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ORIGINAL PAPER

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Expression of the Thomsen-Friedenreich antigen and of its putative carrier protein mucin 1 in the human placenta and in trophoblast cells in vitro

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Abstract The Thomsen-Friedenreich (TF) antigen (or, more precisely, epitope Galβ1–3GalNAcα-O-) has been known for a long time as a carcinoma-associated antigen. In normal tissues the occurrence of TF antigen is restricted to a few immunologically privileged areas. Here we report on the identification of the TF epitope and its putative carrier protein mucin 1 (MUC1) in human placental tissue, on isolated trophoblast cells in vitro and on trophoblast tumour cell lines BeWo and Jeg3. Cryosections of placental and decidual tissues of the first, second and third trimester were double stained with monoclonal antibodies directed against the TF epitope (IgM) and against MUC1 (IgG). In the first trimester of pregnancy we found strong expression of TF antigen and MUC1 at the apical side of the syncytiotrophoblast directed towards the maternal blood. This expression was consistent in the second trimester of pregnancy, and to a lesser degree in the third trimester. In addition, we found positive staining for TF antigen and MUC1 on extravillous trophoblast cells in the decidua during the first and second trimester of pregnancy. Trophoblast tumour cells of the cell line BeWo, which form a syncytium in vitro, were also positive for TF antigen and MUC1, whereas Jeg3 cells, which are unable to form a syncytium, expressed only MUC1. Freshly isolated trophoblast cells from first trimester placentas showed strong staining for MUC1; however, only a few of these cells (less than 1%) were positive for TF antigen, and might consist of digested fragments of the syncytium. In summary, TF antigen and MUC1 are expressed by the syncytiotrophoblast at the feto-maternal interface and by extravillous trophoblast cells invading the decidua, whereas villous cytotrophoblast cells in situ as well as freshly isolated trophoblast cells from first trimester placentas only express MUC1 but not TF antigen.

Keywords Thomsen-Friedenreich · Mucin 1 · Placenta · Trophoblast cells · Decidua

Introduction

The Thomsen-Friedenreich (TF) antigen (or, more precisely, epitope) has been known for a long time as a tumour-associated antigen (Springer 1984). The presence of TF antigen during an early fetal phase, its absence in non-carcinomatous postfetal tissues and its association with carcinomas suggest that TF antigen is a stage-specific oncofetal carbohydrate antigen. The TF antigen is a carbohydrate epitope related to blood group antigens and consists of galactose-β1–3*N*-acetylgalactosamine (Galβ1–3GalNAc-). In epithelial cells, the TF epitope is carried by mucin 1 (MUC1), which belongs to a family of highly glycosylated proteins present on the apical surface of many glandular epithelial cells. On tumour cells MUC1 is post-translationally modified resulting in incomplete *O*-glycosylation and exposing the TF epitope.

In normal adult human tissues TF epitopes occur only in limited amounts (Cao et al. 1996). The TF antigen is, however, expressed by fetal epithelia and mesothelia (Barr et al. 1989). In addition it is also found on transferrin isolated from human amniotic fluid (van Rooijen et al. 1998) and produced by the trophoblast (Streu et al. 2000). Carbohydrate structures identical to those found on the feto-maternal interface also play a primary role in the initial gamete binding (Oehninger et al. 1998). For-

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eign embryonic cells such as the syncytiotrophoblast in the placenta may be protected by surface expression of oligosaccharide sequences that suppress immune effector cell action in a manner not dependent upon classic major histocompatibility (MHC) recognition (Clark et al. 1996). Widely used models for in vitro studies in placental research are primary cultures of trophoblast cells (Blaschitz et al. 2000) as well as trophoblast tumour cell lines (King et al. 2000). Here we report on the immunohistochemical identification of the TF-carbohydrate antigen and of its carrier protein MUC1 in the human placenta at different stages of gestation, in isolated trophoblast cells and in trophoblast tumour cells of the cell lines BeWo and Jeg3.

Materials and methods

Tissue samples

Samples of human placentas were obtained from first trimester (16 placentas, 6th to 12th week of gestation), second trimester (16 placentas, 14th to 26th week of gestation) and third trimester (15 placentas, 27th to 40th week of gestation). The study was approved by the ethics committee of the University of Rostock.

Materials

Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}, antibiotic and antimycotic solutions, Ultroser G and trypsin-EDTA were obtained from GibcoBRL Life Technologies (Paisley, UK). Trypsin type 3 and DNase I were obtained from Sigma (Taufkirchen, Germany) and HEPES buffer (1 M) and inactivated fetal calf serum (FCS) came from Biochrom (Berlin, Germany).

Trophoblast cell purification and cell culture

Trophoblast cells were isolated from three different human first trimester placentas obtained after legal abortion. Tissue dissected from the placenta was minced and transferred to HBSS-HEPES buffer containing trypsin (10,000–130,00 U/mg) and DNase I (0.1 mg/ml). The cell suspensions obtained by three digestion steps were separated from tissue fragments. The first step of trophoblast cell purification was performed on a Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient. Trophoblast cells collected at the interphase were removed, washed and incubated with an anti-CD45 monoclonal antibody (Dianova, Hamburg, Germany) and an anti-CD9 monoclonal antibody (Dianova) followed by rat anti-mouse magnetic MicroBeads (Miltenyi Biotec, Bergisch-Gladbach, Germany). For the depletion of leukocytes, cells were applied to a MiniMACS column (Miltenyi Biotec), and purified trophoblast cells were obtained. Trophoblast identity of the isolated cells was determined morphologically by light microscopy, by their ability to produce specific hormones (hCG, progesterone, hPL) and by immunostaining with a monoclonal mouse anti-cytokeratin 7 antibody (Progen Biotechnik, Heidelberg, Germany). Cells were cultured in DMEM medium with 10% inactivated FCS and with antibiotics and antimycotics.

The chorionic carcinoma cell lines BeWo and Jeg3 were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were cultured in DMEM medium with 10% inactivated FCS without antibiotics and antimycotics.

Immunohistochemistry

For the immunohistochemical characterisation cryosections from first trimester, second trimester and term placental tissue were examined. All samples were fixed in 5% buffered formalin. Antibodies used for the experiments are listed in Table 1. They were diluted to 10 µg/ml with PBS and incubated with the slides overnight at 4°C. After washing, Cy2-labelled goat anti-mouse IgM or Cy3-labelled goat anti-mouse IgG (both Dianova), diluted 1:200, served as second antibody. Alternatively, Cy2-labelled goat anti-mouse IgG and Cy3-labelled goat anti-mouse IgM (Dianova), diluted 1:200, were used as second antibody in some cases. The slides were finally embedded in mounting buffer containing *p*-phenylenediamine as antifading agent (Johnson et al. 1982; Oriol and Mancilla-Jimenez 1983) or 4′,6-diamino-2-phenylindole (DAPI) resulting in green (*p*-phenylenediamine) or blue (DAPI) (Jeppesen and Nielsen 1989) staining of the nucleus. Slides were examined with a Zeiss (Jena, Germany) Axiophot photomicroscope. Digital images were obtained with a digital camera system (CF20DXC; KAPPA Messtechnik, Gleichen, Germany) and saved on computer.

Immunocytochemistry

BeWo, Jeg3 and isolated trophoblast cells were grown on three-well multitest slides (Roth, Karlsruhe, Germany) to subconfluency, dried, wrapped and stored at –80°C. After thawing, cells were briefly fixed with formalin (Merck, Darmstadt, Germany; 5% in PBS, 5 min) and permeabilised with digitonin (Ysat, Wernigerode, Germany; 6 µg/ml in PBS, 15 min). Both treatments do not interfere with binding even in cases where epitopes are known to be sensitive to formalin (Cao et al. 1997a). Antibodies were diluted

Table 1 Antibodies used in the study. (*CK* Cytokeratin, *Sup.* supernatant, *MUC1* epithelial mucin 1, *TF* Thomsen-Friedenreich antigen, *LCA* leucocyte common antigen)

Antigen	Antibody	Isotype	Concentration/ dilution	Source	Reference
CD 9 (p24)	CBL 560	Mouse IgG	1:100	Dianova	
CD 45 (LCA)	CBL 124	Mouse IgG	1:100	Dianova	
CK 7	Ks 7.18	Mouse IgG	1:50	Progen Biotechnik	
CK (broad range)	A45 B/B3	Mouse IgG	10 µg/ml	Own antibody; Cy3-labelled by Micromet	Kasper et al. (1987)
Glycodelin A	A87-D/F4	Mouse IgG	Sup. 1:5	Own antibody	Mylonas et al. (2000)
MUC1 (CD227)	A76-A/C7	Mouse IgG	Sup. 1:5	Own antibody	Price et al. (1998)
MUC1 (CD227)	BC3	Mouse IgM	5 µg/ml	ISOBM TD-4 Workshop 1996	Price et al. (1998)
MUC1 (CD227)	214D4	Mouse IgG	5 µg/ml	ISOBM TD-4 Workshop 1996	Price et al. (1998)
TF (CD176)	A78-G/A7	Mouse IgM	Sup. 1:5	Own antibody	Karsten et al. (1995)

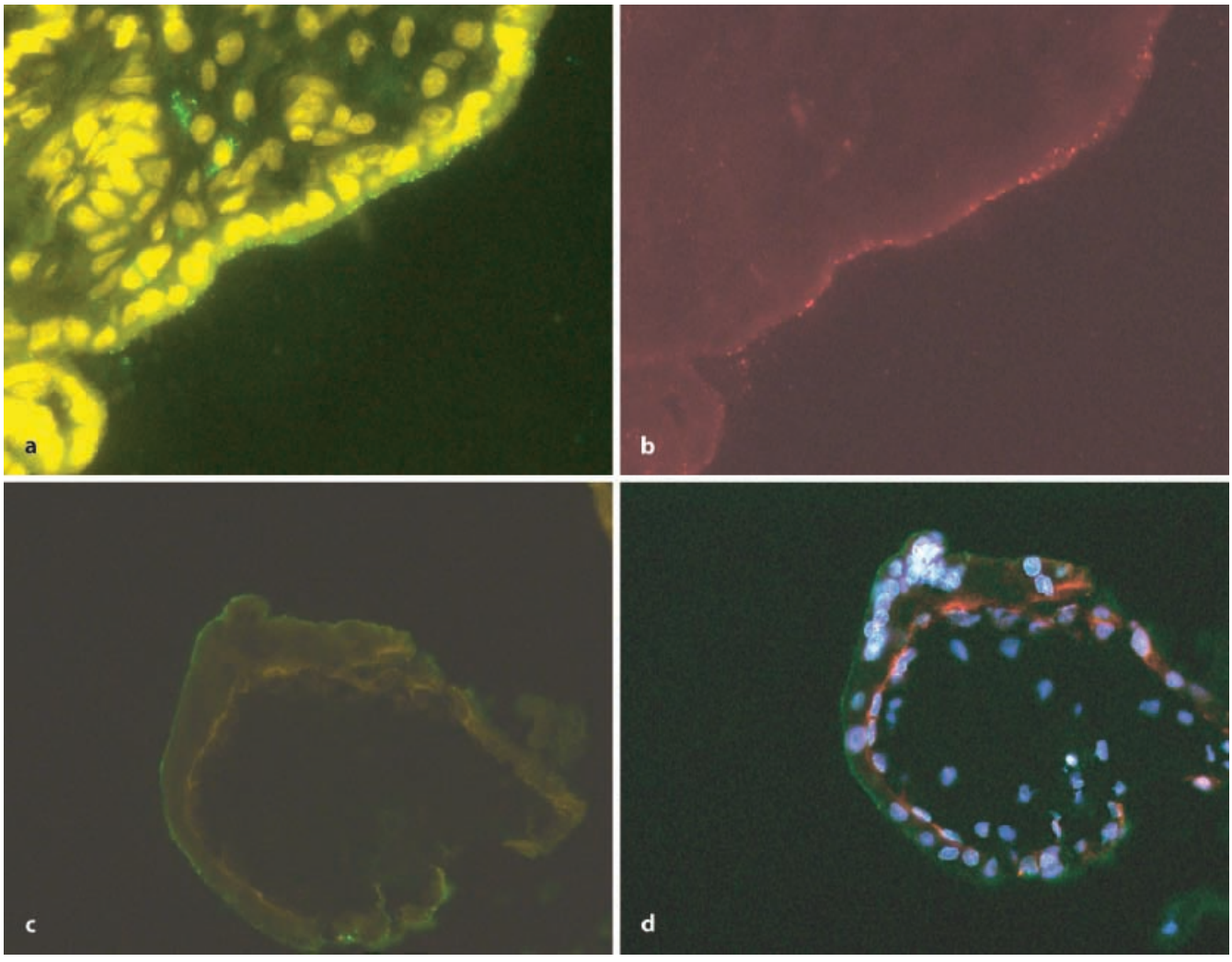

Fig. 1 Demonstration of the Thomsen-Friedenreich (TF) antigen in the syncytiotrophoblast layer of the placenta, 21st week of pregnancy (**a**; A78-G/A7, Cy2-labelled second antibody). The antifading agent *p*-phenylenediamine was added producing the yellowish-brown fluorescence in the nuclei. MUC1 is double stained with TF antigen in the syncytiotrophoblast (**b**; A76-A/C7, Cy3-labelled second antibody), 63× lens. TF antigen is expressed at the apical cell surface of the syncytiotrophoblast that is directed towards the maternal blood (**c**), 40× lens. Double staining of the TF antigen (**d**; A78-G/A7, Cy2-labelled second antibody) and cytokeratin (A45-B/B3, Cy3-labelled) in the syncytiotrophoblast, 10th week of pregnancy. 4′,6-Diamino-2-phenylindole (DAPI) was added producing the blue reaction in the nucleus. Triple filter excitation, 40× lens

to 10 µg/ml with cell culture medium, and the slides were further treated as described in the immunohistochemistry section.

Deglycosylation

As control experiments of the carbohydrate nature of the epitopes recognised by the anti-TF antibodies periodate treatment was performed. Periodate oxidation of tissue sections was performed according to Woodward et al. (1985), as described in Cao et al. (1997b). This treatment destroys glycotopes containing galactose, *N*-acetylgalactosamine, or fucose, but does not damage peptide epitopes.

Results

Thomsen-Friedenreich antigen and MUC1 in the syncytiotrophoblast

We observed a strong expression of both TF antigen and MUC1 in the syncytiotrophoblast layer of the placenta in all three trimesters of pregnancy after staining with the anti-TF antibody A78-G/A7 and with the anti-MUC1 antibody A76-A/C7, respectively. Simultaneous expression of TF antigen and MUC1 on the same section was examined by co-incubation with A78-G/A7 (IgM) and A76-A/C7 (IgG) followed by class-specific second antibodies. Results are documented in Fig. 1a, b. Although MUC1 was more broadly distributed on the syncytiotrophoblast than TF antigen, expression of TF antigen was restricted to MUC1-positive sites in agreement with the assumed carrier function of MUC1 for the TF epitope. Figure 1c shows that the expression of TF on the surface of the syncytiotrophoblast is directed to the maternal blood side. In addition, TF antigen-positive trophoblast cells expressed cytokeratin (Fig. 1d). The expression of the TF antigen in the syncytiotrophoblast decreased with gestational age, which confirms its nature as an oncode-

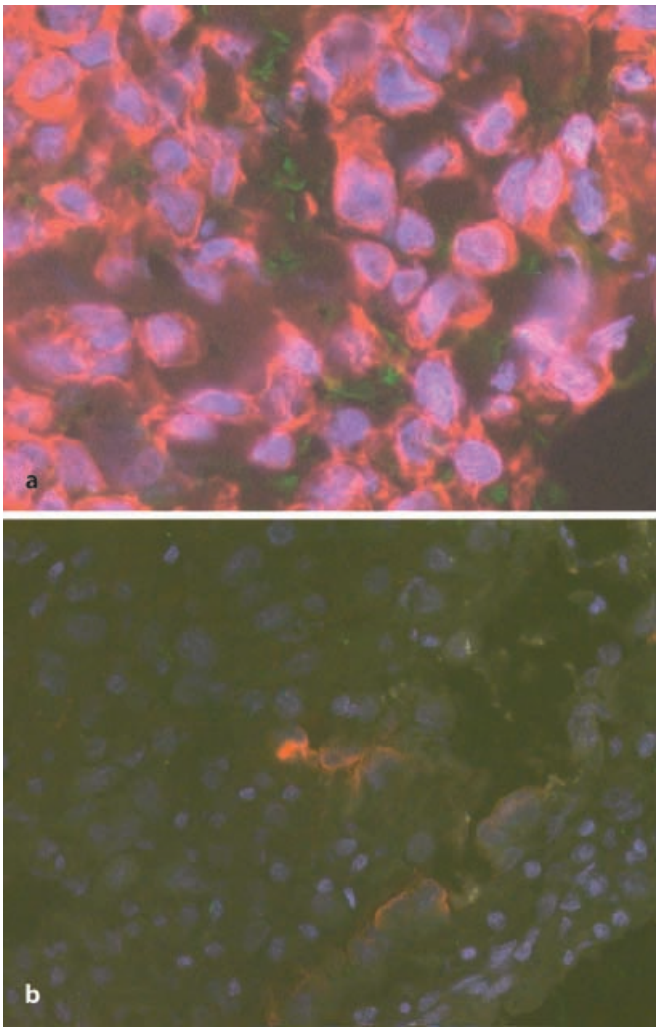

Fig. 2a, b Double staining of the TF antigen (A78-G/A7, Cy2-labelled second antibody) and cytokeratin (A45-B/B3, Cy3-labelled) in the decidua, 10th week of pregnancy, counterstaining with DAPI. Triple filter excitation (**a**), 63× lens. After deglycosylation with sodium periodate decidua cells are positive for MUC1 (A76-A/C7, Cy3-labelled second antibody) and negative for TF antigen (A78-G/A7, Cy2-labelled second antibody) (**b**), counterstaining with DAPI. Triple filter excitation, 40× lens

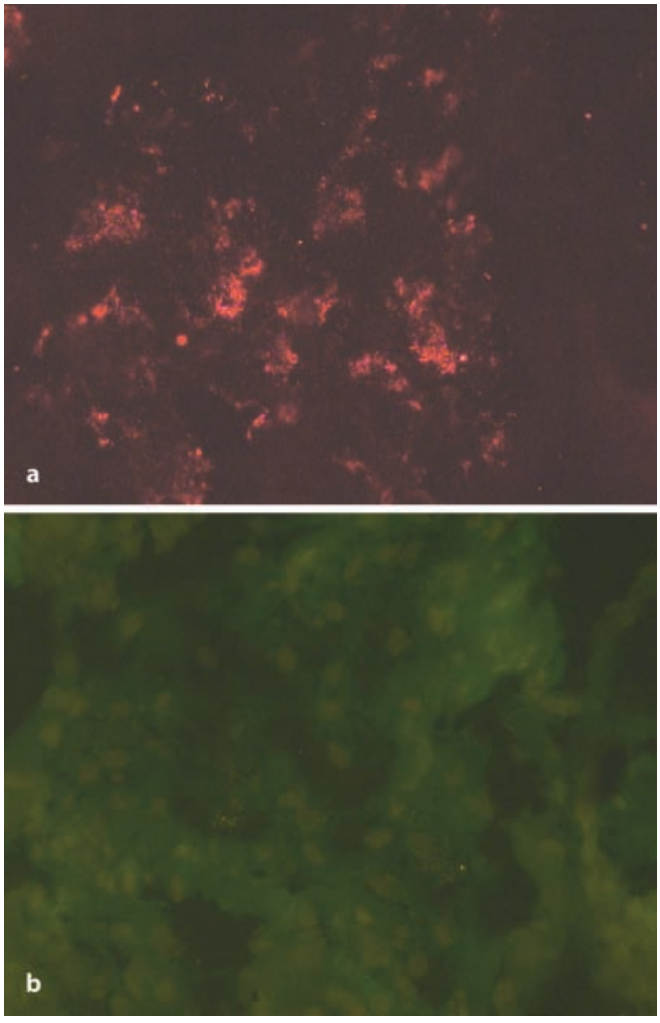

Fig. 3 TF antigen-positive decidua cells (**a**; A78-G/A7, Cy3-labelled second antibody) do not express CD45 (**b**; CBL 124, Cy2-labelled second antibody), 10th week of pregnancy, 40× lens. The brownish counterstaining of nuclei in **b** is due to the addition of the antifading agent, *p*-phenylenediamine, to the embedding medium. This is also the case with Figs. 4, 5a, 6b and 8b

velopmental marker. In the first trimester of pregnancy 80% to 100% of the syncytiotrophoblast expressed the TF epitope. Also in the second trimester of pregnancy 70% to 90% of the syncytiotrophoblast expressed the TF epitope. In the third trimester of pregnancy, only 16% to 33% of the whole syncytiotrophoblast showed a positive staining for the TF antigen.

Thomsen-Friedenreich antigen and MUC1 in the decidua

The decidua showed positive staining for both TF antigen and MUC1. However, the TF epitope in cells of the decidua was detectable only in the first and second trimester of pregnancy. For the identification of the TF antigen-positive cell type, slides were co-incubated with the anti-TF antibody A78-G/A7 (IgM) and the anti-cytokeratin (CK) antibody A45-B/B3 (IgG, Cy3-labelled). TF antigen-positive cells showed a positive CK expression (Fig. 2a). After deglycosylation with sodium periodate slides were co-incubated with anti-TF antibody A78-G/A7 and with the anti-MUC1 antibody A76-A/C7. Figure 2b demonstrates that cells in the decidua after deglycosylation were only positive for MUC1 and not for TF antigen.

Additional slides were co-incubated with the anti-TF antibody A78-G/A7 (IgM) and the anti-CD45 antibody CBL 124 (IgG). TF antigen-positive cells were CD45 negative (Fig. 3a, b). A third set of slides was incubated with the anti-TF antibody A78-G/A7 (IgM) and the anti-glycodelin A antibody A87-D/F4 (IgG). Glycodelin A-positive cells of the decidua were TF antigen negative (data not shown).

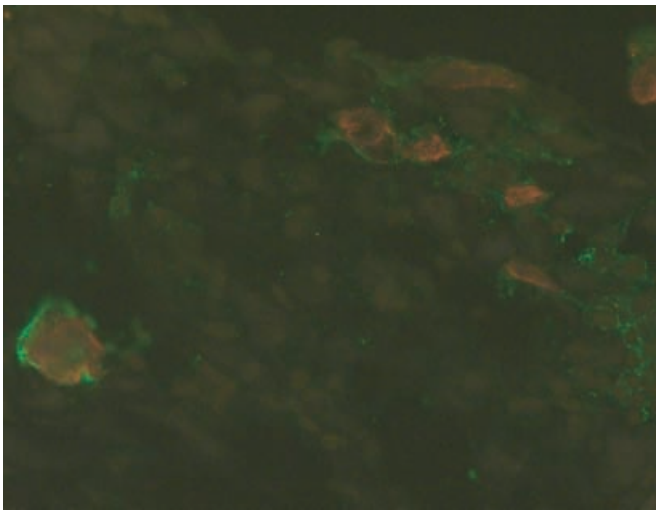

Fig. 4 Double staining of MUC1 (A76-A/C7, Cy2-labelled second antibody) and cytokeratin (A45-B/B3, Cy3-labelled primary antibody) in the decidua, 21st week of pregnancy. Triple filter excitation, 40× lens

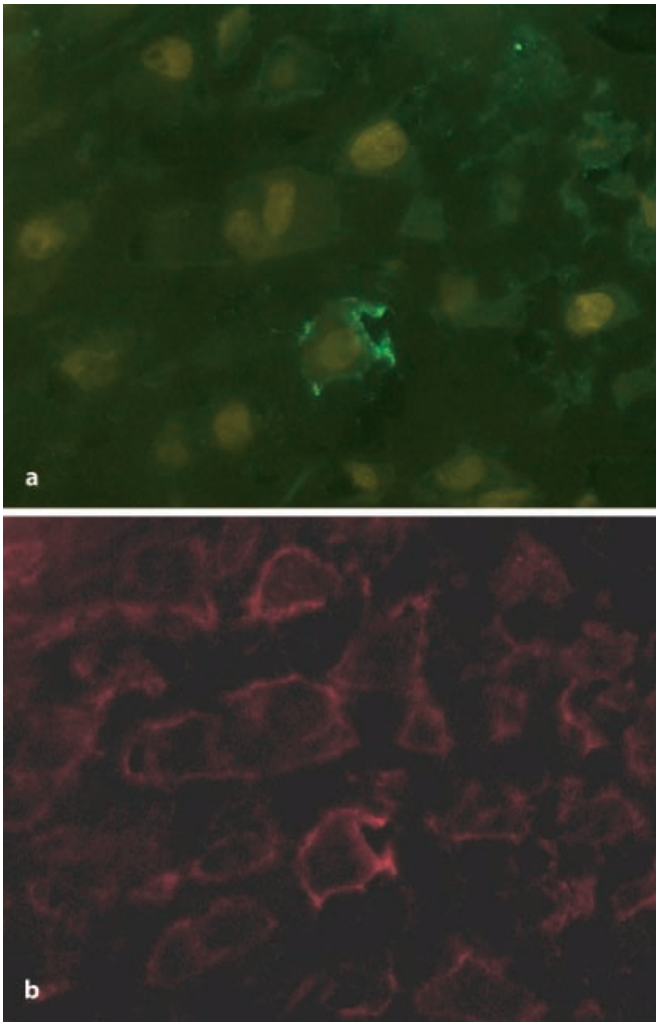

Fig. 5 Partial double staining of MUC1 (**a**; A76-A/C7, Cy2-labelled second antibody) and CD9 (**b**; CBL 560, Cy3-labelled second antibody) in the decidua, 10th week of pregnancy, 63× lens

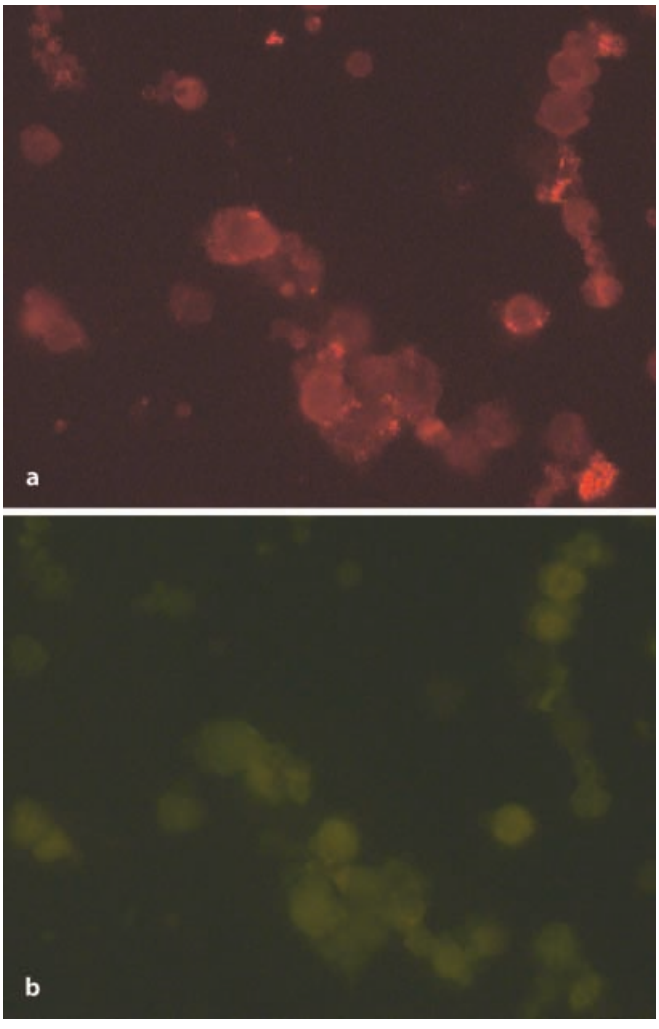

Fig. 6 Expression of MUC1 on freshly isolated trophoblast cells (**a**; A76-A/C7, Cy3-labelled second antibody); these cells do not express TF antigen (**b**; A78-G/A7, Cy2-labelled second antibody), 63× lens

For the identification of MUC1-positive cells, slides were co-incubated with the anti-MUC1 antibody BC3 (IgM) and the anti-CK antibody A45-B/B3 (IgG). Other slides were simultaneously incubated with the anti-MUC1 antibody BC3 (IgM) and the anti-CD9 antibody CBL 560 (IgG). MUC1-positive cells showed expression of CK (Fig. 4) and CD9 as well (Fig. 5a, b).

Thomsen-Friedenreich antigen and MUC1 in isolated trophoblast cells and in trophoblast tumour cell lines BeWo and Jeg3

Isolated trophoblast cells from the first trimester of pregnancy showed strong expression of MUC1 (Fig. 6a) and no expression of the TF antigen (Fig. 6b). We could also detect multinucleated cell conglomerates at a ratio of less than 1% of the total cell number on the slides which were TF antigen positive (data not shown). Trophoblast

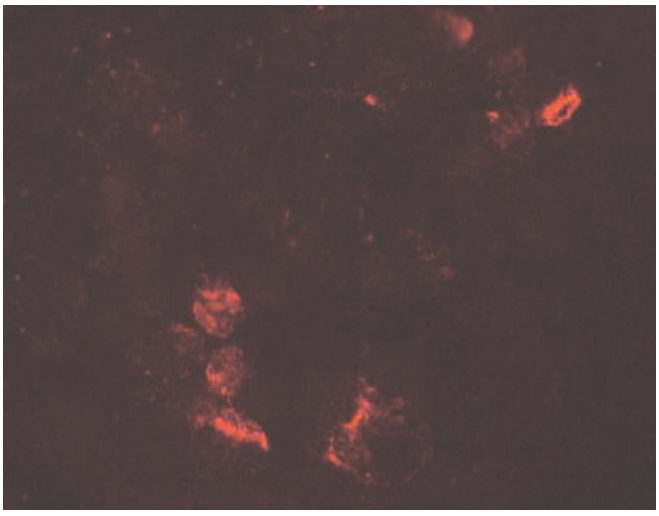

Fig. 7 Expression of MUC1 on trophoblast cells of the malignant cell line Jeg3 (A76-A/C7, Cy3-labelled second antibody), 40× lens

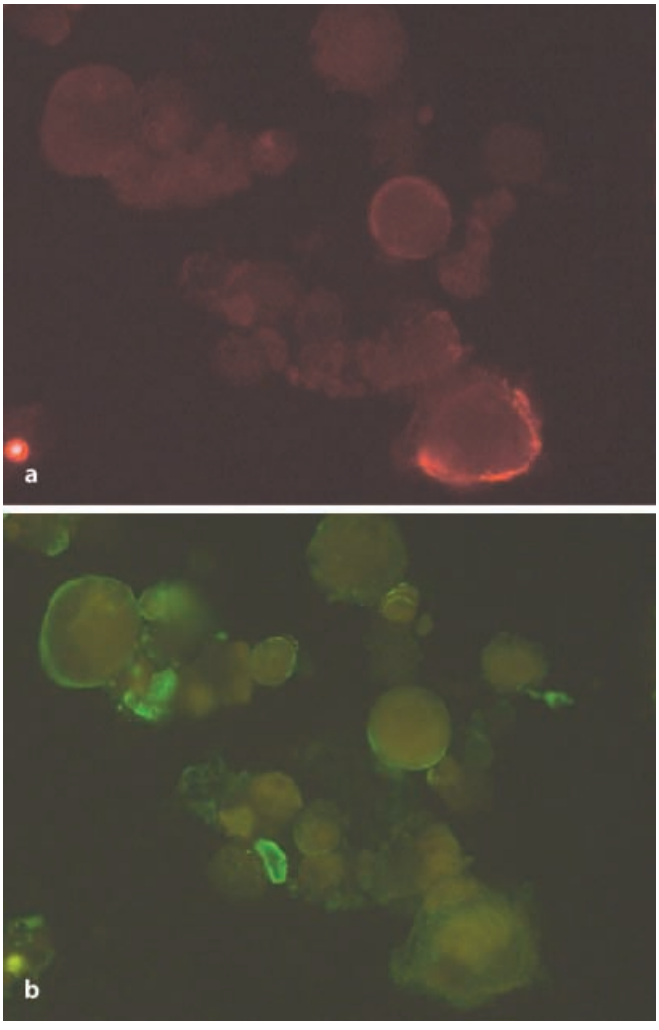

Fig. 8 Expression of MUC1 by trophoblast tumour cells of the cell line BeWo (**a**; A76-A/C7, Cy3-labelled second antibody); these cells also express TF antigen (**b**; A78-G/A7, Cy2-labeled second antibody), 40× lens

tumour cells of the cell line Jeg3 also showed strong staining for MUC1 (Fig. 7), but no expression of TF antigen. Another trophoblast tumour cell line, BeWo, stained positive for both MUC1 (Fig. 8a) and TF antigen (Fig. 8b), but no obvious co-expression was observed.

Discussion

The presented data demonstrate the expression of the TF epitope and its putative carrier protein MUC1 in the syncytiotrophoblast of the placenta during all stages of pregnancy and in cells of the decidua in the first and second trimester of pregnancy. The expression of the TF antigen, which is considered to be an oncodevelopmental marker, resembles changes in glycosylation that occur during neoplastic transformation (Cao et al. 1997a). Trophoblast cells and carcinoma cells share a number of phenotypic similarities. These include the reduction or loss of MHC antigen expression. The syncytiotrophoblast, which is surrounded by maternal blood, lacks HLA class I proteins (Pröll et al. 1999). Although this fact contributes to the protection of the fetus from the mother's immune system, especially the development of alloreactive T cells, it creates another problem. The absence of MHC potentially renders the trophoblast sensitive for natural killer (NK) cell recognition and lysis (Mullen 1998). However, the syncytiotrophoblast is invulnerable to NK cell-mediated lysis (Avril et al. 1999). To explain this, Clark et al. (1996, 2001) discuss a species-recognition system formed by a diversity of receptors and complementary glycoprotein ligands. This glycoprotein ligand system could explain how lytic responses are avoided by the MHC-negative syncytiotrophoblast. Expression of the TF-carbohydrate antigen by the syncytiotrophoblast could play a role as a part of such a protecting system.

During the invasion of the trophoblast into the decidua, phenotypic changes of epithelial cells are observed. As already stated, the formation of the placenta resembles in many respects the invasion of malignant tumours. At the implantation site, fetus-derived trophoblast cells invade deeply into the maternal decidua. Within the decidua, the contact between fetal and maternal cells is most intimate in the first trimester of pregnancy (Hammer et al. 1999). Although the syncytiotrophoblast remains positive for the TF epitope during the whole period of pregnancy, cells in the decidua showed a positive staining for TF antigen only in the first and second trimester of pregnancy, suggesting a possible role of TF antigen in cell contact or trophoblast invasion.

For the identification of the cell type of the TF antigen- and MUC1-positive cells, a panel of additional antibodies was employed. The TF antigen-positive cells showed positive cytokeratin and CD9 staining, and were CD45 and glycodelin A negative. On the basis of these results we conclude that TF antigen-positive cells are not leukocytes including macrophages and large granular lymphocytes, which are present in human endometrial tissue and in the decidualised endometrium in pregnancy

(Vince and Johnson 2000). In addition, decidualised endometrium cells, which are glycodelin A positive (Mylonas et al. 2000), do not express TF antigen or MUC1. This suggests that the TF antigen- and MUC1-positive cells found in the decidua are extravillous trophoblast cells. Extravillous trophoblast cells have been shown to express cytokeratin and CD9 (Blaschitz et al. 2000), a pattern that is consistent with the data presented here. This strengthens the suggestion of an extravillous trophoblast origin of these TF antigen-expressing cells.

Pregnancy is often compared with a successful transplant in that the semiallogenic trophoblast cells escape recognition and destruction by the mother's immune system. Our study shows that the TF epitope is expressed by extravillous trophoblast cells only in that phase of pregnancy where an intimate contact of maternal and fetal cells in the decidua takes place. In contrast, the trophoblast and especially the syncytiotrophoblast is surrounded by maternal immune cells in all trimesters of pregnancy. This correlates with our observation that the TF antigen on the syncytiotrophoblast declines but continues throughout pregnancy.

Mucin 1 and TF antigen were expressed by isolated and purified trophoblast cells of first trimester placentas in a different pattern compared to trophoblast tumour cell lines. Trophoblast cells were purified by Percoll gradient centrifugation followed by depletion of CD9 and CD45-positive cells. The resulting trophoblast cell fraction expressed CK7 and produced the trophoblast-specific hormones hCG, hPL and progesterone (Jeschke et al. 2000). Isolated cytotrophoblast cells showed no expression of the TF epitope but were positive for MUC1. On one slide we detected a multinucleated conglomerate of trophoblast cells with a positive TF antigen staining. We assume that this was a digested part of the syncytium. The percentage of such TF antigen-positive cell conglomerates in isolated cytotrophoblast cells was in any case less than 1%. Remnants of the syncytium, if any, could be removed by incubation with anti-TF antibodies followed by the addition of anti-mouse IgG coated magnetic microbeads and magnetic separation.

In addition, we analysed the expression of TF antigen and MUC1 in the choriocarcinoma cell lines Jeg3 and BeWo. The cells of the cell line Jeg3, which was established by Kohler and Bridson (1971), resemble cytotrophoblast cells in some respects (Dibbelt et al. 1994). Our results support this view. Jeg3 cells showed positive staining for MUC1 but no expression of the TF epitope, as it was found on isolated cytotrophoblast cells from first trimester placentas. The cell line BeWo was established by Pattillo et al. (1968). In contrast to Jeg3, cultures of BeWo choriocarcinoma cells contain two coexisting phenotypes: a cytotrophoblast-like and a syncytiotrophoblast-like phenotype (Grümmer et al. 1990). We found with these cells a moderate staining for MUC1 but a strong expression of TF antigen. This pattern very likely represents the syncytiotrophoblast-like phenotype because the syncytiotrophoblast in vivo also strongly expresses TF antigen. But TF antigen and MUC1 were only partially co-expressed in BeWo cells.

In summary, during human gestation the pancarcinoma TF antigen is expressed in the placenta. It decreases with gestational age in agreement with its nature as an oncodevelopmental marker. Although this study did not include functional experiments, it is obvious that the TF epitope is almost exclusively expressed at the maternofetal interface during those stages of pregnancy where the most intimate contact between maternal and fetal cells takes place, or where the contact between maternal blood cells and fetal tissue is most intense, and we hypothesise that TF antigen may play a functional role at this peculiar site.

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