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Synthetic peptide fragment (65–76) of monocyte chemotactic protein-1 (MCP-1) inhibits MCP-1 binding to heparin and possesses anti-inflammatory activity in stable angina patients after coronary stenting

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Abstract
Objective and design The peptide from C-terminal domain of MCP-1 (Ingramon) has been shown to inhibit monocyte migration and possess anti-inflammatory activity in animal models of inflammation and post-angioplasty restenosis. Here, we investigate the effect of Ingramon treatment on blood levels of acute-phase reactants and chemokines in patients after coronary stenting and the mechanisms of Ingramon anti-inflammatory activity.
Subjects Eighty-seven patients with ischemic heart disease (IHD) who faced the necessity of coronary angiography (CA) were enrolled. In 67 patients, one-stage coronary stenting was performed; 33 of them were treated with Ingramon in addition to standard therapy. Twenty patients underwent CA only.
Methods High-sensitivity C-reactive protein (hsCRP) and fibrinogen blood levels were detected routinely. The chemokine concentration in plasma was measured by enzyme-linked immunosorbent assay (ELISA) or cytometric bead array-based immunoassay. Intracellular Ca^{2+} levels and cell surface integrin exposure were assayed by flow cytometry. MCP-1 dimerization was studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). MCP-1–heparin binding was assessed with a biosensor and ELISA.
Results and conclusions Ingramon treatment was accompanied by less pronounced elevation of hsCRP and fibrinogen levels and decreased MCP-1 concentration in plasma in patients after coronary stenting. Ingramon had no effect on MCP-1 interaction with cell receptors or MCP-1 dimerization, but inhibited MCP-1 binding to heparin. The anti-inflammatory activity of the peptide may be mediated by an impaired chemokine interaction with glycosaminoglycans.

Keywords Inflammation · Chemokines · MCP-1 · Glycosaminoglycans · Peptide inhibitors

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Introduction

Inflammation is a key factor in the development of many chronic diseases including autoimmune disorders, Alzheimer's disease, renal pathology, atherosclerosis, and post-angioplasty restenosis [1]. Among numerous inflammatory mediators, chemokines (i.e., small basic proteins with chemotactic activity) control leukocyte migration into tissues, and cell activation and differentiation [2, 3]. The

so-called homing chemokines regulate the distribution of leukocytes in organs of the lymphoid system, whereas inflammatory chemokines are responsible for attracting leukocytes to inflammatory sites. The inflammatory chemokine monocyte chemotactic protein-1 (MCP-1, CCL2), being a major chemoattractant for monocytes, can also stimulate migration of activated T-cells, natural killer cells, and dendritic cells [3]. Several experimental and clinical studies have proved the impact of MCP-1 on the pathogenesis of cardiovascular disease [4, 5]. The involvement of MCP-1 in atherogenesis has been demonstrated in experiments on transgenic mice deficient in the MCP-1 gene or its receptor CCR2 [6, 7]. An increased level of MCP-1 in blood of patients with ischemic heart disease (IHD) as well as high CCR2 expression by circulating monocytes of hypercholesterolemic patients confirm the participation of MCP-1 in atherogenesis in humans [8, 9]. MCP-1 is not detected in vessel wall with no signs of atherosclerosis, though its content in atherosclerotic lesions is significant; in plaques, MCP-1 is produced by macrophages, and smooth muscle and endothelial cells [4]. It was shown earlier that plasma levels of MCP-1 are elevated in patients with acute coronary syndromes (unstable angina) and that CCR2-positive mononuclear cells are abundant in plaque specimens obtained by directed atherectomy of culprit lesions [9–11].

Percutaneous balloon angioplasty and stenting are widely used procedures for treatment of IHD patients with stenotic lesions. Restenosis, i.e., renarrowing of the blood vessel lumen after angioplasty, occurs in 5–40% cases after bare metal stent (BMS) implantation [12] and in 4–5% cases after implantation of drug-eluting stents (DES) [13]. The key processes in development of restenosis are adhesion and migration of leukocytes to the damaged vessel wall, followed by migration and proliferation of vascular cells and bone marrow precursors, with excessive synthesis of extracellular matrix, leading to neointimal growth. There is strong evidence that MCP-1 stimulates neointima formation. Thus, in animals, balloon injury of arteries leads to induction of MCP-1 synthesis by smooth muscle cells. In turn, inactivation of MCP-1 by specific antibodies inhibits neointima growth [14]. The relation of increased plasma MCP-1 levels with restenosis formation after percutaneous interventions (PCI) has been demonstrated in several clinical studies [15, 16].

Stent implantation into coronary artery induces an inflammatory reaction of the vessel wall which is manifested by systemic elevation of acute-phase reactants and the levels of some cytokines. There is strong evidence that elevated levels of C-reactive protein (CRP) before PCI with implantation of BMS or DES are associated with negative prognosis [17, 18]. Saleh et al. [19] have demonstrated that increased serum CRP after PCI is an independent predictor of death or nonfatal myocardial infarction, which is not associated with the degree of myocardial injury during the procedure.

The important role of MCP-1 in inflammatory diseases inspired investigators to develop MCP-1 antagonists. Unlike chemical compounds, viral proteins, and antibodies, peptide inhibitors are not allergenic, have no side-effects, and are safe even when administrated repeatedly. Reckless and Grainger [20] have demonstrated that the peptide corresponding to the amino acid 51-62 sequence of MCP-1 abrogated leukocyte migration stimulated by MCP-1 and other chemokines. We also initiated a search for chemokine inhibitors. After analysis of MCP-1 structure and calculations with Peptide Companion software, which is commonly used to reveal protein antigenic determinants, we synthesized a series of peptide fragments of MCP-1 and studied their functional activity in the Boyden chamber assay and in animal models of inflammation. One of the tested peptides, representing residues 65–76 of MCP-1 (Asp-His-Leu-Asp-Lys-Gln-Thr-Gln-Thr-Pro-Lys-Thr), suppressed MCP-1-dependent migration of human monocytes in the Boyden chamber assay as well as leukocyte accumulation in mice "air pouches" induced by MCP-1 and lipopolysaccharide (LPS); the maximal inhibition level was achieved at the dose of 35 μg/kg. The peptide activity was comparable to that of an earlier described peptide representing residues 51–62 of MCP-1 [21, 22]. The peptide accumulated at the inflamed limb in rats and showed relatively low degradation level in human plasma in vitro, where it was stable for at least 24 h [23, 24]. The peptide activity was further tested in models of inflammation in rodents and nonhuman primates, and in a rat model of post-angioplasty restenosis [23, 25]. In rats, intramuscular injection of the peptide suppressed subcutaneous recruitment of monocytes and granulocytes induced by LPS. A similar effect on LPS-mediated inflammation was observed in *Macaca fascicularis* when the peptide was injected intravenously. Daily administration of the peptide to rats subjected to experimental angioplasty delayed formation of neointima. The peptide was preliminarily named "Ingramon" (inhibitor of granulocyte and monocyte migration).

Here, we provide new data on clinical trials of Ingramon in patients with coronary stenting, and on the mechanisms of the peptide's anti-inflammatory action.

Patients and methods

Patients

Sixty-seven patients with IHD and stable angina II–III functional class selected for coronary angioplasty with implantation of one or two stents were included. Sirolimus-eluting stents (SES) were mainly used, but in 12 patients BMS

were also implanted (one SES + one BMS). Patients with unstable angina, acute or chronic inflammatory diseases, or renal or hepatic insufficiency were excluded. All patients received standard therapy including clopidogrel, aspirin, beta-blockers, lipid-lowering drugs, and if required angiotensin-converting enzyme (ACE) inhibitors, calcium antagonists, nitrates, and hypoglycemic drugs. Thirty-three patients who gave informed consent to participate in the trial received Ingramon intravenously at 35 μg/kg in six injections (before stent implantation, after 6 h, then two injections at 24-h intervals, and two injections at 48-h intervals), in addition to the standard therapy. The dose of Ingramon and the regimen of injections were established earlier in animal models of inflammation and neointimal growth after experimental angioplasty [23, 25]. The control group consisted of 34 patients who received the standard therapy. The patient groups were comparable in main anamnestic characteristics, concomitant diseases, and total number of implanted stents (Table 1). In patients treated with Ingramon, more BMS were implanted (10 versus 2). To study the influence of right femoral artery puncture on blood levels of inflammatory markers, a group of patients ($n = 20$) who underwent coronary angiography (CA) without endovascular interventions was examined. The study was approved by the Russian Ministry of Health and Local Ethical Committee.

CA was performed using the Coroscop-33 apparatus (Siemens, Germany), and subsequent computer analysis of angiograms was carried out with Hicor (Siemens, Germany). Artery stenosis which exceeded 50% of the proper diameter was considered hemodynamically significant. After predilatation of the stenosed section by balloon catheter (with pressure 6–8 atm), stents were implanted (with pressure 10–14 atm). Stenting was considered successful if the residual stenosis was 10% or less of the proper diameter of the artery.

Blood samples

Blood samples were obtained before the intervention, at first, second, and seventh days, and 1 month after it. Samples of serum and citrate plasma were stored at −70°C. The concentration of MCP-1 in blood plasma was measured by ELISA using a commercial kit (Bender MedSystems). Serum levels of high-sensitivity CRP (hsCRP) were detected by nephelometry (Behring nephelometer, Marburg GmbH). The fibrinogen content in plasma was determined by Clauss method. The concentration of the chemokines interleukin-8 (IL-8), interferon-gamma inducible protein (IP-10, 10 kDa), and monokine induced by gamma-interferon (MIG) in plasma samples was measured by flow cytometry (FACSCalibur, Becton–Dickinson Immunocytometry Systems) with cytometric bead array-based immunoassay (BD Biosciences) according to the manufacturer's instructions.

Peptide synthesis

Ingramon was synthesized using the standard fluorenylmethyloxycarbonyl (Fmoc) strategy of solid-phase peptide synthesis using an Applied Biosystems 431A automatic peptide synthesizer and purified by preparative high-performance liquid chromatography (HPLC) to 96% purity.

Analysis of MCP-1-induced intracellular Ca^{2+} influx in THP-1 cells and Mac-1 externalization by human monocytes in whole blood

THP-1 promonocytic cells (American Type Culture Collection, ATCC) were grown in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 20 μM 2-mercaptoethanol, 100 U/ml penicillin and streptomycin.

Table 1 Clinical and anamnestic characteristics of the patients

	Ingramon	Controls	CA
Patients (*n*)	33	34	20
Males	28 (84.8%)	3 (88.2%)	16 (80%)
Age (years)	59.5 ± 8.5	57.5 ± 7.3	58.5 ± 7.6
Myocardial infarction in anamnesis	18 (54.5%)	17 (50%)	10 (50%)
Arterial hypertension	27 (81.8%)	28 (82.3%)	14 (73.6%)
Type 2 diabetes mellitus	5 (15.2%)	6 (17.6%)	3 (15.0%)
Blood cholesterol level >4.5 mM	23 (69.7%)	22 (64.7%)	13 (65%)
Obesity	10 (30.3%)	11 (32.3%)	6 (30%)
Smoking	10 (30.3%)	10 (29.4%)	6 (30%)
One SES stent implanted	17 (51.5%)	21 (61.8%)	
Two SES stents implanted	6 (18.2%)	11 (32.4%)	
One SES + one BMS implanted	10 (30.3%)	2 (5.8%)	
Length of stented segments (mm)	27.4 ± 12.4	32.2 ± 12.5	
Diameter of stents (mm)	2.81 ± 0.62	2.75 ± 0.73	

Cells were washed in Hank's balanced salt solution (HBSS) and resuspended in dye loading solution consisting of HBSS with addition of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2.5 mM probenecid (water soluble, Molecular Probes), and 2 μM Fluo-4 acetoxymethyl (AM) ester (Molecular Probes). Cells were incubated for 30 min at 37°C, washed in HBSS with 2.5 mM probenecid, and then incubated for an additional 30 min at room temperature in HBSS with 2.5 mM probenecid to allow complete de-esterification of intracellular AM esters. Then cells were stimulated with 10 nM MCP-1 (recombinant human protein, R&D Systems). The responses were measured using a FACSCalibur flow cytometer with CellQuest software (BD Immunocytometry Systems).

Blood samples were obtained from healthy volunteers in citrate anticoagulant. Probes were incubated with 5 nM MCP-1 ± Ingramon, 1 mg/ml (714 μM), for 10 min at 37°C. Leukocytes were then stained with FITC-CD14 (for monocyte typing) and PE-CD11b monoclonal antibodies (BD Biosciences), and erythrocytes were lysed with FACS lysing solution (BD Biosciences). Antibody binding was estimated by flow cytometry.

Chemical cross-linking of MCP-1 oligomers

Recombinant human MCP-1 (carrier free, R&D Systems) was dissolved in complete phosphate-buffered saline (PBS) at 5 μg/ml (576 nM). To fix the oligomers, the samples were incubated with 2 mM disuccinimidyl suberate (DSS, Sigma) for 30 min at room temperature as described earlier [26]. Ingramon was added at concentration of 100 μg/ml (71 μM). Cross-linked products were subjected to 15% SDS-PAGE. Proteins in gels were either stained with silver or transferred onto nitrocellulose membranes. The membranes were incubated with biotinylated rabbit anti-human MCP-1 antibody (BD Pharmingen) and streptavidin–peroxidase conjugate (Sigma). The protein bands were identified using a chemiluminescent detection (ECL, Amersham). In control experiments, aprotinin (a protein with comparable molecular weight to MCP-1) was used instead of MCP-1.

Analysis of chemokine binding to heparin using the Picoscope® label-free biosensing device and ELISA

The Picoscope® biosensor was developed and fabricated in the Biophotonics Laboratory of the A.M. Prokhorov General Physics Institute, Russian Academy of Sciences. The device was successfully tested for recording the dynamics of interactions between various protein molecules. Picoscope® permits quantitative registration of changes of average optical thickness of a molecular layer on a surface (i.e., the product of the refraction index n and the geometric thickness of the layer Δd) at picometer scale [27]. Microscopic glass slips used as sensor chips were silanized and then covered with biotin. After streptavidin immobilization (50 μg/ml), the following substances were successively pumped along the glass surface at flow rate of 5 ml/min: biotinylated heparin (100 μg/ml), MCP-1 in complete PBS with 0.15% bovine serum albumin (BSA, pH 7.4), followed by anti-MCP-1 monoclonal antibody (20 μg/ml, clone 5D3-F7; BDPharmingen) to confirm binding of MCP-1 to heparin. Ingramon (or control amino acid mixture) solution was added to the solution of MCP-1. The volume of test sample was 30–40 μl, and each solution was pumped for 5–10 min. The results of real-time measurements of molecular layer thickness in these experiments are presented in sensograms.

Then, 96-well plates (Costar) precoated with streptavidin (1 μg/well) were incubated with biotinylated heparin (400 μg/ml) which was obtained by biotinyl-Σ-aminocaproic acid chemical linking to heparin (Sigma). Solutions of MCP-1 or MCP-1 + Ingramon in complete PBS with 0.15% BSA (pH 7.4) were then added to the appropriate wells, and plates were incubated for 1 h at 37°C. Separate wells were incubated with Ingramon solution (150 μg/ml) for 1 h at 37°C and washed twice with PBS prior to MCP-1 addition. In a series of experiments, IL-8 (recombinant human protein, R&D Systems) was used instead of MCP-1. MCP-1/IL-8 binding was detected by sequential addition of mouse anti-human MCP-1/IL-8 monoclonal antibody (both from BD Pharmingen) and rabbit polyclonal antibody against mouse immunoglobulins conjugated with horseradish peroxidase (Imtek). The substrate solution contained *o*-phenylenediamine and hydrogen peroxide. Optical density was measured at wavelength of 492 nm by 96-well spectrophotometer (Uniplan, Picon).

Statistical analysis

Data are presented as mean ± standard deviation (SD) or as median and quartiles (25th–75th percentile). Student's t tests for dependent and independent variables were applied for intra- and intergroup comparisons, respectively. Since the distribution pattern of hsCRP and chemokine values was not normal, the W–Wilcoxon and Mann–Whitney U tests were used. Differences were considered statistically significant at $p < 0.05$.

Results

Ingramon administration decreases the levels of acute-phase reactants and MCP-1 in blood of patients after coronary artery stenting

Preclinical testing and phase I clinical trials in healthy volunteers demonstrated the safety of Ingramon, and a

Table 2 Concentrations of acute-phase proteins and chemokines in blood plasma/serum

	Before	1st day	2nd day	7th day	1 month
hsCRP (μg/ml)					
Control	1.93 (1.41–2.70)	3.99*,& (2.93–5.57)	6.08*,& (4.72–7.35)	3.60*,& (2.72–4.26)	1.56 (0.99–2.12)
Ingramon	1.28 (0.83–1.85)	1.96*,# (1.36–2.24)	2.32*,# (1.95–3.36)	1.61*,# (1.07–2.11)	0.98 (0.73–1.19)
CA	1.30 (0.92–1.69)	1.87* (1.67–2.97)	3.03* (2.27–3.4)	2.13* (1.50–2.51)	1.01 (0.85–1.17)
Fibrinogen (mg/ml)					
Control	3.60 ± 0.69	4.38 ± 0.72*,&	4.58 ± 0.66*,&	4.37 ± 0.88*,&	3.84 ± 0.57
Ingramon	3.38 ± 0.55	3.51 ± 0.66*,#	3.84 ± 0.92*,#	3.80 ± 0.92*,#	3.30 ± 0.50
CA	3.58 ± 0.93	3.75 ± 0.58*	4.09 ± 0.68*	4.08 ± 0.53*	3.61 ± 0.64
MCP-1 (pg/ml)					
Control	100.7 ± 30.4	92.5 ± 34.9	91.4 ± 40.8	99.9 ± 33.5	97.4 ± 36.8
Ingramon	98.9 ± 48.3	83.2 ± 33.2*,#	85.2 ± 46.4*,#	95.6 ± 45.4	90.7 ± 53.8
CA	92.6 ± 34.6	91.1 ± 41.5	94.8 ± 39.1	96.1 ± 44.7	92.9 ± 11.2
IL-8 (pg/ml)					
Control	19.0 (8.14–24.0)	18.9 (11.1–44.0)	15.7 (10.8–27.0)	11.50 (8.15–22.50)	6.4 (5.5–12.6)
Ingramon	17.8 (12.0–25.0)	14.5 (13.8–21.0)	19.3 (9.1–28.0)	9.1 (6.1–22.2)	6.25 (5.50–8.20)
MIG (pg/ml)					
Control	204 (193–345)	219 (203–280)	239 (188–268)	206 (133–234)	203 (129–254)
Ingramon	237 (163–299)	276 (234–355)	272 (203–294)	264 (169–400)	250 (198–294)
IP-10 (pg/ml)					
Control	336 (313–349)	311 (292–438)	341 (322–413)	206 (133–234)	203 (129–254)
Ingramon	293 (238–350)	285 (242–352)	259 (203–294)	264 (169–400)	250 (198–294)

* $p < 0.05$ versus initial values in group

# $p < 0.05$ versus control

& $p < 0.05$ versus CA

clinical trial of the peptide in patients with coronary stenting was launched. Ingramon i.v. injections at the established dose were well tolerated by patients. No side-effects were observed.

The interventions led to a significant increase of circulating hsCRP and fibrinogen levels in all three groups of patients that reached maximal values at the second day with a gradual decrease and return to baseline values after 1 month (Table 2). hsCRP and fibrinogen blood concentrations on the first, second, and seventh days after intervention were significantly higher in patients with coronary stenting who received standard therapy (control group), as compared with patients who underwent CA without stenting. In comparison with the control group, in the group of patients who received Ingramon the hsCRP concentration was significantly lower at first, second, and seventh days after coronary stenting; the fibrinogen content in plasma was also decreased at the first, second, and seventh days after the intervention. We did not observe any changes in MCP-1 concentration after CA/stenting, but revealed a slight but significant decrease on the first and second days after Ingramon administration. No changes of the levels of other chemokines, such as the main attractant for neutrophils IL-8, as well as the alternative attractants for lymphocytes and monocytes MIG and IP-10, in the blood of patients after coronary stenting were found, and Ingramon infusions had no effect on these parameters (Table 2).

Ingramon does not impair MCP-1-induced intracellular Ca^{2+} influx in THP-1 cells or Mac-1 externalization by human monocytes in whole blood

To investigate the mechanisms of anti-inflammatory activity of the peptide, we first focused on receptor-mediated effects of MCP-1. In accordance with published data [28], MCP-1 induced a rapid and transient increase in intracellular Ca^{2+} concentration in promonocytic THP-1 cells (Fig. 1). Ingramon had no influence on intracellular Ca^{2+}, alone or in the presence of MCP-1. MCP-1 binding to its receptor is known to induce externalization of cell adhesion molecules, in particular β2-integrins by blood monocytes [28]. The study of membrane density of β2-integrins Mac-1 (CD11b/CD18) on monocytes with and without addition of exogenous MCP-1 did not reveal any effect of the peptide on Mac-1 externalization (Fig. 1). Thus, we concluded that Ingramon did not impair the MCP-1–receptor interaction.

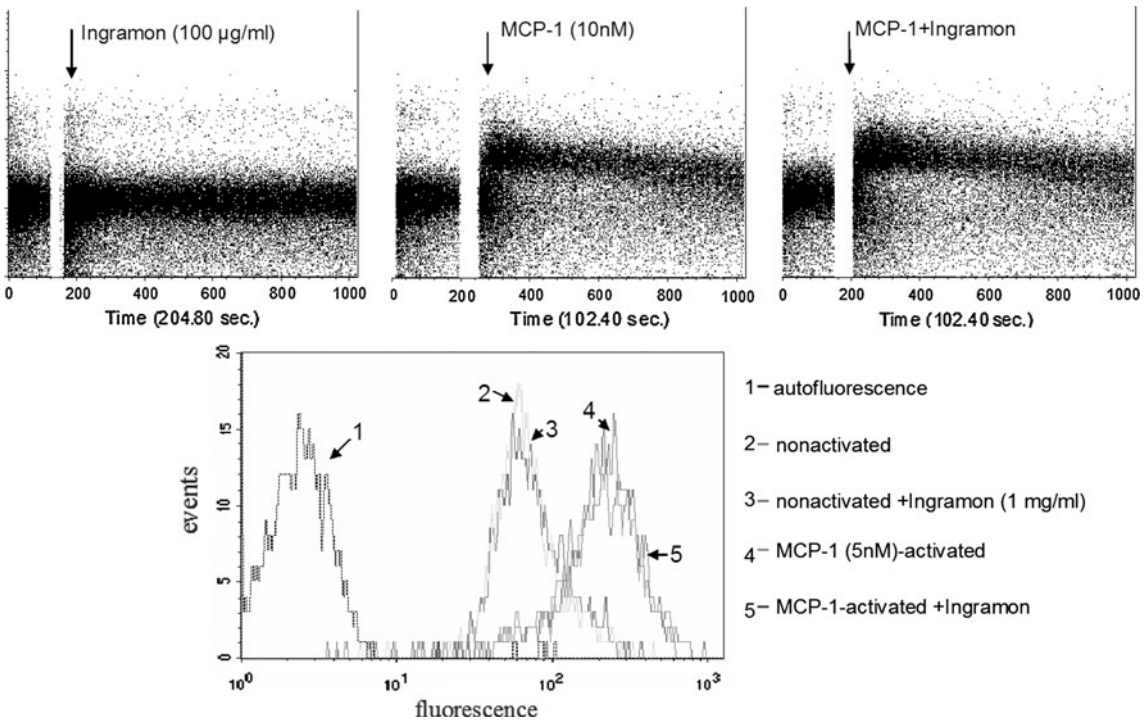

Fig. 1 Ingramon does not suppress MCP-1-induced intracellular Ca^{2+} mobilization in THP-1 cells or Mac-1 integrin externalization by monocytes. FACS analysis of intracellular Ca^{2+} in THP-1 cells (*upper panel*) and membrane CD11b molecules on blood monocytes (*lower panel*)

The chemotactic activity of MCP-1 and other chemokines in vivo is closely related to their ability to bind glycosaminoglycans (GAGs) on the cell surface and extracellular matrix and to form oligomers. This creates a chemotactic gradient and guides cell migration [30, 31]. Therefore, we assumed that Ingramon could interfere with MCP-1 binding to GAGs and/or oligomerization.

Ingramon does not affect MCP-1 dimerization

It has been demonstrated earlier that MCP-1 forms dimers in vitro [30, 31]. To stabilize the dimeric form of MCP-1 by cross-linking it was treated with DSS. Untreated MCP-1 migrated upon SDS-PAGE as a single band of approximately 9 kDa (Fig. 2). Cross-linking of MCP-1 with DSS resulted in the appearance of a ~18-kDa band, indicating dimerization of MCP-1. In contrast, DSS treatment of the control protein aprotinin (~6.5 kDa), which does not form dimers, did not lead to appearance of a slower migrating band. No significant changes on electrophoregrams or immunoblot were revealed when Ingramon was added to the MCP-1 solution.

Ingramon inhibits MCP-1 and IL-8 binding to heparin

Preliminary results obtained in studies of the influence of the peptide on MCP-1–heparin interaction have been published earlier [32]. In experiments with the Picoscope® biosensor, an increase of optical thickness of the molecular layer Δd (nm) consisting of MCP-1 and anti-MCP-1 monoclonal antibody was observed with increasing MCP-1 concentration (Fig. 3a). The MCP-1–heparin dissociation constant calculated in Scatchard's coordinates was 0.44 μM, consistent with data on low-affinity binding of MCP-1 to GAGs [31]. In the absence of MCP-1, no binding of monoclonal antibody or increase of optical thickness was detected.

The effect of Ingramon on MCP-1–heparin interaction was investigated by pumping the MCP-1 solution through the second channel. Addition of 100 μg/ml Ingramon to the MCP-1 solution was associated with very low response to pumping the antibody ($\Delta d \approx 0.15$ nm) even at the highest MCP-1 concentration (5 μg/ml). A control mixture of amino acid compounds of Ingramon did not affect the binding of MCP-1 to heparin (not shown).

On ELISA, dose-dependent inhibition of MCP-1 (576 nM) binding to heparin by Ingramon was observed (Fig. 3b). Maximum inhibition was achieved at peptide concentration of 150 μg/ml. Further increase of the peptide concentration was not accompanied by increased inhibitory effect on MCP-1–heparin interaction. It should be noted that the maximum suppression of leukocyte migration was observed in vivo at the same concentration of the peptide [22, 23]. The inhibitory activity of the peptide was

preserved when it was added to the wells before MCP-1 (Fig. 3c). Accordingly, the peptide was suggested to bind preferentially to heparin, not to MCP-1. The differences in the degree of inhibition of MCP-1–heparin binding in ELISA and the biosensor analysis were probably due to the unequal area (30 and 1 mm^2, respectively) and sorption properties of the sensor surfaces as well as different modes of substance application (stationary versus flow-through).

The potent neutrophil chemoattractant IL-8 can also interact with heparin and other GAGs [33]. In ELISA, Ingramon was also able to inhibit IL-8–heparin binding, although less effectively than MCP-1–heparin complex formation (Fig. 3d).

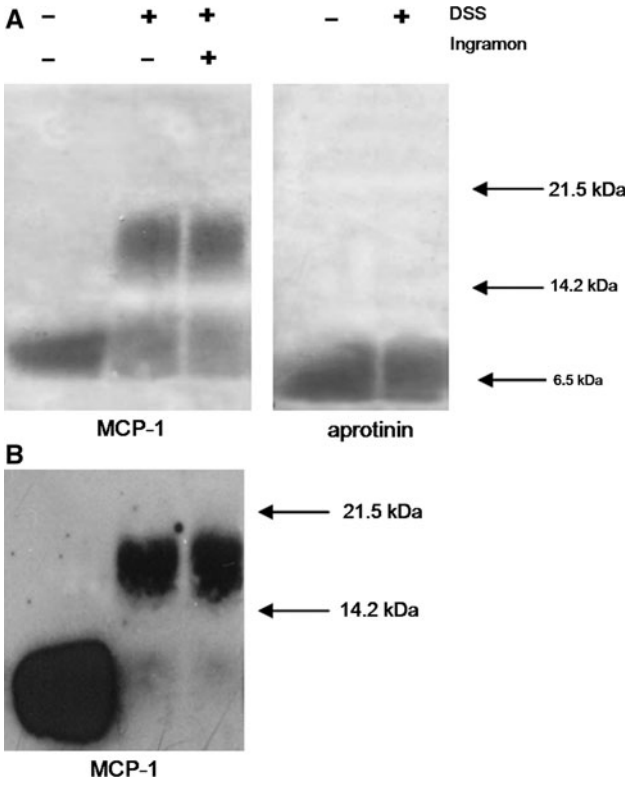

Fig. 2 SDS-PAGE (**a**) and Western blot analysis (**b**) of MCP-1 dimerization in solution. Carrier-free MCP-1 and aprotinin, both at 5 μg/ml in PBS, were cross-linked with 2 mM DSS. Ingramon was added at concentration of 100 μg/ml

Discussion

At present, chemokine-mediated recruitment of inflammatory cells to vessel wall is considered to be one of the main mechanisms involved in the development of arterial stenotic lesions. Excessive leukocyte accumulation in an atherosclerotic plaque can lead to its rupture and thus to development of acute coronary syndrome. Numerous investigations support an important role of inflammation in development of IHD and its complications. A number of studies have shown a relation between high levels of inflammatory markers in plasma and negative prognosis after PCI in terms of increased frequencies of cardiac death, myocardial infarction, and restenosis formation. Angioplasty was shown to trigger leukocyte migration to vascular wall, causing intimal hyperplasia [34]. Thus, therapeutic targeting of the chemokine network can provide an effective tool to combat atherosclerosis and prevent restenosis [35].

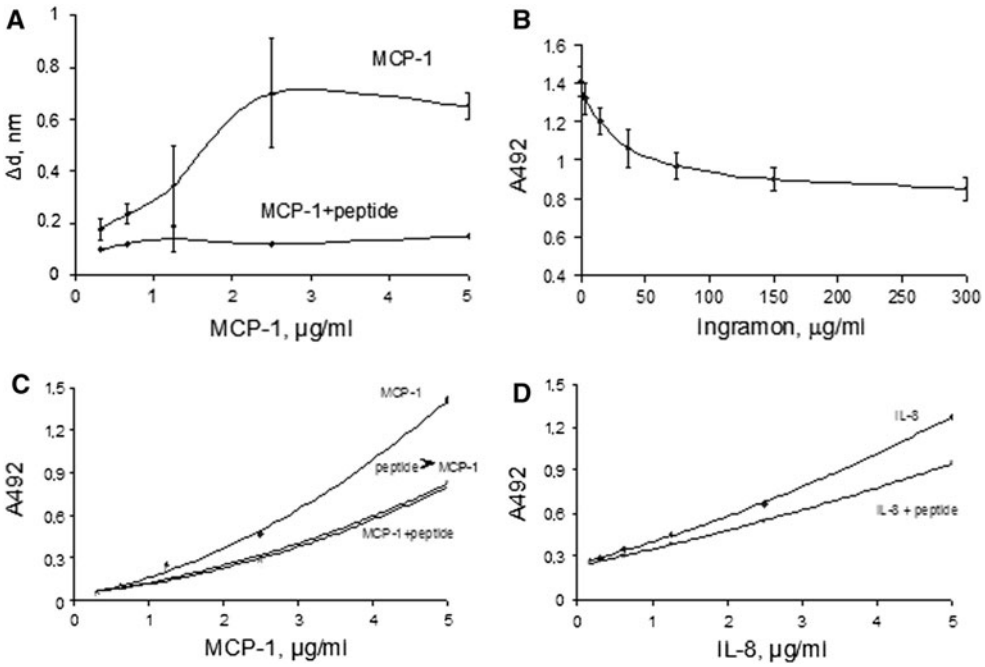

Fig. 3 Ingramon impairs binding of MCP-1 and IL-8 to heparin. **a** Biosensor analysis of optical thickness after pumping of MCP-1 + peptide (100 μg/ml) and anti-MCP-1 antibody. **b** ELISA of MCP-1 (5 μg/ml, 576 nM) interaction with heparin in the presence of increasing concentrations of Ingramon. **c** Binding of MCP-1 to heparin by Ingramon was inhibited when the peptide (150 μg/ml) was added before or simultaneously with MCP-1 (results of one of three independent experiments are shown). **d** Ingramon (150 μg/ml) inhibits IL-8–heparin binding (results of one of three independent experiments are shown)

During our previous studies we designed, synthesized, and tested several peptide fragments of chemokine MCP-1, a powerful chemoattractant for monocytes and other leukocyte subsets. Peptide of the 65–76 MCP-1 amino acid sequence (Ingramon) showed a significant inhibitory effect on leukocyte accumulation at sites of LPS-induced inflammation in rats and primates [11]. It should be noted that peptide administration effectively suppressed migration of not only monocytes but also granulocytes. In a rat model of post-angioplasty restenosis, peptide-treated animals showed significant delay of neointimal growth, which was manifested as less pronounced neointima thickening and larger area of vessel lumen observed in 1 week after the intervention [25]. The anti-inflammatory action of Ingramon was detectable with i.v. and i.m. administrations and was not species specific. This pilot clinical trial revealed that Ingramon may possess an anti-inflammatory effect in patients with coronary artery stenting.

Polypeptide chain of chemokine MCP-1 contains 76 amino acid residues with two intramolecular disulfide bridges (Cys11–Cys36 and Cys12–Cys52). The spatial (tertiary) structure of the MCP-1 molecule as well as of the other members of the CC chemokine family consists of a flexible N-terminal fragment, a rigid middle part composed of three antiparallel β-layers, and a C-terminal α-helix [36]. The N-terminal fragment of MCP-1 participates in the interaction of MCP-1 with G-protein coupled receptors CCR2 in monocytes and CCR4 in T-cells [37]. It is now established that many chemokines are able to dimerize. MCP-1 in solution forms dimers through interaction of amino acids in the N-terminal domain [25]. MCP-1 mutants with impaired dimerization are not active in vivo, despite their ability to bind and activate their cognate receptors in vitro [31, 38, 39]. The activity of chemokines in vivo also depends on their binding to GAGs of the endothelial surface and extracellular matrix [40] as this prevents proteolytic degradation of chemokines and induces their oligomerization, which is necessary for formation of a stable gradient of chemokine concentration [41]. As was shown earlier, the basic amino acids from the C-terminal domain, primarily Lys58 and His66, are responsible for MCP-1's interaction with GAGs. Mutations at these sites were associated with inability of MCP-1 to bind to heparin and induce leukocyte chemotaxis in vivo [42].

According to our data, Ingramon does not impair MCP-1-mediated cellular effects, i.e., MCP-1-induced Mac-1 β2-integrin exposure by blood monocytes and intracellular Ca^{2+} mobilization in monocytic cells. In addition, it does not influence the ability of MCP-1 to form dimers in vitro. These data indicate that Ingramon does not interfere with MCP-1–receptor binding or MCP-1 dimerization. At the same time, Ingramon blocks MCP-1 binding to heparin in vitro, as demonstrated by the biosensor analysis and ELISA. Ingramon represents the C-terminal fragment of MCP-1 and contains three basic amino acids (His66, Lys69, and Lys75) which can participate in GAG binding, thus competing with MCP-1. According to our preliminary data, Ingramon also inhibited (although less efficiently) heparin binding of IL-8, a major granulocyte chemoattractant. As in the case of MCP-1, the basic amino acids from C-terminal domain of IL-8 (Arg60, Lys64, Lys67, and Arg68) were shown to be involved in GAG binding [43]. We think that Ingramon may block to some degree the action of several proinflammatory chemokines, which contain the conserved heparin-binding domains. This could explain the impairment of both granulocyte and monocyte recruitment in animal models of inflammation, and non-species-specific activity of the peptide.

Currently, there is no doubt that circulating levels of inflammatory mediators correlate with the extent of vessel wall damage and are linked to prognosis of IHD and endovascular interventions. We have identified a significant increase in hsCRP and fibrinogen concentrations in blood of patients in the early period after stenting in comparison with the CA group. In accordance with published data [16, 44, 45], the levels of these acute-phase reactants did not depend on the type of implanted stent. Blood concentration of MCP-1 and other chemokines did not rise after the intervention, probably due to their predominantly paracrine action and more complex mechanisms of synthesis and elimination. Ingramon administration was associated with less pronounced elevation of hsCRP and fibrinogen levels in blood after the intervention. MCP-1 concentration in plasma was reduced. We speculate that Ingramon inhibits chemokine-stimulated migration of leukocytes to the damaged vessel wall, thereby impairing production of MCP-1 and other proinflammatory cytokines. Ultimately, this is manifested in the systemic reduction of blood concentration of inflammatory markers.

In conclusion, when added to a standard therapy, Ingramon positively affects the established risk factors for IHD and its complications. Further investigation is required to determine the effect of Ingramon on long-term prognosis in patients with IHD.

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