The Journal of Immunology

RESEARCH ARTICLE | JULY 01 2005

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J Immunol (2005) 175 (1): 237–245.
https://doi.org/10.4049/jimmunol.175.1.237

Spermine, a Natural Polyamine, Suppresses LFA-1 Expression on Human Lymphocyte

Kuniyasu Soda,[1]* Yoshihiko Kano,* Takeshi Nakamura,* Keizo Kasono,† Masanobu Kawakami,† and Fumio Konishi*

Natural polyamines, spermine, spermidine, and putrescine, play a pivotal role in the regulation of gene expression; therefore, the age-dependent decreases and the disease-dependent increases in polyamine synthesis suggest a possible contribution of polyamines to the age-related and disease-associated changes in cellular function. In this study, we examined the effects of polyamines on the cellular function and the expression of adhesion molecules on human PBMCs from healthy volunteers. Flow cytometry revealed that PBMCs cultured with spermine decreased mean fluorescent intensities (MFIs) of CD11a and CD18 in the lymphocyte light-scattered region, but not in the monocyte region. This suppression was observed in a dose- and time-dependent manner and found nonspecifically on all cell subsets we tested ($CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, $CD45RA^+$, $CD45RO^+$, $CD4^+CD45RA^+$, $CD4^+CD45RO^+$, $CD8^+CD45RA^+$, $CD8^+CD45RO^+$). The decreases of CD11a and CD18 MFIs were accompanied by the decrease in adherent capacity of PBMCs to HUVECs. Spermine did not hinder cell activities or cell viability. Among 42 healthy volunteers (mean, 49.5 years old; from 26 to 69), blood spermine levels inversely correlated with the CD11a MFIs of cells in the lymphocyte region ($r = -0.48$; $p = 0.001$), but not with those in the monocyte region. The effects of spermidine seemed weaker than those of spermine, and blood spermidine levels had no correlation with CD11a MFIs of the lymphocyte region. Putrescine had no effect on the expressions of membrane molecules. Polyamines, especially spermine, decrease LFA-1 (CD11a/CD18) expression on human lymphocyte and adhesion capacity of PBMCs to HUVECs. ***The Journal of Immunology,*** **2005, 175: 237–245.**

The natural polyamines, putrescine, spermidine, and spermine, are polycations found in every living cell in high micromolar to low millimolar quantities (1). Polyamines not only stabilize cellular nucleic acids and membranes, but also play a pivotal role in the regulation of gene expression both by altering DNA structure and by modulating protein kinases and transcription factors (2–5). When young, growing cells have increased polyamine synthesis and higher intracellular concentration; however, with aging, the enzymatic activities for polyamine synthesis and the ability to synthesize polyamine decrease (6, 7). The age-dependent decrease in polyamine synthesis suggests the possible contribution of polyamines to the age-associated changes in the cellular functions.

It is well known that the aging is accompanied by the progressive increase in a proinflammatory status (8, 9). Several predisposing factors represent the biologic background favoring the susceptibility to age-dependent inflammatory diseases (10–13). One of them is age-dependent increases in the expression of several adhesion molecules (13, 14). Adhesion molecules are membrane-bound proteins that play a crucial role not only in cell adhesion but also in mediating cell-cell interactions, and their enhanced expression results in an augmented capability of cell adhesion and activation (15, 16). The adhesion and activation of immune cells mediates inflammation (17, 18). Therefore, the sustained overexpression of adhesion molecules of the immune cells, such as PBMCs, in the elderly is considered to contribute the progression of chronic inflammatory diseases. In fact, not only the expression of adhesion molecules in immune cells but also their corresponding cellular function (i.e., cell adhesion) was enhanced in the aged subjects (12, 13).

Polyamines are synthesized in cells; however, recent studies have brought to light the importance of polyamines from extracellular sources (19, 20). Cells, including monocyte and lymphocyte, absorb polyamines from their surroundings (21, 22), and the majority of the circulating polyamines are contained in erythrocyte and leukocytes (23). Increased polyamine concentrations in leukocytes (including monocytes and lymphocytes), as well as erythrocytes, of patients suffering from cancer and other diseases in which polyamine synthesis is active have been reported (24, 25). Because circulating PBMCs contribute the immune functions of the whole body, the modulation of their cellular functions may directly affect the systemic immune functions.

The increases in blood polyamine concentrations of patients suffering from cancer are dramatically reduced when tumors are removed (24). In contrast, suppressed immune functions often observable in cancer patients are sometimes normalized when a tumor is removed (26–29). Deficient immunity against cancer cells in cancer patients is sometimes attributed to a decreased adhesion of immune cells (30, 31). And the decreased cellular adhesion is mainly attributed to the decrease in the expression of adhesion molecules (32, 33).

Age-dependent decrease in polyamine synthesis and the increased blood polyamine levels in cancer patients suggest the possible involvement of polyamines in the cellular functions and the expression of adhesion molecules of immune cells. In this study, we test the effects of polyamines on the expression of adhesion molecules in PBMCs and the corresponding cellular functions by adding polyamines directly in vitro, and examine the correlations

Departments of *Surgery and †Internal Medicine, Omiya Medical Center, Jichi Medical School, Saitama, Japan

Received for publication June 23, 2004. Accepted for publication April 15, 2005.

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[1] Address correspondence and reprint requests to Dr. Kuniyasu Soda, Department of Surgery, Omiya Medical Center, Jichi Medical School, 1-847 Amanuma, Saitama-city, Saitama 330-0834, Japan. E-mail address: soda@omiya.jichi.ac.jp

0022-1767/05/$02.00

among age, blood polyamine levels, and the expression of adhesion molecules in human PBMCs.

Materials and Methods

Isolation and preparation of PBMCs

Human PBMCs were obtained from healthy volunteers, and were isolated by density gradient centrifugation, using separate-L (Muto Pure Chemicals). Isolated PBMCs were washed three times by PBS(−). PBMCs were resuspended in RPMI 1640, supplemented with 10% heat-inactivated human serum AB (Wako Pure Chemicals), 0.1% L-glutamine, and 0.01% penicillin-streptomycin (Invitrogen Life Technologies) at a concentration of 7×10^5 cells/ml. Freshly prepared spermine (spermine tetrahydrochloride), spermidine (spermidine trihydrochloride), or putrescine (1,4-butanediamine dihydrochloride) (Wako Pure Chemicals) was added to human PBMCs at various concentrations. All of the PBMCs cultured in the culture plate were then harvested by gentle pipetting.

In some experiments, PBMCs cultured overnight in polyamine-containing culture medium were collected, washed three times with 50 ml of PBS(−) to remove extracellular polyamines, and resuspended in polyamine-free culture medium.

Determination of polyamine concentrations in whole blood and cultured PBMC

Human PBMCs were cultured with polyamines for 24 or 72 h and harvested. After washing three times by abundant PBS(−) to remove extracellular polyamines, PBMCs were resuspended in PBS(−) at a concentration of 7×10^6 cells/ml. After cell degradation by sonication and a freeze-thaw cycle, samples were added to 5% sulfosalicylate at a ratio of 1:1. For determination of whole-blood polyamine concentrations, a 0.5 ml of heparinized whole blood were diluted with 1.0 ml of distilled water, and samples were added to 5% sulfosalicylate. A 1-ml sample of supernatant was used for polyamine measurement by HPLC.

Postcolumn derivatization of the eluted polyamines with *o*-phthalaldehyde yielded fluorescent products detectable at a wavelength of 455 nm after excitation at 340 nm (FP-920; JASCO).

Correction index to eliminate the differences in the cell amount among individuals

Because the majority of the polyamines are contained in blood cells, we used the following formula to eliminate the differences in the hematocrit and the blood cell count among individuals. Because the polyamine concentration in leukocytes is 200–800 times higher than that in erythrocytes, the effects of the leukocyte cell count were also taken into consideration (23).

Corrected blood polyamine = whole-blood polyamine concentration (μM)/((hematocrit × correction index)/100)

Correction index = 1 + ((leukocyte count (/μl) × *X*)/(erythrocyte count (/μl))

X in the correction index is 200 or 400 for spermidine, and 500 or 800 for spermine.

Flow cytometric analysis

PBMCs cultured with polyamine or freshly isolated from healthy volunteers were fixed in 2% paraformaldehyde for 10 min at 4°C. Abs (5 μl per 500,000 cells) were added to cells suspended in PBS(−) containing 0.1% BSA. The following Abs were used for single-color analysis: FITC-conjugated anti-human CD11a, PE-anti-CD11b, PE-anti-CD11c, FITC-anti-CD18, PE-anti-CD31, PE-anti-CD49d, PE-anti-CD49e, PE-anti-CD54, and FITC-ViaProbe (BD Pharmingen); rhodamine 1 (RD1)[2]-anti-CD3, PE-cyanin 5 tandem anti-CD4, PE-cyanin 5 tandem anti-CD8, RD1-anti-CD19, and RD1-anti-CD45RA (Beckman Coulter); and FITC-anti-CD11a and PE-anti-CD45RO (Caltag Laboratories). For single-color analysis, $3–5 \times 10^4$ cells gated in the lymphocyte and monocyte light-scattered regions were analyzed, and for two- and three- color analyses, $3–7 \times 10^4$ cells gated in the same regions were analyzed. A FACScan flow cytometer (FACSCalibur) with analysis software (CellQuest) was used for analyses.

For the experiment to evaluate the correlation between blood spermine levels and CD11a mean fluorescent intensities (MFIs) of PBMCs in healthy volunteers, 100 μl of whole blood was incubated with 20 μl of FITC-conjugated anti-human CD11a at 4°C for 30 min. RBC were lysed with lysing solution (0.826% (w/v) NH_4Cl, 0.1% (w/v) $KHCO_3$, 0.0037% (w/v) EDTA). The cells were washed with washing solution (0.2% (w/v) BSA, 0.1% (w/v) NaN_3, 10% PBS). A FACSCalibur flow cytometer (BD Biosciences) was used to quantify CD11a expressions in the area of lymphocyte and monocyte populations.

Preparation of HUVEC

Human umbilical cords were obtained from healthy pregnant women who consented to provide them immediately after parturition. The umbilical vein was cannulated, and the vessel was perfused two to three times with PBS(−) to remove any blood cells. The distal end of the cord was cross-clamped, and the vein was filled with 0.2% (w/v) collagenase (Worthington Biochemical) in PBS(−), and then the proximal end was clamped. After incubation at room temperature for 20 min, the clamps were removed and the contents of the vein were recovered by perfusion with PBS(−). The cells were collected by centrifugation at 400 × *g* (600 rpm) for 5 min at 4°C, and the pellet was suspended in a medium for an HUVEC culture, which was composed of an MCDB 131 medium (Sigma-Aldrich) supplemented with 10% of FBS (Invitrogen Life Technologies), 1% penicillin-streptomycin, and 10 ng/ml human fibroblast growth factor-basic (PeproTech). The cells were cultured at 37°C in an atmosphere of 5% CO_2 in a culture plate with a 10-cm diameter. HUVECs were used between the third and fourth passages for the experiments.

Assay of PBMCs' adhesion to HUVECs

HUVECs were seeded in 96-well plates and grown to confluency. Human PBMCs cultured with or without polyamines were washed thoroughly, and resuspended to 5×10^6 cells/ml in RPMI 1640 with 10% FBS. The fluorescent probe 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) (Molecular Probes) was added to the PBMCs at a final concentration of 5 μM, and PBMCs were incubated at 37°C for 1 h. PBMCs (100 μl at 1×10^7 cells/ml) were added to each well of cultured HUVECs. After incubation at 37°C for 30 min, the culture plate was placed upside down at room temperature for 30 min. Fifty microliters of 50 mM Tris-HCl and 1% SDS was added, and the fluorescence intensity was measured at a wavelength of 538 nm after excitation at 485 nm. For the estimation of cell number per well, a serial dilution of PBMCs labeled with BCECF-AM were seeded into 96-well plates, and the fluorescence intensity of each well was measured.

Cell viability and cell activation assay

PBMCs in culture medium were cultured 3–4 h in the presence of 0.35 mg/ml MTT (Sigma-Aldrich). After color changed, the supernatant was removed, and 100 μl of isopropyl alcohol containing 12 M HCl was added to the wells. The absorption at 570 nm and 690 nm was determined with an automated enzyme immunoassay analyzer (Titertek Multiskan; Flow Laboratories).

For lymphocyte activation tests, PBMCs that had been cultured overnight with 100 μM spermine were washed thoroughly (three times with 50 ml of PBS(−)), and the cells were cultured in RPMI 1640 with 10% FBS containing 20 μg/ml PHA (Difco Laboratories) or 7 μg/ml Con A (Sigma-Aldrich) for 64 h. PBMCs were cultured for an additional 8 h after adding [^{3}H]thymidine (Amersham Biosciences). Radioactivity in PBMCs was measured (LKB-1205; LKB; Skatron). A stimulation index was calculated using the radioactivity of stimulated PBMCs divided by that of nonstimulated PBMCs.

Statistical analysis

Data are expressed as mean ± SE of several experiments indicated as *n* (number). In addition to the actual figures, some of the data from the analyses of flow cytometry, adhesion to HUVECs, cytotoxic activities, and cell activation by lectins were expressed as a percentage compared with that of control cells, i.e., those cultured in 0 μM polyamine in each experiment. Group means were compared by paired *t* test, and a *p* value of <0.05 was considered significant. The relationship between whole-blood polyamine concentrations and the expression of CD11a was examined by means of linear regression analysis.

Results

Cultured human PBMCs can take up polyamines

Determination of cellular polyamine concentrations using HPLC revealed that when cells were cultured with spermine, spermidine, or putrescine, only the concentration of the exogenously added polyamine was elevated (Fig. 1). The concentration of spermine in

[2] Abbreviations used in this paper: RD1, rhodamine 1; MFI, mean fluorescent intensity; BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester.

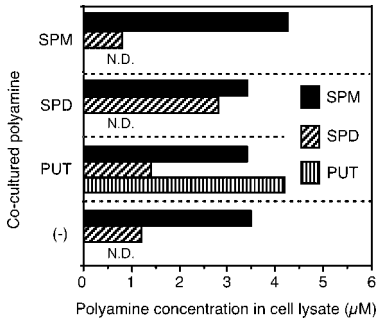

FIGURE 1. Cultured human PBMCs can take up polyamines. Cells cultured overnight with one of the three polyamines (indicated along the ordinate) were washed thoroughly with PBS and suspended to 7×10^6 cells/ml in PBS. Concentration of polyamines was measured by HPLC. The data represent the actual figures of one experiment in which cells were cultured for 20 h with 500 μM of one of the polyamines. The bars indicate the concentrations in cell suspension. The findings were confirmed by two additional experiments. SPM, Spermine; SPD, spermidine; PUT, putrescine; (−), no polyamine; N.D., not detectable.

cells cultured overnight with 500 μM spermine was 122.3 ± 1.2% (significantly higher than control at $p = 0.003$; $n = 3$) and the concentration of spermidine in cells cultured overnight with 500 μM spermidine was 251.3 ± 28.0% ($p = 0.033$; $n = 3$), when compared with those of cells cultured with no polyamine. Interestingly, the concentration of spermidine in cells cultured with spermine tended to decrease (68.9 ± 1.4%; $n = 3$). The changes in the composition of the intracellular polyamines did not differ between cells cultured overnight and those cultured for 72 h. Namely, only the concentration of the exogenously added polyamine was elevated irrespective of the length of cell culture. Additionally, polyamine concentration of culture medium in which cells after washing to remove extracellular polyamine were cultured was under detectable levels.

Spermine and spermidine, but not putrescine, suppress the MFIs of CD11a and CD18 staining in vitro

Flow cytometric examination revealed that spermine (SPM 3 days) treatment decreased the MFI of CD11a staining of cells in the lymphocyte light-scattered region in a dose-dependent manner (Table I; Fig. 2*a*). No decrease in CD11a expression by spermine treatment was observed in a 24-h culture (SPM 1 day) (Fig. 2*b*). However, when these cells were cultured for additional 48 h in polyamine-free culture medium (SPM 1 day + Wash + 2 days), a decrease in CD11a MFI became apparent and was similar to that observed on cells cultured with the spermine for over 72 h (Fig. 2*b*). Spermine treatment did not decrease the percentage of cells expressing CD11a (Table I). Histograms revealed that the decrease in CD11a MFI was due to a decrease in the number of cells expressing bright CD11a (Fig. 3). The ratio of CD11a-bright cells in CD11a-positive cells decreased in a spermine dose-dependent manner. When assuming the ratio of the number of bright CD11a cells to the number of CD11a-positive cells in cultures without a polyamine level (0 μM spermine) of 100%, the percentage of the ratio decreased to 93.9 ± 1.2% in cultures containing 100 μM spermine ($p = 0.02$ to control; $n = 7$) and 84.6 ± 2.2% in those containing 500 μM ($p < 0.01$ to control; $n = 7$).

The effect of spermine on various membrane molecules in the lymphocyte region is shown in Table I. The MFIs for the CD18 staining of lymphocytes cultured with spermine were also decreased (Fig. 2*c*). The MFIs of other stained membrane molecules in the lymphocyte region were not changed. Although the percentage of cells expressing CD11b, CD11c, CD49d, or CD54 was not changed, the percentages of CD31-positive and CD49e-positive cells in cultures with 500 μM were slightly lower than those in cultures without the polyamine (Table I).

Unlike in the lymphocyte light-scattered region, spermine treatment did not have a significant effect on the MFIs of CD11a or CD18 staining in the monocyte region (Table II). As shown in Table II, in the monocyte light-scattered region, spermine treatment had no effect on either the MFIs of any adhesion molecules, nor the percentage of cells positive for any adhesion molecules. Spermine seemed not to be cytotoxic to PBMCs, because the percentage of cells expressing ViaProbe in whole cultured cells was not changed (99.6 ± 0.3% ($n = 3$; 100 μM) and 101.2 ± 6.2% ($n = 3$; 500 μM)).

Spermidine treatment resulted in effects similar to those observed with spermine. When compared with cells cultured with 0

Table I. *The effect of spermine on the expression of adhesion molecules in the lymphocyte region*[a]

	Spermine Concentration (μM)		
	0	100	500
MFI			
CD11a ($n = 12$)	50.7 ± 3.3	49.0 ± 3.3*	46.1 ± 3.2*†
CD11b ($n = 9$)	26.0 ± 2.6	25.2 ± 2.2	26.2 ± 2.3
CD11c ($n = 9$)	19.6 ± 1.7	20.3 ± 1.9	19.7 ± 1.8
CD18 ($n = 6$)	18.8 ± 2.9	17.5 ± 3.0	16.8 ± 2.8*
CD31 ($n = 6$)	54.1 ± 4.7	53.9 ± 4.4	52.4 ± 3.9
CD49d ($n = 6$)	114.8 ± 7.2	118.6 ± 6.7	119.5 ± 8.2
CD49e ($n = 6$)	45.2 ± 1.0	45.0 ± 1.8	45.7 ± 1.5
CD54 ($n = 6$)	35.2 ± 7.3	35.1 ± 7.2	35.0 ± 7.8
Percentage of expressing cells			
CD11a ($n = 12$)	94.7 ± 2.5	95.0 ± 2.5	94.9 ± 2.7
CD11b ($n = 9$)	6.1 ± 0.9	5.8 ± 1.0	6.4 ± 1.1
CD11c ($n = 9$)	4.1 ± 0.6	3.9 ± 0.5	3.9 ± 0.5
CD18 ($n = 6$)	93.4 ± 2.2	90.7 ± 2.4	90.9 ± 2.4
CD31 ($n = 6$)	46.2 ± 6.6	45.5 ± 6.7	45.1 ± 6.5*
CD49d ($n = 6$)	93.5 ± 1.0	93.6 ± 1.0	93.7 ± 1.0
CD49e ($n = 6$)	87.3 ± 2.7	86.3 ± 2.9	86.0 ± 2.9*
CD54 ($n = 6$)	76.3 ± 6.3	77.8 ± 6.5	77.1 ± 6.9

[a] Changes in MFI and in the percentage of cells expressing one of the membrane molecules on cells in the lymphocyte light-scattered region. Thirty to 50 thousand cells gated in the lymphocyte and monocyte light-scatter regions were counted. Data are presented as mean ± SE.
*, $p < 0.05$ vs 0 μM; †, $p < 0.05$ vs 100 μM.

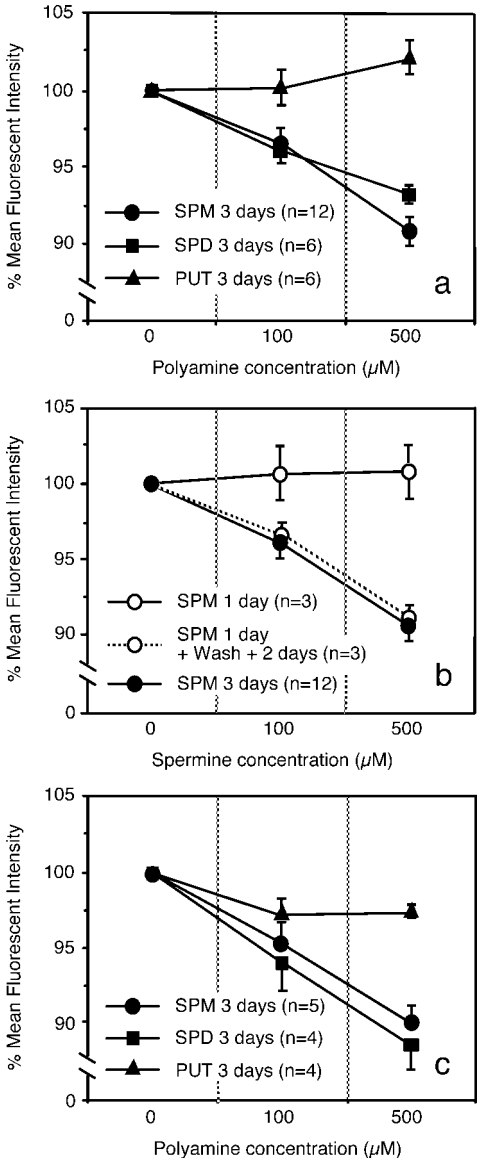

FIGURE 2. Spermine and spermidine, but not putrescine, suppress CD11a and CD18 MFIs on cells in the lymphocyte light-scattered region in a dose- and time-dependent manner. *a*, Cells cultured for 3 days (72 h) either with spermine (SPM), spermidine (SPD), or putrescine (PUT) (at the indicated concentrations) were harvested, and the expression of CD11a on cells in the lymphocyte light scattered region was measured by flow cytometry. *b*, Cells cultured either with 0, 100, and 500 μM SPM were harvested, and the expression of CD11a in the lymphocyte region was measured by flow cytometry. SPM 1 day: Cells cultured overnight (16–20 h) with SPM. SPM 1 day + Wash + 2 days: Cells cultured overnight with SPM were washed by PBS and subsequently cultured in polyamine-free culture medium for additional 48 h. SPM 3 days: Cells cultured for 72 h with SPM. c, Cells cultured for 3 days (72 h) either with SPM, SPD, or PUT (at the indicated concentrations) were harvested, and the expression of CD18 in the lymphocyte region was measured by flow cytometry. Data are mean ± SE from *n* experiments.

μM polyamine, cells treated with either 100 or 500 μM spermidine exhibited decreases in the MFIs of CD11a and CD18 staining on cells in the lymphocyte area (Fig. 2, *a* and *c*). However, spermidine did not have ability to change the MFIs of CD11c-, CD31-, CD49d-, and CD49e-stained cells. By contrast, putrescine did not change the expression of the membrane molecules tested (CD11a, CD11c, CD18, CD31, CD49d, and CD49e) (Fig. 2, *a* and *c*).

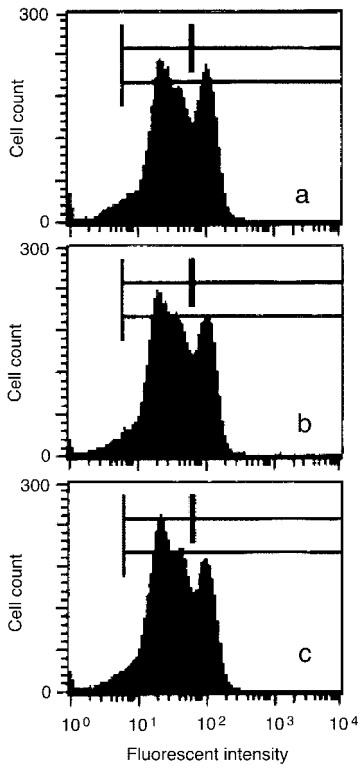

FIGURE 3. Spermine suppression of CD11a MFI was due to the decrease of cells expressing bright CD11a. The expression of CD11a on PBMCs cultured for 72 h either with 0, 100, or 500 μM spermine was measured by flow cytometry. Result is a representative of several separate experiments. *a*, Cells cultured with 0 μM spermine. *b*, Cells cultured with 100 μM spermine. *c*, Cells cultured with 500 μM spermine.

Polyamines have been shown to inhibit the synthesis of proinflammatory cytokines normally released upon stimulation (34). Such decreases should result in an attenuation of the cytokine-enhanced expression of adhesion molecules (i.e., CD31, CD54). When PBMCs were treated with 10 ng/ml LPS, a potent stimulator for proinflammatory cytokines, and cultured in the presence of spermine, the MFI of stained CD54 and the percentage of cells staining positive for CD31 and CD54 were significantly decreased (data not shown). Moreover, when PBMCs from a volunteer infected with a virus, which was revealed soon after the blood was drawn, or if cells cultured in a culture medium contaminated with microbial organisms were used for analyses, the results were quite different from those obtained in the rest of this study. Culturing the PBMCs did not itself enhance the MFI of CD11a staining, because intensities observed in PBMCs cultured for 3 days were lower (78.2 ± 13.0%; $n = 5$) than those in freshly isolated PBMCs.

We further evaluated which cell subpopulation in the lymphocyte area is responsible for the spermine-induced suppression of CD11a MFI and the decrease in the number of bright CD11a cells. Two-color staining revealed that CD11a MFI and the percentage of bright CD11a cells on cells positive for CD3, CD4, CD8, CD19, CD45RA, or CD45RO were all suppressed in a spermine dose-dependent manner (Table III). Similarly, three-color staining revealed that the percentage of bright CD11a-positive cells among $CD4^+CD45RA^+$, $CD4^+CD45RO^+$, $CD8^+CD45RA^+$, or $CD8^+CD45RO^+$ cells decreased in a spermine concentration-dependent manner (Table IV).

Table II. *The effect of spermine on the expression of adhesion molecules in the monocyte region*[a]

	Spermine Concentration (μM)		
	0	100	500
MFI			
CD11a (n = 12)	111.0 ± 12.9	108.1 ± 13.7	101.5 ± 13.8
CD11b (n = 9)	237.0 ± 38.3	214.5 ± 33.1	210.0 ± 27.0
CD11c (n = 9)	249.3 ± 38.9	266.5 ± 38.6	256.4 ± 33.5
CD18 (n = 6)	117.6 ± 23.2	100.4 ± 17.8	101.2 ± 17.4
CD31 (n = 6)	376.5 ± 105.0	381.7 ± 108.2	360.0 ± 108.9
CD49d (n = 6)	209.9 ± 70.0	209.2 ± 59.2	222.8 ± 63.4†
CD49e (n = 6)	244.0 ± 53.4	216.5 ± 41.2	225.8 ± 50.8
CD54 (n = 6)	733.9 ± 197.7	704.1 ± 199.7	698.5 ± 201.9
Percentage of expressing cells			
CD11a (n = 12)	89.7 ± 6.9	89.2 ± 6.6	89.2 ± 6.5
CD11b (n = 9)	59.2 ± 5.6	55.9 ± 4.6	58.1 ± 3.3
CD11c (n = 9)	61.6 ± 5.3	61.6 ± 4.4	61.2 ± 3.6
CD18 (n = 6)	87.8 ± 9.4	87.6 ± 9.1	90.2 ± 6.2
CD31 (n = 6)	64.9 ± 14.7	66.1 ± 13.6	65.8 ± 13.2
CD49d (n = 6)	88.5 ± 3.0	87.0 ± 3.6	86.0 ± 3.6
CD49e (n = 6)	65.9 ± 11.3	68.7 ± 9.6	68.6 ± 10.4
CD54 (n = 6)	76.8 ± 14.5	77.7 ± 13.9	77.3 ± 13.8

[a] Changes in MFI and in the percentage of cells expressing one of the listed membrane molecules on cells in the monocyte region. Data are presented as mean ± SE.

†, $p < 0.05$ vs 100 μM.

Spermine and spermidine, but not putrescine, decreased adhesion characteristics of human PBMCs to HUVECs

Fig. 4 summarizes the results of the several experiments. The percent change in the number of adherent cells was relative to the control (cells cultured without polyamine). The percentage of the adherent cell number per loaded cell number in the control group was 10.3 ± 0.2% (ranging from 2.2 to 16.7%; n = 23). Culturing PBMCs with spermine or spermidine for 3 days decreased their ability to adhere to HUVECs (Fig. 4). No such effect was observed on cells cultured with putrescine. To examine the possible effects of oxidation products from a minute amount of spermine adherent to the cell membrane or remained in the culture medium after cell wash, we tested the adherent capacity of cells cultured overnight with 500 μM spermine and found that the decreased adhesion was not observed on these cells (the *next to right-most* bar in Fig. 4). However, when these PBMCs were washed to remove extracellular polyamines and were subsequently cultured in polyamine-free culture medium for an additional 48 h, a decrease in their adherent capacity for HUVECs became apparent and comparable to those cultured for 72 h with 500 μM spermine (the *right-most* bar in Fig. 4).

MTT assay revealed that the decreased adhesion was not due to decreased cell viability or activity, because the MTT assay using whole cultured cells confirmed that spermine concentrations of up to 1 mM were not toxic for up to 80 h. Separate experiments using trypan blue exclusion also confirmed that spermine, spermidine, and putrescine concentration up to 1 mM were not toxic to human PBMCs during an 80-h treatment.

Table III. *The effect of spermine on the expression of CD11a among various subsets in the lymphocyte region*[a]

	Spermine Concentration (μM)		
	0	100	500
% MFI (actual figures)			
CD3 (n = 6)	100 (164.7 ± 18.5)	93.6 ± 1.7*	90.2 ± 2.0*†
CD4 (n = 6)	100 (143.1 ± 13.1)	92.7 ± 2.2*	90.0 ± 2.4*
CD8 (n = 6)	100 (275.4 ± 21.0)	94.6 ± 1.5*	89.4 ± 2.5*†
CD19 (n = 6)	100 (94.1 ± 7.1)	90.7 ± 4.2*	82.8 ± 5.1*
CD45RA (n = 6)	100 (214.9 ± 16.1)	92.8 ± 1.5*	89.1 ± 2.7*
CD45RO (n = 6)	100 (195.3 ± 20.4)	93.9 ± 1.6*	91.0 ± 2.0*
% Change of CD11a-bright cells (actual percentage among subsets)			
CD3 (n = 6)	100 (17.8 ± 4.1)	90.7 ± 2.7* (16.4 ± 3.9)	88.8 ± 3.7* (16.0 ± 3.9)
CD4 (n = 6)	100 (12.0 ± 1.8)	85.1 ± 4.7* (10.1 ± 1.6)	82.7 ± 5.1* (9.9 ± 1.7)
CD8 (n = 6)	100 (25.6 ± 3.4)	96.9 ± 0.9* (24.9 ± 3.5)	94.1 ± 2.0* (24.1 ± 3.3)
CD19 (n = 6)	100 (1.27 ± 0.21)	89.6 ± 8.8* (1.10 ± 0.20)	75.0 ± 9.1* (0.94 ± 0.21)
CD45RA (n = 6)	100 (35.3 ± 3.1)	90.9 ± 1.4* (32.0 ± 2.6)	91.0 ± 9.1* (32.0 ± 2.8)
CD45RO (n = 6)	100 (19.5 ± 4.4)	86.0 ± 2.3* (16.7 ± 3.7)	86.7 ± 1.8* (16.9 ± 3.9)

[a] Thirty to 70 thousand cells gated in the lymphocyte and monocyte light-scattered regions were counted. Change in MFI and in the number of CD11a-bright cells among cells expressing one of the membrane molecules listed above. The values of cells cultured with spermine were relative to the control (the percentage of the same cells cultured without spermine). Parenthetical values indicate the actual values of the MFI and the percentage of CD11a-positive cells. Data are presented as mean ± SE.

*, $p < 0.05$ vs 0 μM; †, $p < 0.05$ vs 100 μM.

Table IV. *The effect of spermine on the bright CD11a cell count in CD4$^+$ and CD8$^+$ lymphocyte*[a]

		% Change of CD11a-bright Cells (actual percentage among CD4- or CD8-positive cells)		
		Spermine concentration (μM)		
		0	100	500
CD4$^+$ cell	CD45RA$^+$bright CD11a$^+$ ($n = 6$)	100 (7.5 ± 1.0)	65.0 ± 10.3* (5.0 ± 1.2)	60.3 ± 11.9* (4.7 ± 1.3)
	CD45RO$^+$bright CD11a$^+$ ($n = 6$)	100 (16.1 ± 3.5)	98.7 ± 12.8* (12.3 ± 2.6)	80.4 ± 5.9* (12.4 ± 2.6)
CD8$^+$ cell	CD45RA$^+$bright CD11a$^+$ ($n = 6$)	100 (56.5 ± 4.1)	91.1 ± 3.2* (52.1 ± 5.2)	89.6 ± 4.4 (51.3 ± 5.6)
	CD45RO$^+$bright CD11a$^+$ ($n = 6$)	100 (28.9 ± 4.5)	91.4 ± 1.9* (26.4 ± 4.3)	90.3 ± 2.9* (25.9 ± 4.2)

[a] Thirty to 70 thousand cells gated in the lymphocyte light-scattered region were counted. Percentage change in the number of bright CD11a cells cultured with spermine was relative to the control (the percentage of the same cells cultured without spermine). Parenthetical values indicate the actual values of the percentage of cell subpopulations indicated among CD4$^+$ or CD8$^+$ cells. Data are presented as mean ± SE.

*, $p < 0.05$ vs 0 μM.

Spermine enhanced cell activation by lectins

PBMCs cultured overnight with spermine were washed thoroughly by PBS(−) to remove spermine in the culture medium, and these spermine-rich PBMCs were examined for cell activation by lectins such as PHA and Con A. PBMCs cultured with 100 μM spermine showed higher tritiated thymidine uptake upon stimulation with PHA (127.1 ± 11.6%; $n = 3$), or with Con A (131.2 ± 10.5%; $n = 3$) than those cultured with no spermine.

A previous report indicated that the presence of polyamines in culture medium supplemented with FBS inhibited mitogen-stimulated cell activation (35). Similarly, PBMCs cultured overnight in medium containing FBS and 100 μM spermine exhibited extensive decreases in cellular activities upon PHA stimulation (1.3 ± 0.2%; $n = 3$).

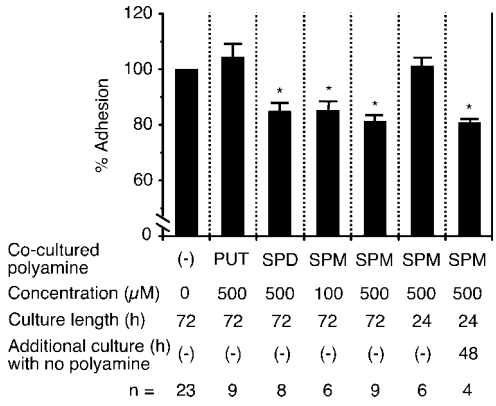

FIGURE 4. PBMCs with decreased CD11a MFI by spermine and spermidine lose their adherent capacities to HUVECs. Freshly isolated PBMCs from healthy volunteers were cultured for indicated hours (culture length) in RPMI 1640 supplemented with 10% human serum and one of the polyamines at indicated concentrations. PBMCs were then washed thoroughly to remove extracellular polyamines. The PBMCs incorporating a fluorescent probe (BCECF-AM) were cocultured with HUVECs. After removing any nonadherent cells, the numbers of adherent cells were estimated by measuring the fluorescence intensity. The bars indicate the number of adherent PBMCs relative to the control (PBMCs cultured with no polyamine: *left-most bar*). The actual percentage of the adherent cell number per loaded cell number in the control group was 10.3 ± 0.2% (ranging from 2.2 to 16.7%; $n = 23$). *Right-most bar*, Cells cultured overnight with 500 μM spermine were washed with PBS to remove extracellular spermine, and were subsequently cultured for 48 h in culture medium containing no polyamines. Cocultured polyamine: (−), no polyamine was added; SPM, spermine; SPD, spermidine; PUT, putrescine. Concentration, The concentration of polyamine cultured with PBMCs. Culture length, The length (hours) of time during which PBMCs were cultured with polyamine. Additional culture with no polyamine, The length of time (hours) during which PBMCs were cultured without a polyamine after culturing the PBMCs with a polyamine. *n*, Number of experiments. *, $p < 0.01$.

Inverse correlation between blood spermine levels and CD11a MFIs

The correlation between CD11a expression and whole-blood polyamine concentration was studied among 42 healthy male volunteers. Their ages ranged from 26 to 69, with a median age of 49.5. Among them, blood spermine levels were inversely correlated with the CD11a MFIs of lymphocytes ($r = -0.475$; $p = 0.0013$), and this inverse correlation was also confirmed when spermine concentrations were corrected by the correction formula with correction indexes of 500 ($r = -0.451$; $p = 0.002$) (Fig. 5*a*) and 800 ($r = -0.420$; $p = 0.005$). The inverse correlation between spermine concentrations and the percentages of bright CD11a cells in lymphocytes was not quite as significant ($r = -0.360$; $p = 0.0185$) as that observed between spermine levels and CD11a MFIs. Like in vitro experiments, in the monocyte light-scattered region, no correlation was found between spermine levels and CD11a MFIs ($r = -0.227$; $p = 0.150$) (Fig. 5*b*). Despite the high degree of positive correlation between spermine and spermidine concentrations ($r = 0.524$; $p = 0.0003$), no correlation was found between blood spermidine levels and the CD11a MFIs in the lymphocyte region. Age had a positive correlation with the CD11a MFIs ($r = 0.478$; $p = 0.001$) (Fig. 5*c*), but the blood spermine and spermidine levels differed widely among individuals (*d*).

At the time of blood draw and 6 mo later, all volunteers were confirmed to have no pathologies possibly contributing to enhance or influence the CD11a expression or increase blood polyamine concentrations, such as neoplastic, infectious, autoimmune, or inflammatory diseases.

Discussion

LFA-1, which consists of an α_L chain (CD11a) and a β_2 chain (CD18), mediates adhesion and migration of leukocytes in immune and inflammatory processes by binding to its ligand, ICAMs, on endothelial cells (15, 16). Increased adhesion of leukocytes to endothelial cells through LFA-1/ICAM-1 binding is needed for the activation of immune function and responsible for the progression of inflammation (17, 36, 37). Selective inhibition of LFA-1 function, and consequently, of immune function and inflammation, can be achieved using a mAb against LFA-1, or a synthetic small molecule derived from compounds mimicking ICAM-1 (18, 38–40). Therefore, LFA-1 is an important molecular target, and modulating LFA-1 function can control cellular immunity and inflammation. In this study, we provide the first evidence that spermine suppresses the MFIs of CD11a and CD18 of human lymphocyte. The result of the study is not necessarily enough to rule out the

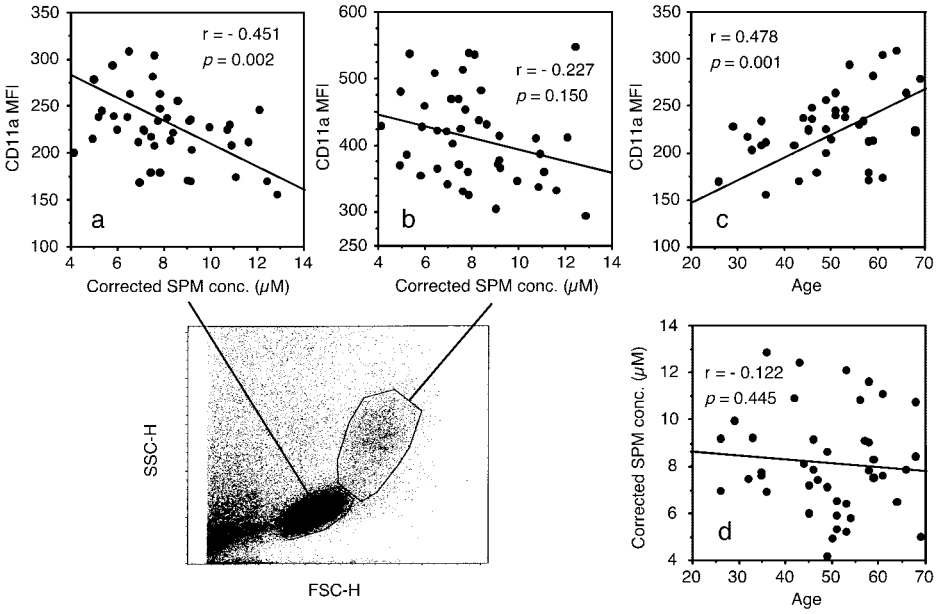

FIGURE 5. Spermine suppresses, but aging enhances the expression (MFIs) of CD11a of lymphocytes in healthy volunteers. Blood samples were collected from 42 healthy volunteers. Concentration of polyamines was measured by HPLC, and the expression of CD11a was determined by flow cytometry. Blood spermine (SPM) levels were corrected by the formula indicated in the text. Correlations among age, blood SPM levels, and CD11a expression were determined. *a*, The inverse correlation was found between CD11a MFIs of cells in the lymphocyte light-scattered region and the corrected blood SPM concentrations ($r = -0.451$; $p = 0.002$). *b*, Correlation between CD11a MFIs of cells in the monocyte light-scattered region and the corrected blood SPM concentrations ($r = -0.227$; $p = 0.150$). *c*, Age had a positive correlation with the CD11a MFIs of lymphocytes ($r = 0.478$; $p = 0.001$). *d*, Correlation between the corrected SPM concentration and age ($r = -0.122$; $p = 0.445$).

involvement of adhesion molecules other than CD11a and CD18 in the spermine-induced decrease in adhesion capacity; however, spermine-induced decrease in CD11a and CD18 expression was accompanied by the decrease in adhesion capacity of PBMCs to HUVECs, one of the very beginning and important events that provoke inflammation.

The intracellular concentrations of spermine in PBMCs cultured overnight with 500 μM spermine were 1.2–1.3 times higher than those cultured without the polyamines. The concentrations of spermine and spermidine in PBMCs from patients with a pathology in which polyamine synthesis is active have been reported to be 1.1–1.5 times higher than those from healthy subjects (25). These indicate that the increase of spermine concentrations in PBMCs cultured overnight with up to 500 μM spermine represent the in vivo condition. Spermidine decreased the expression of CD11a and CD18 and the adhesion characteristics of PBMCs in vitro. However, its effects seem to be weaker than those of spermine, because spermidine concentrations in PBMCs cultured overnight with 500 μM spermidine increased beyond the clinical settings (25). Its weak effect was further supported by the findings that blood spermidine levels had little correlation with the CD11a MFIs of lymphocytes among healthy volunteers.

The inverse correlation between the MFIs of CD11a on lymphocyte and the spermine concentrations among 42 healthy volunteers confirmed the spermine-dependent suppression of LFA-1 expression on lymphocyte. In human blood, the majority of the blood polyamines are located in blood cells, such as leukocytes and erythrocytes (23, 41). A previous report and our preliminary experiment showed that the polyamine concentrations in leukocytes reflect those in whole blood and in erythrocytes (41). Therefore, in the experiment to examine the correlation between the polyamine levels and the MFIs of CD11a staining, we used polyamine concentrations in whole blood, instead of in PBMCs, because such a close correlation had been shown. In this regard, we used the correction index to eliminate the difference in the hematocrit and leukocyte count among individuals. The inverse correlation between CD11a MFIs in the lymphocyte region and spermine concentrations was observed either when whole-blood spermine concentrations or corrected spermine concentrations were applied.

The possibility that spermine interferes with binding of fluorescent Abs to CD11a epitope can be denied, because no suppression was observed among cells cultured for 24 h with spermine, despite the findings that lysate of these cells had increased spermine concentration. Moreover, the fact that cells cultured overnight with spermine and subsequently cultured with no polyamine after removing polyamine from cultures had lower CD11a MFIs further negates the possibility.

The suppression of the LFA-1 expression and adhesion capacities by spermine and spermidine appeared not to be due to the nonspecific cellular damage mediated by toxic substances derived from the oxidation of polyamines by amine oxidases contained in human serum or FBS in the culture medium (35). MTT assays, trypan blue exclusions, and the staining of ViaProbe showed that spermine and spermidine are not toxic to cultured human PBMCs when cultured in a culture medium supplemented with human serum (34). Moreover, cells cultured with polyamines in this condition showed an increase in cell proliferative responses to lectins. And the following two results of the experiments further confirmed no or the minimal, if any, involvement of oxidation products in the expression of adhesion molecules and cellular function. First, spermine suppressed the MFIs of CD11a staining in a dose- and a time-dependent manner. Despite the fact that the concentrations of polyamines in a culture medium in which PBMCs were cultured after three cell washings were under the detectable levels, the MFIs of CD11a on PBMCs cultured overnight with spermine and subsequently cultured in a polyamine-free culture medium for an additional 48 h decreased comparably to those on cells cultured for 72 h with spermine. If the suppression were induced by the oxidation products, the CD11a MFI on lymphocyte that had been cultured overnight with 500 μM spermine and were subsequently cultured in a polyamine-free culture medium for 48 h should not be lower than those in cells cultured 72 h with 100 μM spermine or similar to those with 500 μM. Second, if the spermine cleaved to the cell membrane or remained in the culture medium after the cell washes were oxidized by oxidases contained in the FBS, and the adherent capacities of PBMCs to HUVECs were suppressed by the oxidation products, the adherent capacity of cells cultured overnight with 500 μM spermine should be suppressed in a comparable

manner to those of cells cultured 72 h with 500 μM spermine. However, decreased adhesion was not observed from cells cultured overnight with 500 μM spermine. Only PBMCs cultured in the conditions by which LFA-1 expression was suppressed had decreased adhesion capacities to HUVECs. These strongly suggest that the effects of spermine and spermidine on the expression of membrane molecules and on the adhesion capacities are considered to not be due to the oxidation products by serum amine oxidase contained in the FBS, but rather due to the increased concentration of intracellular or membrane-bound polyamines.

LFA-1 is one of the molecules involved in the lectin-induced T cell proliferation (42). However, despite the fact that spermine suppresses LFA-1 expression on lymphocytes, spermine-rich cells had a higher proliferative response to lectin. Upon stimulation with mitogen, lymphocytes resume DNA synthesis and proliferation, as well as polyamine synthesis. Polyamines are essential for cell proliferation, because inhibitors of polyamine synthesis reduce the rate of DNA replication and synthesis (43, 44). In addition to their de novo polyamine synthesis, cells, including PBMCs, can take up polyamines from their surroundings (21, 22). And one of the first effects of the lectin-induced signal is to stimulate the polyamine uptake in sufficient amounts to sustain the cell growth (45). Therefore, despite the LFA-1 suppression, the increase in the lymphocyte proliferation upon stimulation with lectin is considered due to the abundant availability of polyamines. Additionally, this observation supports the findings that spermine in the culture medium was not at all toxic to cultured PBMCs.

The concentration of tissue and blood polyamines is generally higher in the young and growing than in older subjects (7, 46, 47), and aging is associated with a progressive increase in the proportion of cells expressing high levels of LFA-1 (13, 14). The age-dependent increase in LFA-1 expression is found to be due to the increased percentage of bright CD11a among a certain cell subset (48). The results of our in vitro study seemed to demonstrate our initial hypothesis that the age-dependent decrease in the intracellular polyamine concentrations contributes to the age-dependent increase in LFA-1 expression. However, the suppression of CD11a expression is not confined to any single-cell subpopulation, yet it was observed nonspecifically in all cell subpopulations we tested. Recent studies suggest the involvement of changes in the DNA methylation pattern in the mechanisms of aging (49–51). And the age-dependent increase in the LFA-1 expression is considered due to the age-dependent methylation change (51, 52). Because the effects of polyamines on the LFA-1 expression are different from those of aging, the mechanisms by which polyamine affects LFA-1 expression seem different from those associated with age-dependent methylation change.

The trend of a slight decrease in the blood spermine concentration with aging was observed among 42 volunteers; however, the concentrations of either spermine or spermidine did not have an inverse correlation with age (53). Blood cells can take up polyamine from the surrounding environment, and the largest source in the body is the intestinal lumen (19–22). Because our volunteers had no pathologies that influence blood polyamine levels, the wide differences in polyamine levels among individuals reflect the differences in polyamine intake from diets and the environment for polyamine synthesis in the body. This difference in polyamine intake, as well as changes in the environment for polyamine synthesis, directly affects blood polyamine levels (20, 24, 25). These suggest possible therapeutic or preventive implications of polyamine against age-dependent increases in chronic inflammatory condition.

Disclosures

The authors have no financial conflict of interest.

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