Histol Histopathol (2009) 24: 1213-1222

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Expression pattern alterations of the serine protease HtrA1 in normal human placental tissues and in gestational trophoblastic diseases

Daniela Marzioni[1], Alexia Quaranta[1], Teresa Lorenzi[1], Manrico Morroni[1], Caterina Crescimanno[2], Michele De Nictolis[3], Paolo Toti[4], Giovanni Muzzonigro[5], Alfonso Baldi[6], Antonio De Luca[7] and Mario Castellucci[1]

[1]Department of Molecular Pathology and Innovative Therapies, Polytechnic University of Marche, Ancona, Italy, [2]Institute of Sciences of Formation, Kore University of Enna, Enna, Italy, [3]Department of Pathological Anatomy and Histopathology, General Hospital, Ancona, Italy, [4]Department of Human Pathology and Oncology, University of Siena, Siena, Italy, [5]Department of Urology, Polytechnic University of Marche, Ancona, Italy, [6]Department of Biochemistry "F. Cedrangolo", Section of Pathologic Anatomy, Second University of Naples, Naples, Italy and [7]Department of Medicine and Public Health, Human Anatomy, Second University of Naples, Naples, Italy

Both Daniela Marzioni and Alexia Quaranta contributed equally to this work as first authors.

Summary. HtrA1 is a secreted protein which behaves as a molecular chaperone at low temperatures and as a serine protease at high temperatures. When the placenta escapes the normal growth control mechanisms, which are present during normal pregnancy, it may develop trophoblastic diseases, such as hydatidiform mole and choriocarcinoma. The aim of the study is to investigate the expression of HtrA1 in these gestational trophoblastic diseases and evaluate whether different HtrA1 expression might be associated with increasingly severe forms of disease.

We used immunohistochemistry to assess the expression of HtrA1 in normal human placenta, hydatidiform mole (partial and complete) and choriocarcinoma. In addition to that we used the western blotting technique to quantify HtrA1 immunoreaction in normal human placentas. The most striking finding of our investigation is the decrease in immunostaining of this protease with increasing severity of gestational trophoblastic disease. For instance, in partial and complete moles HtrA1 is weakly expressed in the trophoblast. Moreover, absence of immunoreaction for HtrA1 is observable in the choriocarcinoma cells.

In conclusion, we suggest that HtrA1 may play an important role in the pathogenesis and progression of hydatidiform moles and choriocarcinomas, and that HtrA1 may play an important role during the normal development of the placenta, as well as in trophoblastic diseases.

Key words: HtrA1, Hydatidiform mole, Placenta, Immunohistochemistry, Western blotting

Offprint requests to: Daniela Marzioni, Ph.D., Dep. Molecular Pathology and Innovative Therapies, Faculty of Medicine, Marche Polytechnic University, Via Tronto, 10/a, I-60020 Ancona, Italy. e-mail: d.marzioni@univpm.it

Introduction

HtrA (High temperature requirement factor A), also known as DegP, was initially identified in *E. coli* as a heat shock-induced envelope-associated serine protease (Pallen and Wren, 1997). Generally, it behaves as a molecular chaperone at low temperatures and as a serine protease at high temperatures (Spiess et al., 1999; Krojer et al., 2002). HtrA1 is the first identified member of the human HtrA protein family, a widely conserved family of serine proteases (Zumbrum and Trueb, 1996; Hu et al., 1998; Clausen et al., 2002). The other members are: HtrA2 (Faccio et al., 2000; Gray et al., 2000), HtrA3 (Nie et al. 2003a), and HtrA4. In humans, the four HtrA homologues appear to be involved in several important functions, such as cell growth, apoptosis, and inflammatory reactions, and they control cell fate via regulated protein metabolism (Clausen et al., 2002).

HtrA1 is a secreted protein involved in the degradation of extracellular matrix (ECM) proteins (Clausen et al., 2002). HtrA1 has been implicated in the pathology of several diseases, such as arthritis (Grau et al., 2006) and in tumor progression and invasion (Baldi

et al., 2002; Shridhar et al., 2002). Recent reports suggest that HtrA1 plays a protective role in various malignancies because of its tumor suppressive properties (Shridhar et al., 2002; Nie et al., 2003b). Studies have shown that HtrA1 is down-regulated in cancerous tissue as compared with normal tissue, and that over expression results in the inhibition of tumor cell growth and proliferation both *in vitro* and *in vivo* (Baldi et al., 2002).

Several points of evidence confirm that the transcription of the HtrA1 gene is highly regulated during development and in adult tissues (De Luca et al., 2003), suggesting that HtrA1 may exert its functions not only on neoplastic cells, but also under physiological conditions.

Correct placenta formation and function are essential for the development of the mammalian fetus. The human placenta is a rapidly growing and differentiating organ where growth factors (GFs), their receptors, (Marzioni et al., 2005) and the remodeling of extracellular matrix components (Castellucci et al., 1991) play a fundamental role. During placental development, various subsets of trophoblast originate from the trophectoderm of the blastocyst, including the villous trophoblast, which covers the placental villi, and the extravillous cytotrophoblast of cell islands and cell columns. Cell islands and cell columns are composed of a stratified/multilayered core of extravillous cytotrophoblast surrounded by a sleeve of syncytiotrophoblast (Muehlhauser et al., 1995; Benirschke et al., 2006). Cell islands are free-ending structures in the intervillous space, whereas cell columns are responsible for the attachment of placental villi to the basal plate, forming the so-called anchoring villi. From the most distal parts of these villi, extravillous trophoblastic cells invade the decidua (Damsky et al., 1992).

De Luca and coworkers (2004) have recently demonstrated in first and third trimester specimens that HtrA1 is expressed in human placenta. It can be assumed that HtrA1 acts on different targets, such as intracellular growth factors or ECM proteins, to favor the correct formation/function of the placenta (De Luca et al., 2004). The regulation of the balance between cell proliferation and cell death in the placenta is essential in order to allow the necessary morphological and functional changes (Smith et al., 1997; Lea et al., 1999, Levy et al., 2000). When the trophoblast escapes the normal growth control mechanisms that are present during normal pregnancy, gestational trophoblastic diseases with various degrees of malignancy may develop, such as hydatidiform mole, and choriocarcinoma (Mazur et al., 1994). We hypothesized that the expression of HtrA1 might be altered in these gestational trophoblastic diseases and that different degrees of alteration might be associated with increasingly severe forms of disease. To verify this hypothesis, we used immunohistochemistry and western blotting techniques to assess the expression of HtrA1 in normal human placenta, hydatidiform mole (partial and complete) and choriocarcinoma.

Material and methods

Tissues

The protocol of the study was approved by the University's Ethical Committee. Informed consent was obtained from all patients. Placental tissues were obtained from 24 pregnant women at different gestational ages. Tissues were immediately collected from: 11 women undergoing voluntary termination of pregnancy at 9 (n=4), 10 (n=3), 11 (n=2) and 12 (n=2) weeks of gestation (first trimester); 5 women undergoing elective abortion for fetal or maternal indications at 15 (n=2) and 16 (n=3) weeks of gestation (second trimester); and 8 women undergoing caesarean section at 38 (n=4) and 39 (n=4) weeks of gestation (third trimester). To the best of our knowledge, there was no pathology affecting placental structure or function.

Specimens of 8 partial moles (15-33 weeks post-menstruation), 10 complete moles (10-13 weeks post-menstruation) , 8 choriocarcinomas (detected 4-6 months after gestation) and 8 term placentas at 38 (n=4) and 39 (n=4) weeks of gestation were collected from the pathology files of the Departments of Pathological Anatomy of the University of Siena (Italy) and from the Marche Polytechnic University (Ancona, Italy). All the moles and choriocarcinomas were documented with clinical and histopathological data. Frozen pathological samples were not available for western blotting.

Western blotting and Immunohistochemical Analysis

Tissue preparation for western blotting

Two biopsies from each normal placenta (first, second and third trimester of gestation) were randomly collected for Western blotting analysis. The biopsies and NIH3T3 fibroblasts were frozen in liquid nitrogen and stored at -80°C until use.

Tissue preparation for immunohistochemistry

Tissue blocks from normal and pathological placentas were fixed in 4% neutral buffered formalin for 24 hours at 4°C. The tissues were rapidly and routinely processed for paraffin embedding at 56°C, as previously described (Muehlhauser et al., 1993). Paraffin sections (3 µm) were cut and stretched at 45°C, allowed to dry and stored at 4°C until use.

Western blotting

Tissue lysates of normal placental specimens were obtained after complete potter homogenization (Ultra-Turrax T8, IKA®-WERKE, Lille, F) in lysis buffer

containing 1% NP40, 20 mM TRIS-HCI, pH 8, 137 mM NaCl, 10% glycerol, 1 mM $MgCl_2$, 1 mM $CaCI_2$, 5 mM EGTA, 10mM EDTA and freshly added protease inhibitors (Pefabloc SC; Complete™). Protein concentrations were assessed with Bradford protein assay (Bio-Rad Laboratories; Bradford, 1976). Equal amounts of proteins (100 µg) were denaturated with 1x sample buffer (Laemmli, 1970), boiled for 5 minutes, and fractionated on 10% SDS-polyacrilamide gels (SDS-PAGE). Blots were first incubated with 6% dry-fat milk (BioRad) in TBS-T 0.5% and then with the affinity-purified rabbit polyclonal antiserum raised against a purified bacterially expressed glutathione-S-transferase (GST)- HtrA1 (aa 363-480) human fusion protein (Baldi et al., 2002) diluted 1:500. After washing, blots were incubated with anti-rabbit secondary antibody conjugated to horseradish peroxidase (Amersham Italia srl, Milano, Italy) diluted 1:5000. Detection of bound antibody was performed with the ECL-Western blotting detection kit (Amersham) according to the manufacturer's instructions. Fibroblasts were used as positive control (Zumbrum and Trueb, 1996). Levels of HtrA1 protein were assessed by densitometric analysis using Chemidoc and Quantity-One program (Bio-Rad Laboratories). The relative quantities were expressed as the ratio of densitometric reading for HtrA1 protein to ß-actin.

Results were presented as mean ± SD. HtrA1 protein levels (~50kDa and ~30kDa forms) of the first vs the second, the second vs the third, the first vs the third trimester of gestation were tested using analysis of variance (ANOVA) (PRISM version 4 for windows: GraphPad Software Inc, San Diego, CA); $p<0.05$ was considered statistically significant.

Immunohistochemistry

Paraffin sections were deparaffinized and rehydrated via xylene and a graded series of ethyl alcohol, and treated with 0.1% trypsin (Sigma Chemical Co, St Louis, MO, USA) in PBS for 5-10 min at 37°C. To inhibit endogenous peroxidase activity, sections were incubated for 50 min with 3% hydrogen peroxide in de-ionized water. Sections were then washed in PBS. To block nonspecific background, the sections were incubated for 1 hr at room temperature (RT) with: a) PBS-6% non fat dry milk (BioRad; Hercules CA), for the detection of HtrA1; b) normal goat serum diluted 1:75, for the detection of pan-cytokeratin; c) normal horse serum, diluted 1:75, for the detection of vimentin.

Sections were then incubated overnight at 4°C with the following primary antibodies: a) HtrA1, affinity-purified rabbit polyclonal antiserum raised against a purified bacterially expressed glutathione-S-transferase (GST)- HtrA1 (aa 363-480) human fusion protein (Baldi et al., 2002) diluted 1:50 in PBS-3% non fat dry milk; b) rabbit anti-human pan-cytokeratin (Zymed, San Francisco, USA) diluted 1:200 in PBS, used to identify trophoblastic cells; c) mouse anti-vimentin (DakoCytomation, Glostrup, DK) diluted 1:30 in PBS, used to identify decidual cells. After washing in PBS, the sections were subsequently incubated with goat anti-rabbit biotinylated antibody (for HtrA1 and pan-cytokeratin antibodies) and horse anti-mouse biotinylated antibody (for anti-vimentin antibody) diluted 1:200 (Vector Laboratories). The peroxidase ABC method (Vector Laboratories) was performed for 1 hr at RT and 3',3'diaminobenzidine hydrochloride (Sigma, St Louis, MO, USA) as chromogen was used.

Sections were counterstained in Mayer's haematoxylin, dehydrated and mounted with Eukitt solution (Kindler GmbH and Co., Freiburg, Germany). Specificity tests were performed by: (i) by omitting the primary antibody in the immunostaining procedure; (ii) by incubating sections with the antiserum saturated with homologous antigen (for this procedure, the antibody was incubated with a 10-fold excess of GST fusion protein, for 48 h); or (iii) by replacing the primary antibody with the pre-immune serum.

The level of HtrA1-staining per field (x200) at light microscopy was calculated and compared in different specimens by two separate observers (D.M. and M.C.) in a double- blind fashion and was described as score 0 (negative), score 1 (modest), score 2 (intense) and score 3 (striking). An average of 22 fields was observed for each specimen. All values were expressed as mean ± SD, and differences were compared using Student's t-test.

Results

Western blotting

We found two forms of HtrA1 expressed as a native protein of ~50kDa and autocatalytic product of ~30kDa. Fibroblasts known to express high levels of HtrA1 protein (Zumbrum and Trueb, 1996) were used as positive control (Fig. 1a, line 1). As shown in Fig. 1a bands representing the predicted native form (~50kDa) and autocatalytic product (~30kDa) of HtrA1 protein were detected in the first, second and third trimester of gestation. Interestingly, for the first time, we observed that both bands were always present in normal placentas (Fig. 1a). Furthermore, the levels of HtrA1 expression (~50kDa and ~30kDa forms) were analyzed densitometrically. The total levels of HtrA1 protein (~50kDa form + ~30kDa form) were gradually decreased during gestation (i.e. first, second and third trimester of gestation) and the differences of HtrA1 expression were statistically significant in the three placental groups analyzed (Fig. 1b). In addition, we separately analyzed the two forms of HtrA1 (Fig. 1c). The expression levels of the ~50kDa form, as well as the ~30kDa form of HtrA1 protein gradually decreased during gestation (Fig. 1c), and the differences of ~50kDa HtrA1 expression in the different trimesters of gestation

were statistically as significant as the differences of ~30kDa HtrA1 expression (Fig. 1c). The proportion of ~50kDa form with respect to total HtrA1 protein level was statistically higher in the first vs the second and the second vs the third trimester of gestation (Fig. 1c). The proportion of ~30kDa form with respect to total HtrA1 immunoreactivity was not significant in any trimester of gestation (Fig. 1c).

Immunohistochemical analysis

Representative HtrA1 immunostaining of the placenta during normal gestation is shown in Fig. 2a-f. Examples of HtrA1 immunostaining in pathological tissues (partial and complete moles and chorio-carcinomas) are shown in figure 4 (a-e). The scoring of total HtrA1 expression as well as the evaluation of HtrA1 immunostaining in different compartments in normal placentas and pathological placental tissues are shown in Figs. 3 and 5, respectively.

Normal tissues

First trimester. In specimens from the first trimester of gestation, immunostaining for HtrA1 was generally found in both layers of villous trophoblast, syncytiotrophoblast and cytotrophoblast. In particular, HtrA1 was regularly expressed in the cytotrophoblast (Figs. 2a, 3a,b), whereas the syncytiotrophoblast was irregularly labeled in different fields of the same section, showing negative tracts for HtrA1 staining (Figs. 2a, 3a,b). Syncytial knots were mainly positive for HtrA1.

The villous stroma, as well as the fetal vessel walls, showed immunostaining for HtrA1 in some placental villi (Fig. 2a).

Some degenerated villi with fibrinoid depots showed a more intense immunostaining for HtrA1 in the syncytiotrophoblast and cytotrophoblast when compared

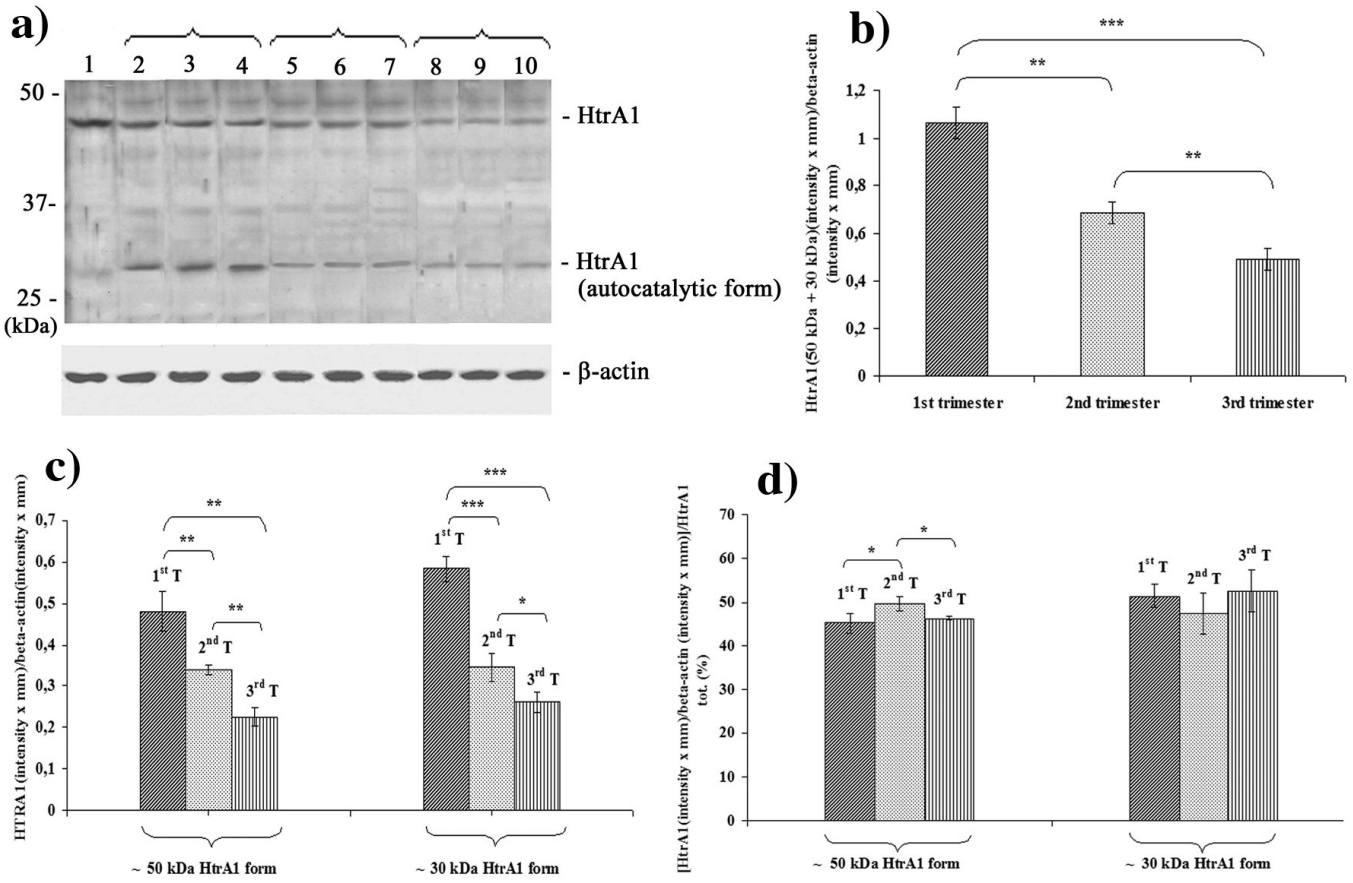

Fig. 1. Representative quantitative Western blotting of HtrA1. **a.** NIH3T3 fibroblasts as positive controls in lane 1; first trimester placentas in lines 2-4; second trimester placentas in lines 5-7; third trimester placentas in lines 8-10. ~50kDa: HtrA1 native protein; ~30kDa: autocatalytic product of HtrA1. ß-actin: housekeeping protein. **b-d.** Densitometric analysis of ~50kDa and ~30kDa HtrA1 bands relative to that of ß-actin. **b.** Total levels gradually decrease from the first to the third trimester placental tissues. **c.** HtrA1 protein levels of ~50kDa and ~30kDa forms decrease during pregnancy. **d.** Proportion of the ~50kDa and ~30kDa forms relative to total HtrA1 protein level. 1stT: first trimester placentas; 2ndT: second trimester placentas; 3rdT: third trimester placentas. * p<0.05, ** p<0.01,* ** p<0.001.

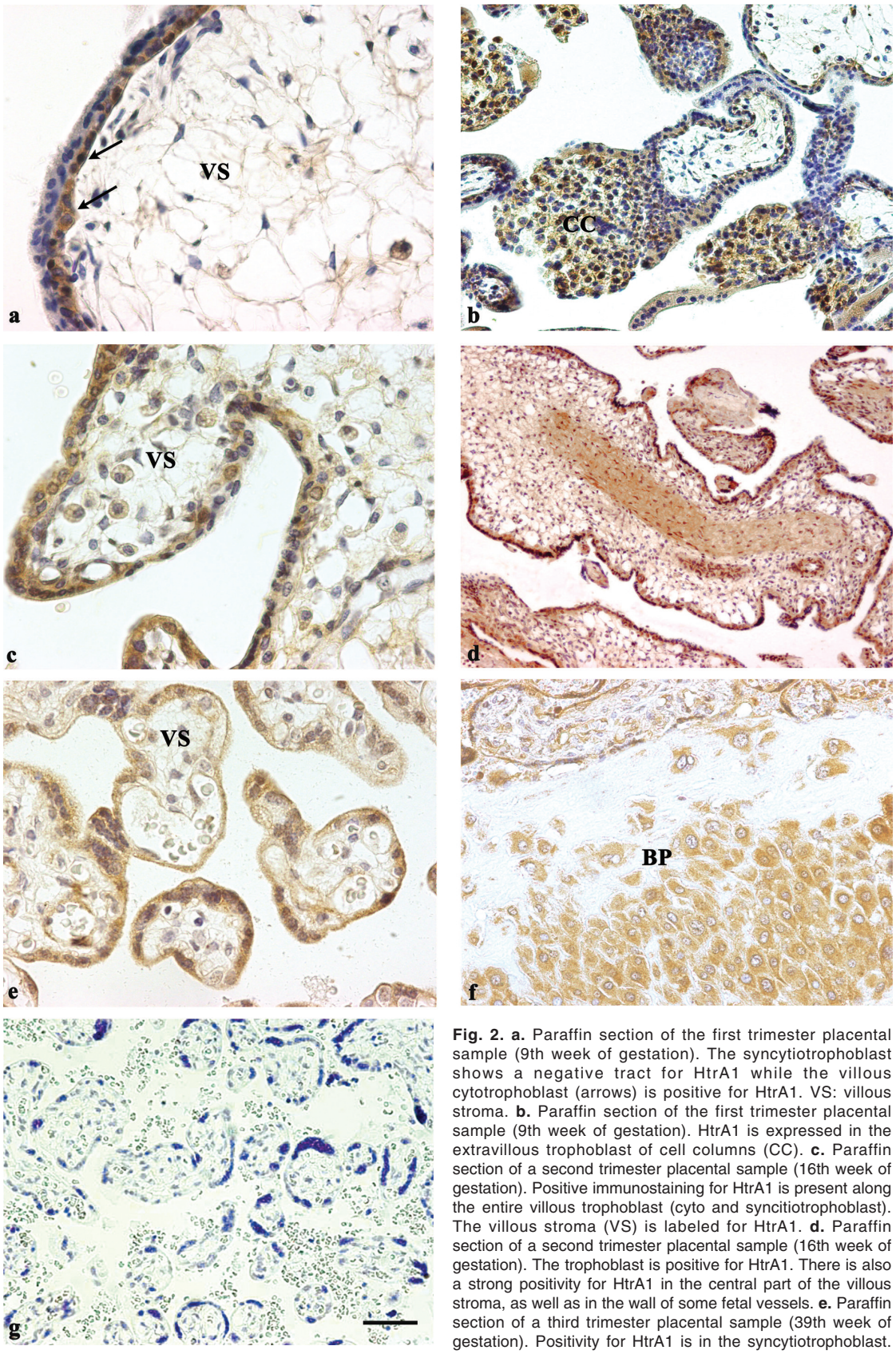

Fig. 2. a. Paraffin section of the first trimester placental sample (9th week of gestation). The syncytiotrophoblast shows a negative tract for HtrA1 while the villous cytotrophoblast (arrows) is positive for HtrA1. VS: villous stroma. **b.** Paraffin section of the first trimester placental sample (9th week of gestation). HtrA1 is expressed in the extravillous trophoblast of cell columns (CC). **c.** Paraffin section of a second trimester placental sample (16th week of gestation). Positive immunostaining for HtrA1 is present along the entire villous trophoblast (cyto and syncitiotrophoblast). The villous stroma (VS) is labeled for HtrA1. **d.** Paraffin section of a second trimester placental sample (16th week of gestation). The trophoblast is positive for HtrA1. There is also a strong positivity for HtrA1 in the central part of the villous stroma, as well as in the wall of some fetal vessels. **e.** Paraffin section of a third trimester placental sample (39th week of gestation). Positivity for HtrA1 is in the syncytiotrophoblast. **f.** Paraffin section of a third trimester placental sample (39th week of gestation). Cells of the basal plate (BP) are immunolabeled for HtrA1. **g.** Paraffin section of a third trimester placental sample (39th week of gestation). Negative control. Bars: a, c, e, 27 μm; b, d, f, g, 54 μm.

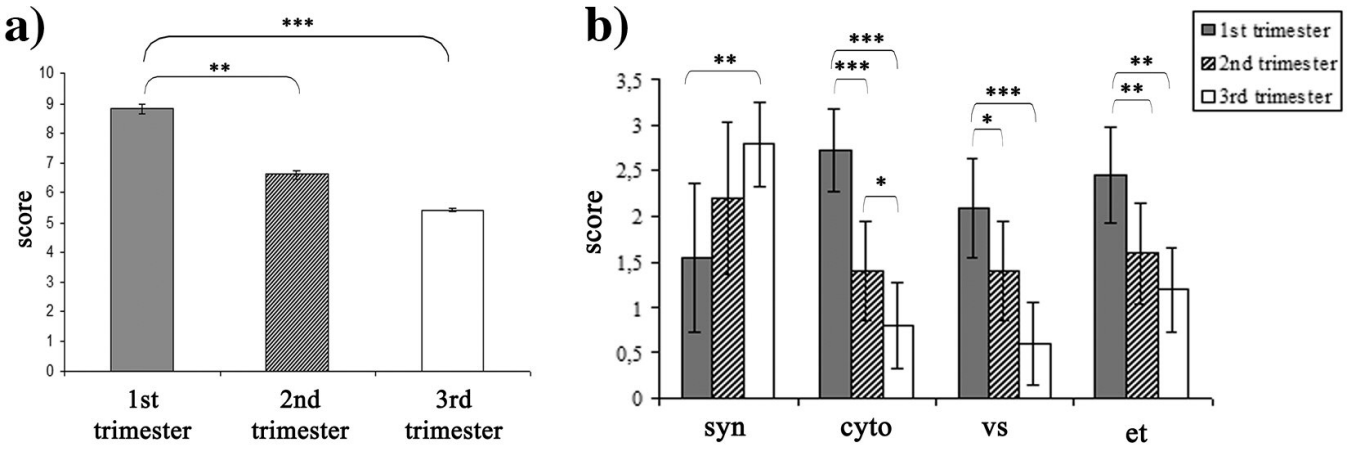

Fig. 3. a. Total HtrA1 immunostaining in the first, second and third trimester of gestation. The gestational time course of syncytiotrophoblast (syn), villous cytotrophoblast (cyto), villous stroma (vs) and extravillous trophoblast (et) for HtrA1 immunostaining is shown in **(b)**. Ordinate: immunoreactivity scored as described in Materials and Methods. * p<0.05, ** p<0.01, *** p<0.001.

with the trophoblastic covering of physiological villi.

The cytoplasm of extravillous trophoblast and extracellular matrix of cell islands and cell columns were labeled for HtrA1 (Fig. 2b; Figs. 3a,b).

Second trimester. In placentas of the second trimester, HtrA1 was expressed by both the villous trophoblast (syncytiotrophoblast and cytotrophoblast; Figs. 2c, 3a,b) and extravillous trophoblast of the basal plate (Fig. 3a,b). Syncytial knots were mainly positive for HtrA1.

Although in specimens of the second trimester the villous stroma was weakly immunostained in the majority of the placental villi, several villi showed a strong positivity for HtrA1 in the central part of the villous stroma (Figs. 2d, 3a,b) or in the fetal vessel walls, or in both of these locations.

Third trimester. Placental tissues (freshly collected and from pathology files) showed a more intense positivity for HtrA1 in the syncytiotrophoblast than in cytotrophoblast (Figs. 2e, 3a,b). Syncytial knots were mainly positive for HtrA1.

In the villous stroma some small blood vessels showed a moderate positivity. The other components of the villous stroma were weakly stained or negative for this protein (Figs. 2e; Figs. 3a,b). In the basal plate, the extravillous trophoblast (identified by staining with the antibody to cytokeratins), as well as the decidual cells (identified with the antibody to vimentin), were positive for HtrA1 (Figs. 2f, 3a,b).

Controls

No staining was observed in any of the samples when the HtrA1 primary antibody or the secondary antibody was omitted (Fig. 2g). Specificity tests showed negative results.

Pathological Tissues

Partial mole

The expression of HtrA1 in the trophoblast of pathological villi (Figs. 4a, 5a,b) was weaker than in the normal chorionic villi. The stroma was mainly negative (Fig. 4a). Cell islands and cell columns showed a modest, homogeneous positive staining.

Complete mole

The trophoblastic collections (or trophoblastic hyperplasia) were labeled for HtrA1, showing an irregular and weak staining pattern (Fig. 4b). In particular, the cytotrophoblast was prevalently negative (Figs. 4b, 5a,b). The stromal tissue was not immunoreactive for HtrA1 and the extravillous cytotrophoblast showed an irregular modest positivity (Figs. 4b,c, 5a,b).

Choriocarcinoma

Most of the neoplastic tissue (Fig. 4d), identified by pan cytokeratin staining (Fig. 4e), was mainly negative for HtrA1.

Discussion

Cytotrophoblastic cells form a cell layer underneath the syncytiotrophoblast. The villous cytotrophoblastic layer is nearly complete during early pregnancy but later

becomes discontinuous. The villous cytotrophoblast supports the growth and regeneration of the syncytium (Benirschke et al., 2006). According to previous findings (De Luca et al., 2004) our results show that HtrA1 shifts its expression from cytotrophoblastic cells to syncytiotrophoblast from the first to the third trimester

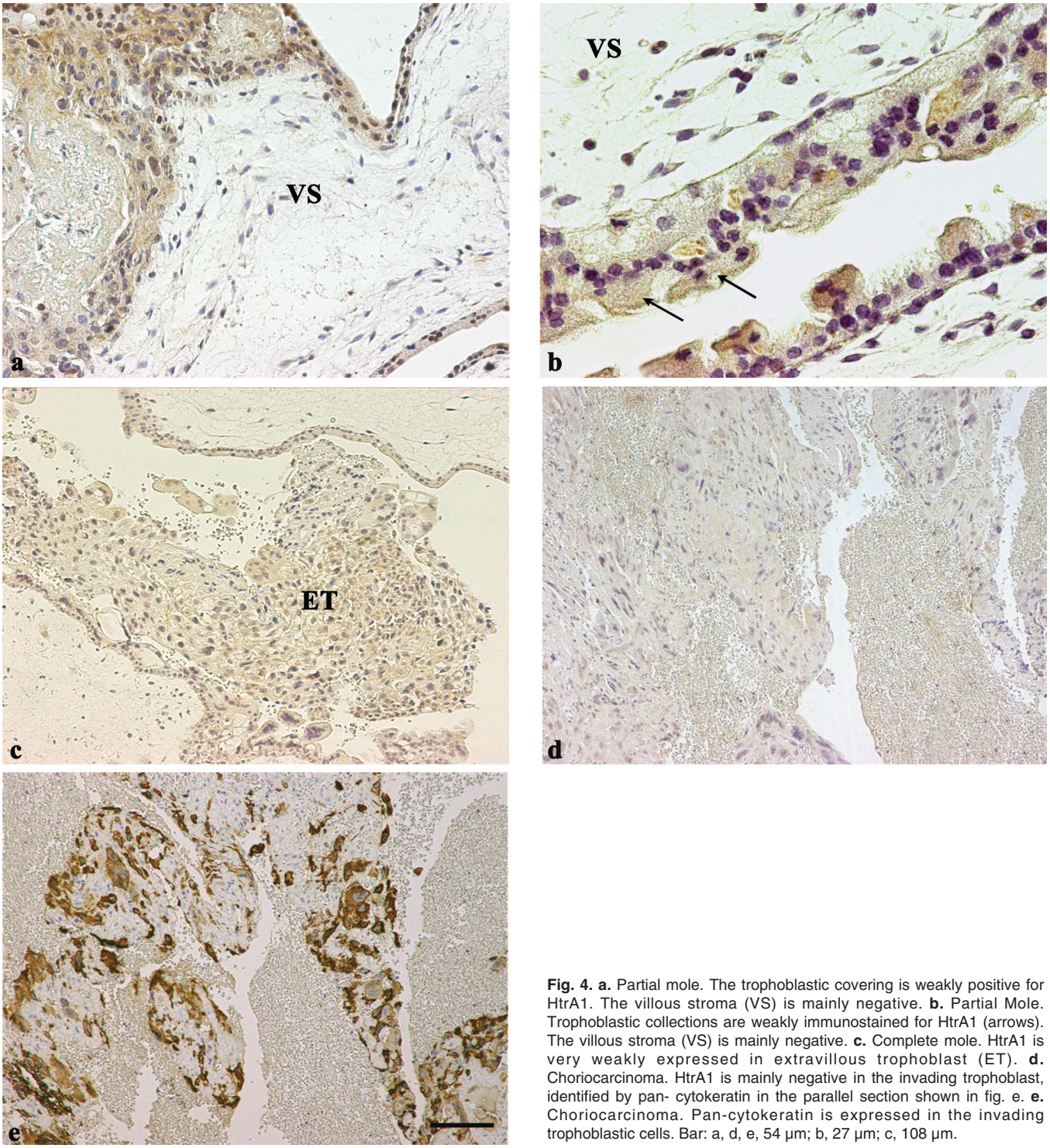

Fig. 4. a. Partial mole. The trophoblastic covering is weakly positive for HtrA1. The villous stroma (VS) is mainly negative. **b.** Partial Mole. Trophoblastic collections are weakly immunostained for HtrA1 (arrows). The villous stroma (VS) is mainly negative. **c.** Complete mole. HtrA1 is very weakly expressed in extravillous trophoblast (ET). **d.** Choriocarcinoma. HtrA1 is mainly negative in the invading trophoblast, identified by pan- cytokeratin in the parallel section shown in fig. e. **e.** Choriocarcinoma. Pan-cytokeratin is expressed in the invading trophoblastic cells. Bar: a, d, e, 54 μm; b, 27 μm; c, 108 μm.

of gestation respectively. This suggests an involvement of HtrA1 in the balance of cytotrophoblastic cell proliferation and their differentiation into syncytiotrophoblast. In addition, we observed that various placental villi showed strong HtrA1 immunostaining in their stromal compartment in the first and second trimester of gestation in accordance with HtrA1 secretive properties. The localization of HtrA1 in the stroma of these villi suggests a possible action of this protease in the remodeling of the villous core during placental development and differentiation, particularly occurring in the first and second trimesters of gestation (Castellucci et al., 1990). The villous stroma was mainly negative for HtrA1 in the third trimester placentas. Interestingly, our western blotting data show a significant decrease of HtrA1 protein during gestation. These western blotting data are not in contrast with previous morphological data suggesting an increase of this protein in third trimester specimens of placenta (De Luca et al., 2004). Indeed, De Luca and coworkers (2004) examined the expression level of HtrA1-stained syncytiotrophoblast and cytotrophoblast without examining stroma and extravillous trophobast HtrA1 immunostaining. For our western blotting analysis we used placenta lysates taken as a whole, consequently the stroma (positive in the first and second trimesters, negative in the third trimester) and extravillous trophoblast HtrA1 expression (positive during gestation) added to the villous trophoblast positivity for this protein revealed that total HtrA1 protein density decreases from the first to the third trimester of gestation. In addition, our immunohistochemical analysis of different placental compartments, i.e. syncytiotrophoblast, villous cytotrophoblast, villous stroma and extravillous trophoblast, confirm our western blotting data, as well as previous data concerning HtrA1 immunostaining in the first and third trimester placentas (De Luca et al., 2004). In addition, for the first time our western blotting data demonstrate that placental tissues express HtrA1 ~50kDa native protein and ~30kDa autocatalytic product during gestation. Recently, Chien and coworkers (2006) have observed that the autocatalytic product of HtrA1(~30kDa autocatalytic product) is involved in cell death through its serine protease activity. Interestingly, an increasing body of evidence shows that apoptosis is a normal constituent of trophoblast turnover during pregnancy (Huppertz et al., 1998) and that in the villous trophoblast the processes of apoptosis and differentiation, at least in part, share the same route (Huppertz and Kingdom, 2004). These data suggest that the ~30kDa HtrA1 form (autocatalytic product) detected in our experiments could be related to the apoptotic processes occurring during normal gestation.

During implantation and placental development, the trophoblast invades the uterine wall, i.e., the endometrium and the uterine blood vessels. In the first half of pregnancy most of the invasive processes of the trophoblast are performed by the extravillous trophoblast developing from the cell columns (Benirschke et al., 2006). We show that the extravillous cytotrophoblastic cells of cell columns express HtrA1 in physiological gestation, suggesting a potential role of this protease during implantation and placental development. In fact, it has recently been established that HtrA1 expression pattern is developmentally regulated at the decidual-trophoblast interface during the formation of mice placenta (Nie et al., 2005). The invasive capacity of the extravillous trophoblast shows striking similarities with that displayed by malignant cells during tumor invasion. However, trophoblast invasion of the endometrium is tightly regulated during normal pregnancy (Crescimanno et al., 1996). Interestingly, HtrA1 has been suspected to function as a tumor suppressor in melanomas (Baldi et al., 2002), ovarian cancer (Chien et al., 2004) and human

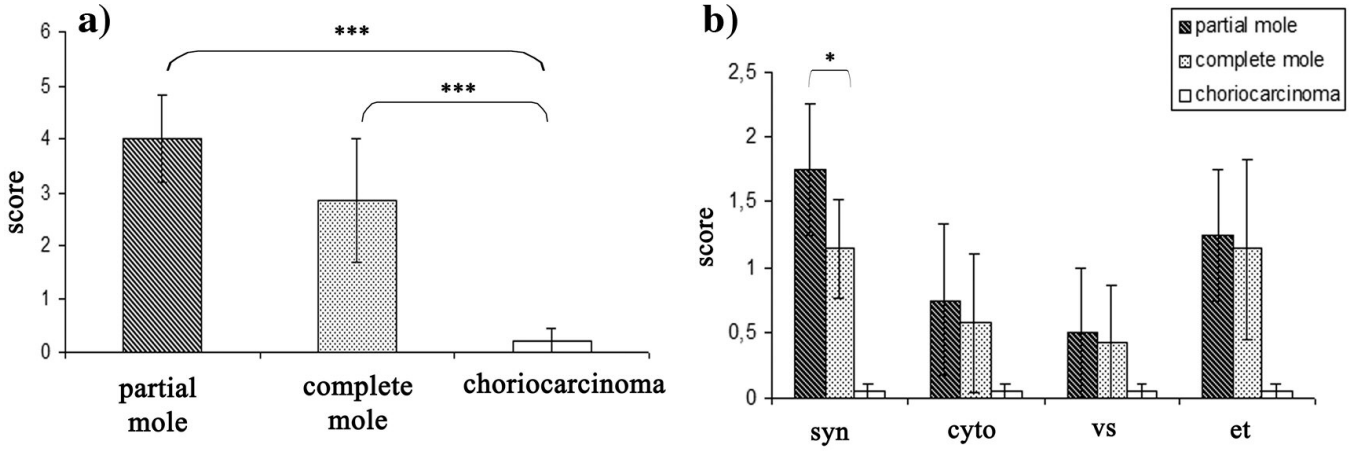

Fig. 5. a. Total HtrA1 immunostaining in partial mole, complete mole and choriocarcinoma. Immunostaining of syncytiotrophoblast (syn), villous cytotrophoblast (cyto), villous stroma (vs) and extravillous trophoblast (et) for HtrA1 in partial mole, complete mole and choriocarcinoma is shown in **(b)**. Ordinate: immunoreactivity scored as described in Materials and Methods. * p<0.05, ** p<0.01, *** p<0.001.

endometrial cancers (Bowden et al., 2006). Baldi and coworkers (2002) showed that PRSS11, a gene encoding HtrA1, is down regulated in a metastatic cell line. In addition, their results suggest that down-regulation of PRSS11 and HtrA1 expression may represent an indicator of melanoma progression (Baldi et al., 2002). In the light of these findings, we analysed the expression of HtrA1 in hydatidiform mole and choriocarcinoma. The most striking findings of our investigation concern the decrease in the immunostaining of this protease with increasing severity of gestational trophoblastic disease. For instance, in partial and complete moles HtrA1 is weakly expressed in the trophoblast. Moreover, choriocarcinoma cells show an absence of immune reaction. These data support previous findings showing that HtrA1 protein expression decreases with the increasing grades of endometrial carcinomas (Bowden et al, 2006). In conclusion, we suggest that HtrA1 could play an important role in growth, differentiation and apoptosis processes during normal development of the placenta, as well as in the increasing of severity of trophoblastic diseases.

Acknowledgements. This study was supported by grants from Italian Ministry of the University and Research to M.M., grants from Marche Polytechnic University to D.M., M.C., M.M., grants from Futura Onlus and Second University of Naples to A.D. and A.B.

References

Baldi A., De Luca A., Morini M., Battista T., Felsani A., Baldi F., Catricalà C., Amantea A., Noonan D.M., Albini A., Natali P.G., Lombardi D. and Paggi M.G. (2002). The HtrA1 serine protease is down-regulated during human melanoma progression and represses growth of metastatic melanoma cells. Oncogene 21, 6684-6688

Benirschke K., Kaufmann P. and Baergen R. N. (2006). Pathology of the Human Placenta. 5th ed. Springer. New York.

Bowden M.A., Di Nezza-Cossens L.A., Jobling T., Salamonsen L.A. and Nie G. (2006). Serine proteases HtrA1 and HtrA3 are down-regulated with increasing grades of human endometrial cancer. Gynecol. Oncol. 103, 253-260.

Bradford M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Castellucci M, Scheper M, Scheffen I, Celona A and Kaufmann P. (1990). The development of the human placental villous tree. Anat. Embryol. 181, 117-28.

Castellucci M., Classen-Linke I., Muehlhauser J., Kaufmann P., Zardi L. and Chiquet-Ehrismann R. (1991). The human placenta: a model for tenascin expression. Histochemistry 95, 449-458.

Chien J., Staub J., hu S.I., Erickson-Johnson M.R., Couch F., Smith D.I., Crowl R.M., Kaufmann S.H. and Shridhar V. (2004). A candidate tumor suppressor HtrA1 is downregulated in ovarian cancer. Oncogene 23, 1636-1644.

Chien J., Aletti G., Baldi A., Catalano V., Muretto P., Keeney G.L., Kalli K.R., Staub J., Ehrmann M., Cliby W.A., Lee Y.K., Bible K.C., Hartmann L.C., Kaufmann S.H. and Shridhar V. (2006). Serine protease HtrA1 modulates chemotherapy-induced cytotoxicity. J. Clin. Invest. 116, 1994-2004.

Clausen T., Southan C. and Ehrmann M. (2002)The HtrA family of proteases: implications for protein composition and cell fate. Mol. Cell. 10, 443-455.

Crescimanno C., Foidart J.M., Noël A., Polette M., Maquoi E., Birembaut P., Baramova E., Kaufmann P. and Castellucci M. (1996). Cloning of choriocarcinoma cell shows that invasion correlates with expression and activation of gelatinase A. Exp. Cell. Res. 227, 240-251.

Damsky C.H., Fitzgerald M.L. and Fisher S.J. (1992). Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. J. Clin. Invest. 89, 210-222.

De Luca A., De Falco M., Severino A., Campioni M., Santini D., Baldi F., Paggi M.G. and Baldi A. (2003). Distribution of the serine protease HtrA1 in normal human tissues. J. Histochem. Cytochem. 51, 1279-1284.

De Luca A., De Falco M., Fedele V., Cobellis L., Mastrogiacomo A., Laforgia V., Tuduce I.L., Campioni M., Giraldi D., Paggi M.G. and Baldi A. (2004). The serine protease HtrA1 is upregulated in the human placenta during pregnancy. J. Histochem. Cytochem. 52, 885-892.

Faccio L., Fusco C. Chen A., Martinetti S., Bonventre J.V. and Zervos A.S. (2000). Characterization of a novel human serine protease that has extensive homology to bacterial heat shock endoprotease HtrA and is regulated by kidney ischemia. J. Biol. Chem. 275, 2581-2588.

Gray C.W., Ward R.V., Karran E., Turconi S., Rowles A., Viglienghi D., Southan C., Barton A., Fantom K.G., West A., Savopoulos J., Hassan N.J., Clinkenbeard H., Hanning C., Amegadzie B., Davis J.B., Dingwall C., Livi G.P. and Creasy C.L. (2000). Characterization of human HtrA2, a novel serine protease involved in the mammalian cellular stress response. Eur. J. Biochem. 267, 5699-5710.

Grau S., Richards P.J., Kerr B., Hughes C., Caterson B., Williams AS., Junker U., Jones S.A., Clausen T. and Ehrmann M. (2006). The role of human HtrA1 in arthritic disease. J. Biol. Chem. 281, 6124-6129.

Hu S.I., Carozza M., Klein M., Nantermet P., Luk D. and Crowl R.M. (1998). Human HtrA, an evolutionarily conserved serine protease identified as a differentially expressed gene product in osteoarthritic cartilage. J. Biol. Chem. 273, 34406-34412.

Huppertz B. and Kingdom J.C.P. (2004). Apoptosis in the trophoblast-Role of apoptosis in placental morphogenesis. J. Soc. Gynecol. Investig. 11, 353-362.

Huppertz B., Kertschanska S., Demie A.Y., Frank H.G. and Kaufmann P. (1998). Immunohistochemistry of matrix metalloproteinases (MMP), their substrates, and their inhibitors (TIMP) during trophoblast invasion in the human placenta. Cell Tissue Res. 291, 133-148.

Krojer T., Garrido F.M., Huber R., Ehrmann M. and Clausen T. (2002). Crystal structure of DegP (HtrA) reveals a protease-chaperone machine. Nature 416, 455-459.

Laemmli U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227, 680-685.

Lea R.G., Riley S.C., Antipatis C., Hannah L., Ashworth C.J., Clark D.A. and Critchley H.O. (1999). Cytokines and the regulation of apoptosis in reproductive tissues: a review. Am. J. Reprod. Immunol. 42, 100-109.

Levy R. and Nelson D.M. (2000). To be, or not to be, that is the

question. Apoptosis in human trophoblast. Placenta 21, 1-13.

Marzioni D., Capparuccia L., Todros T., Giovannelli A. and Castellucci M. (2005). Growth factors and their receptors: fundamental molecules for human placental development. Ital. J. Anat. Embryol. 110, 183-187.

Mazur M.T. and Kurman R.J. (1994). Gestational trophoblastic disease and related lesions. In: Blaustein's pathology of the female genital tract. Kurman R.J. (ed). Springer-Verlag. New York. pp 1049-1093.

Muehlhauser J., Crescimanno C., Kaufmann P., Höfler H., Zaccheo D. and Castellucci M. (1993). Differentiation and proliferation patterns in human trophoblast revealed by c-erbB-2 oncogene product and EGF-R. J. Histochem. Cytochem. 41, 165-173.

Muehlhauser J., Crescimanno C., Kasper M., Zaccheo D. and Castellucci M. (1995). Differentiation of human trophoblast populations involves alterations in cytokeratin patterns. J. Histochem. Cytochem. 43, 579-589.

Nie G.Y., Hampton A., Li Y., Findlay J.K. and Salamonsen L.A. (2003a). Identification and cloning of two isoforms of human high-temperature requirement factor A3 (HtrA3), characterization of its genomic structure and comparison of its tissue distribution with HtrA1 and HtrA2. Biochem. J. 371, 39-48.

Nie G.Y., Li Y., Minoura H., Batten L., Ooi G.T., Findlay J.K. and Salamonsen L.A. (2003b). A novel serine protease of the mammalian HtrA family is up-regulated in mouse uterus coinciding with placentation. Mol. Hum. Reprod. 9, 279-290.

Nie G., Li Y. and Salamonsen L.A. (2005). Serine Protease HtrA1 is developmentally regulated in trophoblast and uterine decidual cells during placental formation in the mouse. Dev. Dyn. 233, 1102-1109.

Pallen M.J. and Wren B.W. (1997). The HtrA family of serine proteases. Mol. Microbiol. 26, 209-21.

Shridhar V., Sen A., Chien J., Avula R., Kovats S., Lee J., Lillie J. and Smith D.I. (2002). Identification of underexpressed genes in early- and late-stage primary ovarian tumors by suppression subtraction hybridization. Cancer Res. 62, 262-270.

Smith S.C., Baker P.N. and Symonds E.M. (1997). Placental apoptosis in normal human pregnancy. Am. J. Obstet. Gynecol. 177, 57-65.

Spiess C., Beil A. and Ehrmann M.A. (1999). A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell 97, 339-347.

Zumbrum J. and Trueb B. (1996). Primary structure of a putative serine protease specific for IGF-binding proteins. FEBS Lett. 398, 187-192.

Accepted March 30, 2009