Simvastatin has deleterious effects on human first trimester placental explants

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BACKGROUND: Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG-CoA reductase), the rate-limiting enzyme of the mevalonate pathway, and have been used successfully in the treatment of hypercholesterolaemia. Animal models have provided evidence for the teratogenic effects of statins on pregnancy outcome. Thus statins are contraindicated during pregnancy. However, conflicting data are available from inadvertent use of statins in human pregnancy. Therefore we decided to explore the effects of simvastatin on the placenta in an in vitro human placental model. METHODS: Human first trimester placental explants that were grown on matrigel were exposed to medium supplemented with simvastatin. Migration of extravillous trophoblast cells was assessed by visual observation. Proliferative and apoptotic events of the trophoblast cells were assessed by immunohistochemical examination using anti-Ki67 and anti-activated caspase-3 antibodies respectively. Hormone levels were measured. RESULTS: Simvastatin sharply inhibited migration of extravillous trophoblast cells from the villi to the matrigel (P < 0.05). Moreover, simvastatin inhibited half of the proliferative events in the villi (P < 0.05) and increased apoptosis of cytrophoblast cells compared to control. Moreover, simvastatin significantly decreased secretion of progesterone from the placental explants (P < 0.01). CONCLUSION: Simvastatin adversely affects human first trimester trophoblast.

Key words: migration/pregnancy/proliferation/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). Statins are contraindicated during pregnancy although very few data are available regarding their use in this period. However, young women with familial hypercholesterolaemia and diabetes mellitus with atherosclerotic cardiovascular diseases are potential candidates for treatment with statin. Also, in the last few years statin treatment has become very common and is even available over the counter in some countries, and since 50% of pregnancies are unplanned, many women may be exposed to it non-deliberately during pregnancy. 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 cytrophoblast (CT) progenitor cells. These cells retain the ability to proliferate, and can differentiate to form syncytiotrophoblast cells (SCT) 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 the extravillous trophoblast cells (EVT cells) (Malassine and Cronier, 2002). EVT 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). Proliferation, apoptosis and migration are key processes that play an important pathophysiological role in determining the structure and function of the placenta (Chakraborty et al., 2002; Murakoshi et al., 2003). Several factors such as insulin-like growth factor (IGF-I), hormones (βhCG) and other proteins (such as ras) are involved in the proliferation, apoptosis and migration processes in the placenta (Sasa et al., 1997; Zygmunt et al., 1998; Srisuparp et al., 2001; Mandl et al., 2002; Chakraborty et al., 2002). Many of these processes depend on HMG-CoA reductase function (Larsson, 1996). Inhibition of HMG-CoA reductase results in reduced levels of cholesterol, which is required for normal development in mammals, and is a precursor in the biosynthesis of hormones that are important for the maintenance of pregnancy (Benahmed et al., 1983; Siperstein, 1984; Henson et al., 1997). In addition, HMG-CoA reductase inhibition also results in decreased concentrations of mevalonate and its many isoprenoid derivatives, 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 G proteins (RAS) and growth factors (Larsson, 1996; Bellosta et al., 2000). Thus statins may influence membrane synthesis, DNA replication, cellular proliferation, growth, metabolism and protein glycosylation that are crucial for normal development of the embryo and the placenta (Larsson, 1996; Bellosta et al., 2000).

Animal models (rats, mice, rabbits) have provided evidence for the teratogenic effects of statins on pregnancy outcome, including fetal death, intrauterine growth retardation, malformations and abortions (Dostal et al., 1994; Masters et al., 1995). Because statins were shown to induce malformations when administered at high doses to rats and mice, and also because discontinuation of lipid-lowering drugs for the relatively short duration of pregnancy was not expected to have long-term impact on women with hypercholesterolaemia, a Food and Drug Administration (FDA) pregnancy category X (very high risk to the human fetus) was established for these agents. However, very few and conflicting clinical and laboratory data are available from inadvertent use of statins in human pregnancy (Hosokawa et al., 2003). Whereas one report of 134 women demonstrated no increase in abnormal outcome of pregnancy above the expected rate in healthy women (Manson et al., 1996), another study found few rare anomalies in fetuses of women who were exposed to statins in the first trimester (Edison and Muenke, 2004). It should be stressed that severe hypercholesterolaemia can adversely affect both women and fetuses (Palinski and Napoli, 2002) and has to be controlled when administered at high dose to rats and mice, and also because discontinuation of lipid-lowering drugs for the relatively short duration of pregnancy was not expected to have long-term impact on women with hypercholesterolaemia, a Food and Drug Administration (FDA) pregnancy category X (very high risk to the human fetus) was established for these agents. However, very few and conflicting clinical and laboratory data are available from inadvertent use of statins in human pregnancy (Hosokawa et al., 2003). Whereas one report of 134 women demonstrated no increase in abnormal outcome of pregnancy above the expected rate in healthy women (Manson et al., 1996), another study found few rare anomalies in fetuses of women who were exposed to statins in the first trimester (Edison and Muenke, 2004). It should be stressed that severe hypercholesterolaemia can adversely affect both women and fetuses (Palinski and Napoli, 2002) and has to be controlled during pregnancy. Currently such women are treated intensively with invasive approaches such as lipidopheresis (Cashin-Hemphill et al., 2000).

Placental damage by statin may harm the fetus. Moreover, as the chorionic villi are derived from embryonic tissue, placental cellular damage may induce damage to the embryo/fetus. Therefore, we decided to explore the effects of simvastatin on the placenta in an in vitro human placental model. The study parameters included cell migration, apoptosis, proliferation, and hormone production.

Materials and methods

Tissue preparation and culture

The study was approved by the ethical committee of Sapir Medical Center. Placental tissues (6–9 weeks gestational age) retrieved from women 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, MA, USA), which had been layered with polymerized Matrigel (BD Biosciences, Bedford, MA, USA). Medium [Dulbecco’s modified Eagle’s medium/Ham’s F-12 1:1, l-glutamine (2 mmol/l), sodium pyruvate (1 mmol/l), antibiotics and fetal calf serum (10%); Biological Industries, Beit-Haemek, Israel] was added to the lower well of the culture dish (bottom medium). Cultures were preincubated overnight in the 5% CO₂ incubator. On the following day medium was added above and below the placental explants. During that time simvastatin 5 µmol/l (simvastatin low) and 10 µmol/l (simvastatin high) (provided by Merck & Co., Rahway, NJ, USA) was added to the upper medium of the placental culture. Simva was dissolved in NaOH 0.1 mol/l, and diluted with the medium to the appropriate concentration. Medium with or without NaOH 0.1 mmol/l 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. Villous explants were inspected daily using an inverted phase-contrast microscope for general cellular integrity, cellular migration and outgrowth. Six placenta were analysed in the study. Hexaplicates of each treatment for every placenta were performed. 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.

Visual migration assessment of human trophoblast

The cultured villi were examined daily under a phase-contrast microscope in order to follow the migration of trophoblastic cells from the villi to the matrigel. Visual assessment of migration was performed in two ways: (i) by counting the number of explants from which EVT cells migrated out of all explants; (ii) by measuring the length of zones demonstrating migration of trophoblastic cells out of explant perimeter, reflecting the proportional number of villi from which EVT cells migrated. [Placental explant was simulated to a clock and divided to 12 parts. The number of sites from which EVT cells migrated out of 12 possible sites was documented daily and the percentage out of 12 parts of the circle was calculated. For example: EVT cells that migrated out of two sites were considered to have 16.6% of migration out of periphery area (2/12*100 = 16.6, Figure 1),] Evaluations (i) and (ii) were preformed daily for each explant. In each of the six experiments, six explants were analysed for every treatment. Presented data are the average of six experiments + SE for control.

Proliferation in the human trophoblast

Paraffin-embedded sections were deparaffinized in xylene, dehydrated in ethanol, rinsed in Phosphate-buffered saline (PBS) and pre-treated with proteinase K. The slides were then immersed in 10 nmol/l (pH 6) sodium citrate buffer and heated in a microwave oven for 15 min

![Figure 1. Measurement of the migration zone. The illustration demonstrates the measuring assay of the migration. Placental explant was simulated to a clock, divided into 12 parts. The number of sites from which extravillous trophoblast (EVT) cells migrated out of 12 possible sites was documented every day and the percentage out of all parts of the circle was calculated. Explant from which EVT cells migrated from two areas (hours 3 and 12), as demonstrated here, was considered to have 16.6% of migration out of periphery area (2/12*100 = 16.6).

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at 700 W. Following PBS rinsing, endogenous peroxide activity was quenched in 1% H$_2$O$_2$ (diluted in PBS) and rinsed with PBS. Samples were covered with normal blocker serum, immersed in PBS, and incubated with the primary monoclonal antibody mouse anti-human Ki67 antigen (mouse IgG$_1$; Zymed Laboratories, San Francisco, CA, USA) for 60 min at room temperature. The slides were then rinsed and incubated again with the biotinylated second antibody. The specimens were covered with horseradish peroxidase conjugated to streptavidin, rinsed with PBS, and covered with AEC–chromogen (biotinylated antibody, horseradish peroxidase conjugated to streptavidin and AEC; Zymed). Sections were counterstained with Mayer’s haematoxylin. Proliferation was analysed in the villous trophoblast cells, excluding SCT. To determine the extent of non-specific immunostaining, primary antibody was substituted with mouse IgG$_1$ (at the same concentrations as the primary antibodies). Presented data are average of six experiments + SE.

**Staining for low molecular weight cytokeratin**

The same procedure was used as for the Ki67 staining. However, pretreatment with proteinase K was not needed and anti-low molecular weight cytokeratin (mouse IgG$_1$; Zymed) was used instead of anti-Ki67.

**Apoptotic events in the human trophoblast**

Apoptotic events were analysed by staining the cells with anti-activated caspase-3. Activated caspase-3-stained cells were detected on paraffin sections in a similar way to the proliferation assay without proteinase K pretreatment, and by using rabbit anti-caspase-3 polyclonal antibody (Biocare Medical, CA, USA) instead of anti-Ki67. Purified normal rabbit immunoglobulin (Zymed) served as isotype control.

**Cell count**

Quantitative analysis of cell proliferation and apoptosis was done by counting stained cells out of trophoblast cells in the villi. Proliferation and apoptosis analysis were performed on three explants for each treatment in six and five experiments respectively. They were assessed in the trophoblast cells in the villi, not including the non-proliferating cells, i.e. SCT and EVT cells that migrated into the matrigel. Twelve/six slices from each treatment in every experiment were analysed for proliferation and apoptosis respectively (four/two slices from each of three randomly chosen wells in every treatment). Average of 1530, 1341, 1783 and 1473 cells and of 789, 956, 901 and 812 cells were counted in the proliferation and apoptosis assays in the control without/with solvent, simvastatin high and simvastatin low treatments of every experiment respectively. Counting was performed under ×400 magnification.

**Screening the upper fluid of the human trophoblast for hormones**

Twenty-four hours following culture, media were collected from the inserts of control and simvastatin (10 µmol/l)-treated explants. Progesterone and hCG levels were measured by Immulite 2000 analyzer using kits L2KPG2 and L2KCG2 respectively.

**Statistical analysis**

Student’s t-tests were employed in analysis of differences between cohorts. P ≤0.05 was considered significant.

**Results**

**Staining for low molecular weight cytokeratins**

To confirm the epithelial origin of EVT cells that have differentiated in vitro, we stained placental explants with antibodies to low molecular weight cytokeratin (Figure 2). EVT cells as well as trophoblast cells in the villi did stain positively.

**Migration of EVT cells from the villi to the matrigel**

Twenty-four hours following the beginning of the experiment the first EVT cells started to migrate from the villi to the matrigel (Figures 2 and 3). No significant difference was observed between the number of explants with migrating EVT cells at that time in the simvastatin-treated trophoblasts as compared to controls (Table I). Significantly, fewer explants with migrating EVT cells were observed after 72 and 96 h in culture in the simvastatin 10 µmol/l-treated group compared to

<table>
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<th>Treatment</th>
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<th>72</th>
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<td>90</td>
<td>90.4</td>
<td>85.7</td>
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*Significantly different from control and control NaOH (P < 0.05).
The effect of simvastatin on human placenta

Proliferation of trophoblast cells in the villi
Proliferating trophoblast cells were analysed by anti-Ki67 antibody in explants that were fixed 96 h following the beginning of the experiment (Figure 5). Higher proliferation rate of trophoblast cells was observed in the control villi (control and control NaOH) in comparison to the simvastatin-treated explants (10 and 5 µmol/l, *P < 0.05 and **P < 0.001 respectively, Figure 6).

Apoptosis of trophoblast cells
Apoptosis of trophoblast cells was analysed by the anti-activated caspase-3 antibody staining in explants that were exposed to simvastatin for 96 h and compared to controls (Figure 7). Increased number of apoptotic events was found in the simvastatin 10 µmol/l-treated explants in comparison to the control explants (P < 0.01, Figure 8). Apoptosis of trophoblast cells in the simvastatin 5 µmol/l-treated explants was higher than in control; however, the results were not significantly different.

Figure 4. Migration of extravillous trophoblast (EVT) cells from the placental explants to the matrigel. First trimester placental explants were exposed to simvastatin for 5 days. After 0, 24, 72 and 96 h, we measured the length of explant zones demonstrating migration of trophoblastic cells out of explants perimeter. The results are percentages of control following 96 h of incubation. Simva Low: simvastatin 5 µmol/l; Simva High: simvastatin 10 µmol/l.

Figure 5. Photograph of the proliferation assay.

Figure 6. Proliferation of trophoblast cells in the villus. First trimester placental explants were exposed to simvastatin for 5 days. Following 96 h of incubation the explants were fixed in 4% buffered formaldehyde, paraffin-embedded, and stained for anti-Ki67 antigen reflecting proliferation of cells. Quantitative analysis of cell proliferation was done by counting stained cells out of trophoblast cells in the villi. Simva Low: simvastatin 5 µmol/l; Simva High: simvastatin 10 µmol/l. *, **Significantly different from Control NaOH (P < 0.05, P < 0.001 respectively). +, ++Significantly different from Control (P < 0.05, P < 0.001 respectively).

Figure 7. Photograph of the apoptosis assay.
Phoblast differentiation (Bauer et al., 2000; Yacobi et al., 2004) were examined in an explant model which has gained a wider spectrum of applications in studies of the human placenta. We used a human first trimester placental explant model (Genbacev et al., 1992) in which the placental explants are located above the matrigel and the EVT cells differentiate and migrate from the villi to the matrigel. Human placental explants contain CT, EVT and SCT cells and the system has the advantage of presenting topological and functional villous—extravillous trophoblast inter-relationships. First trimester explant model has gained a wider spectrum of applications in the last few years and has been used to study the effects of several parameters including oxygen tension and sera with autoantibodies on trophoblast differentiation (Genbacev and Miller, 2000; Yacobi et al., 2002; Bose et al., 2004), and also to analyse the morphological and molecular processes during trophoblast differentiation (Bauer et al., 2004; Nishimura et al., 2004). Migration, proliferation, apoptosis and hormone production can be measured with this system.

We found that simvastatin inhibited migration and proliferation of trophoblast cells and increased CT apoptosis. Also, βhCG and progesterone levels were reduced following incubation of the trophoblast cells with simvastatin. Cholesterol is needed for mammalian cell metabolism and it plays a critical role in the growth of eukaryotic cells. Isoprenes are involved in the initiation of DNA synthesis and thus also regulate mammalian cell growth (Siperstein, 1984; Doyle and Kandutsch, 1988; Larsson, 1996). Indeed, inhibition of cell growth and proliferation by HMG-CoA reductase inhibitors was demonstrated in animals and humans (Minsker et al., 1983; Larsson and Blegen, 1993) and was reversed by adding either cholesterol or mevalonate (Minsker et al., 1983). However, this is the first study to show that statins inhibit the proliferation of human trophoblastic cells. This phenomenon may affect the number and function of cells that are responsible for supporting the embryo throughout pregnancy, especially in the first trimester. We also found that statins inhibit migration of EVT cells. Previous studies have also demonstrated inhibition of human vascular smooth muscle cells and macrophage infiltration by statins, associated with significant reduction of matrix metalloproteinase (MMP)-9 and -2 levels (Luan et al., 2003). MMP-9 and MMP-2 are important regulators of trophoblast cell migration (Bischof et al., 1995) and their inhibition by statins may reduce trophoblast cell migration. Another mechanism for the effect of statins on proliferation and migration may be their modulation of growth factor receptors. Receptors of some growth factors require N-linked glycosylation which is essential for their stability, binding activity and cellular distribution (Wendland et al., 1991). Suppression of HMG-CoA reductase decreases the rate of N-linked glycosylation (Larsson, 1996), and indeed the function of some growth factors such as insulin-like growth factor (IGF) is blocked following inhibition of the enzyme (Calberg et al., 1996; Larsson, 1996). The differentiation pathway of CT cells, which is responsible for their ability to proliferate and migrate, depends on various cytokines and growth factors such as IGF-I and IGF-II (Chakraborty et al., 2002; Mandl et al., 2002; Kabir-Salmani et al., 2003). N-Linked glycosylation regulates IGF-I and -II expression at the cell membrane (Wendland et al., 1991; Calberg et al., 1996); thus its absence may be responsible for the inhibition of CT proliferation and migration induced by statin (Wendland et al., 1991; Calberg et al., 1996). Simva also increased activation of caspase-3 in trophoblast cells. Inhibitors of HMG-CoA reductase can trigger a subset of tumour-derived cells to undergo apoptosis (Wong et al., 2002). Statins may also cause apoptosis of non-malignant cells in humans and animals. Human differentiated monocytes (Vamvakopoulos and Green, 2003), cardiac myocytes and cultured hepatocytes (Rabkin and Kong, 2003; Kubota et al., 2004), as well as rat vascular smooth muscle cells (Cheng et al., 2003) demonstrated increased apoptotic events following exposure to simvastatin, as demonstrated by the increased caspase-3 (Cheng et al., 2003; Kubota et al., 2004). Simva decreased both hCG and progesterone levels in the trophoblast culture. One of the regulators of hCG production by the trophoblast cells is the cytokine IL-1β (Seki et al., 1997). Simva significantly reduced IL-1β mRNA expression and its protein level in primary endothelial cells (Inoue et al., 2000). We suggest that simvastatin may have reduced IL-1β levels in our culture with secondary decrease of hCG production. βhCG is a glycoprotein hormone produced mainly by SCT cells in the first trimester.
placenta (Srisuparp et al., 2001). Its biological function is to stimulate the luteal body to secrete progesterone (Srisuparp et al., 2001). βhCG modulates progesterone production by human trophoblast tissue at early gestational stage, and thus the inhibitory effect on hCG production may also affect progesterone synthesis (Shi et al., 1991; Dhar et al., 2004). Progesterone is essential for the development of endometrial receptivity for blastocyst implantation and pregnancy maintenance (Srisuparp et al., 2001). Both hCG and progesterone may affect motility-related processes. βhCG receptor is expressed on invasive trophoblast cells (Zygmont et al., 1998) and the hormone was found to be an important regulatory factor for invasion (Srisuparp et al., 2001) and migration of trophoblastic cells (Zygmont et al., 1998). Progesterone regulates gelatinase B in trophoblast cells (Shimonovitz et al., 1998) and thus may affect their migration. Hence, the decreased hCG and progesterone levels following simvastatin administration may also contribute to the reduced cell invasion following simvastatin administration. Indeed the decrease of trophoblast culture hormone levels was observed as early as 24 h after experiment initiation, before the major inhibition of EVT migration. It is therefore reasonable to assume that the decreased hormone level may have contributed to the inhibition of trophoblast cell migration.

Our study is the first to suggest that simva adversely affects the placenta. These effects may contribute to failure of the implantation process and be deleterious to the growth potential of the placenta. Impaired implantation and function of the placenta in the first trimester of pregnancy can be responsible for the higher abortion rate and teratogenicity that were observed in animals exposed to statins during pregnancy. Further studies will clarify the mechanisms of simva effect on placental function.

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Benachmad M, Reventos J and Saez JM (1983) Role of plasma lipoproteins in blastocyst implantation and pregnancy maintenance (Srisuparp et al., 2001). Both hCG and progesterone may affect motility-related processes. βhCG receptor is expressed on invasive trophoblast cells (Shimonovitz et al., 1998) and the hormone was found to be an important regulatory factor for invasion (Srisuparp et al., 2001) and migration of trophoblastic cells (Zygmont et al., 1998). Progesterone regulates gelatinase B in trophoblast cells (Shimonovitz et al., 1998) and thus may affect their migration. Hence, the decreased hCG and progesterone levels following simvastatin administration may also contribute to the reduced cell invasion following simvastatin administration. Indeed the decrease of trophoblast culture hormone levels was observed as early as 24 h after experiment initiation, before the major inhibition of EVT migration. It is therefore reasonable to assume that the decreased hormone level may have contributed to the inhibition of trophoblast cell migration.

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