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The Role of Heat Shock Protein 27 in Extravillous Trophoblast Differentiation

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Abstract Trophoblast cells from placental explants differentiate in culture to extravillous trophoblast cells (EVT cells). During trophoblast differentiation heat-shock-protein-27 (HSP27) mRNA and multidrug-resistance-protein-5 (MRP5, transporter of cyclic nucleotides) expression are increased. HSP27 is a regulator of actin filaments structure and dynamic, has a role in cell differentiation and may affect NF-kB activity. In this study we aimed to assess HSP27 level in trophoblast cells and its correlation with motility and differentiation related processes [MMPs activity, nitric oxide (NO), inducible nitric oxide synthase (iNOS), proliferation and MRP5 levels]. We evaluated HSP27 expression in a first trimester human trophoblast explants model designed to assess EVT cells differentiation/migration with/without 6-mercaptopurine (6MP, an EVT inhibitor of migration). We found that HSP27 level is expressed in the nucleous and cytoplasm of non-proliferting villous-trophoblast cells (negative for Ki67) and in the cell periphery and cytoplasm of motile EVT cells. Moreover, 6MP decreased HSP27 nucleous expression that was associated with inhibited MMP2 activity and NO production. Also decreased iNOS expression and increased MRP5 mRNA levels were observed. In conclusion, HSP27 expression is modulated in concordance with migration dependent parameters in trophoblast cells. J. Cell. Biochem. 103: 719–729, 2008. © 2007 Wiley-Liss, Inc.

Key words: 6-mercaptopurine; HSP27; MMP2; NO; MRP5; trophoblast

Successful invasion and remodeling of the uterine vasculature by extravillous trophoblast (EVT) cells are crucial steps in early pregnancy. cytotrophoblast (CT) cells may differentiate into EVT cells which migrate and invade maternal uterine blood vessels [Genbacev et al., 1992; Malassine and Cronier, 2002]. During maturation CT cells switch from a proliferative state in the proximal inner part of the column into an invasive, non-proliferative phenotype at the distal external part of the column [Bulmer et al., 1988; Genbacev et al., 1992]. Decreased trophoblastic invasion may be responsible for preeclampsia, which is an important cause of maternal and fetal mortality. Interestingly, lately it was demonstrated that heat-shock-protein-27 (HSP27) levels are elevated in the placentae of women with preeclampsia in comparison to normal term gestations [Geisler et al., 2004]. HSP27, actively transcribed during trophoblast differentiation, is a downstream regulator of actin filaments structure and dynamics [Landry and Huot, 1995; Morrish et al., 1996; Hirano et al., 2004]. Association between HSP27 levels and MMPs, important facilitators of trophoblast invasion, level, and function has been described [Hansen et al., 2001; Aldrian et al., 2002]. HSP27 may also be involved in NF-kB regulation, which is responsible for transcription of the inducible nitric oxide synthase (iNOS) and resultant nitric oxide (NO) [Guzik et al., 2003; Parcellier et al., 2003]. Interestingly, NO and cGMP are involved in trophoblast motility and apoptosis and may be regulated by multidrug-resistance-protein-5 (MRP5) [Cartwright et al., 1999, 2002; Xu et al., 2004; Dash et al., 2005]. MRP5

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is a transmembrane transport protein for cyclic nucleotides [especially 3′,5′-cyclic GMP (cGMP)] recently suggested to participate in placental differentiation [Xu et al., 2004; Meyer et al., 2005]. One of MRP5 substrates is the immunosuppressive/chemotherapeutic agent 6-mercaptopurine (6MP) which was previously reported by us to inhibit EVT cells invasion into the matrigel [Estlin 2001; Matalon et al., 2005; Ritter et al., 2005]. In this study we aimed to assess HSP27 expression and levels during EVT differentiation in placental explants and after 6MP induced inhibition of migration. Moreover, associations between HSP27 levels, motility, and differentiation related processes (MMPs activity, Ki67, iNOS, and NO) were studied. Also, MRP5 levels (mRNA), which may affect 6MP and cGMP cytoplasmatic concentrations were examined as well.

MATERIALS AND METHODS

Tissue Preparation and Culture

The local ethical committee has approved the use of placental tissues. Placentae (6–8 weeks) retrieved from normal pregnancies terminated legally due to psychosocial causes were used. Placental villi were dissected from the fetal membranes [Genbacev et al., 1992; Matalon et al., 2005]. Explants of 10 mg wet-weight were transferred into culture dish inserts (pore size: 0.4 μm, diameter: 12 mm, Millipore Corporation, Bedford), layered with 200 μl matrigel (BD Biosciences, Bedford). DMEM supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), antibiotics, fetal-calf-serum (10%), was added to the lower well of the culture dish (bottom-medium) and no medium was added above the explants (Biological Industries, Beit-Haemek-Israel). Cultures were preincubated overnight in 5% CO_2 incubator. Twelve to 16 h later bottom-media was replaced and media with/without 6MP in clinically relevant concentrations (0.01 mM in 1 M NaOH, Sigma M7000) was added above the placenta (top-medium). 6MP was dissolved in NaOH 1 M (50 mg/ml), and diluted with the medium to the appropriate concentration. Five placentae were analyzed in the study. Medium with NaOH alone served as control. An additional control of media only was included in three experiments. Indeed, no effects of NaOH were observed in assayed parameters (data not shown). Both media from the insert (top) and from the well (bottom) were replaced 24, 72, and 96 h from initiation of experiments and stored at −80°C. Triplicates of each treatment for each placenta in every time point were preformed. Villi were dissected 24 and 96 h after experiments initiation. Specimens were fixed (4% buffered formaldehyde) and paraffin embedded or freezes in liquid nitrogen for molecular analysis. Previously these cultures were used to analyze the effect of 6MP on trophoblast cell migration, apoptosis, and proliferation [Matalon et al., 2005].

HSP27 Analysis

HSP27 analysis was done by immunohistochemistry methods. 6MP affected HSP27 expression in the villous trophoblast cells (VTCs, whereas, most EVT cells with/without 6MP express HSP27 in the cytoplasm). Protein isolation from the placental explants could reflect the protein levels of both cell populations (villous/EVT cells) and mask the differences in HSP27 levels in the villous cells. However, immunohistochemistry which allows visual separation between different cells reflected solely the HSP27 levels on the VTCs. The immunohistochemistry method is described in the next section.

Immunohistochemistry

Sections were deparaffinized and pretreated with proteinase K (Ki67 staining only), immersed in 10 nM (pH 6) sodium citrate buffer and heated in a microwave oven. Endogenous peroxides activity was quenched in 1% H_2O_2. Then the slides were washed trice with PBS, covered with blocker serum, and incubated with the primary antibody for 20 h in 4°C (mouse anti low molecular weight cytokreatin, IgG1, Zymed, San Francisco, mouse anti Ki67, 0.16 μg/ml, clone 7B11, IgG1 Zymed; mouse anti HSP27, 1.5 μg/ml, clone G3.1, IgG1, Chemicon, CA; rabbit anti iNOS, 2 μg/ml, polyclonal, Novous biologicals, Inc.). Next, slides were washed trice with PBS, incubated with biotinylated antibody (10 min, 20°C), washed trice again and covered with horseradish peroxidase conjugated streptavidin (HRP-SA, 10 min, 20°C), washed trice again with PBS and developed with AEC–chromogen (Biotinylated antibody, HRP-SA, and AEC belongs to "Histostain plus" KIT Zymed laboratories, San Francisco, CA). Sections were counterstained with Mayer's hematoxylin. Isotype matched control antibodies were used to

exclude non-specific staining. No staining was observed in these procedures. For double staining procedure (HSP27 and Ki67) following AEC development of Ki67 staining, slides were washed trice with double distill water, covered with denaturing solution (3 min, 20°C, Biocare Medical, CA), washed trice with PBS and incubated for 2 h (20°C) with the second primary antibody for HSP27. Following PBS washing the slides were incubated with biotinylated antibody, covered with streptavidin conjugated alkaline phosphatase (DBS), washed trice with PBS, and developed with BCIP/NBT–chromogen (chemicon).

Cell Counting

Microscopic evaluation (400×) allowed enumeration of stained cells out of trophoblast cells present in every villous of every slice as detailed in Table I. VTCs were analyzed for HSP27 and iNOS staining, whereas EVT cells (most of them weakly positive for HSP27) were analyzed only for iNOS expression. Syncytiotrophoblast cells were not studied in this research. Experiments were carried out on five different placentae; accept the analysis of HSP27 levels following 24 h exposure that was tested in three placentae. Triplicates of each treatment for each placenta in every time point were preformed. (Since we analyzed 5 placentae we had 15 replicate for each treatment, accept for analyzing HSP27 in 24 h that was preformed nine times). We counted cells in 2 slices from each of the 15 or 9 replicates. In every slice, cells were counted in 5–10 different villi.

Gelatin Zymography

Top media of all placentae were collected for gelatinase activity. Aliquots (20 μl) of top media from 10 mg placental explant culture were electrophoresed at non-reducing conditions in 10% polyacrylamide gels containing 1 mg/ml gelatin type A (Sigma, St. Louis, MO). Gels were washed trice in 2.5% Triton X-100 for gelatinase renaturation and incubated overnight in 50 mM Tris–HCl (pH 7.5) and 5 mM $CaCl_2$. Coomassie blue staining followed by destaining allowed visualization of clear lysis zones. Analysis was done on three randomly chosen explants from each treatment in every experiment ($3 \times 5 = 15$ explants per treatment). MM2 and MMP9 were identified by gelatinase zymogram standards for human MMP2 and MMP9 (Chemicon) that were electrophoresed adjacent to the placental aliquot. Analysis of gel bands intensity was done employing Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA). Evaluation of MMP activity was done by multiplication of band area (area) with light intensity that pass through the band (count) (count × area).

Nitric Oxide Detection

We assessed levels of nitrite, a stable end-product of nitric oxide with Griess reagent (100 μl, 1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4) mixed with 200 μl top medium of three randomly chosen 10 mg placental explants incubated 96 h with/without 6MP in each experiment ($3 \times 5 = 15$ explants/treatment). Absorbance was measured at 550 nM. Nitrite levels were extrapolated from a standard curve of $NaNO_2$.

Semi-Quantitative RT-PCR

Total RNA was extracted from the trophoblast explants employing PURESCRIPT (Gentra #R-5110) according to manufacturer's specifications and reverse transcribed (SuperScript II™, GIBCO BRL #18053-017) with oligo $d(T)_{15}$ primers. Three microliter MRP5 cDNA were amplified in a multiplex reaction with a housekeeping gene (β-actin) as an internal reference of the reaction efficiency. PCR was optimized

TABLE I. Cell Counting of HSP27, Ki67, and iNOS in Human Trophoblast Cells

Ag.	Time[a]	N	Replicate/ treatment	Slices[b]	Villi/slice	Cells in the villi average + STD		EVT in the matrigel/slice	
						Control	6MP	Control	6MP
iNOS	96	5	5 × 3 = 15	30	5	49 + 25	46 + 18	76 + 38	75 + 39
HSP27	96	5	5 × 3 = 15	30	5–10	75 + 47	66 + 41	NC	NC
HSP27	24	3	3 × 3 = 9	18	5–10	87 + 55	79 + 48	NC	NC

N, number of placentae.
NC, not count.
[a]Time from experimental initiation (h).
[b]Slices per treatment (6MP, Control).

(logarithmic phase) at an initial denaturation of 1 min at 95°C followed by 12 cycles of 30 s at 95°C (denaturation), 30 s at 62°C/increment −0.5°C per cycle (annealing), 25 s at 72°C (extension) and 23 cycles of 20 s at 95°C (denaturation), 25 s at 56°C (annealing), 25 s at 72°C (extension). Primers used were: MRP5 sense 5′TCCATGCATTCTCAGCTCAG3′; antisense 5′CGTCT GGCCCAACTTCATTC; β-actin sense 5′GAGACCTTCAACACCCCAG-C3′; antisense 5′GCTCATTGCCAATGGTGA-TG3′. Electrophoresed RT-PCR products were visualized with Gel Doc 2000 and Multi-analyst software (Bio-Rad). RT-PCR products were normalized according to β-actin amplification efficiency and then compared with transcript level of untreated placental explants. Nine controls and seven 6MP treated trophoblast explants were assayed by RT-PCR for MRP5 expression. Quantified results are presented as Mean ± SE.

Statistical Analysis

The non-parametric "Wicoxon matched-pairs signed-ranks test" was used for the analysis of all tested parameters except for the correlation tests. Pearson correlation and the non-parametric spearman tests were used to determine the degree of correlation between two different groups of variables. Statistic analysis was preformed on results of five placentae (for 96 h analysis) except for HSP27 analysis at 24 h that was preformed on three placentae. A $P < 0.05$ was considered significant.

RESULTS

Staining for Low Molecular Weight Cytokeratin

To identify the trophoblast cells in the villi and the EVT cells in the matrigel we stained placental explants with antibodies to low molecular weight cytokreatin (Fig. 1a). EVT cells as well as trophoblast cells in the villi were stained positively.

Differentiation of Trophoblast Cells

In order to characterize CT cells proliferation and differentiation into migratory cells, Ki67 expression, and MMP2/9 activities were analyzed. In trophoblast explant model flattening

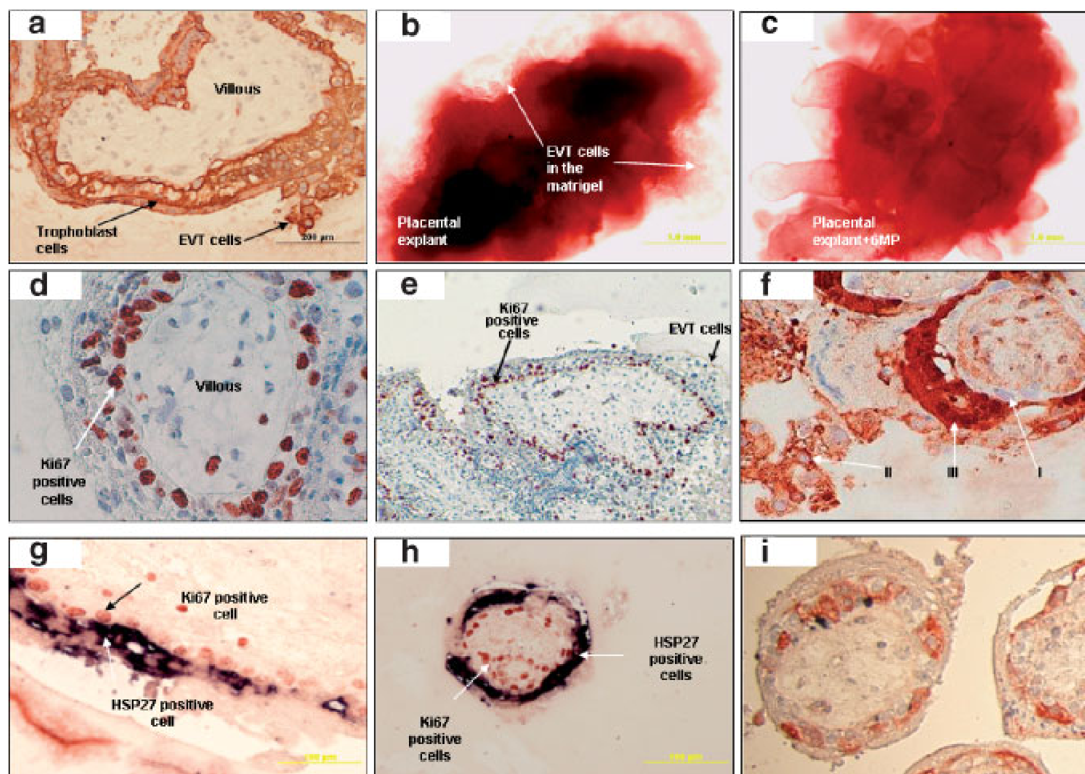

Fig. 1. Photographs demonstrating: cytokeratin expression in trophoblast villi (**a**), trophoblast explants on the matrigel [control (**b**) and 6MP exposed (**c**)], Ki67 staining (**d**,**e**), HSP27 expression in control placental explant (**f**) versus 6MP exposed explant (**i**) and Ki67/HSP27 double staining (**g**,**h**) of placental explants.

and attachment of villous tips to the substrate were observed within the first 12–16 h of culture followed by cell migration into the matrigel 24 h later. The migration continued mostly in the next 2 days of culture as expected (Fig. 1b) [Genbacev et al., 1992]. Following 96 h of culture anti Ki67 monoclonal antibody stained mainly cells in the proximal (inner) side of the villous (Fig. 1d). Some villi displayed Ki67 in CT cells located in the distal part of the villous; however, no staining was observed in the EVT located in the matrigel (Fig. 1e). MMPs were analyzed in placental explants top medium. MMP9 and MMP2 secreted from 10 mg placental explants were identified in the upper placental medium (Fig. 2a). Most of the MMP2 was in its pro-enzyme state. Activity level of MMP2 following zymogram assay was higher then that of MMP9 (Fig. 2b, $P < 0.05$). The activity levels of both enzymes increased during the first 72 h of culture. However, these elevations were not statistically significant due to the high variability of the different placentae.

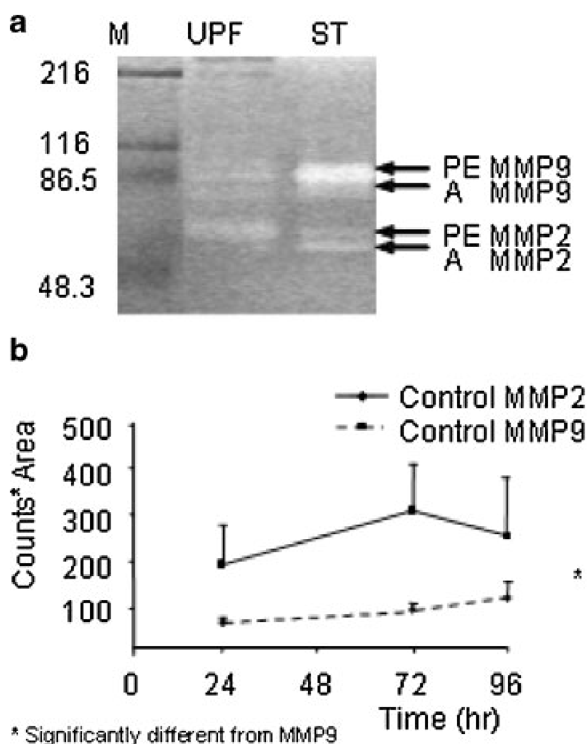

Fig. 2. MMP2/9 activities in control explants. MM2 and 9 were identified in placental upper medium by gelatinases zymogram standards for human MMP2 and MMP9 that were electrophoresed adjacent to the placental aliquot. Representative zymogram test of placental upper fluids 72 h following placental culture (UPF-upper placental fluid, ST-zymogram standard for MMP2/9, PE- pro-enzyme, A-active enzyme (**a**)). Top media from 10 mg placental explants (controls) were collected at different time points and MMPs activities were analyzed by gelatin zymography assay (**b**). Data represent the average ± SE of five independent experiments.

Expression of HSP27 in Trophoblast Cells During Differentiation

During trophoblast differentiation, HSP27 expression of the various VTCs varied. The proximal inner part of the villous was repeatedly negative for HSP27 staining (Fig. 1f,i). These negative cells were usually positive for Ki67 (Fig. 1d). Double staining procedure for HSP27 and Ki67 demonstrated that HSP27 positive cells do not proliferate (Fig. 1g,h), and that the proliferating cells do not express HSP27. However, more then 50% of the villous trophoblast (located in the basal side of the villous between the syncytiotrophoblast and the proliferative VTCs, Fig. 1f-III) and most of the EVT cells in the matrigel stained positively (Fig. 1f-II). Interestingly, most of the trophoblast cells in the villous that expressed HSP27 did so in the nucleus (with/without cytoplasm) (Fig. 3) whereas EVT cells in the matrigel almost always expressed HSP27 in the membrane and the cytoplasm alone (Fig. 1f-II). The number of cells expressing HSP27 in the cytosol increased significantly with time (Fig. 3, $P < 0.05$).

The Effect of 6MP on HSP27 Expression in Trophoblast Cells

In order to evaluate the relationship between HSP27 expression and EVT motility we used 6MP as an inhibitor of trophoblast migration. In our previous study we demonstrated that 6MP inhibited EVT cell migration to the matrigel. In the current study we found that 6MP inhibited HSP27 expression in the trophoblast villous cells [Fig. 1f (control) vs. Fig. 1i (6MP)]. Immunohistochemistry analysis of HSP27 expression demonstrated a reduction of 25% in the number of HSP27 stained cells in the 6MP treated explants after 96 h in culture (Fig. 3, $P < 0.05$). A significant decrease in the number of cells expressing HSP27 in both cytoplasm and nucleus were found (Fig. 3). Interestingly, 6MP did not affect HSP27 staining in the first 24 h (Fig. 3).

Effect of 6 MP on MMPs Activity Level

Since MMPs are important facilitators of trophoblast invasion during motility and because HSP27 was previously demonstrated

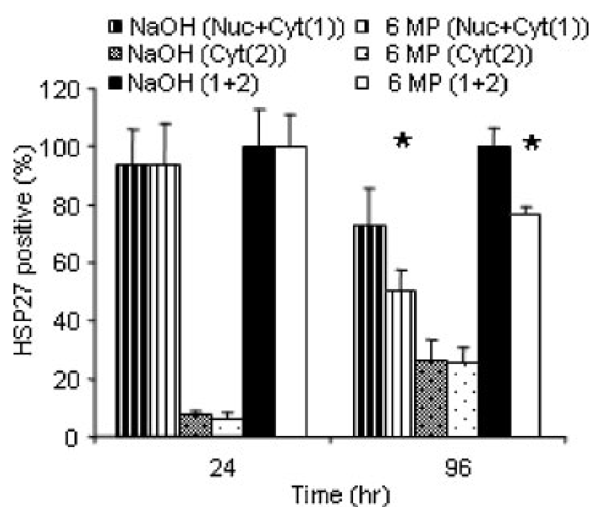

Fig. 3. HSP27 expression in 6MP treated and control explants. First trimester placental explants were exposed to 6MP or the medium alone. Following 24 or 96 h of incubation the explants were fixed in 4% formaldehyde, paraffin embedded, and anti HSP27 staining was preformed. Quantitative analysis of HSP27 expression was done by counting stained cells out of trophoblast cells in the villi (Nuc-Nucleus, Cyt-cytoplasm). Data represent percent of stained cells form total cell count (Nuc+ Cyt) in the control. Data represent the average ± SE of five independent experiments (96 h) and three independent experiments (24 h).

to be associated with MMP's activity [Hansen et al., 2001], we further analyzed the effect of 6MP on MMPs released by trophoblast cells into the medium. Twenty-four hours following establishment of the culture 6MP had no effect on MMPs activity as was tested in the zymogram assay. However, 48 h afterward 6MP inhibited MMP2 activity but did not affect MMP9. Twenty-nine and 44% inhibition of MMP2 activity compared to control were observed following 72 and 96 h of 6MP exposures, respectively (Fig. 4a,b). MMP2 was significantly inhibited following incubation of 72 h ($P < 0.05$) but not 96 h. This can probably be attributed to the high variability in MMP2 activity at the end of the culture period. A significantly positive correlation was found between the effects of 6MP on HSP27 level in the villous to its effect on MMP2 activity (72 h) (Fig. 4c).

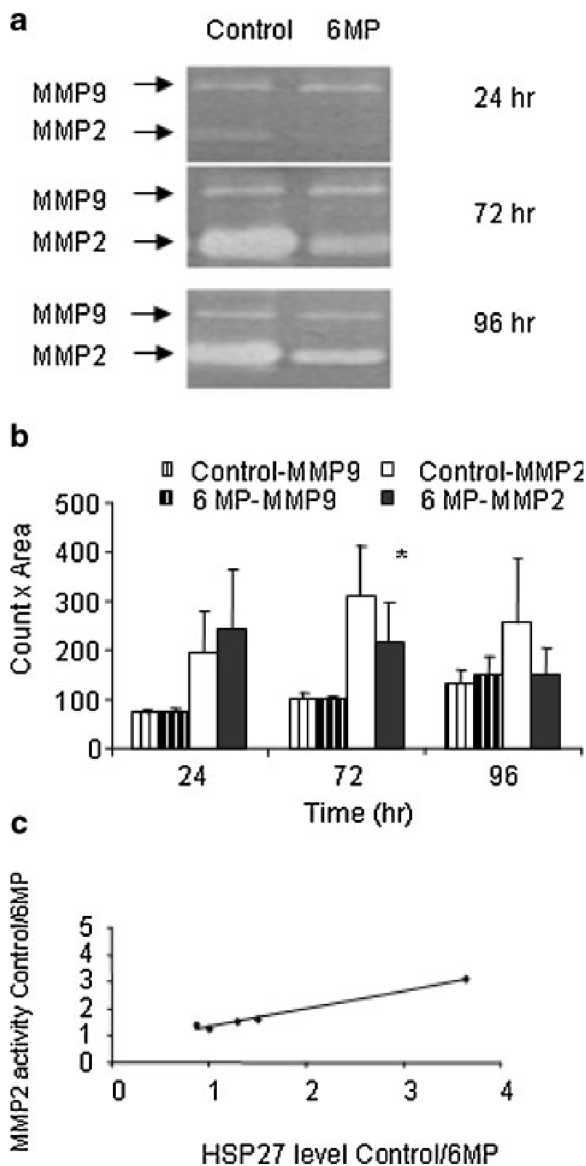

Fig. 4. MMP2 and MMP9 activities in control and 6MP exposed explants. Twenty microliter top media from 10 mg placental explants cultured with/without 6MP were collected at different time points during the experimental period and MMPs were analyzed by gelatin zymography assay. Representative figure of MMP2 and MMP9 activities in control versus 6MP treated explant from the same placentae at different time points (**a**) and average MMPs activities of five different placentae during the experimental period (**b**) as well as the correlation between MMP2 activity and HSP27 expression (values demonstrated are results of the ratio Control/6MP) (**c**) is demonstrated. Data represent the average ± SE of five independent placentae (preformed in triplicate).

Effect of 6MP on NO Level

Since NO is involved in cell migration and may regulate MMPs activity in human trophoblast cells [Kook et al., 2003; Dash et al., 2005] we further analyzed nitrite levels in media of explants treated/not treated with 6MP (96 h). 6MP decreased NO level in all the placentae. A 28% decrease in nitrite levels was observed in 6MP treated explants compared to controls ($P < 0.05$, Fig. 5a).

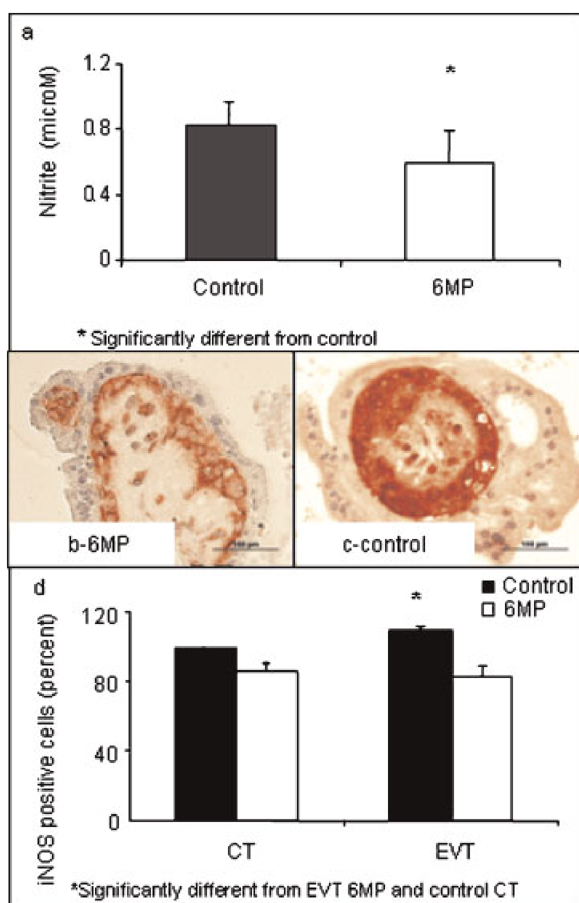

Fig. 5. Analysis of No and iNOS levels. Nitrite levels in 200 μl top media of 10 μg explants treated/not treated with 6MP for 96 h was measured with Griess reagent. The level of nitrite was extrapolated from a standard curve of $NaNO_2$ (**a**). Data represent the average ± SE of five independent experiments. For iNOS analysis first trimester placental explants were exposed to 6MP or to the medium alone. Following 96 h of incubation the explants were fixed in 4% formaldehyde, paraffin embedded, and anti iNOS staining was preformed. Photographs of iNOS expression in placental explants cultured with/without 6MP are represented in (**b** and **c**, respectively). Quantitative analysis of iNOS expression was done by counting stained cells out of trophoblast cells in the villi and the data are in (**d**). Data represent the average ± SE of five independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Effect of 6MP on iNOS Level

Since NO is produced by iNOS and is involved in trophoblast motility [Aktan et al., 2004] we further analyzed the effect of 6MP on iNOS expression. All trophoblast cells stained positive for iNOS except for the syncytiotrophoblast cells. The strongest staining was observed in the control motile EVT in the matrigel. In the trophoblast controls, a higher number of cells were stained in the nuclei (villous trophoblast and EVT) in comparison to the 6MP exposed explants [$P < 0.05$ (Fig. 5b–d)]. Moreover, in the control explants more EVT cells were stained in the nuclei compared to CT cells ($P < 0.05$). However, similar number of EVT and trophoblast villous nuclei stained cells was observed in the 6MP treated explants (Fig. 5d).

Effect of 6MP on MRP5 mRNA Levels

Since most effects of NO are mediated through cGMP we further analyzed the effect of 6MP on MRP5 (cGMP transporter). mRNA was purified from frozen explants of three different experiments. As expected smaller yields of total RNA were purified from the 6MP treated explants in comparison to controls (72.1 vs. 131.7 μg/ml, respectively, $P = 0.006$). This result is consistent with previously published data [Kawahata et al., 1983]. By means of semi-quantitative RT-PCR we tested MRP5 steady state transcript mRNA levels. Higher levels of MRP5 mRNA were found in the 6MP treated explants. Despite the up regulation these results did not reach statistical significance due to the high variability between the different trophoblast explants. Interestingly no migration of EVT cells was observed in 6MP treated explants that had elevated MRP5 levels whereas all the explants excluding one that displayed EVT migration (6MP treated/ not treated) had decreased MRP5 mRNA (Fig. 6a,b).

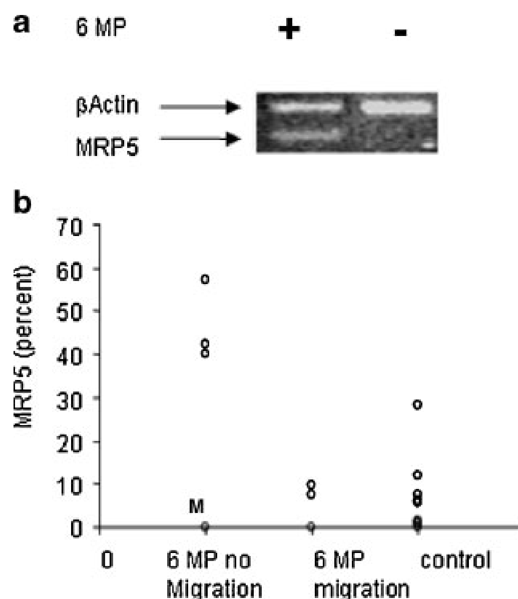

Fig. 6. **A**,**B**: Analysis of MRP5 mRNA levels. Semi-quantitative RT-PCR served for MRP5 mRNA analysis. Amplification of MRP5 was conducted in a multiplex PCR with a housekeeping gene (β-actin) as an internal reference of the reaction efficiency.

DISCUSSION

During implantation EVT differentiate, invade maternal uterine blood vessels [Genbacev et al., 1992] and progressively loose their proliferative capacity [Bulmer et al., 1988]. In order to study HSP27 expression during trophoblast cell differentiation and motility, we used the placental explants culture model in which the placental explants are located above the matrigel and the EVT migrate from the villous into the matrigel [Genbacev et al., 1992]. We found that during the culture period trophoblast cells express HSP27 variably according to their stage of differentiation: professional migratory cells express HSP27 in cell cytoplasm; trophoblast non-proliferating villous cells displayed HSP27 in the nucleus and cytoplasm and proliferating villous cells did not express HSP27. 6MP that inhibited trophoblast cells' migration [Matalon et al., 2005] concomitantly induced a decrease in HSP27, iNOS, NO levels, and MMP2 activity. Also, 6MP increased MRP5 mRNA levels. Finally the effect of 6MP on HSP27 expression correlated positively with its effects on MMP2 activity.

Genbacev et al. (1992) demonstrated that migration of EVT from the villous into the matrigel reliably mimics EVT invasion in vivo. Cytotrophoblast cells in the villous stained positively for Ki67. However, the differentiated EVT cells in the matrigel, like the EVT cells in the uterus, loose their proliferative activity and are Ki67 negative [Bulmer et al., 1988]. Thus, the migratory potential is linked to the differentiation of the EVT cell. We found that the level and localization of HSP27 were affected by the proliferation and differentiation stage of the cells: trophoblast cells in the proximal inner side of the villous were Ki67 positive but did not express HSP27. Inversely, differentiated EVT in the matrigel, negative for Ki67, stained positively for HSP27 in the cell periphery. Similar inverse correlations between HSP27 expression and cell proliferation were demonstrated in human breast and gynecological cancer cell lines that were treated with antineoplastic agents [Vargas-Roig et al., 1997; Tanaka et al., 2004]. Moreover, over expression of HSP27 in laryngeal cancer cells delayed cell growth, compared to control cells [Lee et al., 2007]. Thus, our findings suggest that HSP27 is involved in growth arrest and differentiation of trophoblast cells and that 6MP may have inhibited differentiation of trophoblast cells by reducing HSP27 levels.

Localization of HSP27 in the cell periphery probably reflects the capability of HSP27 to act as an actin cap-binding protein that regulates actin polymerization [Garrido, 2002]. Thus, it is not surprising that the professional migratory EVT cells display HSP27 in their periphery. The positive correlation between 6MP inhibitory effect on HSP27 expression (nuclear and cytosolic) and on migration also supports HSP27 role in trophoblast cell migration regulation and suggests that 6MP affected trophoblast motility through HSP27 regulation. HSP27 was previously demonstrated to be associated with MMP's activity. Indeed, HSP27 is a downstream effectors of p38 MAP kinase-mediated matrix MMP2 activation and cell invasion in human prostate cancer [Xu and Bergan, 2006]. Moreover, genistein (cancer chemopreventive drug) inhibits MMP2 activation and prostate cancer cell invasion by blocking the MAPKAPK2—HSP27 pathway [Xu and Bergan, 2006]. Since MMPs are important facilitators of trophoblast invasion and because HSP27 is associated with MMP's activity we further analyzed the effect of 6MP on MMPs activity. Indeed 6MP reduced MMP2 activity that paralleled the decrease in HSP27 expression. The selective effect of 6MP on MMP2 activity with no effect on MMP9 strengthens the previous suggestion that MMP2 is the main gelatinase responsible for the invasive ability of trophoblast cells [Staun-Ram et al., 2004]. Of note was the similarity in the timeframe of MMP2 activity and HSP27 expression that supports the assumption that HSP27 does regulate motility related processes or that common pathways are involved in regulation of both HSP27 and MMP2.

These findings, as well as the known regulation of trophoblast MMP2 by NO [Novaro et al., 2001, 2002] and the established importance of NO in migratory processes [Kook et al., 2003; Lee et al., 2005] prompted us to examine the effect of 6MP on NO levels in the explants culture media. Indeed, 6MP induced significant reduction of NO levels.

The mechanisms by which 6MP inhibited EVT cell migration and decreased NO levels are not clear. However, increased apoptosis and inhibition of motility by 6MP were observed in other biological systems and involved cGMP which mediate most effects of NO [Elferink

et al., 1997; Hortelano and Bosca, 1997]. GMP mediated rearrangement of the cytoskeleton and involvement in apoptosis dynamics were previously demonstrated [Gorodeski, 2000; Dreiza et al., 2005]. Moreover, the 6MP transporter MRP5 is also a transmembrane transport protein for cGMP [Meyer et al., 2005]. Induction of MRP5 following 6MP exposure was demonstrated herein. Similar induction of MRP5 mRNA following exposure to chemotherapeutic agent was previously reported [Yoshida et al., 2001]. MRP5 may play a biological role in cGMP nucleotides cellular signaling. Indeed, a role for MRP5-mediated cGMP efflux in the regulation of NO [Xu et al., 2004] and in trophoblast differentiation has already been suggested [Meyer et al., 2005]. It was also suggested that induction of MRP5 facilitates elimination of cGMP from the trophoblast cells [Meyer et al., 2005].

The mechanism underlying the 6MP modulation of HSP27 levels is unclear. Interestingly, previous studies demonstrated a regulatory effect of cyclic nucleotides on HSP expression via gene promoter [Choi et al., 1991].

We showed a positive correlation between 6MP's effect on HSP27 level, motility, and NO production. We also demonstrated decreased iNOS nuclear expression in 6MP treated explants concomitant with reduced HSP27. Involvement of iNOS and NO in migration of trophoblast cells was already suggested [Cartwright et al., 2002; Dash et al., 2005]. Expression of iNOS that generates NO is regulated by NF-kB transcription factor [Aktan, 2004]. Interestingly, it was shown that HSP27 may be involved in I-Kbα proteasomal degradation, which is the major NF-kB inhibitor, and its inactivation occurs through proteasomal degradation [Parcellier et al., 2003]. Thus, we hypothesize that 6MP's effect on HSP27 may be responsible for decreased NO production and inhibition of trophoblast migration through NF-Kb regulation as suggested in Figure 7.

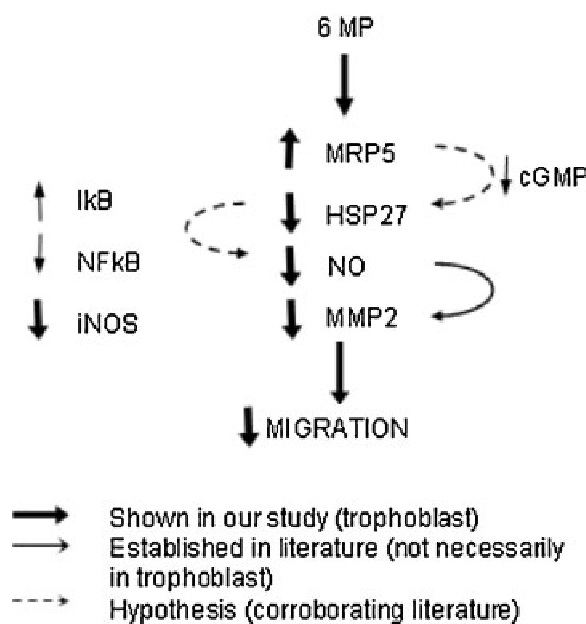

Fig. 7. Schemes which suggest the following hypothesize: 6MP's effect on HSP27 is responsible for the decreased NO production and the inhibition of trophoblast migration through NF-Kb regulation.

Conclusions

We demonstrated that HSP27 levels increase in differentiated trophoblast cells and that placental exposure to 6MP (known inhibitor of EVT invasion) is associated with reduction in trophoblast HSP27, iNOS, NO, and MMP2 levels. Moreover, HSP27 levels are correlated with trophoblast MMP2 activity. The association between HSP27, MMP2 activity, NO levels and motility suggests that HSP27 is involved in the regulation or shares common pathways with theses processes.

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