Dominant-negative mutation of monocyte chemoattractant protein-1 prevents vulnerable plaques from rupture in rabbits independent of serum lipid levels

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Abstract

Active inflammation is an important feature of vulnerable plaques, and monocyte chemoattractant protein-1 (MCP-1) is a key chemokine that promotes monocyte–endothelium binding and initiates inflammation. We aimed to determine whether dominant-negative mutation of MCP-1 could reverse atherosclerotic lesion progression and prevent vulnerable plaques from rupture regardless of serum lipid levels. The mutant MCP-1 was produced by deletion of the N-terminal amino acids 2 to 8 (7ND), and a eukaryotic expression vector pIRES-EGFP-7ND was constructed. The transwell chamber was used to assay chemotaxis of monocytes *in vitro*. Thirty New Zealand white rabbits underwent balloon-induced abdominal aortic endothelial injury and were randomly divided into control group without gene intervention (group A, $n = 10$), pIRES-EGFP-7ND treatment group (group B, $n = 10$) and empty vector treatment group (group C, $n = 10$). All rabbits were fed a diet of 1% cholesterol for 8 weeks, and then group A rabbits were killed, whereas groups B and C rabbits received an intramuscular injection of pIRES-EGFP-7ND and an empty lipofectamine, respectively, and remained on a high cholesterol diet for 4 weeks. At the end of week 12, groups B and C rabbits underwent pharmacological triggering by injection with Chinese Russell's viper venom and histamine. Serum lipids and inflammatory markers were measured, and high-frequency ultrasonography and intravascular ultrasound imaging were performed. Immunohistochemistry and RT-PCR were used to examine expression of inflammatory markers in the plaques. *In vitro* transfection of pIRES-EGFP-7ND resulted in a significant inhibition of monocyte chemotaxis ($P < 0.05$) and *in vivo* transfection of pIRES-EGFP-7ND significantly increased the thickness of the fibrous caps and decreased plaque vulnerability index. The incidence of plaque rupture in group B was 0% as compared with 56% in the empty vector treatment group ($P < 0.05$). The serum levels and expression of inflammatory markers were significantly reduced in group B. In conclusion, pIRES-EGFP-7ND transfection effectively inhibits plaque inflammation, reverses plaque progression and prevents vulnerable plaques from rupture. These therapeutic effects are independent of serum lipid levels and demonstrate that inhibition of plaque inflammation alone without lipid lowering can stabilize vulnerable plaques.

Keywords: vulnerable plaque • inflammation • monocyte chemoattractant protein • gene therapy

Introduction

Atherosclerosis is a complex and perpetuating inflammatory disease involving the aorta and its major branches, and recent studies have demonstrated that active inflammation is an important feature of vulnerable plaques. Accumulation of monocytes/macrophages

has been noted in the intima of early atherosclerotic lesions, and these inflammatory cells may promote progression of atherosclerosis by producing and releasing various cytokines, chemokines and growth factors. Among these mediators, monocyte chemoattractant protein-1 (MCP-1) is a key chemokine that activates monocytes/macrophages by promoting leucocyte–endothelium binding and migration to sites of inflammation [1]. Although a number of cytokines have been found to participate in the inflammatory process of plaque vulnerability, MCP-1 as an upstream chemokine in the atherosclerotic inflammatory pathway has been consistently reported to have high expression in vulnerable plaques, and the

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intensive infiltration of macrophages in plaques with active inflammation could be, to a large extent, due to the chemotactic effects of MCP-1. Therefore, targeting MCP-1 may be an effective approach to the inhibition of plaque inflammation and stabilization of vulnerable plaques regardless of serum lipid levels.

Jarnagin *et al.* first demonstrated that dominant-negative mutation of MCP-1 derived by deletion of the N-terminal amino acids 2 to 8 of the human MCP-1 (7ND) resulted in a deficiency in chemotaxis but retained affinity to the CCR-2 receptor [2]. Subsequent studies confirmed that gene transfer of the MCP-1 mutant significantly inhibited monocyte activation/infiltration and reduced the severity of restenosis in rabbits after arterial balloon injury or stent placement [3]. However, whether dominant-negative mutation of MCP-1 can actually reduce plaque vulnerability and disruption is still unknown. In the present study, we hypothesized that the MCP-1 mutant is effective in attenuating plaque inflammation, reversing plaque progression and preventing plaques from rupture regardless of serum lipid levels. We constructed a eukaryotic expression vector of green fluorescence protein (GFP)-fused human MCP-1 mutant (pIRES-EGFP-7ND), which was transfected into human embryonic kidney (HEK) 293 cells to examine the effects on monocyte chemotaxis *in vitro* and then into rabbits with vulnerable plaques to investigate changes in circulating biomarkers and plaque morphology *in vivo*.

Materials and methods

Plasmid expression vectors

Recombinant PCR was used to construct human 7ND cDNA, with a wild-type MCP-1 cDNA used as a template and N-terminal amino acids 2 to 8 of the human MCP-1 deleted. By use of gene cloning techniques, the MCP-1 mutant was inserted into the Xho I and Mlu I sites of the pIRES-EGFP expression vector plasmid to form a eukaryotic expression vector of GFP-fused human MCP-1 mutant (pIRES-EGFP-7ND) whose gene expression is controlled by the immediate early enhancer/promoter of the cytomegalovirus. Human MCP-1 cDNA was a generous gift from Dr. Yoshimura (National Cancer Institute, Frederick, MD, USA).

In vitro transfection

The vector pIRES-EGFP-7ND was transfected into HEK 293 cells by lipofectamine mediation. Forty-eight hours after transfection, the 7ND expression in the transfected cells was examined by fluorescence microscopy and ELISA and the mRNA expression shown by RT-PCR.

Chemotaxis test

Monocytes were extracted by centrifugation of a blood sample from a normal rabbit, and 0.1 ml monocyte suspension (4×10^4 /ml) was instilled into the transwell chamber to assay monocyte chemotaxis. Monocytes

were stimulated by Hank's fluid (Hank's fluid group), cell supernatant fluid after pIRES-EGFP-7ND transfection (7ND group) and activated rabbit serum containing a potent chemotactic factor complement C5a (C5a group) as described previously [4]. Then, the number of migrated monocytes and the distance travelled by monocytes in 10 high-power microscopic fields were measured and the values averaged.

Animal model

Thirty male New Zealand white rabbits weighing 2.0 to 3.0 kg underwent balloon-induced abdominal aortic endothelial injury and were randomly divided into control group (group A, $n = 10$), pIRES-EGFP-7ND treatment group (group B, $n = 10$) and an empty-vector treatment group (group C, $n = 10$). All rabbits were fed a diet of 1% cholesterol for 8 weeks, then group A rabbits were killed, and group B and C rabbits underwent injection into the right hind limb with previously reported plasmid doses at the end of week 8 [5]. On day 3 after transfection, one rabbit in group B was killed to observe the expression of 7ND in injection sites under a fluorescence microscope. The expression of 7ND in the rabbits serum was monitored by ELISA before and at days 1, 3, 7, 14 and 28 after transfection. Four weeks after transfection, all rabbits underwent pharmacological triggering as described [6]. In brief, rabbits were injected intraperitoneally with 0.15 mg/kg Chinese Russell's viper venom, and then 30 min. later, with 0.02 mg/kg histamine (Sigma, St. Louis, MO, USA). High-frequency ultrasonography and intravascular ultrasound (IVUS) imaging were performed before and after pharmacological triggering to study morphological changes of the abdominal aortic plaques. Rabbits were euthanized 24 hrs later for pathological studies. All animal care and experimental protocols complied with the Animal Management Rule of the Ministry of Public Health, People's Republic of China (documentation 55, 2001) and the Animal Care Committee of Shandong University and were approved by the Animal Care Committee of Shandong University.

Biochemical studies

Blood samples were collected from all rabbits at the beginning of the experiment and before rabbits were killed. Serum levels of total cholesterol (TC), triglycerides (TGs), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were measured by enzymatic assays. Serum levels of high-sensitive C-reactive protein (hs-CRP), interleukin-8 (IL-8), IL-18, matrix metalloproteinase-1 (MMP-1) and P-selectin were assayed by use of a highly sensitive ELISA kit (R&D Laboratory Research, Chicago, IL, USA). A human MCP-1 ELISA kit (R&D Laboratory Research) was used to measure the plasma concentration of 7ND released from the skeletal muscles after pIRES-EGFP-7ND transfection, and a rabbit MCP-1 ELISA kit (R&D Laboratory Research) was used to measure the plasma concentration of MCP-1 in rabbits.

Ultrasonographic studies

High-frequency ultrasonography

A high-frequency duplex ultrasonographic system (HP SONOS 5500, Andover, MA, USA) and a 7.5-MHz transducer were used to detect atherosclerotic plaques in the abdominal aorta before and after pharmacological triggering. After the aortic longitudinal and transversal axis views were scanned, the aortic diameter at end-diastole (Dd) and the maximal

intima-media thickness (IMT) were measured by two-dimensional echocardiography, and the aortic peak velocity (Vp), mean velocity (Vm) and velocity-time integral (VTI) were recorded by pulsed Doppler technique.

Integrated backscatter analysis

Ultrasonic integrated backscatters from the aortic wall and the atherosclerotic plaques were analyzed by acoustic densitometry technique. The mean ultrasonic intensity (All) of the aortic intima and adventitia in normal segments and in the atherosclerotic plaques were measured, and the corrected All (Allc%) was derived by calculating the ratio of All of the intima to All of the adventitia in both normal segments and plaques.

Intravascular ultrasound studies

IVUS studies were performed before and after pharmacological triggering by use of a 3.2F catheter containing a single rotating element transducer of 40 MHz connected to an IVUS system (Galaxy, Boston Scientific Corporation, Fremont, CA, USA). The catheter was withdrawn from the aortic arch to the abdominal aorta by use of a motorized pullback device at a constant speed of 0.5 mm/sec. The following parameters were measured from the abdominal aortic cross-sectional images: external elastic membrane area (EEMA), lumen area (LA), plaque area (PA = EEMA-LA) and plaque burden (PB% = PA/EEMA $\times$ 100%). The IVUS images were reviewed by two independent observers, and values were averaged for data analysis.

Histopathology and immunohistochemistry analysis

Rabbits were euthanized by overdose of intravenous pentobarbital. The abdominal aorta was dissected and excised to observe the occurrence of plaque rupture and thrombosis. Tissue samples 2-cm long were taken from the abdominal aorta of the three groups of rabbits. Abdominal aortic segments were fixed in 4% formaldehyde, and some segments were embedded in paraffin and cut into 5- μ m-thick sections for staining with haematoxylin and eosin or picosirius red or for immunohistochemical staining. Cryosections were cut into 6- μ m-thick sections for Oil-red O staining. Some segments were fixed in 3% glutaral and post-fixed with 1% osmium tetroxide, and the intimal surface of the abdominal aorta was examined by scanning electronic microscopy (TEM-100, Electron Co., Tokyo, Japan) under 15-kV accelerating voltage. For ultrastructural examination, samples were fixed with 0.2 mol/l glutaraldehyde and osmium tetroxide and embedded into epon by standard procedures. Ultra-thin sections were cut and doubly stained with uranyl acetate and lead citrate. The ultrastructure of the smooth muscle cells (SMCs) was observed on transmission electron microscopy (JEM-1200EX, Electron Co.).

The primary antibodies included monoclonal antibodies against rabbit macrophages (RAM11, Lab Vision Neomakers, Fremont, CA, USA) diluted to 1:400 to identify macrophages; SMC actins (Boshide, Wuhan, China); and mouse anti-MMP-1, MMP-3, MMP-12, P-selectin and MCP-1 monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100 to detect MMP-1, MMP-3, MMP-12, P-selectin and MCP-1, respectively.

Histopathological slides were analyzed by use of a computer-assisted morphometric analysis system (Image-Pro Plus 5.0, Media Cybernetics,

Cambridge, MA, USA). The fibrous cap thickness and IMT were measured at 10 equidistant points around the cap in each slice; three slices per section were measured and values were averaged, and the ratio of fibrous cap thickness to IMT was calculated. The area of positive staining of lipids, collagen, iron and macrophages was expressed as a percentage of the staining area divided by the plaque area in at least 10 high-power fields ($\times 400$). The vulnerability index was (macrophage staining% + lipid staining%) / (SMC% + collagen fibre%) [7]. Plaque rupture was defined on histopathological observation as fibrous cap disruption with luminal thrombosis or buried fibrous cap within a plaque [8].

Molecular biological studies

Tissue samples were frozen with use of liquid nitrogen. Total RNA was extracted, and mRNA expression of MCP-1, MMP-1, MMP-3, MMP-12 and P-selectin in plaques was examined by quantitative real-time PCR with use of LightCycler (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's instructions. The mRNA sequences were obtained from GenBank (Bethesda, MD, USA). Quantitative values were obtained from the threshold cycle value (Ct), the point at which a significant increase of fluorescence is first detected. The transcript number of glyceraldehyde 3-phosphate dehydrogenase was quantified as an internal control. Experiments were performed in triplicate for each data point. Quantitative values were obtained from the Ct, the point at which a significant increase of fluorescence is first detected [9]. The results of RT-PCR were confirmed by gel electrophoresis.

Statistical analysis

We carried out all statistical analyses with SPSS, v11.0 (SPSS Inc, Chicago, IL, USA). Quantitative variables are expressed as means $\pm$ SD. Variables with normal distributions were compared by independent Student's t-test for between group differences; variables with skewed distribution were log transformed before the t-test. We used paired t-test to compare data within animals at different time points. Chi-squared analysis was used to compare categorical data. Two-tailed $P < 0.05$ was considered statistically significant.

Results

Plasmid expression vectors and *in vitro* transfection

The N-terminal amino acids 2 to 8 of the human MCP-1 were successfully deleted by recombinant PCR technique, and DNA sequencing confirmed the deletion of N-terminal 126-146 bases in the human MCP-1 cDNA. The recombinant eukaryotic expression vector pIRES-EGFP-7ND was successfully constructed by gene cloning technique and transfected into the HEK 293 cells. Forty-eight hours after transfection, fluorescence microscopy revealed HEK 293 cells with green fluorescence, with the transfection efficiency estimated at 80%. The mRNA and protein expression of 7ND in transfected HEK 293 cells

was detected by RT-PCR and ELISA technique, respectively, with the protein expression of 7ND measured as 50 ± 12 pg/ml.

Chemotaxis test

The mean number of migrating monocytes measured in 10 high-power microscope fields in the Hank's fluid group, 7ND group and C5a group was 7 ± 4 , 12 ± 9 and 50 ± 15 , respectively, with a significant difference between the C5a and 7ND groups ($P < 0.05$) and no difference between the Hank's fluid and 7ND groups. The mean distance travelled by monocytes was 61.92 ± 12.3 , 65.9 ± 10.6 and 104.3 ± 12.5 μm , respectively, with the mean distance for both the Hank's fluid and 7ND groups significantly lower than that for the C5a group ($P < 0.05$) and no difference between the Hank's fluid and 7ND groups.

Serum lipid profile and inflammatory markers

Only one rabbit in group C died of excessive anaesthesia during the experiment. Compared with group A rabbits, group C rabbits showed a lower level of HDL-C, whereas groups B and C rabbits showed higher levels of LDL-C (all $P < 0.05$) before being killed (Table 1). Groups B and C rabbits did not differ in TC, TGs, HDL-C and LDL-C. The serum levels of hs-CRP, IL-8, IL-18, MMP-1 and P-selectin in group C on day 28 after gene transfection were significantly higher than those in groups A and B before euthanasia (all $P < 0.05$) (Table 2). Likewise, compared with group A, group B showed lower levels of hs-CRP and IL-18 before being killed (all $P < 0.05$, Table 2).

Expression levels of 7ND and MCP-1 in rabbits

In group B, green fluorescence protein expression was detected in the skeletal muscles at the end of day 3 after transfection, for an estimated transfection efficiency of 70%, and were still visible at the end of day 28 after transfection (Table 3). The plasma level of human MCP-1 in group B showed a prominent surge one day after transfection of pIRES-EGFP-7ND, reached a peak of more than 300 times higher than the baseline level on day 3, remained high on days 7 and 14 and gradually declined until day 28, when only a low level of expression was detectable. In contrast, in group C, the plasma level of human MCP-1 was not detected throughout this experiment and that of MCP-1 showed little variation throughout this experiment and was similar in groups B and C.

High-frequency ultrasonography

The IMT in group B was remarkably lower than that in groups A and C (all $P < 0.05$), and the AIIc% in group B was significantly higher than that in group C ($P < 0.05$) (Table 4). However, the three groups did not differ in Dd, Vp, Vm and VTI.

Intravascular ultrasonography

The values of EEMA, PA and PB% in group B were significantly lower than those in groups A and C (all $P < 0.05$, Table 5), with no difference among the groups in LA.

Histopathology

Compared with groups A (102 ± 52 μm) and C (160 ± 40 μm), group B showed a significantly thicker fibrous cap of aortic plaques (221 ± 20 μm ; all $P < 0.05$), with a remarkably thinner aortic IMT (590 ± 192 μm) than in groups A (980 ± 328 μm) and C (890 ± 321 μm ; all $P < 0.05$). Consequently, the ratio of fibrous cap thickness to IMT was significantly larger in group B (0.38 ± 0.10) than in groups A (0.16 ± 0.14) and C (0.18 ± 0.12 ; all $P < 0.05$). After pharmacological triggering, only rabbits in group C showed abdominal aortic plaque rupture (5/9, 56%) and there was a significant difference between the two groups in terms of the occurrence of plaque disruption ($P < 0.05$).

Electronic microscopy

Scanning electronic microscopy depicted few monocytes in the abdominal aortic section in group B and dense and mature monocytes with prominent pericellular pseudopods in groups A and C (Fig. 1). Transmission microscopy demonstrated an intact nucleus of SMCs in group B and a highly irregular nucleus with condensed heterochromosomes around an incomplete nucleus membrane in groups A and C (Fig. 1).

Immunohistochemistry

The positive staining area for α -actin was significantly higher in group B than in groups A and C ($12.46\% \pm 3.54\%$ versus $6.4\% \pm 2.17\%$ and $6.92\% \pm 4.31\%$, respectively, all $P < 0.05$), as was the positive staining with sirius red ($14.23\% \pm 4.56\%$ versus $10.52 \pm 6.45\%$ and $9.23\% \pm 4.44\%$, respectively, all $P < 0.05$). On the other hand, the positive staining area for RAM11 was significantly lower in group B ($6.23\% \pm 3.80\%$) than in groups A ($25.41 \pm 12.90\%$) and C ($25.68\% \pm 9.80\%$) (all $P < 0.05$), as was the positive staining area with Oil-red O ($10.52\% \pm 7.14\%$ versus $17.87\% \pm 8.31\%$ and $15.87\% \pm 12.56\%$, respectively, all $P < 0.05$). As a result, the vulnerability index for group B was significantly lower than that for groups A and C ($0.63\% \pm 0.12\%$ versus $3.13\% \pm 1.81\%$ and $2.29\% \pm 0.51\%$, respectively, all $P < 0.05$). The percentage of positive stained cells for MCP-1, MMP-1, MMP-3, MMP-12 and P-selectin in group B was remarkably reduced as compared with that in group C (all $P < 0.05$, Table 6, Fig. 2) and the expression of MCP-1, MMP-1 and P-selectin in group B was significantly lower than that in group A (all $P < 0.05$, Table 6).

Table 1 Serum lipid profile in rabbits before euthanasia

Groups	TC (mmol/l)	TGs (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)
Group A (<i>n</i> = 10)	20.23 ± 6.03	2.03 ± 1.56	1.28 ± 0.15	17.45 ± 3.5
Group B (<i>n</i> = 9)	27.27 ± 7.52	2.98 ± 1.31	1.12 ± 0.25	22.80 ± 5.12*
Group C (<i>n</i> = 9)	26.54 ± 7.05	3.03 ± 0.87	1.03 ± 0.11*	24.13 ± 4.90*

**P* < 0.05, versus group A. Group A, control group without treatment; group B, pIRES-EGFP-7ND treatment and group C, empty-vector treatment. TC, total cholesterol; TGs, triglyceride; HDL-C, high-density lipoprotein and LDL-C, low-density lipoprotein.

Table 2 Serum inflammatory markers in rabbits before euthanasia

Groups	hs-CRP (ng/ml)	IL-8 (pg/ml)	IL-18 (pg/ml)	MMP-1 (ng/ml)	<i>P</i> -selectin (ng/ml)
Group A (<i>n</i> = 10)	80.7 ± 21.2	7.9 ± 3.2	70.3 ± 12.7	24.8 ± 12.7	15.3 ± 2.7
Group B (<i>n</i> = 9)	37.7 ± 10.6*	4.9 ± 3.8	28.5 ± 12.3*	24.1 ± 9.1	12.7 ± 4.8
Group C (<i>n</i> = 9)	120.3 ± 24.8*†	12.8 ± 4.9†	92.5 ± 24.9†	58.7 ± 18.2†	20.5 ± 5.4†

**P* < 0.05 versus group A and †*P* < 0.05 versus group B.

Group A, control group without treatment; group B, pIRES-EGFP-7ND treatment and group C, empty-vector treatment.

hs-CRP, high sensitive C-reactive protein; IL-8, interleukin-8; IL-18, interleukin-18 and MMP-1, matrix metalloproteinase-1.

Table 3 Serum concentrations of human MCP-1 (HuM) and rabbit MCP-1 (RM) in groups B and C before and after gene transfection (pg/ml)

Groups	Baseline		Day 1		Day 3		Day 7		Day 14		Day 28	
	HuM	RM	HuM	RM	HuM	RM	HuM	RM	HuM	RM	HuM	RM
Group B (<i>n</i> = 9)	<20	39 ± 8	250 ± 22	47 ± 4	326 ± 18	46 ± 5	298 ± 26	46 ± 8	204 ± 17	42 ± 7	60 ± 13	48 ± 5
Group C (<i>n</i> = 9)	0	44 ± 3	0	49 ± 4	0	50 ± 6	0	45 ± 7	0	45 ± 8	0	52 ± 7

Group B, pIRES-EGFP-7ND treatment and group C, empty-vector treatment group.

Table 4 Ultrasonographic measurements in rabbits before euthanasia

Groups	IMT (mm)	Dd (mm)	Vp (cm/sec.)	Vm (cm/sec.)	VTI (cm/sec.)	AIIC(%)
Group A (<i>n</i> = 10)	1.03 ± 0.21	3.54 ± 0.20	86.43 ± 12.42	61.2 ± 12.01	8.02 ± 2.42	78.9 ± 11.34
Group B (<i>n</i> = 9)	0.79 ± 0.12*	3.62 ± 0.21	89.2 ± 21.30	65.10 ± 9.23	8.67 ± 2.39	77.0 ± 10.22
Group C (<i>n</i> = 9)	1.32 ± 0.26*†	3.60 ± 0.28	93.01 ± 25.68	68.91 ± 11.37	9.10 ± 4.22	53.6 ± 12.52†

**P* < 0.05 versus group A and †*P* < 0.05 versus group B.

Group A, control group without treatment; group B, pIRES-EGFP-7ND treatment and group C, empty-vector treatment.

IMT, intima-media thickness; Dd, end-diastolic diameters; Vp, peak velocity; Vm, mean velocity; VTI, velocity-time integral and AIIC(%), corrected averaged ultrasonic intensity (AII).

Table 5 Intravascular ultrasound measurements in rabbits before euthanasia

Groups	LA (mm ²)	EEMA (mm ²)	PA (mm ²)	PB (%)
Group A (n = 10)	6.92 ± 4.87	12.49 ± 1.54	5.57 ± 1.2	44.60 ± 1.11
Group B (n = 9)	6.12 ± 1.68	8.11 ± 1.05*	1.99 ± 0.35*	24.53 ± 1.51*
Group C (n = 9)	7.2 ± 1.98	14.07 ± 3.62†	6.87 ± 2.21†	48.83 ± 2.42††

* $P < 0.05$ versus group A and † $P < 0.05$ versus group B.

Group A, control group without treatment; group B, pIRES-EGFP-7ND treatment and group C, empty-vector treatment.

LA, lumen area; EEMA, external elastic membrane area; PA, plaque area and PB: plaque burden.

Fig. 1 Electronic microscopic images. Scanning electronic microscopy shows few monocytes cells in abdominal aortic section in group B rabbits ($\times 1500$) (A) and plentiful mature monocytes with prominent pericellular pseudopods in group C ($\times 1500$) (B). Transmission electronic microscopy shows an intact albeit slightly injured nucleus in smooth muscle cells in group B ($\times 5000$) (C) and a highly irregular nucleus with condensed heterochromosomes around incomplete nucleus membrane in group C ($\times 5000$) (D).

RT-PCR analysis

The mRNA expression of MCP-1, MMP-1, MMP-3, MMP-12 and P-selectin in the aortic plaques in group B was significantly lower than that in group C (all $P < 0.05$, Table 7), and the expression of MCP-1, MMP-3 and P-selectin in group B was significantly lower than that in group A (all $P < 0.05$, Table 7).

Discussion

The major finding of the present study is that dominant-negative mutation of MCP-1 blocked the signalling pathway of MCP-1 and resulted in a remarkable inhibition of monocyte chemotaxis *in vitro* and a significant reduction of plaque inflammation and vulnerability *in vivo* in rabbits. These molecular and cellular effects translated into a successful prevention of plaque disruption, even in the presence of endothelial injury, hyperlipidaemia and pharmacological triggering. To the best of our

knowledge, this is the first study to show that directly targeting MCP-1 can prevent plaques from progressing to the point where they can be induced to rupture by external triggering.

MCP-1, as a single peptide chain of 76 amino acids, is a major chemotactic molecule in the vascular wall excreted by SMCs, macrophages and endothelial cells [10, 11]. Once bound to its receptor, CCR-2, a member of the G-protein coupling receptor family, MCP-1 can activate the intracellular mitogen-activated protein kinase signalling pathway leading to chemotactic effects and production of a number of cytokines. The key role of MCP-1 in the atherosclerotic process has been firmly established in many studies. Aiello *et al.* reported that MCP-1 accelerated atherosclerosis in apolipoprotein E-deficient mice and was extensively expressed in macrophages in atherosclerotic plaques [12]. Gu *et al.* found that LDL receptor/MCP-1-deficient mice given a cholesterol-rich diet displayed 83% less lipid deposition in the aorta than did LDL receptor-deficient mice with the wild-type MCP-1 allele on the same diet. The MCP-1-deficient mice also had less prominent aortic accumulation of macrophages, which reflects the relative paucity of MCP-1-derived monocyte chemoattraction [13]. Gosling *et al.*

Fig. 2 Histopathological assays. Immunohistological staining of the abdominal aortic cross-section showing dense positive α -smooth muscle cells in group B (A) and sparse positive α -smooth muscle cells in group C (B). Sirius-red staining shows abundant collagen in group B (C) and less collagen in group C (D). Immunohistological staining shows few RAM11-positive cells in group B (E) and ample RAM11-positive cells in group C (F). Oil-red O staining shows a low level of lipids in group B (G) and a high level in group C (H). Masson trichrome staining of the abdominal aortic cross-section in group C rabbits shows a huge thrombus arising from a ruptured plaque (I). Immunohistological staining shows little staining for MCP-1, MMP-1, MMP-3, MMP-12 and P-selectin in group B (J, L, N, P, R, respectively) and dense staining in group C (K, M, O, Q, S, respectively). (Bars = 30 μ m, except for Fig. 2C and D, where bars = 100 μ m)

Table 6 Expression of inflammatory markers by immunohistochemistry in rabbits (%)

Groups	MCP-1	MMP-1	MMP-3	MMP-12	P-selectin
Group A (n = 10)	16.87 ± 7.93	17.21 ± 3.23	13.20 ± 10.20	8.98 ± 6.01	11.43 ± 2.30
Group B (n = 9)	8.24 ± 4.58*	12.09 ± 4.72*	9.23 ± 8.93	7.23 ± 2.48	7.05 ± 2.06*
Group C (n = 9)	24.60 ± 8.93 [†]	23.20 ± 5.98 [†]	18.21 ± 10.10 [†]	13.04 ± 5.68 [†]	13.05 ± 6.21 [†]

**P* < 0.05 versus group A and [†]*P* < 0.05 versus group B.

Group A, control group without treatment; group B, pIRES-EGFP-7ND treatment and group C, empty-vector treatment.

MCP-1, monocyte chemoattractant protein-1; MMP-1, matrix metalloproteinase-1; MMP-3, matrix metalloproteinase-3 and MMP-12, matrix metalloproteinase-12.

Table 7 mRNA expression of inflammatory markers in rabbits (%)

Groups	MCP-1	MMP-1	MMP-3	MMP-12	P-selectin
Group A (n = 10)	48.27 ± 10.24	12.78 ± 9.56	19.08 ± 8.38	13.80 ± 9.02	20.32 ± 6.28
Group B (n = 9)	21.30 ± 16.04*	16.33 ± 9.45	10.75 ± 6.23*	8.22 ± 6.24	11.93 ± 5.67*
Group C (n = 9)	53.86 ± 7.23 [†]	38.43 ± 11.00 [†]	22.45 ± 9.85 [†]	16.59 ± 9.46 [†]	22.34 ± 5.78 [†]

**P* < 0.05 versus group A and [†]*P* < 0.05 versus group B.

Group A, control group without treatment; group B, pIRES-EGFP-7ND treatment and group C, empty-vector treatment.

MCP-1, monocyte chemoattractant protein-1; MMP-1, matrix metalloproteinase-1; MMP-3, matrix metalloproteinase-3 and MMP-12, matrix metalloproteinase-12.

found that the absence of MCP-1 appeared to confer substantial protection against macrophage recruitment and atherosclerotic lesion formation without affecting lipoprotein metabolism in MCP-1-deficient mice [14]. Schechter *et al.* also found that MCP-1 can induce coagulation, which may enhance thrombosis in atherosclerotic plaques [15]. For these reasons, MCP-1 has emerged as an important target in the gene therapy of atherosclerosis.

MCP-1 has two major functional domains – chemotactic and receptor binding. Jarnagin *et al.* first demonstrated that deletion of the N-terminal amino acids 2 to 8 of the human MCP-1 resulted in a deficient chemotactic effect but unchanged receptor affinity [2], which allowed for the use of 7ND as a therapeutic tool. Subsequent studies confirmed that blockade of MCP-1 attenuated neointima formation after balloon injury or stenting and initiation and progression of *de novo* atherosclerotic lesions [3, 16]. Recently, a Japanese group reported that 7ND gene transfer to apoE^{-/-} mice significantly inhibited monocyte infiltration and activation, reduced the severity of atherosclerotic lesions and increased the contents of collagen and elastin in plaques [17, 18]. However, whether the MCP-1 mutant treatment can actually reverse atherosclerotic lesion progression and prevent vulnerable plaques from rupture has never been proved. In the present study, we successfully used recombinant PCR and gene cloning to delete N-terminal amino acids 2 to 8 of the human MCP-1 and construct the recombinant eukaryotic expression vector pIRES-EGFP-7ND.

Our *in vitro* study further demonstrated that pIRES-EGFP-7ND can be transfected into HEK 293 cells with high transfection efficiency and that 7ND significantly reduced the number of migrating monocytes and the distance travelled by monocytes, which indicates that dominant-negative mutation of human MCP-1 resulted in loss of its chemotactic effects.

In the present study, 1 day after intramuscular injection of pIRES-EGFP-7ND in rabbits, the 7ND proteins secreted by the transfected cells were released into the circulatory blood and peaked at a level more than 300 times higher than the baseline level on day 3, remained at a high level for 2 weeks and gradually decreased to a low level up to day 28. Green fluorescence protein expression in the skeletal muscles showed similar dynamic changes. These results indicated that one intramuscular injected dose of pIRES-EGFP-7ND can produce effects for as long as 4 weeks. Such remarkable changes in plasma concentration of human MCP-1, however, were not repeated in the plasma levels of MCP-1 in rabbits, which remained stable, although the mutant 7ND would compete with the rabbits' MCP-1 in combining with the CCR-2 receptors.

An intriguing finding in this study was that 4 weeks after pIRES-EGFP-7ND transfection, even in the presence of continued high-cholesterol diet, plaque inflammation, burden and vulnerability in group B rabbits were greatly attenuated as compared with those in groups A and C, which suggests that pIRES-EGFP-7ND treatment not only halted the progression of atherosclerotic

lesions but also reversed the pathological process. The exact mechanisms underlying this finding is unclear but may be related to the marked inhibition of monocyte–endothelium binding, proliferation and migration of SMCs and oxidative stress in mature plaques.

Consistent with the hypothesis driving this study, transfection of pIRES-EGFP-7ND into group B rabbits greatly protected against plaque burden, inflammation and disruption, as shown by the following evidence: first, after 4-week transfection, ultrasonographic measurements of IMT, EEMA, PA and PB% were greatly decreased and those of AIIc% increased; pathologically, the fibrous cap became thicker and the IMT thinner; second, serum levels of hs-CRP, IL-8, IL-18, MMP-1 and P-selectin 4 weeks after gene transfection were significantly reduced and the vulnerability index as well as positive staining for MCP-1, MMP-1, MMP-3, MMP-12 and P-selectin was significantly lowered; fourth, the mRNA expression of MCP-1, MMP-1, MM-3, MMP-12 and P-selectin in the plaques was consistently decreased; and finally, no rabbits in group B developed plaque disruption, which indicates successful prevention of rupture. A notable finding in this study is that transfection with pIRES-EGFP-7ND did not affect serum lipid level and only moderately reduced lipid contents in plaques. Nonetheless, this gene therapy induced an extraordinary anti-inflammatory effect, which led to stabilization of plaques. These results are in contrast to the situation with statin therapy, in which the major mechanism of plaque stabilization is due to the lipid-lowering effects by statins [19], and lends support to the fascinating concept that inhibition of plaque inflammation alone without lipid lowering can stabilize vulnerable plaques [20].

Our study contains several limitations. First, the sample size in each of the three animal groups was small, and further studies involving a larger animal sample and other types of animals such as apoE^{−/−} mice are warranted to confirm our results. Second, IVUS was used in this study to measure the extent of vessel remodelling

and plaque formation *in vivo* and endothelial injury, and luminal thrombosis may have been produced by IVUS catheter manipulation. Such a possibility, however, is trivial because the IVUS catheter is much smaller than the abdominal aortic lumen, and no luminal thrombosis occurred in groups A and B rabbits. Third, the incidence of plaque rupture after pharmacological triggering in group C rabbits was relatively low because of the simplicity of our methods in inducing plaque vulnerability, which may explain in part the absence of plaque rupture in group B rabbits. Therefore, the therapeutic effects of dominant-negative mutation of MCP-1 in animals with a high incidence of plaque disruption require further investigation. Finally, other gene interventions against MCP-1, such as mRNA interference, possibly effective in alleviating the chemotactic effects of MCP-1, as well as the relative efficacy of MCP-1 mRNA interference and 7ND in preventing vulnerable plaques from rupture, remain to be clarified. In conclusion, pIRES-EGFP-7ND transfection provides a novel and effective approach to the inhibition of monocyte chemotaxis, attenuation of plaque inflammation, reversal of plaque progression and prevention of plaque disruption. These therapeutic effects are independent of serum lipid levels and demonstrate that inhibition of plaque inflammation alone without lipid lowering can stabilize vulnerable plaques.

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