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Expression of Markers of Regulatory $CD4^+CD25^+foxp3^+$ Cells in Atherosclerotic Plaques of Human Coronary Arteries

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The content of marker foxp3 of regulatory T cells and chemokines in atherosclerotic plaques of human coronary arteries was measured by the polymerase chain reaction. *In vitro* migration of regulatory $CD4^+CD25^+foxp3^+$ cells in the $CD4^+$ lymphocyte population from healthy donors was studied after treatment with chemokines I-309, IP-10, and SDF-1. mRNA for the factor foxp3 and chemokines SDF-1, I-309, and MIP-1β were found in the majority of samples from atherosclerotic plaques. SDF-1 induced maximum migratory response of $CD4^+CD25^+foxp3^+$ cells.

Key Words: *human regulatory T cells; atherosclerosis; chemokines; migration*

Regulatory $CD4^+CD25^+foxp3^+$ T cells (Treg) constitute a pool of lymphocytes exhibiting suppressor activity towards populations of effector T cells and monocytes/macrophages. These cells are involved in the pathogenesis of chronic inflammatory and tumor diseases, autoimmune reactions, and antiviral immunity [1,6,7]. The role of Treg in human atherogenesis remains unknown. CD25 expression was detected in clusters of $CD4^+$ lymphocytes in human atherosclerotic plaques [8]. After discovery of foxp3 protein, a specific marker of regulatory T cells, and generation of antibodies to this protein, localization $foxp3^+$ cells in atherosclerotic plaques of human large arteries was demonstrated immunohistochemically [2].

This work was designed to reveal regulatory T cells in atherosclerotic plaques in the intima of human coronary arteries and to identify chemokines recruiting Treg into injured vascular wall. To this end, the expression of foxp3 and chemokines stimulating migration of Treg was studied in samples of human atherosclerotic plaques and migration activity of Treg from peripheral blood of healthy donors was studied *in vitro* in the presence of some chemokines.

MATERIALS AND METHODS

Intima samples of the coronary artery was sampled in patients with stable angina pectoris during endarterectomy (n=11). The samples were thoroughly washed with physiological saline and frozen in liquid nitrogen. Frozen samples were stored at -70°C. For morphological study, the segments were embedded in a freezing medium (O.C.T. Compound, Tissue-Tek®).

RNA was isolated from the intima of human coronary arteries by means of acid phenol-chloroform extraction with RIBO-zol'-A kit (AmpliSens). Total RNA

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TABLE 1. Characteristics of Primers

Nucleotide sequence		b.p.	Primer annealing temperature
FOXP3	CAGCACATTCCCAGAGTTCCT	150	61°C
	CGTGTGAACCAGTGGTAGAT		
IP-10	CCACGTGTTGAGATCATTGC	205	60°C
	CCTCTGTGTGGTCCATCCTT		
I-309	TTTCTTTCCATTGTGGGCTC	109	60°C
	AGCAGATCCTCTGTGACCTAGC		
MIP1-α	TGCAACCAGTTCTCTGCATC	197	60°C
	TTTCTGGACCCACTCCTCAC		
MIP1-β	AAGCTCTGCGTGACTGTCCT	210	60°C
	GCTTGCTTCTTTTGGTTTGG		
MDC	AGGACAGAGCATGGCTCGCCTACAGA	336	68°C
	TAATGGCAGGGAGGTAGGGCTCCTGA		
TARC	ACTGCTCCAGGGATGCCATCGTTTTT	269	72°C
	ACAAGGGGATGGGATCTCCCTCACTG		
SDF-1	GCCATGAACGCCAAGGTCGTGGT	314	60°C
	CCTCGAGTGGGTCTAGCGGAAAG		
β_2-microglobulin	GATGAGTATGCCTGCCGTGTG	113	55°C
	CAATCCAAATGCGGCATCT		

concentration was estimated by absorption at 260 nm. The reverse transcription reaction with REVERTA kit (AmpliSens) was conducted immediately after isolation of RNA to obtain cDNA. The polymerase chain reaction (PCR) was performed using an Eppendorf Mastercycler Personal amplifier. The reaction mixture consisted of PCR buffer (Universal PCR-buffer, Omega), DEPC-H_2O (Sileks), cDNA, 5 U antibody-blocked Taq polymerase (Taq-gold, Omega), 0.1 mM dATP, 0.1 mM dTTP, 0.1 mM dGTP, and 0.1 mM dCTP (Sileks), and 0.2 μM each specific primer (Syntol). Sequences of primers are presented in Table 1. Each cycle of amplification (n=15-35) included the following stages: denaturation at 95°C for 40 sec; annealing at the optimal temperature (for a specific primer) for 45 sec; and chain elongation at 72°C for 45 sec (including the first denaturation at 95°C for 5 min). The final stage of the last cycle was performed at 72°C for 2 min. The number of cycles and cDNA concentration in the sample were standardized by the expression of β_2-microglobulin. cDNA from human leukocytes or endothelial cells served as the positive control. DEPC-H_2O was added to negative control (instead of cDNA). The amplicons were analyzed by the standard method of gel electrophoresis in 2% agarose gel.

Mononuclear cells were obtained from the leukocyte mass of healthy donors (Laboratory of Blood Transfusion, Russian Cardiology Research-and-Production Complex) by centrifugation in a Histopaque density gradient (Sigma-Aldrich, ρ=1.077 g/ml). The population of $CD4^+$ lymphocytes was obtained by immunomagnetic separation using a commercial kit $CD4^+$

TABLE 2. Gene Expression in Samples of Atherosclerotic Plaques

Sample/ factor	1	2	3	4	5	6	7	8	9	10	11
FOXP3	+	+	+	+	-	-	+	+	+	+	+
IP-10	+	+	-	-	-	-	-	+	-	-	-
I-309	+	-	+	-	+	+	+	+	+	-	+
MIP-1α	-	-	-	+	+	-	-	+	+	-	+
MIP-1β	+	+	-	+	+	+	-	+	+	+	+
MDC	+	-	-	-	-	-	-	-	-	-	-
TARC	-	+	-	-	+	+	-	+	+	-	-
SDF-1	+	+	+	-	+	+	+	+	-	-	+

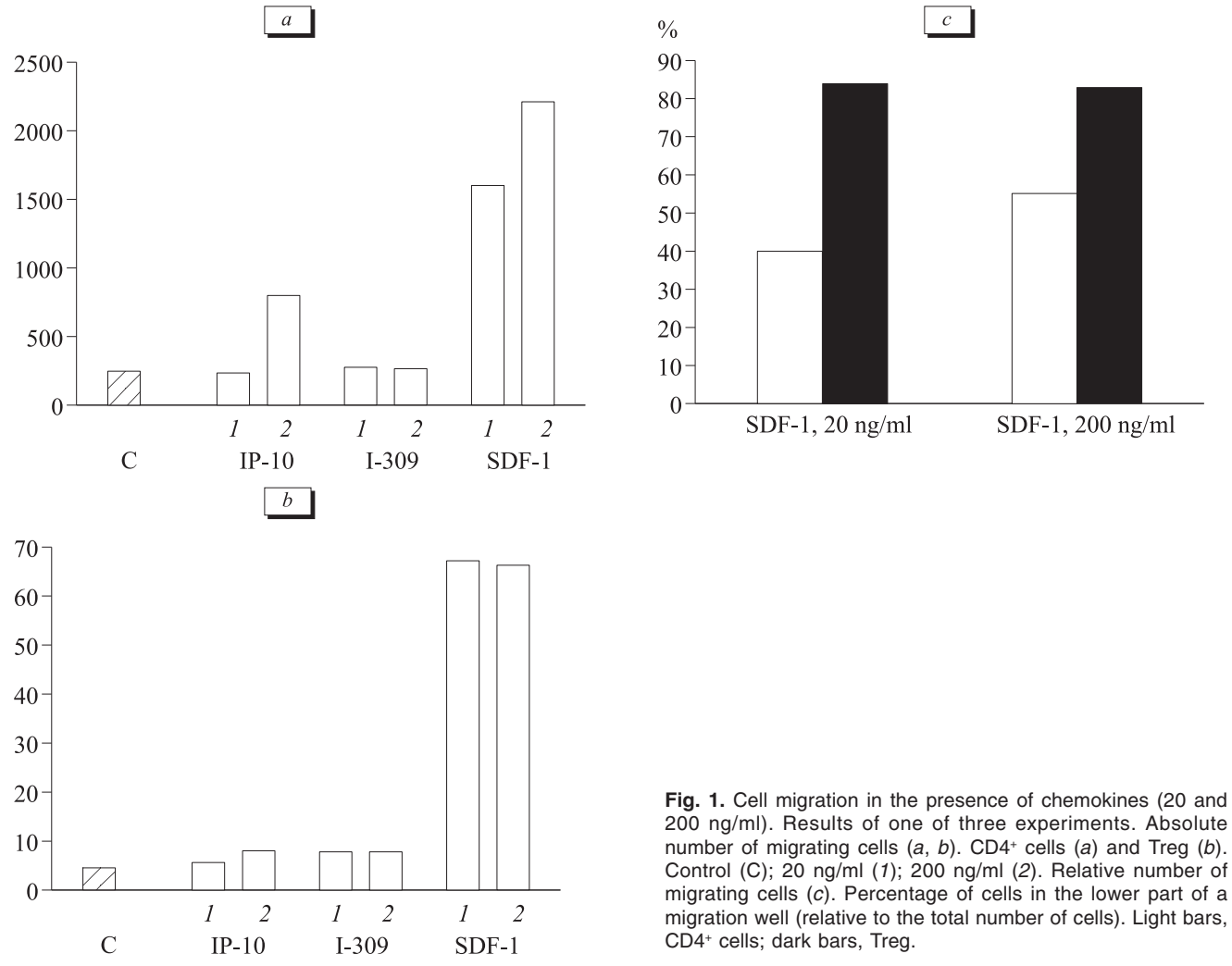

Fig. 1. Cell migration in the presence of chemokines (20 and 200 ng/ml). Results of one of three experiments. Absolute number of migrating cells (*a*, *b*). $CD4^+$ cells (*a*) and Treg (*b*). Control (C); 20 ng/ml (*1*); 200 ng/ml (*2*). Relative number of migrating cells (*c*). Percentage of cells in the lower part of a migration well (relative to the total number of cells). Light bars, $CD4^+$ cells; dark bars, Treg.

Isolation kit (MiltenyiBiotec). Lymphocytes were cultured in RPMI-1640 medium at 37°C and 5% CO_2 for 3 days. The medium contained 5% inactivated pooled human serum, 10 mM Hepes, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine, 20 μM mercaptoethanol, 1% pyruvate, and 1% mixture of nonessential amino acids (GibcoBRL). Culturing was performed in the presence of human recombinant interleukin-2 (Ces145Ser, 4 ng/ml, R&D systems).

The migration response of Treg in the population of $CD4^+$ cells was studied using a TransWell system for 24-well plates (pore size 5 μ, CorningNY). Lymphocytes (20 million cells per ml) were resuspended in Hanks solution (PanEco) with 1% human serum and 10 mM Hepes (Hanks-Hepes-1% HS). The cell suspension (150 μl) was placed in the upper part of the TransWell system. The lower part was filled with a solution of Hanks-Hepes-1% HS (500 μl) containing chemokines I-309, IP-10, and SDF-1 (R&D systems). The plates were put in a CO_2 incubator for 2 h. The cells migrating to the lower part of the TransWell system were counted in a Goryaev chamber. FITC-labeled monoclonal antibodies CD4-FITC (BDBiosciences) and CD25-PE (MiltenyiBiotec) were used for cell typing. The intracellular protein foxp3 was detected with eBioscience kit, which consisted of PC5-labeled rat monoclonal antibodies to human foxp3, isotypic control, and reagents for cell fixation and permeabilization. Binding of antibodies and cells was studied by means of flow cytofluorometry (FACSCalibur, BD Immunocytometry Systems).

RESULTS

Lipofibrous and fibrous plaques were found in the intima samples. Calcification was revealed in the majority of plaques. PCR analysis revealed mRNA for the factor foxp3 (Treg marker) in 9 plaques. The majority of plaques contained mRNA for chemokines SDF-1, TARC, I-309, and MIP-1β. mRNA for chemokines IP-10, MIP-1α, and MDC was found in less than 50% samples (Table 2).

The migration response of $CD4^+$ cells was studied *in vitro* in the presence of chemokines SDF-1, I-309,

and IP-10 (20 and 200 nM). SDF-1 was most potent in stimulating the migration of cells. IP-10 had a weak chemotactic effect, which was observed at high concentration of the chemokine. I-309 did not stimulate cell migration. Studying the relative number of migrating Treg in this model system showed that SDF-1 mainly affects migration of foxp3$^+$ cells (relative to the total population of CD4$^+$ lymphocytes, Fig. 1).

PCR analysis of foxp3 expression revealed the presence of Treg in atherosclerotic plaques of human coronary arteries. The samples also contained mRNA for some chemokines that can stimulate Treg migration. There are no data on the existence of an unique chemoattractant for Treg [3,5]. For example, homing chemokines (*e.g.*, ELC) are involved in cell migration from the thymus to secondary lymphoid organs. MDC, TARC, I-309, MIP-1, IP-10 and other agents are involved in cell migration to target organs. The chemokine SDF-1 is constitutively expressed in organs of hemopoiesis and lymphopoiesis. This chemokine plays a regulatory role in the migration and homing of CXCR4-positive immune cells (SDF-1 receptor), including Treg [9]. Previous studies revealed increased SDF-1 content in sites of acute or chronic inflammation (*e.g.*, atherosclerotic plaques) [4]. In the present study, we did not evaluate a wide range of chemokines that can recruit Treg into the atherosclerotic artery. Our results indicate that at least SDF-1 causes migration of regulatory T cells to the injured vascular wall.

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