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Heparin Inhibits Proliferation and Migration of Patient-Derived Vascular Cells Only at Non-physiological Concentrations Due to Antagonistic Pathways

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Authors' contributions

This work was carried out in collaboration between all authors. Author VSE designed the study, wrote the protocol, participated in statistical analysis and wrote the manuscript drafts. Author IA did the cell culture experiments and participated in statistical analysis. Authors JL and TJ both contributed to the design of the study and the analyses. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: Experimental animal models have demonstrated inhibition of intimal hyperplasia processes by heparin. These effects have not been observed in clinical trials. Diverging experimental conditions are the most conspicuous reasons. This study was designed to test if heparin inhibits intimal hyperplasia processes on patient-derived vascular cells at clinically relevant concentrations.
Study Design: Comparison of heparin effects on patient-derived vascular cells in culture.
Place and Duration of Study: Department of Biomedicine, University of Bergen, Norway between

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2012 and 2014.
Methodology: Vascular cells isolated from patient biopsies were treated with heparin and evaluated for effects on proliferation, migration and cell signaling using image based cell enumeration, real time migration monitoring and flow cytometry.
Results: Reduced proliferation and migration in vascular cell cultures could only be detected after addition of high non-physiological heparin concentrations. In cultures stimulated by human fibroblast growth factor (hFGF), mitogen-activated protein kinase extracellular signal-regulated kinase (MAPK-ERK) phosphorylation was increased at lower heparin concentrations, while it was reduced dose-dependently by heparin in unstimulated cultures.
Conclusion: Heparin inhibits intimal hyperplasia processes in patient-derived VSMC cultures, but only at concentrations exceeding clinical doses. This is due to an increased MAPK-ERK phosphorylation at lower heparin concentrations in the presence of hFGF. MAPK-ERK phosphorylation was influenced by heparin through both inhibitory and stimulatory pathways. These findings can explain the divergence of results between previous *in vitro* and clinical studies and provide a basis for new therapeutic strategies.

Keywords: Heparin; proliferation; migration; signaling; vascular smooth muscle cell; co-culture.

ABBREVIATIONS

BSA : bovine serum albumin
EC : endothelial cell
EDTA : ethylene-diamine-tetraacetic acid
EGM-2 : endothelial cell growth medium-2
FACS : fluorescence-activated cell sorter
FBS : fetal bovine serum
GFP : green fluorescent protein
hFGF : human fibroblast growth factor
IC50 : half maximal inhibitory concentration
ICAM-2 : intercellular adhesion molecule 2
MAPK-ERK : mitogen-activated protein kinase - extracellular signal-regulated kinase
PBS : phosphate buffered saline
PFA : paraformaldehyde
SmBM-2 : smooth muscle basal medium-2
SmGM-2 : smooth muscle growth medium-2
TRITC : tetramethyl-rhodamine isothiocyanate
UEA-I : Ulex europaeus agglutinin I
VEGF : vascular endothelial growth factor
VSMC : vascular smooth muscle cell

1. INTRODUCTION

Intimal hyperplasia is considered the main cause of graft failure following vascular surgery [1,2]. It is induced by endothelial damage leading to proliferation and migration of vascular smooth muscle cells (VSMCs) [3]. Previous experiments have demonstrated heparin to be a strong inhibitor of both proliferation and migration of VSMCs [4-13]. However, clinical studies have failed to demonstrate improved patency by heparin [14,15]. The discrepancy of results is due to several factors, the most conspicuous being diverging experimental conditions. The *in vitro* studies were almost exclusively performed using animal vascular smooth muscle cell lines. The heparin concentrations used exceeded the recommended therapeutic dosages. Results may also have been influenced by the *in vivo* presence of endothelial cells (ECs), as they produce glucosaminoglycans, growth factors and nitrogen oxide involved in VSMC regulation) [7,16-18]. Heparin is thought to exert its inhibition on proliferation and migration of VSMCs by binding to a specific cell membrane heparin receptor [19-21]. Consequently, the mitogen-activated protein kinase - extracellular signal-regulated kinase (MAPK-ERK) pathway, is inhibited.

Heparin is currently rarely used in the treatment of intimal hyperplasia, however further

investigation of its known inhibitory effects may contribute to a deeper understanding of its pathophysiology and unveil new therapeutic principles. This study was designed as an experimental study using human VSMCs to test if heparin inhibits proliferation, migration and MAPK-ERK signaling at clinically relevant concentrations. To address the possible influence of endothelial cells parallel proliferation series of co-cultures were performed.

2. MATERIALS AND METHODS

Approval to establish a human tissue biobank and to perform this study was granted by the Regional Independent Scientific Ethical Committee (REK, Bergen 162.08) and the Norwegian Directorate of Health. All experiments have been performed in accordance with the ethical standards laid down in the 1964 declaration of Helsinki.

Full thickness vessel biopsies were collected from the aorta of six consenting patients undergoing vascular surgery (Table 1). The biopsies and cells were treated and analyzed as described in the following sections. All cells used in the experiments were maximum fourth passage.

2.1 Patient-derived Vascular Cell Isolation

Cell isolation was based upon a method described by Bryan and D'Amore [22]. Tissue samples were surgically divided into tunica media and tunica intima, and treated separately. The tissue was finely cut and mixed with phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, MO) to separate cells from surrounding tissue. The cell suspension was centrifuged at 515 x g for 5 minutes and the pellet resuspended in 0.2% collagenase bovine serum albumin (BSA; Sigma-Aldrich). The cells were then incubated for 30 minutes in 5% CO_2 at 37℃ before 0.25% Trypsin Ethylene-diamine-tetraacetic acid (EDTA; Sigma-Aldrich) was added. The cells were then incubated under the same conditions for another 15 minutes. The cell suspension was centrifuged at 515 x g for 15 minutes before the pellet was resuspended in 300 µL cell specific growth medium, transferred to a cell culture plate and placed in an incubator. VSMCs from biopsied tunica media were cultured in human fibroblast growth factor (hFGF)-supplemented smooth muscle growth medium-2 (SmGM-2; Lonza, Cologne, Germany) and ECs from the tunica intima in vascular endothelial growth factor (VEGF)-supplemented endothelial cell growth medium-2 (EGM-2; Lonza). After 2-6 days of incubation the proliferating monolayers of cells were examined, sorted and validated.

2.2 Sorting and Validation of Patient-derived Vascular Cells

The cells were sorted with CD31 Dynabeads, Endothelial Cell (Invitrogen 111.55D; Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The cells were washed with PBS and trypsinized with 0.25% Trypsin-EDTA, before 5% fetal bovine serum (FBS; PAA Laboartories, Pasching, Austria) in PBS was added. The cells were then centrifuged at 201 x g for 5 minutes before being resuspended in 0.1% BSA in PBS to yield approximately 2 million cells in each tube. 25 µL of Dynabeads per mL of cell suspension were added before being placed on a carousel and incubated at 4℃ for 20 minutes. Thereafter 0.1% BSA in FBS was added and the tubes placed on ice in the Magnetic Particle Concentrator (Life Technologies) for 2 minutes before the supernatant containing $CD31^-$ VSMCs was collected. The $CD31^+$ cells bound to the beads contained ECs and contaminating fibroblasts. To separate the ECs from the fibroblasts, *Ulex europaeus* agglutinin I (UEA-I)-lectin (Sigma-Aldrich) conjugated to tetramethyl-rhodamine isothiocyanate (TRITC, L4889; Sigma-Aldrich) was added to the cell culture, thereby staining the ECs (Fig. 1). The ECs were sorted twice from the lectin-negative fibroblasts on an Aria fluorescence-activated cell sorter (FACS; BD Biosciences, San Jose, CA; Fig. 2) and verified by expressing CD31, intercellular adhesion molecule 2 (ICAM-2, ab35045; Abcam, Cambridge, UK; Fig. 3) and bound UEA-I lectin (Sigma-Aldrich). The VSMCs were verified by anti-SMC actin M0851 (Dako, Glostrup, Denmark) Western Blot (Fig. 4).

2.3 EC-VSMC co-culture Method

An EC-VSMC co-culture method was applied to study the effects of heparin in a model more representable of *in vivo* conditions. It generates *in vitro* organotypic capillary like structures comprising basement membrane and pericyte enveloped 3D endothelial tubular structures [23]. This approach has previously been used to study several facets of angiogenesis, vascular homeostasis and antivascular compounds [24-31]. Patient endothelial cells

self-assemble into capillary like structures embedded in a locally deposited collagen and laminin rich matrix in the presence of VSMCs (Fig. 5). VSMCs reciprocally express VEGF that drives EC morphogenesis [22]. As ECs stop dividing, only the VSMCs will proliferate and contribute to cell number increases [23].

Table 1. An overview of patient characteristics and comorbidity

Patient	Gender	Age	Aorta	HT	DM	HChol	Smoke	Morbidity
1	Male	22	Thx	Yes	No	No	No	Coarctation
2	Female	58	Thx	Yes	No	No	No	TAA, AVR
3	Male	64	Abd	No	No	No	Earlier	COPD, AAA
4	Female	83	Abd	No	No	No	No	AAA
5	Male	41	Thx	No	No	No	No	TAA
6	Female	62	Thx	Yes	No	No	Earlier	TAA, AVR

Abbreviations: Thx, thoracalis; Abd, abdominalis; HT, hypertension; DM, diabetes mellitus; HChol, hypercholesterolemia; TAA, thoracic aortic aneurysm; AAA, abdominal aortic aneurysm; AVR, aortic valve replacement; COPD, chronic obstructive pulmonary disease

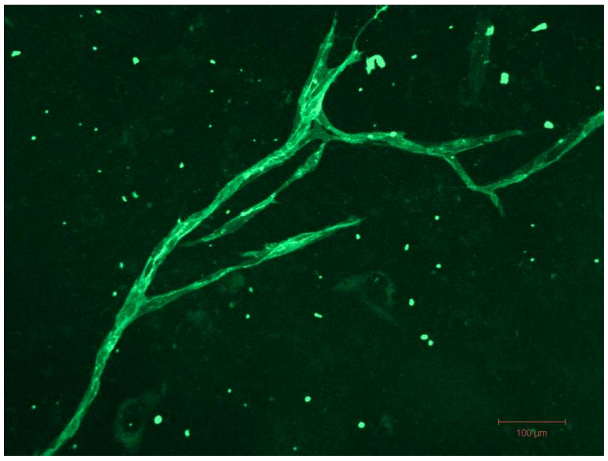

Fig. 1. Micrograph of patient ECs

Staining by green fluorescent protein (GFP) as Ulex europaeus agglutinin I (UEA-I)-lectin conjugated to tetramethyl-rhodamine isothiocyanate (TRITC, L4889) did not provide micrographs of equal clarity. Scale bar: 100 microns

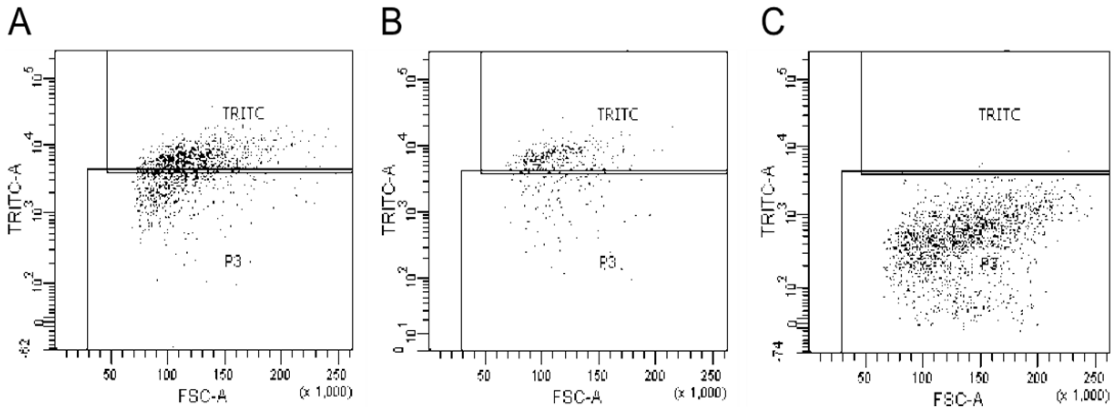

Fig. 2. Separation of ECs from fibroblasts

The ECs were stained by adding Ulex europaeus agglutinin I (UEA-I)-lectin conjugated to tetramethyl-rhodamine isothiocyanate (TRITC, L4889) to the cultures. Dotplots show culture cells before (A) and after (B) sorting. Cells were sorted twice and only the TRITC positive were included in the subsequent analyses. Negative control (C)

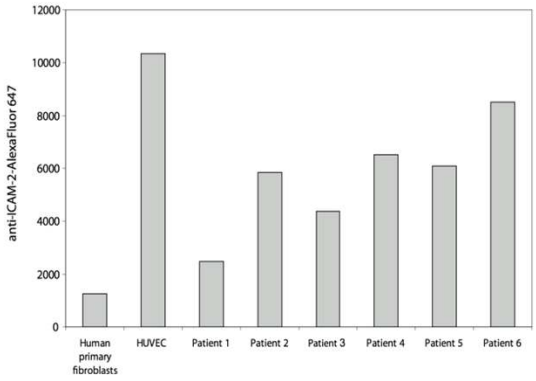

Fig. 3. Verification of ECs

ICAM-2 expression of human fibroblasts (negative control), Human umbilical vein endothelial cells (HUVEC; positive control) and patient cells verified that the cells were indeed ECs

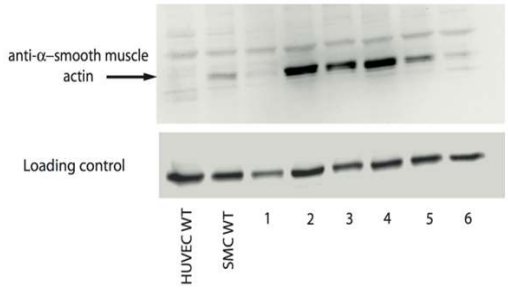

Fig. 4. Verification of VSMCs

Western blot analysis of the patient cells by anti-SMC actin M0851 verified that these were VSMCs. HUVEC (negative control), pooled smooth muscle cells (SMC; positive control) and patient VSMCs (1-6)

2.4 Cell Proliferation Assay

The cells were serum starved over night in SmBM-2 + 0.2% BSA (w/v) before cultures of VSMCs and EC-VSMCs were set up in 96-well plates. The total number of cells seeded out was maintained at 2.5 x 10^3 for both mono- and co-cultures. VSMCs were cultured in hFGF-supplemented SmGM-2 (Lonza). The co-cultures were seeded out in a 50:50 ratio of ECs and VSMCs and grown in EGM-2 (Lonza). All experiments were run in triplicate. All cell cultures were incubated in 5% CO_2 at 37℃ for 2 hours before heparin (100 IU/mL; LEO Pharma, Ballerup, Denmark) was added in the concentrations of 0, 5, 10, 25, 50 and 100 µg/mL. Incubation was further continued for 96 hours (no medium exchanged). Cell numbers were determined at assay endpoints by automated image acquisition and image analysis employing the BD Pathway system 855 (BD Biosciences; Fig. 6). Prior to the analysis the system was set up and calibrated by a company technician. Cell cultures were stained with Hoechst solution 10 ng/mL (B2261; Sigma-Aldrich) for 15 minutes. Images were acquired with 20x objective as 2x2 montages and the total number of nuclei (Hoechst) was quantified using the Montage cell-count algorithm embedded in Attovision v1.6.2 (BD Biosciences). The measurements resulted in a dose-response curve for each cell culture.

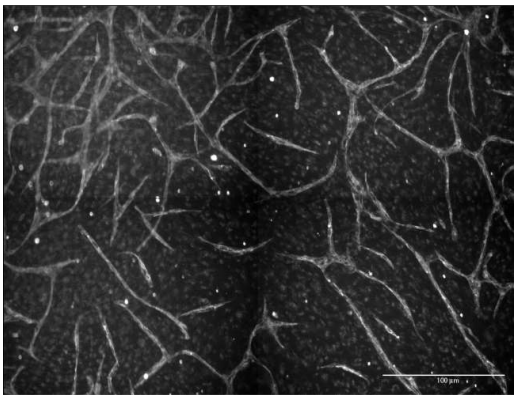

Fig. 5. EC-VSMC co-cultures replicate *In vivo* conditions

Micrographs of the capillary-like network (3 days) formed by ECs co-cultured with VSMCs. 20x objective. Scale bar: 100 microns

2.5 Cell Migration Assay

The xCELLigence™ system (Roche Applied Science, Penzberg, Germany) was used for automated real-time migration monitoring. The CIM-plate 16 (Roche Applied Science) especially developed for migration analyses was used. The system was set up and calibrated according to the producer's specifications. 160 µL of complete SmGM-2 (Lonza) was added to the lower chamber wells of the CIM-plate. The top chamber, containing the membrane and electrodes, was attached before 50 µL smooth muscle basal cell medium (SmBM-2; Lonza) with 0.2% (w/v) BSA (Sigma-Aldrich) was added to the top chamber wells. The plate was then incubated (5% CO_2 at 37℃) for one hour to allow the CIM-plates' membrane surface to reach equilibrium with the media. The spontaneous background activity was measured. 10 000 cells that had been serum starved over night in 100 µL SmBM-2 (Lonza) and 0.2% BSA (Sigma-Aldrich) were added to the top chamber wells together with 0, 5, 10, 25, 50 and 100 µg/mL heparin. Each concentration was run in quadruplicate. The prepared CIM-plates were left in room temperature for 30 minutes for the cells to settle

on the upper side of the membrane. The plates were then loaded into the xCELLigence™ system and the scan period started. Scanning was set to incur every 10 minutes for the first 4 hours and every 15 minutes the next 20 hours. Plateau phase was reached after 100 minutes. The measurements were plotted as the relative change in measured electrical impedance of the chamber-electrodes as cell index (CI).

2.6 Flow Cytometry Analysis of MAPK-ERK Phosphorylation

Cold trypsin phosphorylation specific flow cytometry was used to study the response of the MAPK-ERK pathway to varying concentrations of heparin (Fig. 7A) [32-34]. Patient-derived VSMCs were grown in 6-well culture dishes. The cells were serum starved overnight before stimulated with hFGF (R&D systems Inc., Minneapolis, MN) at the concentration of 100 ng/mL for 15 minutes. The cells were put on ice immediately after stimulation to quench cell signaling. The cells were then washed twice with ice cold PBS before being trypsinized using 0.5% of Trypsin-EDTA at 4℃ to avoid protease-dependent activation of cell signaling responses. 16% paraformaldehyde (PFA) solution (Electron Microscopy Sciences, Hatfield, PA) was added directly to the cell suspension to obtain a final concentration of 1.6% PFA. The cells were incubated in the fixative for 20 minutes at room temperature before being washed with PBS and pelleted before permeabilized in 100% cold methanol (Sigma-Aldrich).

To stain the cells for flow cytometry, the samples were first centrifuged at 448 x g for 5 minutes to remove the methanol and then washed with FACS buffer (1% weight/volume BSA in PBS). Cells were then stained with p44/42 MAPK antibody 4695 (Cell Signaling, Danvers, MA) at a dilution of 1:1000 for 30 minutes at room temperature. The cell suspension was then washed with FACS buffer before goat anti-rabbit IgG-Alexa Fluor 647 (A-21244, Invitrogen; Life Technologies) was added at a concentration of 1:2500 (0.8 µg/mL) as a secondary antibody. The cells were incubated for 1 hour in darkness at room temperature before being washed once more with FACS buffer. In order to verify phosphorylated MAPK-ERK signal specificity, cells were treated with the MEK1 inhibitor PD98059 (Cell Signaling) at a concentration of 30 µM for 3 hours. Samples were run on BD Accuri C6 (BD Biosciences) to determine the phosphorylation levels of MAPK-ERK. Analyses were performed using FlowJo (TreeStar Inc., Ashland, OR) creating outputs as illustrated in Fig. 7B.

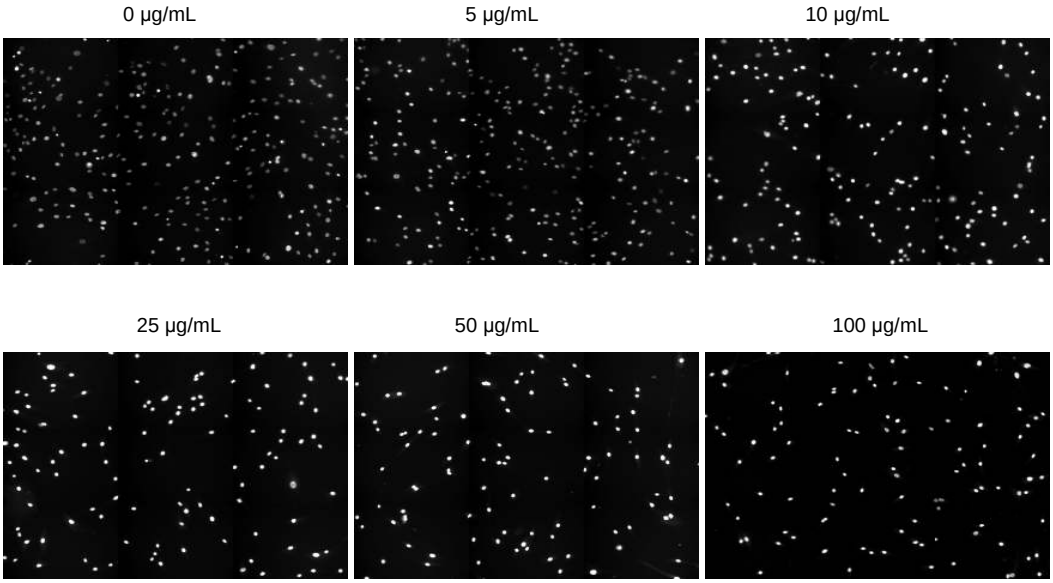

Fig. 6. Micrographs of VSMCs at different heparin concentrations as acquired by the BD Pathway system 855 analysis

Heparin was added in concentrations as labeled above each micrograph. Images were acquired with 20x objective as 2x2 montages and the total number of nuclei (Hoechst) was quantified using the Montage cell-count algorithm embedded in Attovision v1.6.2. The measurements resulted in a dose-response curve for each cell culture

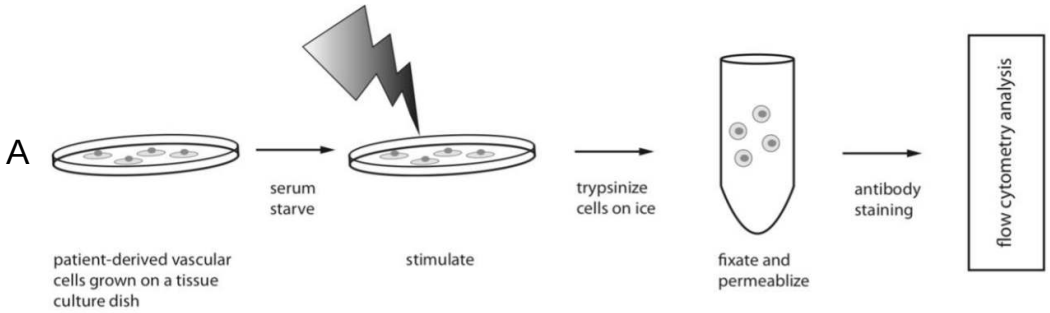

Fig. 7A. Schematic overview of the flow cytometry approach

Adherent patient-derived VSMCs were harvested by a cold trypsinization approach to minimize protease activated cell signalling. Recovered cells were fixed and permeabilized, and stained with anti-pERK antibodies followed by fluorescently conjugated (AlexaFluor 647) secondary antibodies. Flow cytometry was conducted to quantify the anti-pERK dependent fluorescence levels in individual patient VSMCs

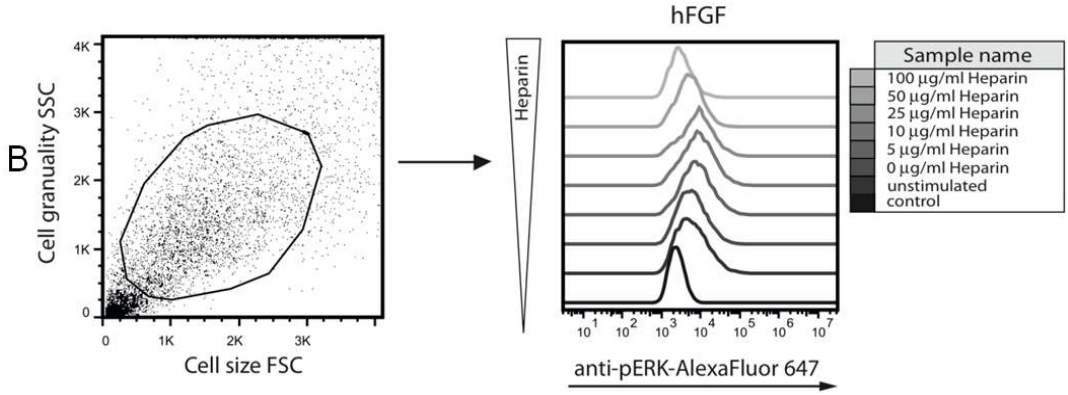

Fig. 7B. Flow cytometry analysis of MAP kinase pathway activation in hFGF stimulated VSMCs

Dotplot shows anti-pERK stained VSMCs within a forward/side side scatter viability gate quantified by fluorescence intensity measurements of anti-pERK-Alexa 647 in the FL4 channel. Histogram shows pERK levels in hFGF (100 ng/mL) stimulated patient VSMCs treated with increasing concentrations of heparin

2.7 Statistical Analysis

To detect differences of proliferation, migration and signaling as a function of concentration, linear regression analysis of fitted trend lines was used. The trend lines were fitted using the least-squares method and tested for linearity. IC50 was calculated using the fitted trend line formula. For the proliferative assay, each patient culture sample was analyzed in triplicate during 2 separate experiments for each concentration. Data were normalized to untreated control values by calculating the coefficient needed for the automated image-based cell enumeration at 0 µg/mL to equal 100. For the migration assay, each sample was analyzed in quadruplicate. The cell indices at 100 minutes were normalized to untreated control by calculating the coefficient needed to achieve a cell index of 0.35 at 0 µg/mL.

For the flow cytometry, three series were analyzed. The ERK phosphorylation levels were normalized to untreated control at 0 µg/mL to equal 100.

Probability values of $P<0.05$ were considered significant throughout the study. All statistical analyses were done using Excel® for Mac 2011 (Microsoft Corporation, Redmond, WA, USA) and STATA for Mac Statistical Software, Release 12 (StataCorp LP, College Station, TX, USA).

3. RESULTS

3.1 Proliferation of Patient-derived Cell Cultures

To detect the effects of heparin on proliferation of patient-derived VSMC cultures, heparin was added in a wide range of concentrations.

Quantified by image cell enumeration heparin inhibited proliferation dose dependently (*P*<0.001; Fig. 8). The fitted trend line had an R^2 of 0.94. IC50 was reached at 107 µg/mL. Analysis of individual concentrations revealed significant inhibition at 50 and 100 µg/mL (*P*=0.02 and 0.001). Heparin treatment inhibited proliferation of patient-derived EC-VSMC co-cultures in a similar fashion (*P*<0.001; Fig. 1). R^2 of the trend line was 0.81 and IC50 reached at 99 µg/mL. Sub analysis by concentration demonstrated inhibition of cell proliferation at 25, 50 and 100 µg/mL (*P*=0.003, 0.003 and <0.001). No difference was detected when the trend lines of mono- and co-cultures were compared (*P*=0.79).

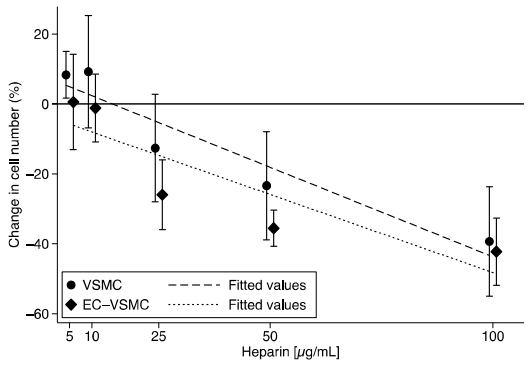

Fig. 8. Proliferative response of patient-derived VSMC monocultures and EC-VSMC co-cultures to heparin

The cell number was determined by automated cell image analysis enumeration. Heparin concentrations (µg/mL) are along the abscissa and the change in cell number (%) along the ordinate. There was a dose dependent inhibition of VSMC and EC-VSMC proliferation by heparin (P<0.001 for both). The fitted trend line of VSMCs had an R^2 of 0.94 and IC50 was reached at 107 µg/mL. Analysis of individual concentrations revealed significant inhibition at 50 and 100 µg/mL (P=0.02 and 0.001). R^2 of the fitted EC-VSMC trend line was 0.81 and IC50 was reached at 99 µg/mL. Sub analysis by concentration demonstrated inhibition at 25, 50 and 100 µg/mL (P=0.003, 0.003 and <0.001). No difference was detected when comparing the fitted trend lines of mono- and co-cultures (P=0.79)

3.2 Migration of Patient-derived VSMCs

Heparin exerted a dose-dependent inhibition of VSMC migration (*P*<0.001; Fig. 9). The fitted trend line (not illustrated) had an R^2 of 0.89 and IC50 was 88 µg/mL. Analysis by concentration showed significant inhibition at 25, 50 and 100 µg/mL (*P*=0.001, 0.006 and 0.005).

3.3 MAPK-ERK Phosphorylation of Patient-derived VSMCs

MAPK-ERK phosphorylation decreased dose-dependently by heparin in unstimulated cultures of VSMCs (*P*<0.001; Fig. 10). IC50 of the fitted trendline was 61 µg/mL and had an R^2 of 0.91. When stimulated by hFGF (100 ng/mL) there was an increase of MAPK-ERK phosphorylation at 5 µg/mL (*P*=0.02) with a subsequent dose-dependent decrease (*P*<0.001). IC50 of hFGF-stimulated cultures was 98 µg/mL with an R^2 of 98.1

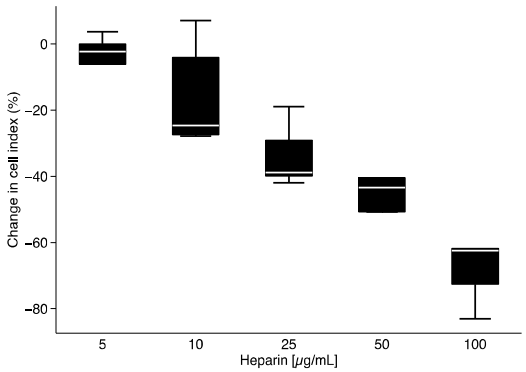

Fig. 9. Migratory response of patient-derived VSMC monocultures to heparin

Patient-derived VSMCs were monitored for cell migration using the xCELLigence™ system in the presence of heparin. Results were plotted as normalized means of the relative change in electrical impedance measured by chamber electrodes, denominated cell index. The change was relative to untreated controls. Following initial calibration for activity (see Material and Methods), cell migration increased for 100 minutes in a time dependent fashion before reaching plateau. There was significant dose-dependent inhibition of migration by heparin (P<0.001). IC50 was 88 µg/mL. Analysis by concentration showed significant inhibition at 25, 50 and 100 µg/mL (P=0.001, 0.006 and 0.005)

4. DISCUSSION

Heparin inhibited proliferation and migration in patient-derived VSMC cultures, but only at concentrations exceeding clinical doses. In concentrations commensurate with clinical administration it did not exhibit inhibitory effects. Previous proliferative studies found in medical literature have predominately used a heparin concentration of 100 µg/mL [10,35]. As full clinical heparinization is achieved at serum concentrations of 2-7 µg/mL (Felleskatalogen AS, Norway), these experiments have provided results of limited clinical value.

Serum concentrations of heparin may differ from actual tissue concentrations surrounding vascular cells. This may be a reason for the different effects of heparin used clinically and in cell cultures. Exact quantification of this difference was addressed by Lovitch and Edelman [36]. Using bovine cells and rats, it was estimated that the required arterial concentration of heparin to inhibit intimal hyperplasia maximally after injury *in vivo* was 0.3 mg/mL (300 µg/mL). This was approximately triple the heparin concentration needed for a similar response in cell culture of the present study. This illustrates that the culture results reflect the cellular response, while the *in vivo* results reflect the tissue response. Therefore, it may be argued that the present study ought to have included even lower concentrations of heparin. However, the serum-tissue concentration ratio in humans was not known and neither was the dose-response curve of heparin on human VSMCs. Based upon this it was decided to use the concentrations described in Materials and Methods.

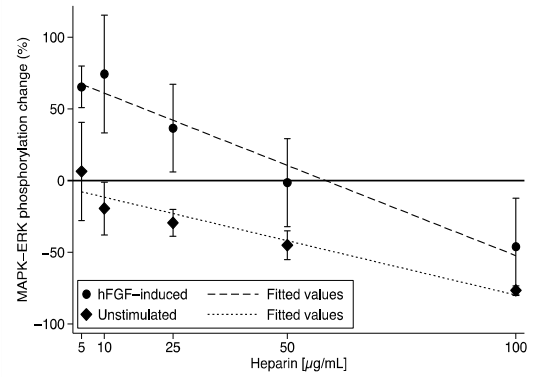

Fig. 10. Effects of heparin on unstimulated and hFGF-induced MAPK-ERK phosphorylation of patient-derived VSMCs

MAPK-ERK phosphorylation of patient-derived VSMCs treated by heparin as assessed by flow cytometry. Heparin concentrations (µg/mL) are along the abscissa and MAPK-ERK phosphorylation change (%) relative to untreated control along the ordinate. There was an increase of MAPK-ERK phosphorylation at lower concentrations when induced by hFGF (100 ng/mL). MAPK-ERK phosphorylation of unstimulated cultures was inhibited dose-dependently. This suggests that the cellular response to heparin is based on the activation-ratio between a stimulatory hFGF-dependent pathway and an inhibitory hFGF-independent pathway

The need for high heparin concentrations to prevent intimal hyperplasia was also reported by Edelman and Karnovsky [37]. In a rat restenosis model they found that heparin exacerbated intimal hyperplasia except when continuously administered via osmotic pumps at high doses. In their study, Chan et al. also reported pro-proliferative effects of heparin at lower concentrations [35]. They presented a figure illustrating negative proliferative inhibition of heparin at 10 µg/mL using unfractioned heparin on human VSMCs. Lehmann and colleagues terminated a prospective controlled clinical trial, as low-dose heparin therapy appeared to increase the likelihood of restenosis and adverse clinical outcomes [15]. These results could be explained by the MAPK-ERK phosphorylation response to heparin at lower concentrations (Fig. 10). In this study the MAPK-ERK phosphorylation was increased at low heparin concentrations when the cell cultures were stimulated by hFGF (100 ng/mL), while unstimulated cultures were inhibited dose-dependently. Thus, heparin seemed to regulate the MAPK-ERK phosphorylation of VSMCs through a stimulatory hFGF-dependent pathway and an inhibitory hFGF-independent pathway. These observations suggest that the MAPK-ERK response of patient derived VSMCs to heparin relies on the activation-ratio between the two pathways.

It has previously been reported inhibition of aortic rat cell migration by heparin at low concentrations (1-10 µg/mL) [38]. However, this study has demonstrated that patient-derived VSMCs were significantly inhibited only when concentrations of heparin exceeded 25 µg/mL (Fig. 9). This illustrates the problem of directly applying experimental results from other species or cell lines into a clinical setting.

The putative influence of ECs on the heparin responsiveness of VSMCs has been attributed to nitrogen oxide, growth factors and endogenous glucosaminoglycans [7,16,18,39]. This was investigated by employing a vascular cell co-culture system that modeled reciprocal EC-VSMC interactions. It generated *in vitro* capillary-like structures comprising basement membrane and pericyte enveloped three-dimensional endothelial tubular structures. VSMC proliferation was not significantly affected by the presence of ECs in this study (Fig. 8). It can be argued that this result was expected since cells were not damaged, as they would be when performing surgery. Presence of damaged cells could have led to different microenvironmental conditions, producing a different outcome. Due to methodological constraints, it was not possible to introduce reproducible controlled damage corresponding to the actual surgical trauma. In

previous experiments of heparin effects on human VSMCs by Chan and Refson, results were reproducible and significant despite the lack of injury [8,10,35]. Based upon this it was concluded that a setup of cell cultures without introducing injury would provide reproducible and reliable data.

Biopsies from 6 patients were used as substrate for a total of 532 different cell cultures for the proliferative- and 144 for the migratory analyses. All cell cultures were individually analysed. Although the sample was small, it was comprised of actual patients that underwent vascular surgery. The cells isolated were taken from areas near the line of resection, minimizing influence of the actual vascular disease. Thus, these cells should be more suited for analysis than single lines of laboratory grown animal cells used in previous studies. As all biopsies were taken from the aorta, generalization of the results could be problematic. However, Refson and colleagues found that VSMCs harvested from different sites in the same individual responded identically to heparin [10].

5. CONCLUSION

Heparin inhibits proliferation and migration in patient-derived VSMC cultures, but only at concentrations exceeding clinical doses. The proliferative response of VSMCs to heparin was not affected by the presence of ECs. MAPK-ERK phosphorylation was increased at low heparin concentrations when the cell cultures were stimulated by hFGF, while unstimulated cultures were inhibited dose-dependently. Thus, heparin seems to influence patient-derived vascular cells through both a stimulatory hFGF-dependent and an inhibitory hFGF-independent pathway. These findings can explain the divergence of results between previous *in vitro* studies and clinical studies and provide a basis for new therapeutic strategies.

CONSENT

All patients gave their written consent before biopsies were harvested.

ETHICAL APPROVAL

Approval to establish a human tissue biobank and to perform this study was granted by the Regional Independent Scientific Ethical Committee (REK Bergen 162.08) and the Norwegian Directorate of Health.

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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