Fortschritte bei der Austestung antiarteriosklerotischer Pharmaka in Transfilter-Co-Kulturen mit humanen vaskulären Zellen

Dorothea Ilse Siegel-Axel[1], Richard Viebahn[2], Eberhard Ludwig Betz[3], and Karl Rüdiger Karsch[1]
[1]Medical Clinic III, Dept. of Cardiology, [2]Dept. of Surgery, and [3]Institute of Physiology I, Eberhard-Karls University, D-Tübingen

Summary
Excessive proliferation and migration of human arterial smooth muscle cells (haSMC) are prominent features of both primary atherogenesis, as well as restenosis following interventions, such as balloon angioplasty (PTCA) or stent implantation. Thus, in the last two decades many efforts were made to establish a therapeutic strategy with antiproliferative and antimigratory compounds. A great variety of substances belonging to different drug classes were already tested both in vitro and in vivo but there is still no breakthrough in the prevention or treatment of human arterial vessel diseases. Among the choice of less potent compounds, the main reasons for this failure are the use of unappropriate animal models or animal cell cultures allowing no direct transfer of the results to the human situation. For that reason, the more complex transfilter coculture model was developed and established for the cocultivation of human vascular cells and blood cells to imitate the morphology of the arterial vessel wall in vitro. The present paper describes the morphology of fibromuscular-like and atheromatous-like proliferates induced in this model in comparison to human plaques, as well as its practicability for the pre-screening of antiarteriosclerotic compounds. As examples, two chemically different compounds are shown. We found that the described transfilter coculture system is a suitable and well-established model allowing fast and reproducible studies with antiproliferative and antimigratory drugs. The transferral of the results to the human situation on the basis of these complex in vitro-studies seems to be improved when compared to most animal models.

Keywords: 3R, replace, atherosclerosis, restenosis, human vascular cells, transfilter cocultures, antiarteriosclerotic drugs

Zusammenfassung: Die überschießende Proliferation und Migration humaner arterieller glatter Muskelzellen (haSMC) stellen zentrale Ereignisse sowohl bei der primären Atheroskleroseentstehung, als auch bei der Restenoseentwicklung nach Ballonangioplastie (PTCA) oder Stent-Implantation dar. Daher wurden in den letzten zwei Jahrzehnten zahlreiche Anstrengungen unternommen zur Suche bzw. Entwicklung antiproliferativer und antimigratorischer Substanzen, wobei bereits eine Vielzahl von Pharmaka aus den unterschiedlichsten Substanzklassen getestet wurden. Bis heute ist jedoch noch kein Durchbruch bei der Prävention bzw. Behandlung arterieller Gefäßwanderkrankungen gelungen. Gründe dafür sind neben unzureichender Wirksamkeit bzw. ungünstiger chemischer Eigenschaften der Pharmaka vor allem im Einsatz ungeeigneter Tiermodelle oder Zellkulturen mit tierischen Zellen zu suchen, die keine direkte Übertragung auf den Menschen zulassen. Aus diesem Grunde wurde das Transfilter-Co-Kulturmodell entwickelt und für die Anwendung humaner Gefäßwandzellen und Blutzellen etabliert, um den Aufbau der arteriellen Gefäßwand in vitro imitieren zu können. Die vorliegende Arbeit zeigt, wie sich in diesem Modell atheromatöse und fibromuskuläre Proliferate induzieren lassen und inwieweit diese mit humanen Plaques vergleichbar sind. Außerdem wurde die Praktikabilität dieses Systems hinsichtlich der Austestung antiarteriosklerotischer Pharmaka untersucht, was am Beispiel zweier Substanzen mit unterschiedlichen chemischen Eigenschaften aufgezeigt ist. Die Ergebnisse zeigen, daß das Transfilter-Co-Kultursystem ein gut etabliertes und für das Prescreening antiproliferativer und antimigratorischer Pharmaka geeignetes Modell darstellt, das in den meisten Fällen sogar eine bessere Übertragung der Ergebnisse auf den Menschen erlaubt, als viele bisherige Tiermodelle.

1 Introduction

Cell-cell interactions play an important role in nearly each tissue including the arterial vessel wall (Ross, 1993). Nevertheless, most *in vitro* studies are still performed at monocultures of a single cell type, in atherosclerosis and restenosis research predominantly at smooth muscle cells or endothelial cells of rats, rabbits, pigs, or humans (Muller et al., 1992; Schwartz and Liaw, 1993). Coculture systems are suitable models to study communication between two or more different cell types which are neighboured in the arterial vessel wall (Casscells, 1991; Jones, 1979). However, in commercially available coculture systems, as for example the transwell system with inserts hanging into a culture dish, one cell type is cultured on the bottom of the dish, the other on the surface of a filter membrane in a basket (Dunzendorfer et al., 1997). In this system, two cell types communicate by the secretion of soluble factors but not via direct cell-to-cell contacts. To overcome this limitation, the transfilter coculture model was established in the Institute of Physio-

logy I in Tübingen in which different cell types can be cocultured on the opposite sides of a porous, collagen-coated polycarbonate filter (Weber et al., 1986). Through the 5 µm-wide filter pores direct cell-to-cell contacts are possible e.g. via the formation of lamellipodia. In order to imitate the morphology of the arterial vessel wall, smooth muscle cells are seeded on one side and endothelial cells on the other side of the porous filter which resembles the internal elastic lamina allowing also migration of smooth muscle cells (Roth et al., 1993). In the beginning of such transfilter coculture studies, smooth muscle cells from the rabbit aorta were cocultured with bovine endothelial cells because cell culture from human specimens was complicated and expensive (Fallier-Becker et al., 1991). In the meanwhile many progress was made in human cell culture, predominantly of human arterial endothelial cells. The main reasons are the better co-operation with surgeons, well established cell isolation techniques, and improved endothelial cell culture media containing very potent growth and adhesion factors (Axel et al., 1996; Dartsch et al., 1990). The present paper describes the establishment and routine use of isolated human arterial endothelial cells (haEC) and human arterial smooth muscle cells (haSMC) in transfilter cocultures for the pre-screening of antiproliferative and antimigratory compounds. The results of cocultures are compared to those obtained in monocultures under identical culture conditions. Furthermore, cocultivation of haEC with media explants is demonstrated representing a kind of organ culture which is even closer to the *in vivo* situation than frequently passaged cells lacking some of the *in vivo* properties. The most complex transfilter coculture model with media explants, vascular cells, and blood cells was developed to mimic important steps in the development of primary atherosclerosis and restenosis, such as atheromatous and fibromuscular plaque formation (Axel et al., 1996).

2 Material and methods

2.1 Test compounds

Low molecular weight heparin (LMWH, Clivarin®) and fluorescein-labeled low molecular weight heparin (FITC-heparin) were kindly supplied by Knoll (Ludwigshafen) and dissolved in sterile and pyrogen-free water (AMPUWA). Dilutions were made with culture medium. Paclitaxel was purchased from Sigma, dissolved in absolute pure ethanol, and sterile-filtered. To obtain different test concentrations between 10^{-9}-10^{-4} mol/L, serial dilutions of a stock solution (2.5 mmol/L) were prepared with 0.9% NaCl-solution. Each concentration of the vehicle ethanol was also analysed separately for independent antiproliferative or cytotoxic effects.

2.2 Cell isolation and culture

Human arterial smooth muscle cells (haSMC) and endothelial cells (haEC) were isolated from specimens of human iliac arteries which were discarded after liver or renal transplantations, as described previously (Axel et al., 1996; Roth et al., 1993). In brief, adhering fat and connective tissue was removed, the vessel was opened, and pinned on a siliconised preparation dish. HaEC were scraped off mechanically with a cotton rod and subcultured in collagen-coated plastic flasks (Sigma) using the EGM-2 kit without heparin and antibiotics (Cell Systems). HaSMC were obtained by the explant technique and subcultured in plastic culture dishes with Waymouth´s MB 752/1 and Nutrient Mixture Ham´s F12 (1+1, Gibco BRL) supplemented with 10% fetal calf serum (PAA Laboratories) and 100 U/ml penicillin and 100 µg/ml streptomycin.

Smooth muscle cell and endothelial cell origin was proven immunocytochemically using a double staining with specific antibodies against a-smooth muscle actin (Progen) and the von Willebrand factor (Boehringer Mannheim). Both cell types were used in the first three passages in order to preserve most of their *in vivo* properties. Routine stainings with the DNA dye DAPI (4´,6-diamidine-2-phenylindole-dihydrochloride, Boehringer Mannheim) were used to exclude mycoplasm contaminations

2.3 Monocultures

Cells were trypsinised, counted with a Coulter counter and seeded onto six-well plates. After cell attachment (24 hours), the "baseline" number of intact cells and the mitotic index was determined. Then, two modes of drug application were performed: (i) nonstop incubation re-adding the test compound every second day and (ii) single dose application, adding the compound only once for a period of 20 minutes. After 7 days final cell growth was measured by cell counting, mitotic indexes were determined by BrdU-ELISA (Colorimetric Cell Proliferation ELISA, Boehringer Mannheim), and viability of cells by MTT-tests (Sigma). Each test consisted of 6 measurements for each concentration of the test compound (10^{-9}-10^{-4} mol/L) and was performed three times under identical conditions (Axel et al., 1997b).

2.4 Transfilter cocultures with subcultured cells

Polycarbonate filters (Nuclepore, Costar) with a pore size of 5 µm and a growth area of 8 cm^2 were coated with collagen and UV-sterilised according to techniques previously described (Axel et al., 1996). Coated filters were inserted between an inner and an outer polycarbonate frame which were manufactured in the Institute of Physiology I, Tübingen (fig. 2, right side). Then, haEC were seeded onto the "lower" side of the filter at a density of $2.5x10^4$ cells/cm^2.

To study cocultures with proliferative haEC, haSMC ($2.5x10^4$ cells/cm^2) were seeded on the following day onto the opposite side of those filters and both cell types were cocultured for 14 days. By this construction, the two different cell types could be cultured in separate culture media in the given cultivation compartment and cell-cell communication was only allowed through the filter pores. For the cocultivation of confluent haEC with haSMC, haEC were first grown to confluency for about 7 days prior to the seeding of haSMC.

2.5 Detection of growth factors in transfilter cocultures

To study the release of growth factors in transfilter cocultures, conditioned media of the upper and lower compartment were collected after 6,12,24,48, and 72 hours cocultivation. Growth factor concentrations were determined using high sensitive ELISA (PDGF-AB sensitivity: 8.4 pg/ml, 1-10% cross-reactivity with PDGF-BB, TGF-ß1 sensitivity: 5.0 pg/ml; "Quantikine", R&D Systems Inc.). For the measurement of TGF-ß1, samples were subjected to acidification in order to release mature TGF-ß1 from complex forms in active forms (Axel et al., 1997a).

2.6 Antiproliferative or antimigratory compounds

Increasing concentrations of the test compound were added either for 20 minutes or

non-stop exclusively to the lower compartment where haEC were seeded in order to imitate local drug application at the "luminal side" of the vessel via blood. Culture media of both compartments were then replaced by normal, drug-free culture media, and renewal of culture media was performed every third day. After 14 days, cell numbers on both sides of the filters were determined separately by cell counting after disaggregation with trypsin/EDTA and compared to controls which were treated with equivalent concentrations of the vehicle. Each concentration was tested in a total of 3 cocultures (Axel et al., 1997b).

In addition, representative filter pieces were fixed with 4% paraformaldehyde for 3 hours and embedded in araldite (Serva), as previously described (Wolburg-Buchholz et al., 1992). Semithin sections (4 µm) were taken on poly-L-lysine coated cover slips. Cells were then stained with toluidine blue (Merck) and cell numbers on both filter sides were determined by light microscopy. In order to identify haSMC and haEC on both filter sides, immunocytochemical stainings were performed, as previously described (Axel et al., 1996). Labeling of proliferative cells in cocultures was performed using 5-bromo-2'-deoxyuridine (BrdU, 20 µmol/l, Serva).

2.7 Transfilter cocultures with media explants

Instead of isolated and subcultured haSMC media explants from arterial vessel specimens were gently stripped off the adventitia after endothelial cells were removed. Then, 5-7 media pieces were placed on the filter surface without medium since they adhered. Under the stimulatory influence of cocultured haEC first haSMC began to migrate out of the explants after 7 days. Monocytes and LDL-cholesterol were isolated from human blood, as previously described (Axel et al., 1996) and added to the endothelial side. After 28 days filter pieces with explants were fixed with 4% paraformaldehyde for 3 hours and embedded in araldite (Serva). Then, semithin section were made and stained with toluidine blue.

3 Results

3.1 Fibromuscular plaque formation in transfilter cocultures

The transfilter coculture system was designed in order to imitate the morphology of the arterial vessel wall, as shown in figure 1. In order to prove the suitability of this model to study important processes of atherosclerosis and restenosis, transfilter cocultures were first characterised on the morphological and functional level and cell-to-cell interactions were studied in detail.

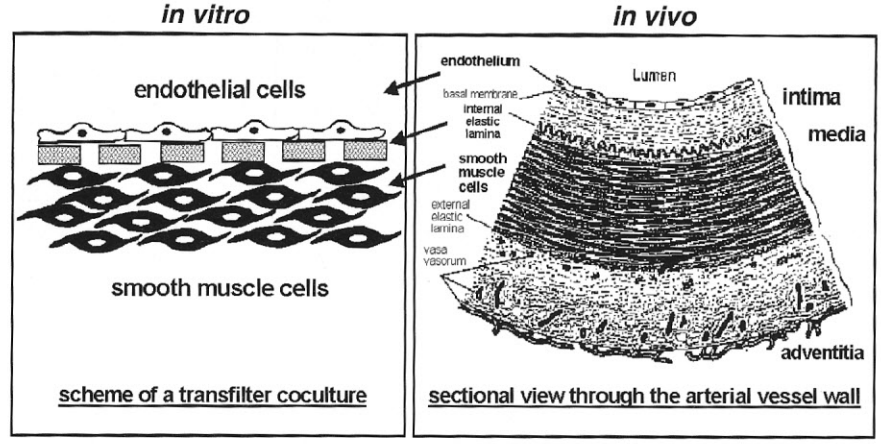

Figure 1: *In vitro* imitation of the arterial vessel wall morphology. The left side shows a transfilter coculture with human arterial endothelial cells and smooth muscle cells seeded on the opposite sides of a collagen-coated, porous filter membrane (pore size: 5 µm). The filter represents the internal elastic lamina of an artery, the smooth muscle cell multilayer mimics the media, and the endothelial cell lining corresponds to the endothelium *in vivo* (right side).

The main finding of the histological studies was that the endothelial cell lining influences smooth muscle cell proliferation and migration significantly depending on the growth stage of haEC. When confluent haEC were cocultured with haSMC, both proliferation of haSMC on the upper filter side, as well as migration from the upper to the lower filter side was inhibited. In contrast, cocultivation of proliferating or mechanically injured haEC with haSMC resulted in a dramatic stimulation of haSMC proliferation and migration with the formation of fibromuscular proliferates beneath the endothelium (fig. 2).

Functional studies by ELISA and Northern blot analysis demonstrated that predominantly platelet-derived growth factor-B (PDGF-B) and transforming Growth Factor-ß1 (TGF-ß1) were secreted significantly depending on the cell type, the cultivation time, and cell density. In contrast to haSMC, haEC showed significant mRNA expression and protein synthesis of the PDGF-B isoform after 12-24 hours cocultivation, predominantly when haEC were in the proliferative growth stage. TGF-ß1 was released by both haEC and haSMC with increasing cultivation time and cell density. The induced fibromuscular proliferates (fig. 2) showed a similar morphology to cell-rich restenotic lesions which were formed in humans after interventions, eg. after balloon angioplasty.

Similar fibromuscular plaques were formed when media explants were cocultured with proliferating endothelial cells. HaSMC grew out of the explant, migrated through the filter pores, and proliferated beneath the endothelium. 6-8 smooth muscle cell layers were found after 14 days cocultivation time.

3.2 Induction of lipid-rich plaques in transfilter cocultures

To imitate the more complex atheromatous plaque formation in humans, monocytes were seeded on the endothelial side of transfilter cocultures containing again media explants. Culture media of both cultivation compartments were supplemented with LDL cholesterol (200 µg/ml) during the total cultivation period of 28 days. Added monocytes adhered to the endothelium and migrated through the endothelium and the filter pores to the opposite filter side where haSMC were seeded. Stainings with specific antibodies showed that monocytes accumulated LDL intracellularly and transformed to lipid-filled macrophages which were similar to human foam cells. Surrounding haSMC and haEC were also filled with lipid leading to a partial cell necrosis. After 28 days cocultivation, characteristic atheromatous-like plaques were formed on the endothelial side which showed a similar

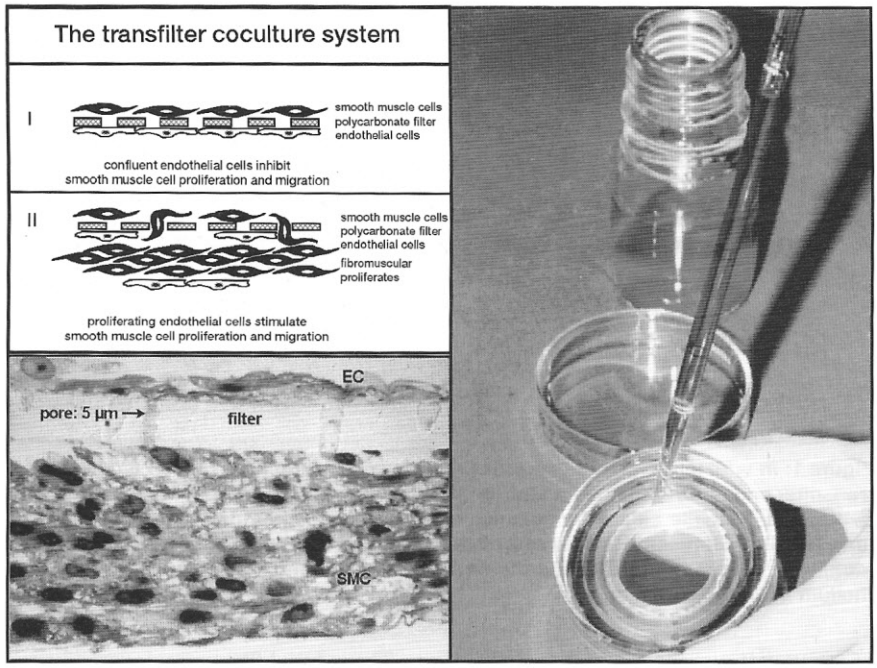

Figure 2: Right side: Photograph of the transfilter coculture system showing the filling procedure of the lower compartment in which haEC were seeded. Left side: The effect of haEC on haSMC-growth. A functionally or mechanically injured endothelial cell lining stimulates haSMC-proliferation and migration significantly leading to the formation of fibromuscular proliferates beneath the endothelium, as shown in a typical toluidine blue stained semithin section.

morphology as lipid-rich plaques in humans with a haSMC-containing fibromuscular cap and a lipid core with necrotic cells, macrophages, and lipid accumulation (fig. 3).

3.3 Pre-screening of antiproliferative and antimigratory compounds

3.3.1 Chemical characteristics and uptake of different compounds

Beside the described basic studies about the development of fibromuscular or atheromatous plaques, the transfilter coculture system was found to be a very suitable model for the pre-screening of growth inhibitory compounds. The following representative examples from two totally different drug classes were chosen in order to demonstrate the main criteria which must be fulfilled for a promising therapeutic concept in the prevention of restenosis in humans: (a) low molecular weight heparin (LMWH, Clivarin®), a potent antithrombotic drug, and (b) the antitumor agent paclitaxel (Taxol®).

Uptake studies using fluorescein-labeling showed that the very lipophilic compound paclitaxel penetrates easily and fastly through cellular membranes within 5 minutes, whereas FITC-labeled LMWH could be detected intracellularly first after 24 hours because of its high hydrophilicity and negative charge.

It is well known that the uptake of hydrophilic, charged compounds can be facilitated enormously by the encapsulation into liposomes. Thus, FITC-LMWH (fig. 4b) was packed into unilamellar cationic liposomes (dioleyl-phosphatidylholin, DOPC,

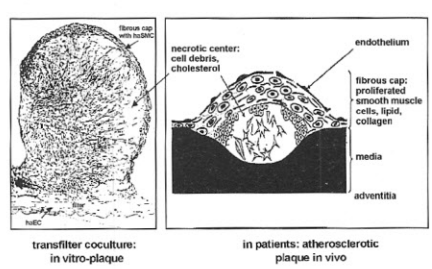

Figure 3: Induction of atheromatous-like plaques *in vitro*. On the left side, a representative semithin section across an *in vitro* plaque is shown and the morphology is compared to typical atheromatous plaques in humans, shown as schematic draft on the right side. The main characteristics are the fibromuscular cap containing haSMC and the lipid core consisting of cell debris, cholesterol, and foam cells.

provided by Dr. Joachim Grammer, Dept. of Surgery, University of Tübingen) which were labeled with a pyren dye (fig. 4a). By this procedure, intracellular uptake could be accelerated enormously. DOPC-encapsulated FITC-LMWH could be detected in the cytoplasm of haSMC after 1-2 hours. But even after continued incubation for several days, no nuclear staining was found indicating that the liposome was degraded and that naked FITC-LMWH was not able to penetrate through nuclear membranes.

3.3.2 Effect of LMWH and paclitaxel on haSMC growth in mono- and cocultures

Cell counting tests and BrdU-ELISA showed that non-stop incubation of haSMC with LMWH in monocultures for 7 days resulted in a dose dependent growth inhibition with a maximum >50% at 10^{-5} mol/L (fig. 5a) whereas no significant inhibition of haSMC-proliferation and migration in transfilter cocultures could be achieved when haEC were present (fig. 5b).

In contrast, paclitaxel was found to be a very potent inhibitor of both haSMC-growth in monocultures (fig. 6a), as well as proliferation and migration in transfilter cocultures (fig. 6b). The IC_{50} was determined at extremely low doses. Non-stop paclitaxel-exposure, as well as single dose application of paclitaxel for 20 minutes on the endothelial side caused a complete (>90%) and prolonged (for 14 days) inhibition of haSMC-growth with an IC_{50} of 2.0 nmol/l. MTT-tests showed that paclitaxel did not exert cytotoxic effects on haSMC and haEC in doses between 10^{-5}-10^{-9} mol/L. Addition of mitogens or the presence of growth-stimulating haEC did not attenuate the inhibitory effects, as demonstrated for LMWH.

The second advantage favouring paclitaxel was that its unique mode of action could be visualised easily: stainings with antibodies against the cytoskeletal filament ß-tubulin showed that paclitaxel caused the formation of disorganised microtubule bundles in the perinuclear region by the enhanced microtubule assembly which were not connected to any microtubule organising centre. Furthermore, cell morphology was altered significantly showing a round, epitheloid instead of an elongated cell shape (data not shown).

4 Discussion

The purpose of the present work was to establish cell culture systems with human vascular cells to imitate main features of both atherosclerosis and restenosis, to study cell-cell interactions between vascular and blood cells, and to utilise these models routinely for the pre-screening of antiproliferative and antimigratory compounds.

In the last decade a great variety of animal studies at rats, rabbits, or pigs were performed to establish a therapeutic strategy for the treatment of atherosclerosis and for the prevention of restenosis after balloon angioplasty (Muller et al., 1992). Despite several promising animal studies showing a reduction of neointima formation after the administration of antiproliferative compounds, the transfer of these protocols to the human situation did not lead to a breakthrough in restenosis therapy. There is still no real therapeutic success in humans leading to a significant reduction of restenosis (Johnson et al., 1999).

Several reasons might be responsible for this failure. First, the compound chosen is not able to penetrate fastly enough into the vascular wall and into target cells (Baumbach et al., 1998), or is blocked by human serum (Underwood et al., 1998) because of its chemical nature. Second, the mode of action is often not known well enough. Accordingly, the drug-induced effects are not potent enough to inhibit cell growth significantly. Third, the test models used are inadequate and did not allow a transfer to the human situation, e.g. when restenosis is induced at small animals, as rats or rabbits, by endothelial denudation at a normal carotid artery which differs enormously from an atheromatous human plaque developed at coronary arteries over centuries (Lafont and Faxon, 1998).

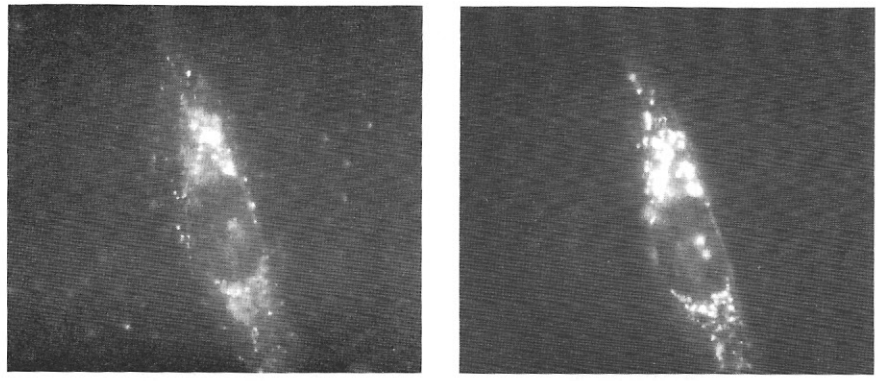

Figure 4: (a) Intracellular uptake of pyren-labeled cationic liposomes into haSMC. After one hour, liposomes accumulate in the cytoplasm and perinuclear region, but not inside the nucleus. (b) Intracellular uptake of FITC-labeled LMWH which was encapsulated into liposomes. A nearly identical pattern is found one hour after transfection, as shown for labeled liposomes, indicating that LMWH was not separated from its vehicle.

To overcome these obvious limitations, the described cell culture models with human vascular cells were developed as prescreening systems. Monocultures with haSMC or haEC were found to be very suitable tools to study penetration of FITC-labeled test compounds across cellular and nuclear membranes, as the two examples with LMWH and paclitaxel showed. Furthermore, time kinetics can be performed easily which provide important information about the duration of cellular uptake (within seconds, minutes or days), solubility problems (need of potent solvents), or possible rebound effects after drug withdrawal. Then, the effects of increasing test concentrations from a wide range can be examined in detail and in statistically sufficient numbers to define the most potent dose, as well as possible cytotoxic effects of the compound. In addition, specific stainings against cytoskeletal proteins, signal transduction protein, or growth factors can provide important information about the mode of action, as well as possible side effects on other cellular proteins (Axel et al., 1997b). The comparison of the results after application of LMWH in monocultures versus transfilter cocultures showed that cocultured haEC attenuated the inhibitory effects on haSMC or that LMWH was not able to penetrate sufficiently and fastly through the endothelial cell lining and the filter pores to reach the target cells. Thus, transfilter cocultures were found to provide further important information about the efficacy of test compounds in regard of the transfer to the *in vivo* situation and seems to be superior to monocultures. In contrast to LMWH, paclitaxel was potent enough to inhibit haSMC-growth in monocultures, as well as in transfilter cocultures because of its high lipophilicity enabling a very fast cellular uptake of both haEC and haSMC.

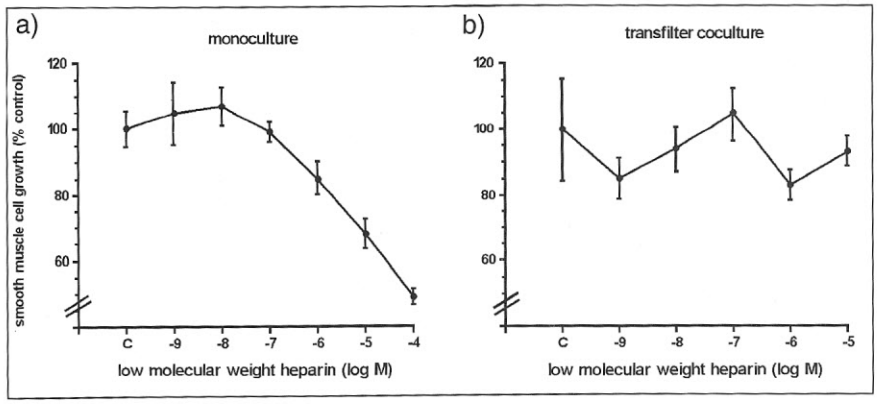

Figure 5: (a) Effect of LMWH on haSMC-growth in monocultures. Dose dependent inhibition of haSMC-proliferation determined after 7 days non-stop application of LMWH. (b) Effect of LMWH on haSMC-growth in transfilter cocultures. LMWH which was applicated exclusively on the endothelial side did not cause any significant effect on haSMC-proliferation and migration determined 14 days after non-stop incubation.

These results showed that the development of the transfilter coculture system might be an important step forward to the replacement of unsuitable animal models because in this model the morphology of the arterial vessel wall can be imitated much better than in monocultures allowing cell communication between vascular cells and blood cells. Furthermore, the establishment of transfilter cocultures with human vascular cells improves the transferability of prescreening tests with growth inhibitory com-

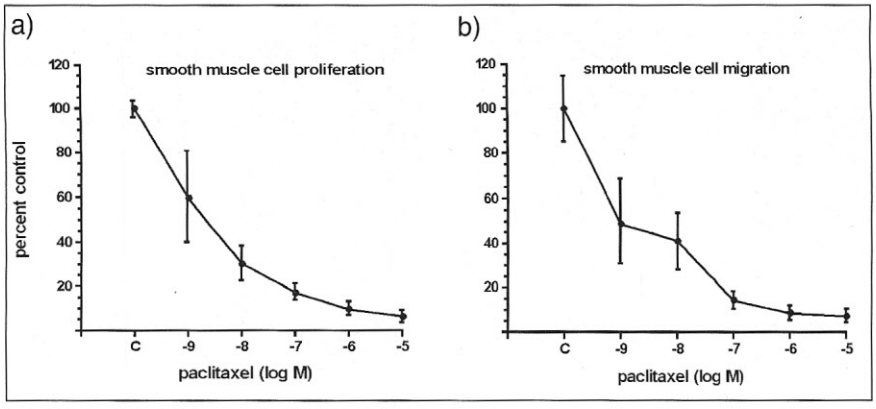

Figure 6: (a) Effect of paclitaxel on haSMC-proliferation on the upper filter side of transfilter cocultures and (b) haSMC-migration across the 5 µm-wide filter porous to the endothelial side. Single dose application of paclitaxel for only 20 minutes caused a prolonged, dose dependent inhibition of both haSMC-proliferation and migration determined 14 days after cocultivation with haEC.

pounds on patients in respect of two aspects: single dose application of the compound exclusively to the endothelial side imitates local drug delivery via blood and the use of vascular and blood cells isolated from human specimens avoids species problems.

The exemplary results with LMWH and paclitaxel demonstrate the practicability of these models for identifying antiproliferative and antimigratory compounds *in vitro* whereas the studies with media explants, monocytes and LDL show that transfilter cocultures are also useful models for basic research about the development of atherosclerosis and restenosis.

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Correspondence to

Dr. rer. nat. Dorothea Siegel-Axel
Medical Clinic III, Dept. of Cardiology
University of Tübingen
Otfried-Müller Str. 10
D-72076 Tübingen
Tel. +49-7071-29 87338
Fax +49-7071-29 3169
E-mail: daaxel@med.uni-tuebingen.de