Original research article

Comparison of chosen activation markers of human monocytes/macrophages isolated from the peripheral blood of young and elderly volunteers

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A B S T R A C T

Background: The immune system of humans is strongly affected by the processes of aging and what is called immunosenescence and inflammaging. Aging processes are also associated with altered macrophage functions and their ability to undergo differential activation. As a result, the risk of macrophage-related disorders like atherosclerosis is increased in the elderly.

Methods: Human monocyte-derived macrophages obtained from young and elderly healthy volunteers were stimulated with either lipopolysaccharide (LPS) or interleukin-4 (IL-4), and the expression of classical and alternative activation markers was assessed. The concentrations of nitric oxide (NO), reactive oxygen species (ROS) and IL-1β were measured in addition to the expression of genes and relevant proteins of inducible nitric oxide synthase, IL-1β, arginase-1 and suppressor of cytokine signaling-1.

Results: We showed that the macrophages isolated from the young generally demonstrated higher responsiveness to introduced stimuli and balanced the classical activation state. The cells from the elderly showed stronger generation of nitric oxide (NO) and reactive oxygen species (ROS), which contribute to stress and damage reactions.

Conclusions: The changes observed in the macrophages isolated from the elderly indicate that these cells could contribute to the development of metabolic disorders like atherosclerosis and diabetes. The cells from the young volunteers are less likely to present such properties.

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Introduction

The alterations associated with aging in humans also affect the immune system, leading to impairment of its functions in a process called immunosenescence [1–3]. Among others, the most strongly pronounced is an increased mild inflammatory state, called inflammaging [3–5]. Immunosenescence contributes to more frequent infections, increased risk of tumorigenesis and auto-immunological disorders [1,2]. Most publications focus on acquired immunity impairment, however, recent papers also indicate that innate immunity is affected by aging [1,6]. Presently, it is known that the count of macrophages in the peripheral blood of the elderly does not decrease [3], but their phenotypic properties like phagocytosis, chemotaxis and respiratory burst production are altered, leading to weakened antimicrobial activity [3,6,7].

The macrophages in humans do not form a homogenous population. They undergo different activation processes depending on the acting stimuli and often demonstrate contrasting functions [8,9]. The macrophages can be divided into two major groups: the classically activated macrophages (M1) and the alternatively activated ones (M2). These groups *in vitro* cover in total five subpopulations: M1a, M1b, M2a, M2b and M2c. Both M1 and M2 macrophages produce characteristic marker substances. The M1 macrophages produce significant amounts of nitric oxide (NO) resulting from increased expression of inducible nitric oxide synthase (iNOS), which is necessary for efficient antimicrobial activity. They also secrete large quantities of a major pro-inflammatory cytokine, interleukin-1β (IL-1β) [10] and reactive oxygen species (ROS) [9]. In contrast, the M2 macrophages demonstrate elevated activity of arginase-1 (Arg1), which is essential in the final stages of inflammatory state quenching [11]. One of the regulators of macrophage activation is the suppressor of

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cytokine signaling-1 (SOCS1); its presence is necessary for upholding the M2 phenotype [12,13]. Its expression in M1 macrophages also reduces their pro-inflammatory response [13].

Different processes of activation are crucial for atherosclerosis and other diseases associated with inflammation and aggravate with aging. The aforementioned diseases are often a consequence of genetic basis, but also are brought by alterations in immune system properties. However, the changes in macrophage activation during aging are not well established and require further investigations, which was the aim of the given study.

Materials and methods

Cell cultures and stimulation

The study was accepted by the Bioethical Committee of the Medical University of Silesia in Katowice, Poland. The investigation conformed to the principles of the Declaration of Helsinki. Human whole blood samples were taken from 21 young and 21 elderly healthy volunteers (Table 1). Monocytes were isolated from the peripheral blood monocytic cells using methods described by Okopień et al. and Flø et al. [14,15]. The mean purity of the obtained monocytes determined by labeling with anti-CD14 fluorescent antibody was 92%. The cultures (1×10^6/ml) were incubated for 48 h in 24-well dishes with medium (control), medium with lipopolysaccharide (LPS) (1 μg/ml) or medium with IL-4 (50 ng/ml) (Sigma–Aldrich Co., USA). Finally, the cells and supernatants were separated, collected and frozen.

Assessment of cell viability

The viability of the cells was examined using the MTT conversion test according to the method developed in the literature [16,17].

Assessment of NO production

The concentration of NO was measured according to the instruction of the manufacturer of the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company, USA). Briefly, initially the cell culture supernatants were diluted 2-fold. Next, the nitrates present in 80-μl samples were reduced into nitrites with the use of nitrate. The nitrites present in the samples were detected using the Griess reaction. After 10 min of incubation, the absorbances at 540 nm were read in the microplate reader (xMark Microplate Spectrophotometer, Bio-Rad Laboratories Inc., USA). Fresh culture medium was used as a blank. The calibration curve based on serial dilutions of sodium nitrite standard was used for calculations. The medium was not changed every time the sample was taken.

Assessment of ROS production

The concentration of ROS in the culture media was measured using the Nitro Blue Tetrazolium (NBT) Reduction Kit (Sigma–Aldrich Co., USA).

Assessment of IL-1β secretion

The concentration of the cytokine was determined by ELISA kit according to the manufacturer's instructions (R&D Systems Inc. assay kit, USA).

Assessment of IL-1β, SOCS1, iNOS and Arg1 messenger ribonucleic acid (mRNA) expression

The expression of the genes was measured by the reverse transcription-quantitative polymerase chain reaction (RT-PCR) method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene. Total RNA was extracted with TRIzol reagent (Sigma–Aldrich Co., USA) according to a previously developed method [18]. Reverse transcription was performed using the Affinity Script QPCR cDNA Synthesis Kit (Agilent Technologies, USA). RT-PCR was performed using SYBR Green QPCR Master Mix with Low ROX (Agilent Technologies, USA). PCR primers used in this study are listed in Table 2.

Assessment of SOCS1 and Arg1 protein expression

The expression of proteins was analyzed with the SDS-PAGE/Western blot method. The concentrations of proteins were assessed using Bradford's reagent [19]. Antibodies for Arg1 and SOCS1 were from Sigma–Aldrich Co. (USA). Antibody for β-actin (reference protein) was from Thermo Scientific (USA). Secondary antibody (1:10,000) was conjugated with horseradish peroxidase (Amersham GE Healthcare, GB). The chemiluminescence was analyzed densitometrically.

Statistical analysis

The normality of continuous variable distributions was checked with Shapiro–Wilk's test. Significant differences between experimental groups were determined using the one-way ANOVA

Table 1
The characteristics of study participants.

	Population	
	Young	Elderly
Number of participants	21	21
Age [years]	28.3 ± 2.4	63.5 ± 3.5
Men/Women	11/10	12/9
Smokers	3 (14.3%)	4 (19.0%)
Body mass index [kg/m^2]	19.6 ± 1.1	21.3 ± 1.2
Total cholesterol [mg/dl]	165.4 ± 17.1	173.1 ± 15.9
LDL cholesterol [mg/dl]	104.1 ± 11.3	110.2 ± 13.2
HDL cholesterol [mg/dl]	47.4 ± 4.2	43.1 ± 3.9
Triglycerides [mg/dl]	116.2 ± 19.3	124.3 ± 15.5
Fasting glucose [mg/dl]	82 ± 8	90 ± 7
HbA$_{1c}$ [%]	5.3 ± 0.3	5.6 ± 0.5
Thyroid-stimulating hormone [IU/l]	1.7 ± 0.8	2.1 ± 0.4
Systolic blood pressure [mmHg]	122 ± 11	125 ± 14
Diastolic blood pressure [mmHg]	65 ± 4	71 ± 4
Hemoglobin [g/dl]	13.9 ± 1.2	13.3 ± 1.1
Leukocytes [1/μl]	5650 ± 1330	7204 ± 983
Blood platelets [1/μl]	255 ± 66	311 ± 132
Alanine aminotransferase [IU/l]	24 ± 14	28 ± 11
Aspartate aminotransferase [IU/l]	16 ± 7	20 ± 8
Creatine kinase [IU/l]	122 ± 22	131 ± 25
C-reactive protein [mg/l]	1.9 ± 0.9	2.2 ± 1.3

The results are presented as mean values with standard deviations.

Table 2
Sequences of primers used in the RT-PCR.

Gene	Sequences of primers
IL-1β	Forward: TCCCCAGCCCTTTTGTTGA Reverse: TTAGAACCAAATGTGGCCGTG
iNOS	Forward: CCATGTCTGGGAGCATCAC Reverse: GCATACAGGCAAAGAGCACA
SOCS1	Forward: GGAAGGAGCTCAGGTAGTC Reverse: AGCTTCGACTGCCTCTT
Arg1	Forward: GACCCTGGGGAACACTACATT Reverse: GTGCCAGTAGCTGGTGTGAA
GAPDH	Forward: GAAGGTGAAGGTCGGAGTC Reverse: GAAGATGGTGATGGGATTTC