53, can efficiently bind the p53-binding element. By binding to DNA, p53 activates genes involved in DNA repair, thus avoiding DNA damage to be carried over to daughter cells. p53 is thus a tumour suppressor. As stated by Levine (1997), p53 is the 'gatekeeper' of the genome. When p53 is mutated or otherwise altered in its expression, as in many cancers, it looses this tumour-suppressor activity and becomes oncogenic (Matsumuto *et al*., 2006). The status of p53 in a cell is thus directly related to the acquisition of an invasive phenotype. A previous study suggested that p53 might regulate trophoblast invasion by up-regulating the expression of matrix metalloproteinase-2 (MMP-2) (Bian and Sun, 1997), but p53 is known to be a multifunctional protein that exerts different effects. p53 could also play a role in cellular differentiation in various cell types (Almog and Rotter, 1997).

Immunohistochemical studies of first-trimester trophoblast have shown that p53 is detectable in the nucleus of cytotrophoblasts and faintly in syncytiotrophoblasts (Haidacher *et al*., 1995; Marzusch *et al*., 1995; Quenby *et al*., 1998). Wt p53 is generally not detectable by immunohistochemistry because of its short half-life; consequently, its presence in cytotrophoblasts suggests that p53 could be overexpressed in these cells to control excessive trophoblastic proliferation in normal placentation (Marzusch *et al*., 1995). Three hypotheses have already been presented to account for the accumulation of p53 in cancer cells: the presence of p53 mutant (Royds and Iacopetta, 2006), the presence of spliced variants (Wu *et al*., 1994; Flaman *et al*., 1996; Courtois *et al*., 2002; Yin *et al*., 2002; Maier *et al*., 2004; Bourdon *et al*., 2005) and the sequestration of p53 in cytoplasm (Aladjem *et al*., 1998; Zaika *et al*., 1999). Another type of regulation of the p53 protein level is its rate of degradation, which is known to be mediated by the Mdm-2–ubiquitin–proteasome degradation pathway (Haupt *et al*., 1997; Kubbutat *et al*., 1997). The aim of this study was to understand why p53 is overexpressed in cytotrophoblastic cells (CTB).

Materials and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), Hanks' balanced salts and antibiotics mixture (penicillin and streptomycin) were products of Invitrogen (Basel, Switzerland). Fetal bovine serum (FBS) was from Biochrom AG (Oxoid AG, Basel, Switzerland). Complete mini cocktail inhibitor tablets were from Roche (Basel, Switzerland). BIO-RAD protein assay and Trans-Blot transfer medium were from Bio-Rad (Munich, Germany). Hybond-N+ membrane, rainbow-stained protein molecular weight (MW) markers and enhanced chemiluminescence (ECL) western blotting detection system were from Amersham Biosciences (Buckinghamshire, UK). Goat polyclonal glyceraldehyde-3-phosphatedehydrogenase (GAPDH)-specific antibody was from Santa Cruz (CA, USA). Mouse monoclonal wt p53-specific antibody clone Pab1620, mouse monoclonal p53 antibody clone Pab240, DO-1 and PAb421 were from Oncogene (Stehelin, Basel, Switzerland). Mouse monoclonal vimentin-specific antibody clone V9 and mouse monoclonal cytokeratin 7 clone OV-TL 12/30 were from Dako (Dako Schweiz AG, Baar, Switzerland). 'Seize primary mammalian immunoprecipitation' kit was from Pierce (Pierce, Rockford, USA). The proteasome inhibitor MG132 was from Sigma (Sigma-Aldrich, Switzerland).

Immunohistochemistry

First-trimester trophoblasts (n = 10) were obtained from patients undergoing a legal abortion and who gave their written informed consent. Breast cancer

tissue (used as positive control) was obtained as anonymized paraffin sections from our pathology department and used as positives controls. This study was approved by our departmental ethics committee. Tissues were rapidly washed with 0.1 mol/l phosphate buffer at pH 7.4 and fixed for 4–12 h in 4% buffered formalin at 4°C. The specimens were then dehydrated in ethanol and embedded in paraffin wax.

Serial sections of tissue were deparaffinized and rehydrated through graded ethanol. Antigen retrieval was performed by microwave pretreatment in 0.01 mol/l citrate buffer (pH 6.0) for 5 min four times, followed by cooling in a cold water bath. Non-specific binding was blocked with 5% (v/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 20 min at room temperature. The sections were incubated with different primary antibodies specific for p53 (dilution 1/10) in 1% BSA–PBS overnight at 4°C. Sections were then washed with PBS (5 min), and p53 was visualized by further incubations with biotinylated anti-mouse secondary antibody (dilution 1/250), alkaline phosphatase-labelled conjugate (dilution 1/2) and Fast Red substrate (Dako, USA). Counterstaining was performed with hemalun.

CTB purification

Placental tissue was obtained from patients undergoing a legal abortion during the first trimester (7–12 weeks of gestation). Informed written consent was obtained from all patients before their inclusion in the study, for which approval was obtained from the local ethics committee. CTBs were isolated from first-trimester placentas and immunopurified (by negative adsorption on immobilized CD45 antibodies) as described elsewhere (Bischof *et al*., 1995) and grown in DMEM high glucose/F-12 containing 10% FBS and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin) at 37°C in a humidified 5% CO_2 atmosphere. Purity of the final cell preparation was evaluated by immunocytochemistry using cytokeratin-7 as a marker of CTBs and vimentin as a marker of non-epithelial cells. Less than 5% of the cells stained for vimentin, and 95% were cytokeratin-7-positive. Cells were treated or not with MG132 in serum-free medium for 24 h before lysis. Protein concentration was assayed with the Biorad assay.

Subcellular fractionation

Plated CTBs were rinsed in Hanks' balanced salts, trypsinized and collected by centrifugation (800 *g*, 10 min). The pelleted cells were rinsed in ice-cold PBS buffer (0.01 M sodium phosphate, 138 mM NaCl and 2.7 mM KCl, pH 7.4) and collected by centrifugation. The pellets were resuspended in 20 volumes of 10 mM Hepes buffer containing 1.5 mM $MgCl_2$, 10 mM NaCl and 0.5 mM dithiothreitol (DTT) and a Roche inhibitor cocktail tablet pH 7.9. Cell suspensions were incubated on ice for 30 min and collected by centrifugation. The pellet was resuspended in 10 volumes of 10 mM Hepes containing 1.5 mM $MgCl_2$, 10 mM NaCl and 0.5 mM DTT, 0.5% nonidet P40 and homogenized gently by passing the suspension at least five times through a 20-gauge needle fitted to a syringe. Nuclear fraction was obtained by centrifugation at 1000 *g* for 10 min. The supernatant was collected for cytosolic analysis. Nuclei were resuspended by gentle homogenization in 0.88 M sucrose and 3 mM $MgCl_2$ and centrifuged at 2500 *g* for 20 min to remove cell debris. The pellet was resuspended in PBS buffer and stored at –80°C until use. Assay for the cytoplasmic marker enzyme lactate dehydrogenase (LDH) was performed on each nuclear fraction to determine cytoplasmic contamination (Graham, 1993). No LDH activity was detected in our fraction.

LDH assay

Nuclear fraction (4 µg of protein) was incubated in sodium phosphate buffer, 0.1 M, pH 7, with sodium pyruvate (0.125 mg) and NADH (0.125 mg) for 30 min. The assay measured the rate of NADH absorbance decrease at 340 nm.

Immunoprecipitation

Seize primary mammalian immunoprecipitation kit was used for the elimination of antigen contamination which could interfere with the detection of p53. It was performed according to the manufacturer's instructions. The antibodies PAb240, PAb-1620 and DO-1 were used to immunoprecipitate p53 from CTB cell extracts.

Western blot

Proteins were reduced or not (sample buffer containing or not DTT) and denatured by boiling at 100°C for 10 min. Samples were then subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using a 10% running gel. Rainbow-stained MW markers were used as standards. Proteins (40 µg) were electro-transferred to nitrocellulose membranes. Non-specific binding was blocked for 30 min at 37°C with 5% powdered milk in 0.2% NP40 buffer. p53-specific antibodies (diluted 1/1000) were incubated overnight with the nitrocellulose membrane. After washing, the membranes were incubated with the appropriate horse-radish peroxidase (HRP)-linked secondary antibody (2 h, room temperature). After washing, the bands were revealed by chemiluminescence (ECL detection kit). Films were scanned with an Epson Perfection 1 200 Photo scanner, and the surface of the bands was measured by the Kodak 1D Image analysis software (Kodak, Rochester, NY, USA).

Cloning and sequencing of trophoblastic p53

RNA was extracted from CTBs purified from three separate placentas, and RT–PCR was performed. Primers were directed against ATG and Stop codon, amplifying the entire coding region. Amplicons were purified and cloned into pGEM. Inserts were sequenced from three individual clones, and sequences were compared with known human TP53 sequence.

Results

We immunolocalized p53 in first-trimester trophoblast using different antibodies recognizing different epitopes of the p53 protein (Figure 1). As shown in Figure 2, the cytoplasm but not the nuclei of villous and extravillous CTB (Figure 2A) and breast cancer tissue (Figure 2C) is highly positive for p53 protein when Pab240, specific for a mutated p53 conformation, and Pab421 (results not shown) antibodies were used. By contrast, PAb 1620, an antibody specific for the wt conformation of p53 protein, only few nuclei of CTBs were positive (Figure 2B), whereas in breast cancer specimens used as control, most nuclei and some cytoplasmic compartments (Figure 2D) were positive.

To verify the status of p53 in CTB, we cloned and sequenced p53 cDNA of CTB. The observed p53 sequence corresponded to the one published for the wt p53 (results not shown).

To study putative p53 protein isoforms, we immunoblotted cell lysates and subcellular fractionations of CTB using different antibodies recognizing different epitopes of the p53 protein. As shown in Figure 3, all antibodies used recognized four main protein bands (84, 64, 53 and 35 kDa) in whole cell lysates. Intensities of the bands varied with the antibodies.

p53 was then immunolocalized with the DO-1 antibody in subcellular fractionation of CTB. As shown in Figure 4, under reducing conditions, p53 is present both in cytoplasmic and in nuclear fractions in CTB. Similar results were obtained with CTBs from 7, 10 and 11 weeks of gestation (data not shown). The predominant protein isoforms in the cytoplasm were 64- and 53-kDa proteins, whereas the most abundant proteins in the nuclear fraction were 53- and 35-kDa proteins. When western blots were performed under non-reducing conditions, three more high-MW p53 isoforms were observed at 125 and 195 kDa in the cytoplasm and at 220 kDa in the nuclear fraction of CTB.

Immunoprecipitation of p53 from CTB cell extracts with PAb-240, PAb-1620 and DO-1 and immunoblotting with the same p53 antibodies were performed under non-reducing conditions. As shown in Figure 5, immunoprecipitation of p53 with PAb240 revealed two complexes of p53 with apparent MWs of 220 and 195 kDa irrespective of the anti-p53 used to visualize it, whereas only one complex with an apparent MW at 220 kDa was detected when p53 was immunoprecipitated with PAb1620 or DO-1 antibodies. Two minor p53 complexes could be detected when p53 was immunoprecipitated with PAb240 and immunoblotted with PAb-1620 and PAb-240. These complexes had apparent MWs of 125 and 167 kDa.

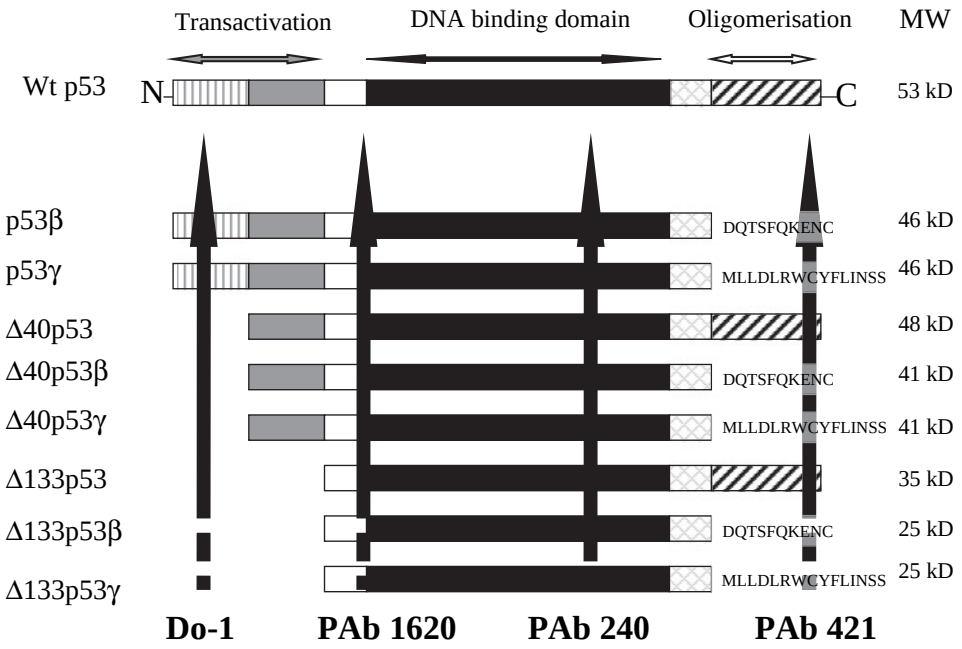

Figure 1. Structure of wild-type (wt) and published isoforms of p53 (Bourdon *et al*., 2005) and epitope specificity of p53 antibodies (DO-1, PAb1620, PAb240 and PAb421).

As p53 is overexpressed in CTB, whereas its expression is generally not detectable because of its short half-life, we next examined the activity of the p53 degradation pathway in CTBs using a proteasome inhibitor. As shown in Figure 6, the degradation pathway of p53 is present and active in CTBs because inhibition of the proteasome increases the p53 levels.

Discussion

Localization studies of p53 in trophoblasts have so far been performed with N-terminus-specific antibodies. As described previously (Haidacher *et al*., 1995; Marzusch *et al*., 1995; Quenby *et al*., 1998), we confirm here that p53 is immunohistochemically detectable in human first-trimester trophoblast. However, while staining is found only in a few nuclei of CTBs with N-terminus-specific p53 antibodies, intense staining in the cytoplasm of villous and extravillous trophoblast was obtained, with the PAb240 antibody recognizing an epitope located at amino acids 213–217 and characteristic of the mutated conformation of the protein. These observations would suggest that two different forms of p53 exist in CTB. Furthermore, because antibodies recognizing the N-terminus of p53 did not localize p53 in the cytoplasm of trophoblasts, but did detect p53 in breast cancer samples, one could conclude that the N-terminal epitope of cytoplasmic p53 might be absent or masked in CTB. However, western blot analysis of cellular extracts of CTB, performed under reducing conditions with four different p53 antibodies recognizing different domains of the protein, revealed four identical proteins with apparent MWs of 84, 64, 53 and 35. By comparison with a western blot performed on SAOS-2 cells that do not contain p53 mRNA, background staining in different cells was observed with different p53 antibodies (pAb240, 246, 1620, 421, 1801 and to a lesser extent Do-1) at ~80 kDa (Bonsing *et al*., 1997). So it is probable that the protein with an apparent MW of 84 kDa observed in western blot of CTBs is non-specific to p53. Several p53 protein isoforms have been described in different cell types (Wu *et al*., 1994; Flaman *et al*., 1996; Courtois *et al*., 2002; Yin *et al*., 2002; Maier *et al*., 2004; Bourdon *et al*., 2005) but never in first-trimester CTB. All described p53 proteins are truncated at the N- or C-terminus (Figure 1). Because all antibodies used here recognized a protein with an apparent MW of 35 kDa, it is unlikely to be an isoform of p53 or a proteolytic fragment as described by Courtois *et al*. (2004). A 35-kDa protein band was detected in three different p53-free yeast strains and thus considered as non-specific (Nickels *et al*., 1997). Although in our hand cloning and sequencing of p53 cDNA from CTB revealed only wt p53 in these cells, our experimental conditions cannot rule out the presence of quantitatively less important splice variants.

A p53 protein with an apparent MW of 64 kDa has already been described as ADP-ribosylated p53 (Wesierska-Gadek *et al*., 1996). ADP-ribosylation could modulate the balance between folded and unfolded species and could stabilize and regulate the biological activity of p53.

Immunoblotting of subcellular fractionations of CTB revealed that p53 (both 53- and 64-kDa p53 proteins) can be observed both in nuclear and in cytoplasmic fractions of these cells. Moreover, under non-reducing conditions, the presence of p53 complexes with an apparent MW of 220 kDa was found in the nuclei of CTB and might represent tetramers of p53 because these complexes disappear under reducing conditions. Tetramerization of p53 seems to be important for the binding of p53 to the DNA-binding elements of its target genes (Weinberg *et al*., 2004). Two other complexes were found in the cytoplasmic and not in the nuclear fractions of CTBs with apparent MWs of 195 and 125 kDa. After immunoprecipitation of p53 under non-reducing conditions, the same complexes were detected in the cellular extracts of CTB using the PAb240 antibody. Because, under these conditions, no protein with an apparent MW of 50 kDa was detected, it is suggested that, in CTB, most p53 protein is complexed. The antibody specific for the N-terminus of p53 did not recognize the 195-kDa complex and two minor complexes (167 and 125 kDa), suggesting that the N-terminal epitope of p53 might be masked by the formation of a protein complex. Other proteins might be involved in these high-MW complexes and could sequester p53 in cytoplasm of CTBs as described in neuroblastoma cells or in embryonic stems cells (Aladjem *et al*., 1998; Zaika *et al*., 1999). Modifications in the p53 structure (conformational

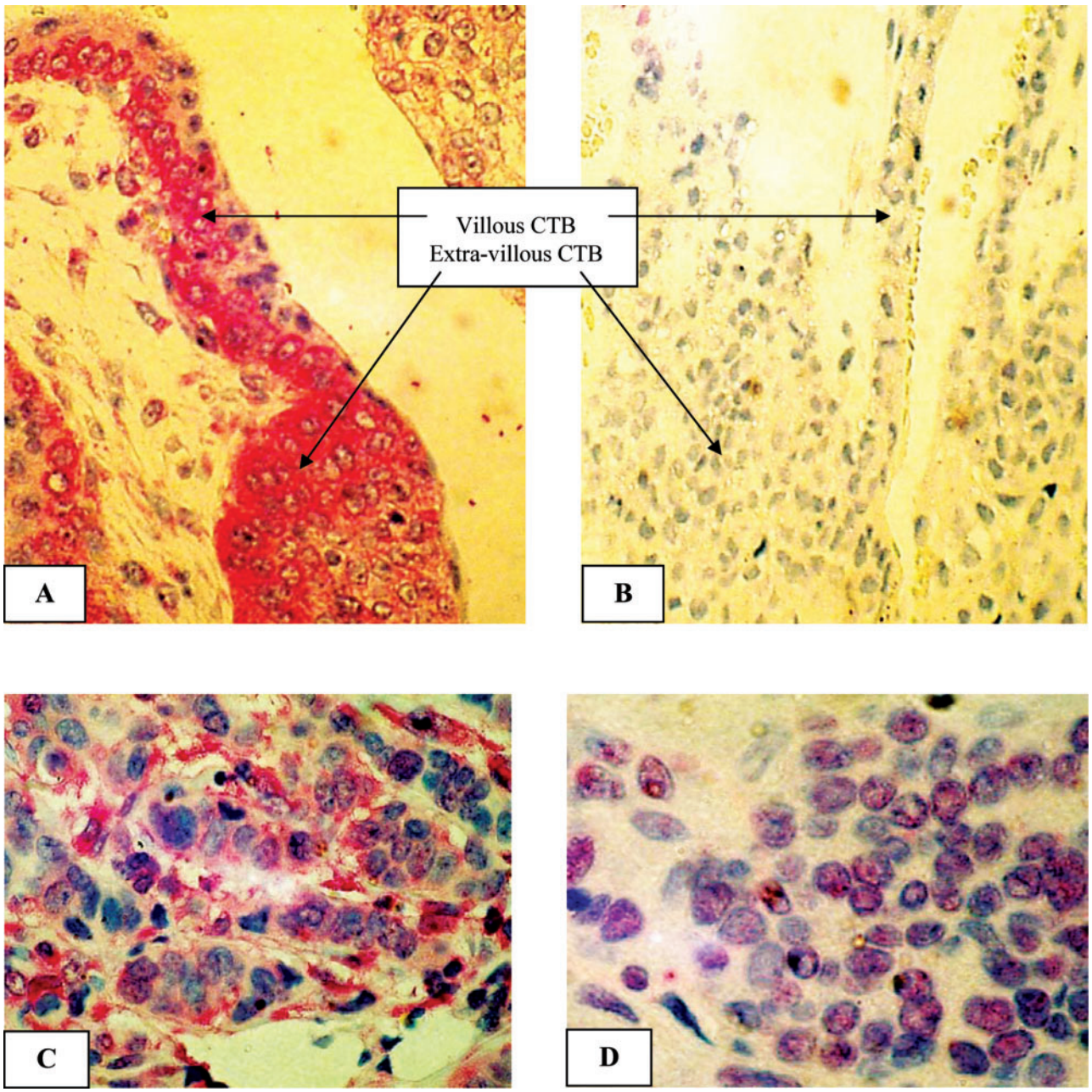

Figure 2. Immunohistochemistry for p53 expression [**A** and **C** clone: PAb240, p53-specific antibody; **B** and **D** clone: PAb1620, wild-type (wt) p53-specific antibody] in first-trimester trophoblast (**A**, **B**) and breast cancer tissue (**C**, **D**). Magnification ×400.

modification or interaction with other proteins) have already been described during differentiation of cells (Almog and Rotter, 1997).

Because the use of proteasome inhibitors in CTB showed that the degradation pathway of p53 is active in these cells, one would speculate that the observed complexes in the cytoplasm could prolong the half-life of p53 in the cytoplasm of CTB.

In conclusion, abundant p53 expression was observed both in nuclear and in cytoplasmic compartments of first-trimester CTB. In normal cells, wt p53 protein is present at very low concentration because of its rapid degradation mainly directed by MDM2 (Harris and Levine, 2005). The high expression level of p53 in the cytoplasm of CTBs and the lack of immunoreactivity of p53 with antibodies recognizing the epitope located at the N-terminus of p53 suggest that, in CTB, the N-terminus of p53 is involved in the formation of complexes with apparent MWs of 195, 167 or 125 kDa that are located in the

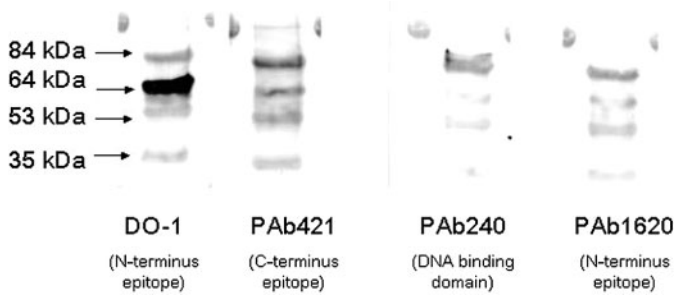

Figure 3. Western blots of whole cytotrophoblastic (CTB) lysates using DO-1, PAb421, PAb240 and PAb1620 antibodies under reducing conditions.

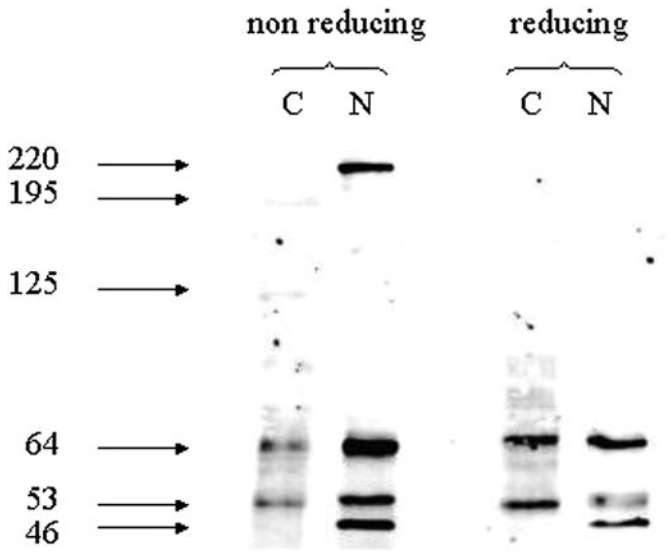

Figure 4. Western blot of cellular fractionations of cytotrophoblastic cells (CTB) performed under non-reducing and reducing conditions and probed with DO-1. C, cytoplasmic fraction; N, nuclear fraction.

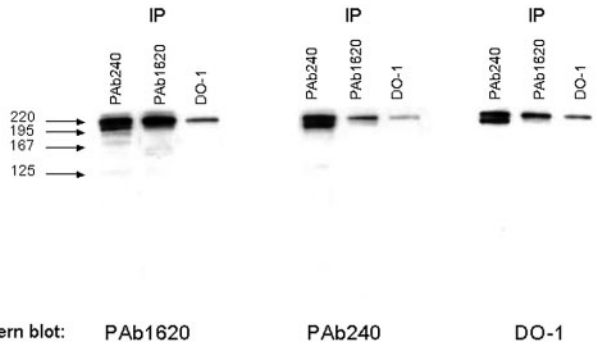

Figure 5. Western blot of immunoprecipitated p53 from cytotrophoblastic cells (CTB) using PAb240 (1), PAb1620 (2) and DO-1 (3) under non-reducing conditions. Western blots were probed with PAb240, PAb1620 and DO-1. IP, immunoprecipitated.

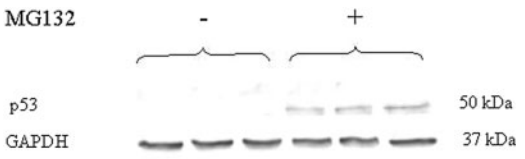

Figure 6. Western blots of cytotrophoblastic (CTB) cell lysates treated or not with MG132 were probed with DO-1 anti-p53 antibody and samples run under reducing conditions. Glyceraldehyde-3-phosphatedehydrogenase (GAPDH) control is shown.

cytoplasm. These complexes could prolong the half-life of p53 in the cytoplasm of CTB. By contrast, in the nuclei of CTB, p53 seems to be present as a tetramer and is recognized by other antibodies than those recognizing the cytoplasmic form. The overexpression of p53, its particular localization, and the presence of p53 high-MW complexes in CTBs suggest that p53 could regulate the invasion of trophoblast. Indeed, MMP-2 is a target gene for p53 (Bian and Sun, 1997), and this enzyme is instrumental in trophoblast invasion during implantation and placentation (Cohen *et al*., 2006). However, the influence of these cytoplasmic p53 high-MW complexes is unknown so far.

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