Growth-modulatory Effects of Heparin and VEGF165 on the Choriocarcinoma Cell-line JEG-3 and its Expression of Heparanase

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Abstract. *Background: Expression of heparanase (HPSE) in tumor cells is strongly associated with invasion, metastasis and angiogenesis. It also plays a key role during pregnancy, in processes of implantation as well as placentation. Vascular endothelial growth factor (VEGF) and heparin are known to alter HPSE expression, with heparin given prophylactically to women with a history of placenta-mediated complications in subsequent pregnancies. Materials and Methods: We examined the growth-modulatory effects of different concentrations of heparin and VEGF on the choriocarcinoma cell-line JEG-3 and the expression of heparanase under VEGF and heparin by proliferation assays, PCR, and western blot. Results: Proliferation of JEG-3 cells was induced by heparin in a dose-dependent manner, whereas highly concentrated VEGF led to a decreased cell proliferation. Both agents did not influence the HPSE-expression. Conclusion: The presumed pregnancy-protecting effects of heparin may partially be due to an increase of trophoblast proliferation and not via regulation of HPSE expression.*

Heparanase (HPSE) is an endo-β-d-glucuronidase, involved in degradation and remodelling of the extracellular matrix (1). Cleaving heparan sulfate side chains is among its mainly examined functions (2). HPSE has been found in placental tissue of both humans (3-5) and animals (6). HPSE is considered to play a key role in processes of implantation. as well as placentation (7). Its expression is not restricted to the placenta as HPSE is expressed in platelets, keratinocytes and activated cells of the immune system under physiological conditions, whereas most normal epithelia stain negative for HPSE (1, 8). HSPE has been described to be over-expressed in both inflammatory (9) and autoimmune diseases and in a variety of cancers (10) with increased rates of tumour angiogenesis (10), invasiveness (11) and metastatic behaviour (12) associated with a HPSE over-expression, which is in fact the case for human choriocarcinoma (13). Recently, there have been reports about an altered expression of the *HPSE* gene after heparin incubation in combination with high glucose in human aortic endothelial cells (14). Heparins themselves are known to exert a wide range of effects beyond anti-coagulation at the placental and decidual level, which has especially been investigated in association with questions of implantation failure and recurrent miscarriages (15).

In women suffering from the anti-phospholipid syndrome (APS), the application of heparin has been shown to increase the live-birth rate in combination with low-dose acetyl salicylic acid. Such a beneficial effect had also been postulated for the isolated application of heparin in patients with other subtypes of early (16-17) and late pregnancy complications (18), such as non-recurrent and recurrent miscarriages, as well as placenta-associated diseases, *e.g.* preeclampsia. It is established that preeclampsia goes along with a reduced invasion of the trophoblast into the maternal decidua (19), implying that a pro-invasive protein, such as HPSE, might play a role in altering this process (20). Additionally, recent reports have described that vascular endothelial growth factor (VEGF), regarded necessary for tumor vascularity and metastasis, shows mutual enhancing effects with HPSE (21). VEGF has been described to alter the expression of pro-invasive matrix metalloproteinases in the choriocarcinoma cell-line JEG-3 (22) and its subtype A (VEGFA) is thought to be secreted by (23) and to stimulate the proliferation of both the trophoblast (24) and choriocarcinoma cells (25).

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As HPSE itself is expressed in the implantation window of non-conceptual cycle endometrium (26), we sought to find out whether heparin and VEGFA may exert pregnancy-protective effects by altering the expression of HPSE.

Taking into account the similarities between the proliferative, migratory and invasive properties of placental and cancer cells (27), immortalized human choriocarcinoma cells served as a surrogate for the primary trophoblast in accordance to other publications (28-32).

Materials and Methods

Cell culture. The human choriocarcinoma cell line JEG-3 (DSMZ, Braunschweig, Germany) was cultured at 37˚C, in a humidified atmosphere with 5 % CO_2. JEG-3 were grown in Ham's F12 (PAA Laboratories GmbH, Cölbe, Germany) supplemented with 10 % fetal calf serum (FCS; PAA Laboratories GmbH) and 1 % (v/v) penicillin/streptomycin (10,000 Units/ml/10 mg/ml; PAA Laboratories GmbH).

Growth experiments. 3,000 cells per well were plated in growth medium on 96-well-plates (Sarstedt AG & Co., Nümbrecht, Germany). Twenty-four h later, the medium was replaced by FCS-free medium. After 1 h various concentrations of heparin (0.01, 0.1, 1, 10, 100 and 1.000 U/ml; Sigma-Aldrich, Steinheim, Germany) or the VEGFA splice variant $VEGF_{165}$ (1, 10 and 100 ng/ml; Reliatech, Wolfenbüttel, Germany), responsible for its biological activity, were added by a further exchange of the media, containing FCS concentrations of 1% and 3.3%. The growth experiments were performed in a general atmospheric incubator with 5% CO_2 and under hypoxic conditions with 2% O_2 and 5 % CO_2 in a hypoxia chamber THC08 124 (Toepffer Lab Systems, Göppingen, Germany). Each experiment was repeated twice with sextuplicates of culture wells. After incubation times of 24, 48 and 72 h the cells were measured in MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) assays as described elsewhere (33).

Expression of heparanase (HPSE) mRNA in JEG-3 under the influence of heparin and $VEGF_{165}$ in normoxia and hypoxia. The mRNA expression of HPSE and its regulation by heparin, $VEGF_{165}$ and hypoxia was evaluated by means of the quantitative reverse transcription polymerase chain reaction (qRT-PCR). JEG-3 were plated on 6-well plated by 600,000 cells per well in growth media. Twenty-four h later, media were replaced by media containing heparin (0.1 U/ml), $VEGF_{165}$ (100 ng/ml) or control medium with 1% or 3.3% FCS for 3 h. Experiments were performed in duplicates. The respective concentrations of heparin and $VEGF_{165}$ were chosen due to their pronounced effects in the proliferation experiments. Total RNA was isolated by using QIAzol (Qiagen, Hilden, Germany). One μg RNA was reverse-transcribed with Superscript-II (Invitrogen, Karlsruhe, Germany) using random-primers. qPCR was performed by using Platinum SYBR Green qPCR Super Mix-UDG (Invitrogen) on the qPCR system Opticon 2 (BioRad, Munich, Germany). To quantify the *HPSE* gene the following primer pair was used according to GenBank Accession No. NM_006665: 5'- TCC TGC GTA CCT GAG GTT TG-3' (forward); 5'-CAA CCG TAA CTT CTC CTC CAC-3' (reverse). Succinate dehydrogenase complex subunit A (*SDHA*; forward: 5'-TGG GAA CAA GAG GGC ATC TG-3' and reverse: 5'-CCA CCA CTG CAT CAA ATT CAT G-3') and hypoxanthine-guanine phosphoribosyltranferase (*HPRT*; forward: 5'-TCA GGC AGT ATA ATC CAA AGA TGG T-3' and reverse: 5'-AGT CTG GCT TAT ATC CAA CAC TTC G-3') were used for normalization. All primers were synthesized at Metabion (Martinsried, Germany). The PCR protocol consisted of 40 cycles of 15 s at 95˚C and 30 s at 60˚C. Each sample was tested in duplicate. The reaction efficiency of every primer pair had been determined using dilution series with a representative pool of cDNAs (Table I) as proposed by Pfaffl (34). Data were further processed with the excel-based program REST-MCS©-version 2 (www.gene-quantification.info).

Table I. *Results of the q-RT-PCR of HPSE in JEG-3 cells, treated with 0.1 U/ml heparin (upper panel) or with 100 ng /ml VEGF165 (lower panel).*

Heparin (0.1 U/ml)	1% FCS		3.3% FCS	
	R	*p*-Value	R	*p*-Value
Atmosphere	0.842	0.665	0.961	0.672
Hypoxia	1.098	0.824	1.449	0.484
VEGF165 (100 ng/ml)	1% FCS		3.3% FCS	
	R	*p*-Value	R	*p*-Value
Atmosphere	0.847	0.834	0.912	0.929
Hypoxia	0.962	0.918	1.103	0.885

R=Relative change of expression of the HPSE gene compared to the untreated control, normalized by the reference genes SDHA and HPRT1.

Immunochemical analysis of heparanase. Total protein (HPSE) was extracted by using the Mammalian Protein Extraction Buffer (GE-Healthcare, München, Germany). Twenty μg of the extractions were electrophoresed and blotted onto a nitrocellulose membrane. The membrane was blocked in TBST-buffer and 5% (w/v) nonfat dry milk and incubated at 4˚C overnight with a 1:4000 diluted monoclonal anti-human heparanase 1 (HPA1) antibody (Clone HP3/17; Acris, Herford, Germany). Following 3 times washing for 5 min in TBST-buffer, a secondary antibody incubation was performed for 1 h at room temperature with the horseradish peroxidase-linked sheep anti-mouse antibody diluted 1: 4000 (NA-931; GE-Healthcare, Munich, Germany). After 3 more washings, the immunoreaction was developed using the immobilon-Western-HRP substrate (Millipore, Schwalbach, Germany) and visualized on hyperfilm-ECL (GE-Healthcare).

Statistics. Statistical analyses of growth experiments were performed with GraphPad-Prism 5 (GraphPad, La Jolla, CA, USA) using the monofactorial ANOVA-variance analysis and Dunnett's *post-hoc* test. A p-value <0.05 was considered as statistically significant. Outliers were identified with the GraphPad Outlier calculator, applying the Grubb's test, and excluded from further analysis.

Results

Influence of heparin on the proliferation of JEG-3 cells. The proliferation of JEG-3 cells was significantly increased by heparin at concentrations of 0.01 to 10 U/ml at atmospheric

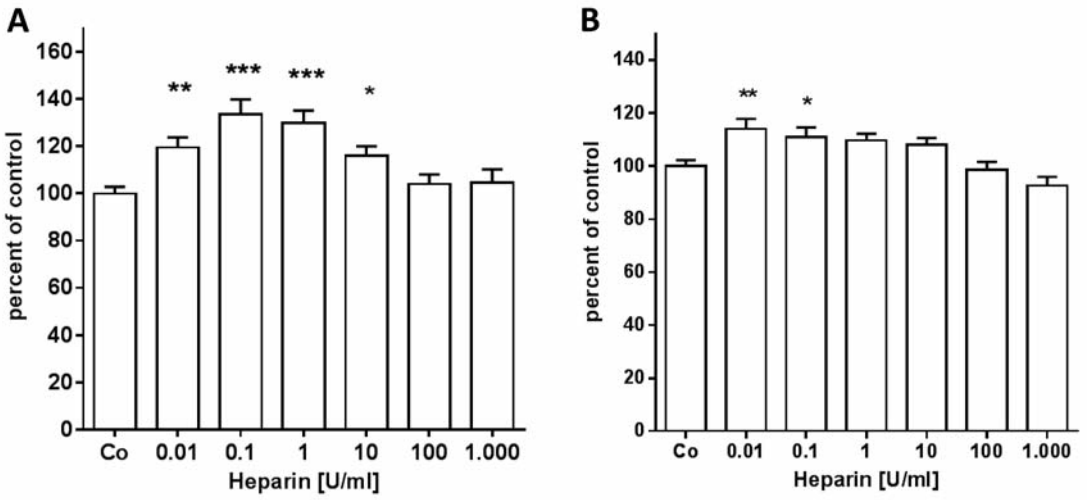

Figure 1. *A) Induction of proliferation of JEG-3 cells by heparin at normoxia and a FCS concentration of 3.3%. B) Under hypoxic conditions and the reduction of FCS to 1%, heparin concentrations of 0.01 and 0.1 U/ml caused a significant induction of cell proliferation. Co, control; *p<0.05; p<0.01; ***p<0.001.*

conditions and a FCS concentration of the medium of 3.3% (Figure 1A). However, heparin concentrations of 100 and 1,000 U/ml showed a return to basic levels of proliferation. Accordingly, heparin also increased cell proliferation at concentrations of 0.01 to 0.1 U/ml at hypoxic conditions and a FCS concentration of the medium of 1% (Figure 1B).

Influence of VEGF165 on the proliferation of JEG-3 cells. The proliferation of JEG-3 cells was significantly decreased by $VEGF_{165}$ at concentrations of 10 and 100 ng/ml at atmospheric conditions and a FCS concentration of the medium of 3.3% (Figure 2A) and at 100 ng/ml in hypoxia (Figure 2B).

HPSE expression. As shown by western blot analysis, HPSE is expressed in JEG-3 cells (Figure 3). No significant differences in the *HPSE* mRNA-expression were detected after incubation with heparin or $VEGF_{165}$ at the respective concentrations (Table I).

Discussion

Heparin, a negatively-charged glycosaminoglycan, has been described to exert anti-metastatic effects on tumor cells (35). However, we were able to show a significant increase of proliferation of choriocarcinoma cells after incubation with heparin at distinct concentrations, a result that was – to the best of our knowledge – not described before. The stimulation of proliferation, depending on the concentration of heparin, is comparable to results from other cell-lines, *e.g.* human osteoblasts (36). In our setting, heparin at most concentrations failed to compensate the anti-proliferative effects of hypoxia but was still able to lead to a significantly increased cell proliferation at 0.01 U/ml.

When reducing both the concentrations of oxygen and FCS to induce a lack of nutrients, heparin at low concentrations again was able to compensate partially the lack of nutrients and promote cell growth. This observation could not be confirmed for higher concentrations of heparin, which may be explained by the fact that heparin can also exert direct cytotoxic effects on tumors cells (35).

It has been shown that heparin may induce trophoblastic invasiveness *in vitro* by altering the expression of matrix metalloproteinases and tissue inhibitors (37), thus possibly promoting a protective effect for the developing pregnancy. However, clinical applications of heparin (s) in pregnancies threatened by miscarriage or complications, such as preeclampsia, have failed to show consistent results (16-18, 38-47). It is especially surprising that heparin may exert a protective effect against placenta-mediated complications when applied in the second trimester of pregnancy (18); when the invasion of the trophoblast is already completed. Nevertheless, our results showing an increase of trophoblastic proliferation may prompt further investigations in the field of heparin application in early pregnancy to prevent late (r) complications in patients at risk.

Heparins have been described as strong inhibitors of heparanase (HPSE) (48) showing to interfere in its transcriptional regulation (14) with chemically modified heparins, such as SST0001, preclinically used to inhibit tumor cell growth and angiogenesis (49). This regulation of HPSE expression by heparin could not be observed in our setting.

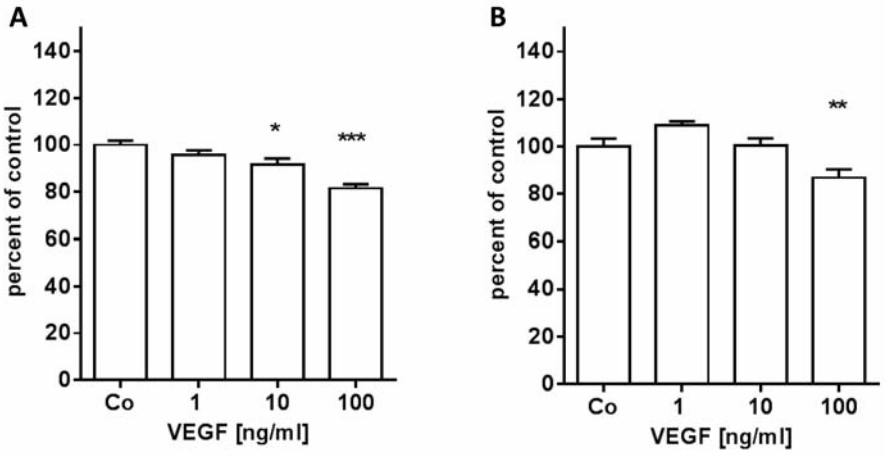

Figure 2. *A) Reduction of proliferation of JEG-3 cells by $VEGF_{165}$ at normoxia and a FCS concentration of 3.3%. B) Under hypoxic conditions, a $VEGF_{165}$ concentration of 100 ng/ml caused a significant reduction of cell proliferation. Co, control; *p<0.05; **p<0.01; ***p<0.001.*

HPSE has been shown to increase VEGF expression in non-malignant and malignant cells (50, 51). However, an enhancing effect of VEGF on the expression of HPSE, as seen in melanoma cell lines (21), could not be demonstrated in our setting.

Hypoxia has been described to induce expression of VEGFA in human choriocarcinoma cells, with VEGF thought to increase the invasive capacity in these cells (52). Interestingly, the splice variant of VEGFA, $VEGF_{165}$, does not lead to a proliferation of JEG-3 cells in our setting; it reduced their growth at higher concentrations. This result seems to stand in contrast to experiments with the BeWo choriocarcinoma cell line where VEGF leads to an increased cell proliferation (25). However, as shown in BeWo cells, endogenous nitric oxide (NO) production induced by exogenous VEGF at a concentration of 10 ng/ml down-regulates the otherwise VEGF-stimulated proliferation. Therefore, our approach with the aforementioned and even higher concentration of VEGF might explain their growth-inhibiting effects on JEG-3 cells.

Since the first characterization of HPSE in the placenta (53) deriving from trophobalstic tissue, some studies have investigated its expression in human placental tissue (2, 4, 54, 55). Placental HPSE has been proven to be expressed in all trimesters of both physiological and pathological pregnancies; however, no findings on its concentrations under the various circumstances were reported (4). Nadir and colleagues (2) proved that placental tissue form early miscarriages over-expresses HPSE. Furthermore, trophoblast cells incubated with exogenous recombinant HPSE show a significantly altered expression pattern of haemostatic factors, such as tissue factor pathway inhibitor 1 and 2. These factors are thought to be involved in early pregnancy complications, such as (recurrent) miscarriages (2). However, as the alterations were detected in early pregnancy tissue and cell culture, the results cannot be automatically attributed to complications in the second or third trimester of pregnancy, such as preeclampsia.

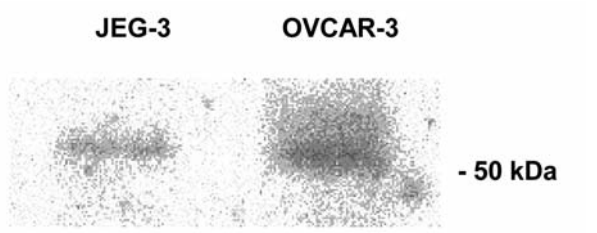

Figure 3. *Protein expression of heparanase (HPSE) in JEG-3 cells as shown in western blot. OVCAR-3 cells are shown as a positive control.*

Since in our setting such a regulation could not be shown, heparin must exert its growth-stimulatory effects through other pathways. On the other hand, as there are striking similarities between the invasion of the trophoblast and that of cancer cells, with the latter stimulated by an increased heparanase expression (10), the otherwise observed reduction of HPSE expression by heparins is lacking in the trophoblast.

Conflicts of Interest

None.

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