Impaired migration of trophoblast cells caused by simvastatin is associated with decreased membrane IGF-I receptor, MMP2 activity and HSP27 expression

S. Tartakover-Matalon1,5, N. Cherepnin1, M. Kuchuk2, L. Drucker1,4, I. Kenis2, A. Fishman3,4, M. Pomeranz3 and M. Lishner1,2,4

1Oncogenetic Laboratory, 2Department of Medicine, 3Department of Obstetrics & Gynecology, Meir Medical Centre, Kfar-Saba and 4Sackler Faculty of Medicine, Tel–Aviv University, Israel

5To whom corresponding should be addressed at: Oncogenetic Laboratory, Meir Medical Centre, 45 Tshernehovski ST. Kfar-Saba, 44281, Israel. E-mail: matalon.shelly@clalit.org.il

BACKGROUND: Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme-A reductase, the rate-limiting enzyme of the mevalonate pathway, and are used successfully in the treatment of hypercholesterolaemia. Statins are contraindicated during pregnancy. Lately, we have shown that simvastatin has adverse affects on human first trimester placental explants’ proliferation and migration. The objective of the present study was to investigate the molecules involved in mediating simvastatin’s effect on trophoblast cell migration. We hypothesized that simvastatin attenuates insulin-like growth factor-I (IGF-I) receptor expression (involved in trophoblast motility), matrix metalloproteinase (MMP) activities, and heat shock protein 27 (HSP27) levels (whose mRNA is actively transcribed during trophoblast differentiation) in trophoblast cells thus consequently effecting their migration. METHODS: Human placental explants were cultured above a matrigel with/without simvastatin (10 \( \mu \)M) for 5 days. In this model, trophoblast migrates from the villi into the matrigel. Western-blot and immunohistochemistry served for analysing HSP27 expression. Immunohistochemistry was used for assessing IGF-I receptor localization. MMPs activity was assayed by gel zymography. RESULTS: Simvastatin reduced IGF-I receptor membranal expression, MMP2 activity and HSP27 expression in trophoblast cells (\( P < 0.05 \)). CONCLUSIONS: The inhibitory effect of simvastatin on trophoblast cell migration is associated with a significant decrease in the tested molecules, which probably contributes to the impaired migration.

Key words: HSP27/IGF-IR/MMP2/simvastatin/trophoblast

Introduction

Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase, the rate-limiting enzyme of the mevalonate pathway, and are used successfully in the treatment of hypercholesterolaemia (Desager and Horsmans, 1996). Inhibition of HMG-CoA reductase results in reduced levels of cholesterol, which is required for normal development in mammals (Benahmed et al., 1983; Siperstein, 1984; Henson et al., 1997). In addition, HMG-CoA reductase inhibition also results in decreased concentrations of the many isoprenoid derivatives of mevalonate, as well as other growth regulating proteins bound to fernesyl and geranylgeranyl residues (Middleton et al., 1984; Larsson, 1996; Bellosta et al., 2000). Major cellular functions depend on isoprenylation including those of G proteins (RAS) and growth factor receptors (Larsson, 1996; Bellosta et al., 2000). Thus, statins may influence membrane synthesis, cellular proliferation and migration, metabolism and protein glycosylation, all crucial for normal development of the embryo and the placenta (Larsson, 1996; Bellosta et al., 2000, Gliemroth et al., 2003).

Indeed, statins are contraindicated during pregnancy although very few data are available regarding the consequences of their use during this period. Animal models (rats, mice, rabbits) have provided evidence for the teratogenic effects of statins on pregnancy outcome (Dostal et al., 1994; Masters et al., 1995). However, conflicting clinical and laboratory data are available from inadvertent use of statins in human pregnancy (Hosokawa et al., 2003). Lately, we have shown that simvastatin has adverse effects on human first trimester placental explants. We demonstrated that simvastatin inhibited proliferation and invasion of trophoblast cells in first trimester placental explants model (Kenis et al., 2005). However, the mechanisms responsible for these effects have not yet been identified.

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During the first trimester of pregnancy, the placenta enables the development of the embryo and the fetus by exchanging ions, metabolites and waste products. The human placenta contains cytotrophoblast (CT) progenitor cells. These cells retain the ability to proliferate and can differentiate to form syncytiotrophoblast cells that perform gas and nutrient exchange for the developing fetus and are responsible for hormonal secretion (Malassine and Cronier, 2002). Alternatively, they may give rise to extravillous trophoblast (EVCT) cells (Malassine and Cronier, 2002). EVCT cells that are released from the CT column progressively migrate and diffusely infiltrate the decidua and invade maternal uterine blood vessels (Chakraborty et al., 2002). Trophoblast cells’ proliferation and invasion are stringently regulated by several proteins including growth factors and proteolytic enzymes (Chakraborty et al., 2002). Many studies established the importance of insulin-like growth factors (IGFs) in the control of trophoblast cell fate demonstrating their influence on proliferation rate, invasion and matrix metalloproteinase (MMP) activity (Mandl et al., 2002; Hills et al., 2004; Kabir-Salmani et al., 2004). Another gene whose mRNA is actively transcribed during trophoblast differentiation is the heat shock protein 27 (HSP27) (Morrish et al., 1996). HSP27 is a downstream regulator of actin filament structure and dynamics (Landry and Huot, 1995). Thus, it modulates motility of endothelial, muscle and fibroblast cells (Piotrowicz et al., 1998; Hedges et al., 1999; Hirano et al., 2004). Recently, we have also demonstrated the existence of a positive correlation between HSP27 levels in trophoblast cells and motility dependent factors such as MMPs activity and migration (Matalon et al., un-published data) and suggested that this molecule is important in the differentiation of EVCT cells to professional migratory cells.

The objective of the present study was to investigate the molecules involved in mediating simvastatin’s effect on trophoblast cell migration. We hypothesized that simvastatin attenuates IGF-I receptor expression (involved in trophoblast motility), MMPs activities, and HSP27 levels in trophoblast cells thus affecting their migration. Therefore, we examined the effect of simvastatin on these parameters in trophoblast cells during their differentiation to migratory trophoblast cells by using the placental explants culture model.

Methods

Tissue preparation and culture

The local ethical committee approved the use of human placental tissues for this study. Placental tissues (6–9 week gestational age) retrieved from normal pregnancies terminated legally due to psychosocial causes were used. The culture techniques were based upon methods that were previously described (Genbacev et al., 1992). In brief, the placental tissue (villi) was dissected from the fetal membranes. Explants of 10 mg wet weight were transferred into Millicell—CM culture dish inserts (Millipore Corporation, Bedford, USA), which had been layered with polymerized Matrigel (BD Biosciences, Bedford, USA). Dubecco’s modified Eagle’s medium (DMEM)/F-12-HAM)1:1, 1-glutamine (2 mM), sodium pyruvate (1 mM), antibiotics and fetal calf serum (10%), (Biological Industries, Beit-Haemek, Israel) were added to the lower well of the culture dish (bottom medium). Cultures were pre-incubated overnight in a 5% CO₂ incubator. On the following day, media was added above (top medium) and below the placental explants. During that time, simvastatin (10 μM) (generously provided by Merck&Co, Rahway, NJ, USA) was added to the upper medium of the placental culture. Simvastatin was dissolved in 0.1 M NaOH, and diluted with the medium to the appropriate concentration. Medium with or without NaOH 0.1 mM served as controls (‘control NaOH’ and ‘control’, respectively). Both media from the insert (top, with/without the drug), and from the well (bottom) were changed 24, 72 and 96 h following the beginning of the experiment, and the collected media were stored at −20°C until processing. Villi were inspected daily using an inverted phase-contrast microscope for general cellular integrity, cellular migration and outgrowth. Five placenta were analysed in the study. Hexaplicates of each treatment for each placenta were preformed. Ninety-six hours following the beginning of the experiments, villi with supporting matrigel were dissected out. The specimens were fixed in 4% buffered formaldehyde and paraffin embedded for immunohistochemical examination.

IGF-IR expression in the trophoblast explants

Paraffin-embedded sections were deparaffinized and washed in PBS. The slides were then immersed in 1 mM (pH 8) EDTA buffer and heated in a microwave oven for 15 min at 700 W. Endogenous peroxidase activity was quenched with 1% H₂O₂ (diluted in PBS). Samples were covered with normal blocker serum and incubated with primary antibody mouse anti IGF-IR (clone 24–31, mouse IgG1, Chemicon international) over night. Next, the slides were incubated with biotinylated second antibody, washed and covered with horseradish peroxidase-conjugated streptavidin, and developed with AEC-chromogen (Biotinylated antibody, HRP-SA and AEC-Zymed laboratories, San Francisco, California). Sections were counterstained with Mayer’s haematoxylin. Isotype matched control antibodies were used to exclude non-specific staining.

Staining for HSP27

The same procedure was used as for the IGF-IR staining. However, pretreatment with 10 nM (pH 6) sodium citrate buffer was preformed instead of the EDTA pretreatment and mouse antihuman HSP27 (clone G3.1, mouse IgG1, Chemicon, CA) was used instead of anti-IGF-IR.

Cell counting

Identification of trophoblast cells in the placental explants has been described previously using immunohistochemistry and antihuman low molecular weight cytokeratin antibody (Kenis et al., 2005). All trophoblast cells in the villi as well as EVCT were stained by the antibody (Kenis et al., 2005). Microscopic evaluation (×400) allowed enumeration of stained cells out of the total trophoblast cells present in the villi. The slides were quantitatively assessed by a person who was blinded to the type of sample being examined. Every test was performed on 3 explants for each treatment and experiment. Thus, thirty slices were counted for every treatment (5 placentae × 3 explants × duplicate for each explant). Ten villi were evaluated in every slice. The average number of cells in one villus was 40.

Gelatin zymography

Twenty-four and 72 hours post culture, top media of all placental explants (10 mg each) were collected for gelatinase activity. Aliquots (20 μl) of top media were electrophoresed at non-reducing conditions in 10% polyacrylamide gels containing 1 mg/ml gelatin type A (Sigma, St Louis, MO). Gels were washed in 2.5% Triton X-100 for gelatinase renaturation and incubated overnight in 50 mM Tris–HCl
after exposure to simvastatin. Trophoblast cells in the cytoplasm versus membrane staining, Figure 2A) was observed. Fied distribution of IGF-IR in the trophoblast cells (cytoplasmatic and membrane staining, Figure 2A) was observed. For MMP analysis, we used MMP9/2zymogram standards for human MMP2 and MMP9 (Cemicon) that were electrophoresed at non-reducing conditions in 10% polyacrylamide gels adjacent to the placental upper fluids.

Western blot analysis

Western blot analysis was carried out to detect MMPs in placental upper fluids and HSP27 in trophoblast explants treated/not treated with simvastatin. For MMP analysis, we used MMP9/2zymogram standard and concentrated placental upper fluids from cultured 10 mg placental explants (vivaspin concentrator, VivaScience). For HSP27 analysis, placental explants treated/not treated with simvastatin were lysed (10 min, 4°C) in a buffer (25 mM Tris (pH 7.5), 1% Triton X-100, 0.5 mM EDTA, 150 mM NaCl, 10 mM NaF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 200 μg/ml Phenyl methyl sulphonym fluoride (PMSF) and a 1:100 phosphatase inhibitor cocktail). Protein was quantified using the Bio-Rad protein assay method (Bio-Rad Laboratories, Hercules, CA, USA). Samples (100 μg protein) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% or 12% polyacrylamide gel for MMPs and HSP27 respectively, and transferred to nitrocellulose filters. Blots were blocked for 3 h in TBST (10 mM Tris–HCl (pH 8), 150 mM NaCl, and 0.05% Tween 20) containing 5% skimmed milk powder. The membranes were incubated with mAb anti-MMP2/MMP9 (1 μg/ml), mAb anti-HSP27 (1 μg/ml) for 24 h 4°C (clones 36006, 36020.111, R&D Systems, clone G3.1 Chemicon, respectively). Detection was performed using a horseradish peroxidase-conjugated secondary Ab (1 h, 20°C). Immuno-reactive bands were visualized using the ECL method according to standard procedures. Isotype matched control antibodies were used to exclude non-specific staining.

Statistical analysis

The Shapiro–Wilk test was used in order to check the distribution of the data. Since all data were normally distributed, paired Student’s-t-test was employed for analysis of the differences between treatments. An effect was considered significant when the P value was ≤0.05.

Results

The effect of simvastatin on IGF-IR expression

As previously shown, simvastatin is an inhibitor of trophoblast cell migration and proliferation (Kenis et al., 2005). Thus, we decided to analyse its effect on IGF-IR, an important regulator of trophoblast cell motility and proliferation (Mandl et al., 2002; Hills et al., 2004; Kabir-Salmani et al., 2004). We analysed the effect of simvastatin on IGF-IR in the trophoblast cells during their differentiation to migratory cells. The number of IGF-IR positive cells was similar in simvastatin exposed explants and controls (Figure 1A); however, a modified distribution of IGF-IR in the trophoblast cells (cytoplasmatic versus membrane staining, Figure 2A) was observed after exposure to simvastatin. Trophoblast cells in the simvastatin-exposed explants expressed IGF-IR mostly in the cytoplasm (85% and 79% cytoplasmatic staining and 15% and 21% membrane staining for EVCT and CT cells), whereas in the control explants, similar levels of cytoplasmatic and membrane expression of IGF-IR expression were observed. These differences in IGF-IR localization were statistically significant (Figures 1B and 2B1 and B2).

MMPs in placental fluids

Gelatinases are released to the placental medium during the culture period. MMP levels in upper placental fluids are very small, thus in order to identify gelatinases by western blotting, samples were concentrated. Only the pre-enzyme configuration (main form of the enzyme in the fluids) could then be found. However, bio-assay using the zymogram technology with MMP9 and MMP2 standards enabled the identification of the gelatinases as MMP9 and MMP2 (Figures 3A and B).
The effect of simvastatin on the level of MMP activity

Trophoblast invasion requires extensive remodelling of the endometrial extracellular matrix, a function largely performed by MMPs. Since simvastatin inhibited invasion of trophoblast cells into the matrigel, we further analysed its effect on MMP levels. Since bio-assay amplified MMPs visualization (as demonstrated in the previous section), we used activity assay for that purpose. MMP2 and 9 activity levels were analysed in fluids collected from 24 and 72 h cultured 10 mg explants with/without 10 \( \mu \)M simvastatin. Reduced MMP2 activity following simvastatin treatment was observed in each of the five experiments that were preformed (on five different placentae). However, high variability in control MMP2 activity was observed in the different placentae. Thus, we normalized the data to one time point to emphasize the similar effects of simvastatin in each of the experiments. Indeed, MMP2 activity level significantly increased during the tested 72 h (Figure 4A); however, MMP9 activity level did not change significantly (Figure 4B). Higher increase of MMP2 activity was demonstrated in the control explants (1240%) in comparison to the simvastatin-exposed explants (370%, Figure 4A). Seventy-two hours following the beginning of the experiment, simvastatin inhibited 40% of MMP2 activity (Figure 4A and C; \( P < 0.05 \)) but had no effect on MMP9 activity level.

The effect of simvastatin on HSP27 expression

Since HSP27 was previously demonstrated to correlate with trophoblast motility related parameters and to associate with MMPs activity level in other biological systems (Aldrian et al., 2002), we further analysed the effect of simvastatin on HSP27 expression. Western blot of proteins extracted from trophoblast explants treated with/without simvastatin demonstrated lower levels of HSP27 in the simvastatin-treated explants (Figure 6A). Moreover, paraffin sections were prepared from five different placenta that were cultured for 96 h with or without 10 \( \mu \)M simvastatin. Sections were stained with anti-HSP27. All EVCT cells in the matrigel expressed HSP27 in the cytoplasm and thus were not counted. However, only a fraction of the trophoblast cells in the villus were positive for HSP27 and most of them were stained in the cytoplasm and the nucleus. Villous trophoblast (VCT) cells that expressed HSP27 in the nucleus with/without simvastatin...
cytoplasmatic staining were counted and normalized to the total number of trophoblast cells in the villus (Figures 5A and 6). Simvastatin inhibited HSP27 expression in the nuclei of trophoblast villi cells (Figures 5 and 6). A reduction of 37% in HSP27 expressing cells was observed in the simvastatin treated explants (P < 0.05).

Discussion

The current study was conducted on a human first trimester placental model (Genbacev et al., 1992) in which the placental explants are located above the matrigel and the EVCT cells differentiate and migrate from the villi to the matrigel. Human placental explants contain CT, EVCT and syncytiotrophoblast cells, and the system has the advantage of presenting topological and functional villous–extravillous trophoblast inter-relationships. Using this model, we have previously shown that simvastatin inhibited migration and proliferation of the villous trophectoderm cells (Genbacev et al., 1992).

Figure 4. Matrix metalloproteinase (MMPs) activity in trophoblast explants treated/not treated with simvastatin. Top media from 10 mg placental explants treated/not treated with simvastatin were collected at different time points (24 and 72 h) and MMPs activities were analysed by a gelatin zymography assay. Assessment of MMP2 (A) and MMP9 (B) activities was achieved by multiplication of band intensity and its area (‘bands’—clear lysis zones, C). MMP activity at 72-h time point was normalized to 100 and other parameters were calculated as percent of this value. Example of a gel lysed by MMPs produced by explants treated/not treated with simvastatin following 72 h is demonstrated in C. Lanes 1–6 demonstrate results of one representative experiment. Lanes 1–3 demonstrate gel lysed by MMPs produced by three placental explants treated with simvastatin, lanes 4–6 demonstrate gel lysed by MMPs produced by three control placental explants that were dissected from the same placenta.

Figure 5. Photographs of placental villi that were stained with mouse anti-human heat shock protein 27 (HSP27) monoclonal antibody. (A) Trophoblast cells negative/positive for HSP27. (B) HSP27 staining of trophoblast cells not treated (Control, B1) treated (Simvastatin, B2) with simvastatin.
of trophoblast cells (Kenis et al., 2005). Here, we explored the molecular pathways that are affected by simvastatin and may modify these important functions of the trophoblast. Indeed, we show that simvastatin inhibits membrane expression of IGF-IR, HSP27 expression and reduced MMP2 activity in the trophoblast cells. Our experiments were carried out in 5% CO₂. The role of oxygen in determining the invasive capacity of trophoblast cells has been widely debated (Genbachev et al., 1996; Newby et al., 2005; James et al., 2006). Different studies demonstrated decreased, increased or even same invasion of first trimester EVT cells in different oxygen concentrations. In our model, more than 80% of the explants released EVCT to the matrigel and thus the effect of external parameters on placental EVCT migration could easily be tested.

Many studies have implicated IGFs in the control of the feto–maternal interface of human pregnancy (Hills et al., 2004). The ability of IGF-I to alter proliferation and migration of trophoblast cells is well established (Mandl et al., 2002; Hills et al., 2004; Kabir-Salmani et al., 2004). It has been reported that first-trimester placental fibroblasts produce IGF-I, which promotes the migration of trophoblast cells in vitro (Lacey et al., 2002). Indeed external supplement of IGF-I induced migration of EVCT cells in culture (Kabir-Salmani et al., 2004). Moreover, IGF-I increased CT cell number in various cell types (Calberg et al., 1996; Siddals et al., 2004). Statins induced reduction in mevalonic acid synthesis results in depletion of dolichyl phosphate that acts as a carbohydrate donor during N-linked glycosylation of membrane targeted proteins (Schenk et al., 2001). N-linked glycosylation is essential for the formation of mature functional IGF-IR since IGF-I α-subunit glycosylation is vital for proreceptors cleavage (Siddals et al., 2004). Here, we observed a reduction in membrane IGF-IR expression in simvastatin treated explants in comparison to controls. Reduced IGF-I membrane expression probably interferes with IGF-I signalling and contributes to the impaired migration capability of the cells. In the future, more studies using additional methods such as western blotting should be done in order to analyse IGF-IR levels in separated proteins of the cytoplasm and nucleus of the trophoblast cells.

Moreover, we demonstrated that simvastatin inhibited MMP2 activity of trophoblast cells. Again, previous studies also showed that inhibition of cell migration by statins is associated with significant reduction of MMPs levels (Luan et al., 2003). Interestingly, IGF-I was found to be one of the regulators of MMP2 synthesis (Zhang and Brodt, 2003; Zhang et al., 2004). Indeed, IGF-I up-regulates MT1-MMP (major activator of MMP2) and MMP-2 synthesis via PI 3-kinase/Akt/mTOR signalling in lung carcinoma cells (Zhang et al., 2004). Moreover, an increase in MMPs (2 and 9) levels was induced by IGF also in trophoblast culture derived form first trimester placentae (Hills et al., 2004). MMP2 plays a critical role in cell invasion by facilitating the degradation of basement membrane type IV collagen, exposing cryptic growth-affecting sites on extracellular matrix proteins (such as proteolysis of fibronectin which affects CT outgrowth (Apin et al., 1999; Mott and Wert, 2004), and increasing the bioavailability of matrix-associated growth factors (Zhang et al., 2003). Indeed, in our study MMP2 level increased dramatically during EVCT differentiation but this increase was significantly abrogated by simvastatin. MT1-MMP and MMP-2 are highly expressed and co-localized in the EVCT of anchoring villi and in CT that had penetrated into the placental bed (Bjorn et al., 1997). Moreover, MMP-2 is a key enzyme in early trophoblast (6–8 weeks) invasion (Staun-Ram et al., 2004) and is an important regulator of trophoblast cell migration (Bischof et al., 1995). Thus we hypothesize that decreased IGF-IR membrane expression diminished the receptor signaling and contributed to the reduced placental MMP2 activity following statin administration.

Interestingly, we also found decreased HSP27 expression in simvastatin-treated villi trophoblast cells. Theses findings are compatible with our previous work that demonstrated decreased trophoblast HSP27 expression associated with inhibited motility. Interestingly, IGF-I increased the degree of HSP27 phosphorylation in MCF-7 cells, suggesting common pathway for theses two molecules (Geier et al., 1997). The phosphorylation-modulated function of HSP27 can contribute to reorganization of the actin cytoskeleton and may affect cell differentiation, migration and proliferation (Landry and Huot, 1995; Mehlen et al., 1997; McMullen et al., 2005). Thus it suggests that HSP27 is involved in the regulation, or shares a common pathway, with IGF-IR and MMP2 activity.

In conclusion, we demonstrated that simvastatin decreased IGF-IR expression on trophoblast cell membranes and reduced HSP27 expression and MMP2 activity. Moreover, this study highlights the importance of HSP27 in trophoblast migration and suggests the involvement of IGF-IR in controlling MMP2 function of trophoblast cells.
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References


Simvastatin affects placental IGFIR, HSP27 and MMP 1167