Gene therapy strategies to target post-interventional vascular remodeling

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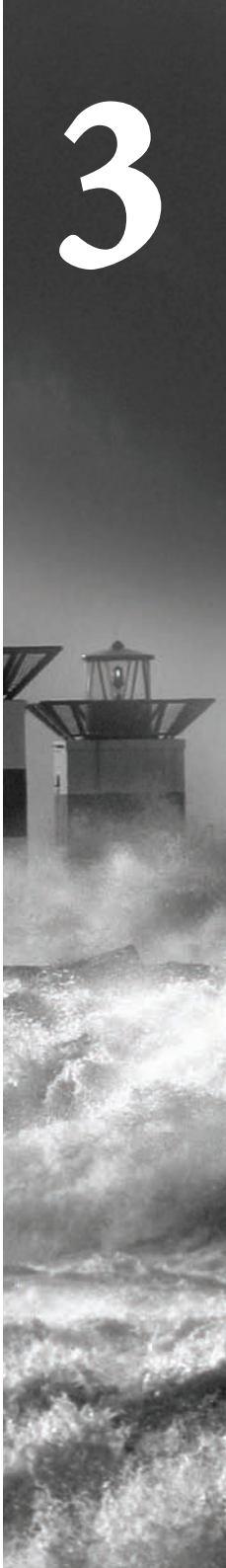

Anti-MCP-1 gene therapy inhibits vascular smooth muscle cells proliferation and attenuates vein graft thickening both *in vitro* and *in vivo*

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Abstract

Objective: Since late vein graft failure is caused by intimal hyperplasia and accelerated atherosclerosis, and these processes are thought to be inflammation driven, influx of monocytes is one of the first phenomena seen in intimal hyperplasia, we would like to provide direct evidence for a role of the MCP-1 pathway in the development of vein graft disease.

Methods and Results: MCP-1 expression is demonstrated in various stages of vein graft disease in a murine model in which venous interpositions are placed in the carotid arteries of hypercholesterolemic APOE*3Leiden mice and in cultured human saphenous vein segments in which intimal hyperplasia occurs.

The functional involvement of MCP-1 in vein graft remodeling is demonstrated by blocking the MCP-1 receptor CCR2 using 7ND-MCP-1. 7ND-MCP-1 gene transfer resulted in 51% reduction in intimal hyperplasia in the mouse model, when compared to controls. In human saphenous vein cultures neointima formation was inhibited by 53%.

In addition, we demonstrate a direct inhibitory effect of 7ND-MCP-1 on the proliferation of smooth muscle cell in human saphenous vein cultures and in smooth muscle cell cultures.

Conclusion: These data, for the first time, prove that MCP-1 has a pivotal role in vein graft thickening due to intimal hyperplasia and accelerated atherosclerosis.

Introduction

Venous bypass grafting is a common treatment for occlusive atherosclerotic vascular disease and establishes revascularization of ischemic tissue. Unfortunately, although primarily successful, it is accompanied by a high incidence of late graft failure (up to 40% after 10 years[1]), leading to a high morbidity and mortality due to re-interventions.

Graft failure is mainly due to vein graft thickening due to intimal hyperplasia and accelerated atherosclerosis. It occurs as a response to altered shear and circumferential stress and loss of endothelial integrity caused by surgery[2]. The process starts with monocyte adhesion and extravasation into the vessel wall, followed by smooth muscle cell (SMC) migration and proliferation, and macrophage accumulation in the intima[3]. Subsequently, lipids accumulate in macrophages resulting in foam-cell formation. Because of the high resemblance with atherosclerotic plaques, this is called accelerated atherosclerosis of the vein graft[4-6].

Several animal models have been developed to study vein graft thickening, including a venous interposition model in the mouse carotid artery[7]. Here, this venous interposition model was used in APOE*3Leiden mice. APOE*3Leiden mice contain the mutant human APOE*3Leiden gene, which leads to a defective clearance of APOE by the LDL receptor, and therefore these mice develop a diet-dependent hyperlipidemia and diet-induced atherosclerosis[8]. When a venous interposition is placed in the carotid artery of these mice, venous thickening with signs of accelerated atherosclerosis develops within 4 weeks[9,10], highly resembling the morphology of the diseased human vein grafts.

Although the exact mechanism of vein graft thickening is unknown, accumulating evidence suggests that it is an inflammation-driven process[11,12]. Monocyte chemoattractant protein (MCP)-1 and its receptor CCR2 are key mediators in vascular inflammation, acting as one of the most potent chemotactic agents to monocytes[13,14]. MCP-1 has been shown to play a pivotal role in spontaneous atherosclerosis and post-angioplasty restenosis. Recently it has been described that blocking of the MCP-1/CCR2 pathway results in reduced atherosclerosis and restenosis by inhibition of monocyte adhesion to the vascular wall and to reduced macrophage content in the atherosclerotic lesion[15-18]. Because of the similarities between restenosis and vein graft disease, we hypothesize that MCP-1 may play a pivotal role in development of vein graft disease.

To prove this hypothesis we use 7ND-MCP-1, a competitive receptor antagonist of the CCR2 receptor. It is created by the deletion of amino acids 2-8 at the N-terminus of human MCP-1[19] and has the potential to block the MCP-1/CCR2 pathway *in vivo*. Recently, 7ND-MCP-1 has been shown to attenuate various disorders both vascular[20-22] and non-vascular of nature[23,24] by blocking MCP-1 mediated monocyte chemotaxis.

In the present study, the role of MCP-1 in vein graft remodeling was assessed. Therefore, the expression of MCP-1 in time was studied in both murine vein grafts and cultured human saphenous veins and the effect of blocking the MCP-1/CCR2 pathway in both models was investigated using 7ND-MCP-1. Furthermore, a direct inhibitory effect of 7ND-MCP-1on smooth muscle cell proliferation was studied. These data, for the first time, prove that the pro-inflammatory cytokine MCP-1 has a pivotal role in vein graft thickening.

Materials and Methods

Mice

Animal experiments were approved by the TNO-animal welfare committee. For all experiments male C57B/6- APOE*3Leiden mice were used. Animals were fed a cholesterol-enriched high-fat diet, containing 1% cholesterol and 0.05% cholate (AB Systems), starting 4 weeks prior to surgery. All mice received water and food ad libitum.

Cholesterol levels in serum were determined 1 week before surgery and at sacrifice. Mice were anaesthetized by Midazolam (5 mg/kg; Roche), Medetomide (0.5 mg/kg; Orion) and Fentanyl (0.05 mg/kg; Janssen).

Vein graft surgery

A venous interposition was placed in the carotid artery as described previously[25]. Grafts, being caval veins of donor mice, were harvested, and preserved in 0.9%NaCl containing 100IU of heparin. In the recipient, the right carotid artery was dissected from its surroundings and cut in the middle. A polyethylene cuff was placed at both ends of the artery. At both ends, the artery was everted around the cuff and ligated. Then, the graft was sleeved over the cuffs and ligated. Pulsations and turbulent blood flow within the graft confirmed successful engraftment.

At time of sacrifice, vein grafts were harvested after 5 minutes *in vivo* perfusion-fixation with formaldehyde (4%), fixated overnight and embedded in paraffin.

7ND-MCP-1 expression vector

Human MCP-1 was modified into 7ND-MCP-1 by deletion of amino acids 2-8 and the 7ND-MCP-1 gene was cloned into the BamH1 (5') and Not1 (3') sites of a plasmid pcDNA3.1 expression vector (Invitrogen), as described before[26]. A pcDNA3.1 plasmid without an insert (pcDNA3.1-empty) was used as the control vector.

Gene transfer by electroporation

Gene transfer of 7ND-MCP-1 was performed one day prior to vein graft surgery by injecting 75µg of plasmid, either pcDNA3-7ND-MCP-1 or pcDNA3.1-empty, into the calf muscles of both legs, followed by electroporation (8 pulses of 10ms, field strength of 200V/cm (Square Wave Electroporator ECM 830, BTX) using Caliper Electrodes). Calf muscles were primed with an intramuscular injection containing 30µl of hyaluronidase (0.45U/µl, Sigma) one hour before electroporation[27]. Electroporation was called successful when the 7ND protein was detectable in serum using a human MCP-1 ELISA kit (Biosource). Protein expression was determined 1 day, 1, 2 and 4 weeks after surgery.

Analysis of intimal hyperplasia formation

Serial perpendicular cross-sections of embedded vessels were made through the entire specimen. All samples were routinely stained with haematoxylin-phloxine-saffron (HPS).

Quantification of vein graft thickening was performed using image analysis software (Qwin, Leica). The thickened vessel wall surface was defined as the total vessel surface subtracted by the luminal surface. For each mouse six equally spaced cross-sections were used to determine vein graft thickening.

The composition of both murine vein grafts and human saphenous veins was visualized by immunohistochemistry. In the murine grafts, the amount of smooth muscle cells (anti-SM α-actin, 1:1600, Roche) and macrophages (AIA31240, 1:3000, Accurate Chemical) was determined as the SM α-actin-positive and AIA-positive area in cross-sections, as a percentage of the total intimal hyperplasia surface and quantified using image analysis software

(Qwin, Leica). MCP-1 expression was determined using an anti-mouse JE/MCP-1 antibody (1:20, BD Biosciences).

Production of 7ND-MCP-1 containing conditioned medium

Human HER 911 were transfected with pcDNA3.1-7ND-MCP-1 or pcDNA3.1-empty by Lipofectamin as described by the manufacturer. Conditioned medium was collected every day and pooled. The 7ND-MCP-1 concentration produced was measured using a human MCP-1 ELISA kit (Biosource). Conditioned medium was diluted with culture medium (DMEM) until a final concentration of 7ND-MCP-1 was reached of 10 ng/ml (approximately 1:100). Medium from the pcDNA3.1-empty transfected HER 911's was collected and diluted in DMEM culture medium in a 1:100 ratio.

Production of purified 7ND-MCP-1 protein

Recombinant 7ND-MCP-1 was purified from serum free conditioned medium from stably transfected CHO-cells. Medium diluted 1:1 with 0.02 M Phosphate-buffer (pH 7.4) was circulated over a SP Sephadex column (Pharmacia) overnight, followed by elution using a NaCl-gradient in 0.02 M Phosphate-buffer (pH 7.4). Recombinant 7ND-MCP-1 containing fractions, as determined by ELISA, were pooled to a final concentration of 28 μg/ml.

Human saphenous vein organ culture

Segments of saphenous veins were obtained from patients undergoing saphenous vein stripping (kindly provided by Dr. H. Stigter, Deaconess Hospital, Leiden, The Netherlands). Healthy looking segments of the stripped veins were put into culture as previously described[28,29]. Segments (n=12 per group) were either exposed to conditioned medium containing 10 ng/ml 7ND-MCP-1 or control conditioned medium. After 4 weeks, segments were harvested, fixed overnight in formaldehyde (4%) and embedded in paraffin. All segments were routinely stained by HPS and neointimal surface was assessed on multiple sections (n=9) per vein segment and quantified using Qwin Image analysis software (Leica).

For detection of proliferating cells by BrdU incorporation, the medium was supplemented with bromo-deoxiuridine (BrdU; 40mmol/l, Sigma) 7 days before harvesting of the vessels. Number of proliferating cells was quantified as the absolute number of BrdU-positive cells per microscopic view

(magnification 100x). MCP-1 was visualized using a monoclonal anti human-MCP-1 (1:65; R&D Systems).

Smooth muscle cell culture and ^{3}H-thymidine incorporation proliferation assay

Human smooth muscle cells, explanted from saphenous veins, were subsequently cultured, characterized and proliferation was measured as earlier described [30]. Briefly, smooth muscle cells were electroporated with plasmids encoding for 7ND-MCP-1, MCP-1 and/or an empty plasmid by Nucleofector Technology (Amaxa Biosystems) according to manufacture's protocol. After electroporation, cells were seeded at a density of at least $2x10^4$ cells/24 well. Next, cells were made quiescent for 48 hours. Methyl-^{3}H-thymidine incorporation (Amersham, 0.25μCu/well) for 16 hours was measured by liquid-scintillation counting. In case purified, recombinant 7ND-MCP-1 and/or recombinant hMCP-1(R&D) was used, after quiescence, 7ND-MCP-1 was given 30 minutes before stimulation with MCP-1. Sixteen hours after stimulation methyl-^{3}H-thymidine was added and incorporation was measured. All experiments were done in triplicate and at least repeated twice.

RNA isolation and PCR procedure

Confluent monolayers of human smooth muscle cells were grown in DMEM and synchronized for 24 hrs. To stimulate smooth muscle cells, DMEM was supplemented with 0.1%FCS, 10% FCS and/or 10% FCS plus TNF-α (5ng/ml). After 40 hours cells were lysed with Tryzol (Invitrogen) and total RNA was extracted using the manufacturer's protocol. Synthesis of cDNA of all samples was performed using Ready-To-Go Beads (Amersham Biosciences). RT-PCR was performed, with gene specific primers for CCR2 (sense 5'CCAACTCCTGCCTCCGCTCTA, antisense 5'CCGCCAAAATAACCGATGTGATAC) on the cDNA samples of the 3 distinctly stimulated smooth muscle cells. Amplification-conditions were: 5 minutes at 94°C, 35 cycles of 1 minute at 94°C, 1 minute at 55°C and 2 minutes at 72°C. PCR-products were run on a 1.2% agarose gel and visualized by ethidium bromide.

Statistical analysis

All data are presented as mean ± SEM. Statistical significance was calculated in SPSS 11.5 for Windows. In both the murine experiments and the smooth muscle cell proliferation experiments overall comparisons between groups were performed with the one way ANOVA. If a significant difference was found, groups were compared to their controls using the Student's T-Test. Regarding the human saphenous vein experiments, 7ND-treated and -untreated segments of an individual patient were compared using the paired T-Test. P-values less then 0.05 were regarded significant.

Results

Expression of MCP-1 in murine vein grafts and human saphenous vein organ cultures

To demonstrate the expression of MCP-1 in murine vein grafts in time, bypass surgery was performed in APOE*3Leiden mice (mean cholesterol levels: 13.1±1.3mmol/l) and animals were sacrificed at various time points after surgery (6 and 24 hours, 7, 14 and 28 days; n=3 per time point).

In the first days after engraftment, MCP-1 was mainly expressed by the remaining endothelial cells. Furthermore, a massive expression of MCP-1 could be detected in the adhering leukocytes. After 7 days, MCP-1 positive cells were detectable in the developing intimal hyperplasia, co-localizing mainly with AIA positive cells, suggesting that this MCP-1 is predominantly expressed by infiltrating macrophages. After two weeks the expression in the intimal hyperplasia decreased and it was scarcely detected after four weeks (Figure 3.1, Panel A).

In addition, MCP-1 expression was analyzed by immunohistochemistry in human saphenous vein organ cultures. From four human saphenous vein cultures, vessel wall specimens were collected at several time points (directly after excision, after 1, 7 and 28 days in organ culture). Hardly any MCP-1 could be detected in human saphenous vein directly after excision. In the cultured human saphenous veins, increased MCP-1 expression was detectable. In the early time points, it was present mainly in the circular smooth muscle cell layer of the media. Besides expression in the media, profound MCP-1 expression was detectable in the developing intimal hyperplasia from day 14 on. (Figure 3.1, Panel B).

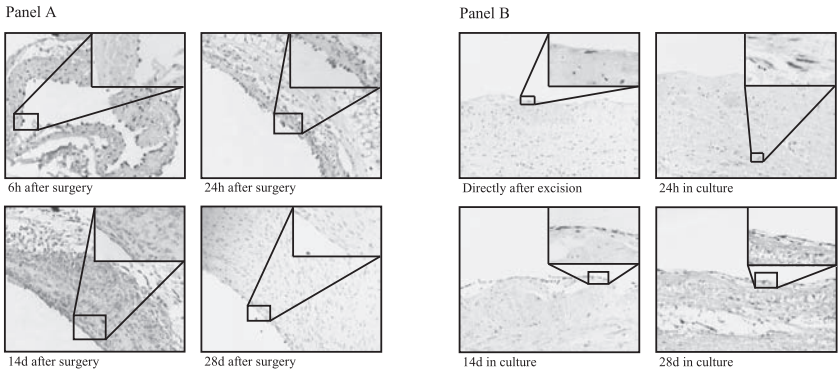

Figure 3.1. Panel A shows representative cross-sections of murine vein grafts harvested after several time points. MCP-1 expression in vein grafts identified by immunohistochemistry was seen mainly in endothelial cells, adhering monocytes and in the infiltrating cells of the developing intimal hyperplasia. Inserts indicate adhering monocytes expressing MCP-1 (6h and 24h) and MCP-1 expression in the developing intimal hyperplasia (14d).
Panel B represents the immunohistochemical detection of MCP-1 in cultured human saphenous veins. MCP-1 is abundantly present in the media at early time points and predominantly in intimal hyperplasia at later time points after 14 and 28 days. Inserts indicate MCP-1 expressing endothelium and smooth muscle cell (directly after excision and 24h) and MCP-1 expression in intimal hyperplasia (28d). Magnification of all pictures 150-600x.

Inhibition of endogenous MCP-1 receptor by 7ND-MCP-1 inhibits vein graft thickening

To study the effect of 7ND-MCP-1 on vein graft thickening, vein graft surgery was performed in APOE*3Leiden mice (n=6 per group). Twelve mice were electroporated one day prior to surgery with either the 7ND-MCP-1 plasmid or the empty plasmid, whereas 6 other were not electroporated. Electroporation of the calf muscle (n=6) with 75 µg of pcDNA3.1-7ND-MCP-1 led to a prolonged expression of 7ND-MCP-1, which was detected in serum. Peak expression (250±79 pg/ml) was reached after 3-7 days and remained high even after 4 weeks (68±21 pg/ml). No 7ND-MCP-1 could be detected in the control (pcDNA3.1-empty) group. Electroporation did not have an effect on the cholesterol levels or body weights of the mice (data not shown).

A significant 51% reduction of vein graft thickening in the 7ND-MCP-1 treated group as compared to the control group and the empty plasmid group (control: 0.63±0.11 mm^2, empty: 0.51±0.05 mm^2, 7ND-MCP-1: 0.31± 0.07 mm^2; p=0.041; Figure 3.2, panel A). Furthermore, the 7ND treated animals showed an increased luminal area when compared to both control groups (control:

0.36±0.06 mm², empty 0.37±0.03 mm², 7ND-MCP-1 0.47±0.06 mm²). However, this difference was not significant (p=0.46).

To study the possible effect of 7ND-MCP-1 on the cellular composition of intimal hyperplasia of the vein grafts immunohistochemical analysis for macrophages and smooth muscle cells was performed. Although vein graft thickening was reduced in the 7ND-MCP-1 treated group, no significant differences were seen in macrophage content in the 7ND-MCP-1 treated vessels (expressed as positive stained area as a percentage of the total area) when compared to the control group (control 22±4%, 7ND-MCP-1 16±4%, p=0.43). Also, no difference was seen in the SM α-actin positive area of the thickened vessel wall (control 39±7%, 7ND-MCP-1 27±9%, p=0.16).

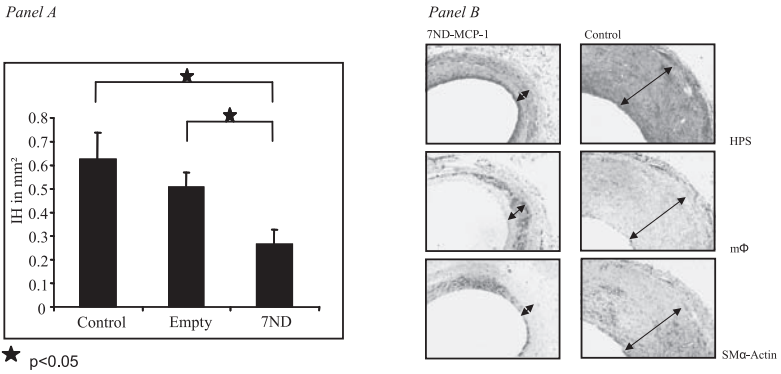

Figure 3.2. Effect of 7ND-MCP-1 gene-transfer on development of intimal hyperplasia in murine vein grafts.

Panel A: Significantly reduced intimal hyperplasia surface is seen in the 7ND-MCP-1-treated group (n=6 per group, p<0.05). Panel B: Immunohistochemical staining for macrophages and smooth muscle cells. No differences in cellular composition of the lesions were observed.

7ND-MCP-1 inhibits neointima formation in human saphenous vein organ cultures

The observation that both smooth muscle cell and macrophage content of the murine lesions was reduced, prompted us to study the effects of 7ND-MCP-1 on the formation of smooth muscle cell-rich lesions in human saphenous vein organ cultures. Segments of human saphenous veins (n=12 per group, from 4 separate patients) were cultured for 4 weeks. Segments exposed to conditioned medium with or without 7ND-MCP-1 (10 ng/ml) were compared.

In all samples a neointima formed within four weeks of culturing. However, quantification revealed reduced neointima formation in human saphenous veins exposed to conditioned medium containing 7ND-MCP-1, as compared to the control counterparts (7ND-MCP-1: 0.42±0.11 mm^2 vs. control: 0.89±0.16 mm^2, p=0.012, Figure 3.3).

Influx of macrophages in the human *ex vivo* model does not occur. Therefore 7ND-MCP-1 most likely may have a direct effect on smooth muscle cells and not via the effect on monocyte chemotaxis. Therefore the effect of 7ND-MCP-1 on proliferation of smooth muscle cells in the human saphenous vein organ cultures was assessed by BrdU staining. In the control vessels 26±2 proliferating cells per microscopic field were detected in the neointima. A significantly lowered number of neointimal proliferating cells was seen in the 7ND-MCP-1 treated vessels (16±2 cell/microscopic field, magnification 100x, p=0.005).

7ND-MCP-1 reduces smooth muscle cell proliferation

Since 7ND-MCP-1 treatment also seemed to have an effect on smooth muscle cell proliferation in the human saphenous vein tissue culture, the direct inhibitory effect of 7ND-MCP-1 on smooth muscle cell proliferation was studied.

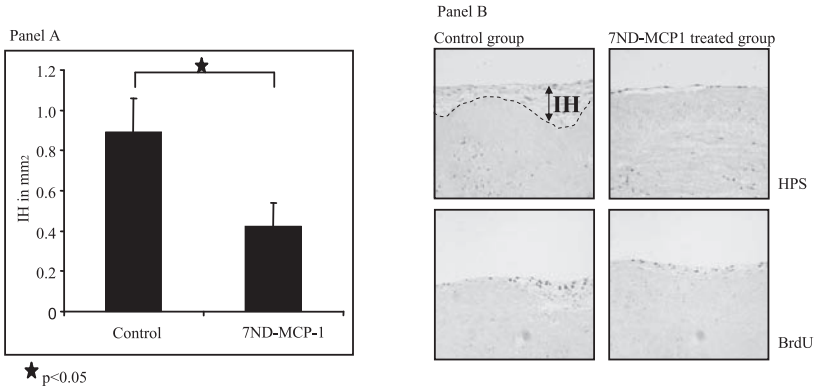

Figure 3.3. Effect of 7ND-MCP-1 on intimal hyperplasia in human saphenous vein (HSV) 28 days in culture.

Panel A: Reduction in intimal hyperplasia (n=12 per group) when exposed to conditioned medium containing 7ND-MCP-1 (p<0.05). Panel B: Representative cross section of human saphenous veins, strong reduction in both intimal hyperplasia surface and BrdU-positive cells can be detected.

First, the presence of the receptor for MCP-1, CCR2, on the human saphenous vein smooth muscle cells was studied by means of mRNA analysis. CCR2 mRNA expression was detectable by PCR in three distinctly stimulated cell cultures (Figure 3.4).

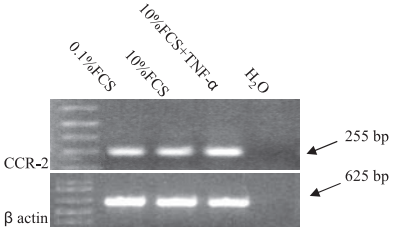

Figure 3.4. RT-PCR of total mRNA of cultured human smooth muscle cell (SMC) for expression of CCR2 mRNA under various culture conditions. Expression is seen under all conditions; however no difference in expression was seen between the various conditions. A: SMC/CCR2, B: SMC/β -actin.

Then, the effect of MCP-1 and 7ND-MCP-1 on smooth muscle cell proliferation was studied in a human venous smooth muscle cell culture. Smooth muscle cell were either transfected with an empty plasmid and/or plasmids encoding for MCP-1 or 7ND-MCP-1.Over-expression of MCP-1 resulted in increased DNA synthesis when compared to mock-transfected smooth muscle cells, as determined by ^{3}H-Thymidine incorporation (Empty $37x10^3 \pm 0.84x10^3$ counts per minute (cpm), MCP-1 $45x10^3 \pm 0.25x10^3$ cpm, $p=0.035$). In addition, when smooth muscle cells over-expressed 7ND-MCP-1, as expected, DNA synthesis was attenuated ($22x10^3 \pm 0.14x10^3$cpm, $P<0.001$), in comparison to mock-transfected cells. When a co-transfection with both MCP-1 and 7ND-MCP-1 plasmids was performed, a similar reduction was observed ($20x10^3 \pm 0.71x10^3$cpm, $p<0.001$, Figure 3.5A).

Next, smooth muscle cells were exposed to either MCP-1 recombinant protein and/or 7ND-MCP-1 protein purified from CHO cells expressing the recombinant 7ND-MCP-1 protein. Exposure to increasing doses of MCP-1 recombinant protein resulted in a dose-dependent increase of DNA synthesis (data not shown).

When smooth muscle cells were exposed to a fixed concentration of MCP-1 (10ng/ml) in combination with increasing concentrations of 7ND-MCP-1, a dose-dependent decrease of DNA synthesis was observed in the ^{3}H-Thymidine assay. The relative reduction in smooth muscle cell proliferation (expressed as percentage of control in which no 7ND-MCP-1 was added (0.3 ng/ml 7ND-MCP-1 added: 76±9%, p=0.07; 1 ng/ml 7ND-MCP-1: 59±3%, p=0.007; 3.3 ng/ml 7ND-MCP-1: 44±7%, p=0.006; 10 ng/ml 7ND-MCP-1: 59±1, p=0.006)) is illustrated in figure 3.5B.

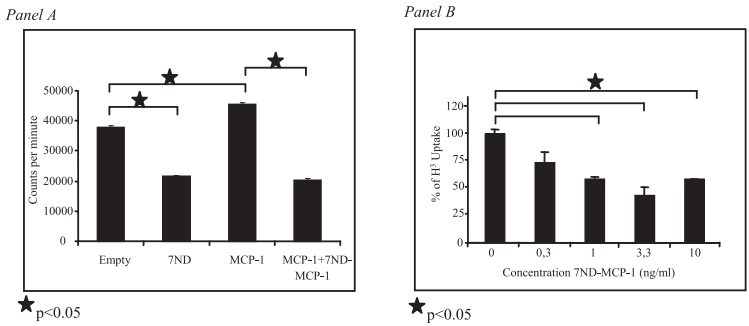

Figure 3.5. Effect of MCP-1 or 7ND-MCP-1 on smooth muscle cell (SMC) proliferation (n=3 per condition).

Panel A: Smooth muscle cell proliferation is increased upon transfection with MCP-1-encoding plasmids. Co-transfection with 7ND-MCP-1-encoding plasmids diminishes this MCP-1 induced proliferation. Transfection with 7ND-MCP-1 alone resulted decreased smooth muscle cell proliferation (for all differences between the groups; $p<0.05$).
Panel B: Dose-dependent inhibition of smooth muscle cell proliferation when cell are exposed to increasing concentrations of 7ND-MCP-1 purified protein (n=3 per condition, smooth muscle cell of all conditions stimulated with a fixed concentration of 10ng/ml recombinant MCP-1).

Discussion

In the present study, the expression and causal involvement of MCP-1 in the development of intimal hyperplasia in a mouse *in vivo* or a human *ex vivo* model of vein graft disease is demonstrated. MCP-1 expression was shown to be present in the murine vein graft and was also detectable in a human saphenous vein organ culture. Blocking the CCR2/MCP-1 pathway, using the receptor antagonist 7ND-MCP-1, resulted in a reduced vein graft thickening in both the murine vein graft and in human saphenous vein segments. Furthermore, we demonstrate that reduced vein graft thickening, besides the effect on monocyte chemotaxis, is caused by a direct anti-proliferative effect of 7ND-MCP-1 on vascular smooth muscle cells.

Vein graft thickening due to development of intimal hyperplasia and accelerated atherosclerosis is the major limitation in the long term survival of patent vein grafts. The mechanism of vein graft thickening development is largely unknown, but it is assumed that it is caused by an inflammatory response to damage of the graft[31,32].

MCP-1 is a well-known pro-inflammatory cytokine and one of the most potent chemoattractant agents for monocytes. Here, we show that MCP-1 is

expressed in vein grafts in an *in vivo* murine model, early after engraftment and expressed mainly by the endothelium and adhering and infiltrating inflammatory cells. Furthermore, in human saphenous vein organ cultures, MCP-1 is predominantly expressed by smooth muscle cells. These data are in line with a report of Stark *et al.*, who showed an enhanced expression of MCP-1 in the healing vein graft which was accompanied by the influx of monocytes[33]. However, this study was performed in normocholesterolemic rats without foam-cell formation in the vein grafts. In the current study, we applied APOE*3Leiden mice on a high-cholesterol diet. These mice have a human-like lipid profile and foam-cell accumulation in the vein grafts does take place. Therefore vein graft morphology in these mice highly resembles what is seen in human vein grafts.

In the processes of spontaneous atherosclerosis and post-angioplasty restenosis, two other disorders characterized by vascular inflammation, the role of MCP-1 is well known. Several clinical studies in humans describe the relation between circulating MCP-1 levels and the risk to develop in-stent restenosis[34,35] and intervention in the MCP-1/CCR2 route results in a reduction of atherosclerosis and post-angioplasty restenosis in several animal models[36-40], Furthermore, in a mouse model of transplantation-induced graft vasculopathy after heterogeneous heart transplantation, 7ND-MCP-1 overexpression significantly reduced accelerated atherosclerosis in the graft tissue[41]. However, the functional role of MCP-1 in the process of vein graft thickening, by intervening in the MCP-1/CCR2 pathway, was never studied. The data provided in this study demonstrate, to our knowledge for the first time, evidence for a pivotal, pro-restenotic role of MCP-1 in vein graft disease. Inhibition of the MCP-1/CCR2 pathway by 7ND-MCP-1 resulted in a significant reduction of vein graft thickening in murine vein grafts.

Since no difference was seen in the cellular composition of treated and untreated grafts, we hypothesized that besides chemotaxis of monocytes also proliferation of smooth muscle cells is diminished by 7ND-MCP-1 exposure. This hypothesis is in line with several reports demonstrating that MCP-1 is a potent mitogenic agent for smooth muscle cells *in vitro*[42,43]. Therefore, the effect of 7ND-MCP-1 was studied in human saphenous vein organ cultures. Intimal hyperplasia in these veins consist mainly of smooth muscle cells and endothelial cells and lacks macrophages[44]. 7ND-MCP-1 reduced intimal hyperplasia in human saphenous vein organ cultures and BrdU staining revealed a significant reduction in proliferating smooth muscle cells in the 7ND-MCP-1 treated vein grafts.

To further asses the direct inhibitory effect of 7ND-MCP-1 on smooth muscle cell proliferation, cultured smooth muscle cells were exposed to MCP-1 and 7ND-MCP-1. MCP-1 exposure resulted in an increased proliferation of smooth muscle cell. Oppositely, smooth muscle cell proliferation was inhibited by exposure to 7ND-MCP-1. These data prove that, indeed, 7ND-MCP-1 directly inhibits smooth muscle cell proliferation, in addition to its known effects on other (inflammatory) cell types present in the vascular lesion.

In conclusion, the present study establishes the important role of the MCP-1/CCR2 pathway in the development of vein graft thickening. Blocking this route (e.g. by 7ND-MCP-1) may be an interesting potential target for therapy in order to overcome the problems of vein graft failure in patients.

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