SPERMINE AND SPERMIDINE INDUCE SOME OF THE IMMUNE SUPPRESSION OBSERVED IN CANCER PATIENTS

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Abstract

The immune function in cancer patients is suppressed, however, the factors that promote this suppression are still obscured. We hypothesized that polyamine is one of the causative factors of the immune suppression, as polyamine levels in blood and tissues are increased in cancer patients. In this study, the effects of increased polyamine concentration on cellular immunity were investigated. For this, human peripheral blood mononuclear cells (PBMCs) from volunteers were cultured in RPMI-1640 medium supplemented with 10% human serum. Freshly prepared polyamine was added to cultured PBMCs at various concentrations, and their effects on the production of tumor necrosis factor (TNF), adhesion capacities, and cytotoxic activities upon stimulation with interleukin-2 were examined. Spermine and spermidine, but not putrescine, immediately...
suppressed lipopolysaccharide-stimulated TNF production in a dose-dependent manner. PBMCs cultured either with spermine or spermidine decreased their ability to adhere to plastic. The decreases in adhesion capacity were observed in a dose-dependent manner, but were not observed in PBMCs cultured for 24 hours with spermine and spermidine. PBMCs cultured overnight with spermine and spermidine decreased their cytotoxic activities. Spermine and spermidine did not affect the cell viability. These suggest that the increased polyamine levels in cancer tissues and in blood may be one of the factors that hinder the immune function of cancer patients.

Introduction

The immune suppression is often observed in cancer patients. Many alterations in immune function, such as impaired cytokine production, attenuated adhesion characteristics of peripheral blood mononuclear cells (PBMCs), and decreased cytotoxic activities of killer cells, are reported (Heriot, Marriott et al., 2000; Shirai, Watanabe et al., 1990; Blindar' and Zubrikhina 1998; Hersh, Gschwind et al., 1982). The mechanism of the immune suppression is considered due to the increased production of the immunosuppressive factors from cancer tissue; however, these factors are not fully characterized yet.

The natural polyamines putrescine, spermidine, and spermine are ubiquitous polycations found in every living cell. Their biosynthesis is active in cells of which growth is active, such as cancer cells, and the elevated levels of polyamine in cancer tissues in comparison to those of normal surrounding tissues have been reported (Loser, Folsch et al. 1990; Linsalata, Russo et al., 1993; Canizares, Salinas et al., 1999). It has been believed that polyamines are synthesized in cells as needed, however, recent studies have brought to light the importance of polyamines from extracellular sources. The polyamine concentration of red blood cells and white blood cells is increased in cancer patients, and the increase was considered due to the polyamine uptake by blood cells from cancer cells (Nishiguchi, Tamori et al. 2002; Cooper, Shukla et al., 1978; Moulinoux, Quemener et al., 1989). Similarly, cultured white blood cells have been shown their capability of taking up polyamines from culture media (Zhang, Borovikova, et al. 1999; Walters and Wojcik 1994).
The increase in polyamine concentrations in leukocyte of cancer patients dramatically reduced when tumors are removed (Uehara, Shirakawa et al., 1980). One interesting finding is that altered immune functions are sometimes 'normalized' when a tumor is removed (Rampone, Rampone et al. (2001). Here, we examine the immunomodulatory effects of polyamines on peripheral blood mononuclear cells (PBMCs) by adding polyamines directly in the absence of enzymes that oxidize polyamines and produce cytotoxic substances.

Materials and Methods

Isolation and preparation of peripheral blood mononuclear cells (PBMCs)

Human PBMCs from healthy volunteers were isolated by density gradient centrifugation using separate-L (Muto Pure Chemicals Co. LTD., Tokyo, Japan). PBMCs were re-suspended in RPMI-1640, supplemented with 10% heat-inactivated human serum AB (Wako Pure Chemical Industries LTD., Osaka, Japan), 0.1% L-glutamine (Invitrogen Corp., CA, USA), and 0.01% Penicillin-Streptomycin (Invitrogen). Spermine (spermine tetrahydrochloride), spermidine (spermidine trihydrochloride), or putrescine (1,4-butanediamine dihydrochloride) (Wako Pure Chemical) was added to human PBMCs at various concentrations.

TNF induction and assay

Escherichia coli endotoxin (LPS; Sigma Chem. Co., MO, USA) was added to a final concentration of 100 ng/ml to stimulate TNF production from cultured human PBMCs. Freshly prepared spermine, spermidine, or putrescine was added to human PBMCs cultured in 96-well at various concentrations 1 hour before LPS stimulation. Four hours after LPS stimulation, supernatants were harvested. TNF levels were estimated by enzyme-linked immunosorbent (ELISA) assay, using a human TNF-α ELISA kit (Biosource International Inc., CA, USA). Fifty µl of incubation buffer was added to each well that was pre-coated with anti human TNF, and then 200 µl of supernatants and standard hTNF solution was added to each well in triplicate. After 2 hours of incubation at room temperature, the ELISA plate was washed 3 times with wash reagent, 150 µl of pre-diluted anti-human TNF reagent was added to each well, and the
plate was incubated at room temperature for 2 hrs. Following another plate wash, 100 µl of substrate reagent was added to each well for the enzymatic color reaction. After the reaction was stopped with a stop solution, the plates were read using a single wavelength (450 nm) on an automated EIA analyzer (Titertek Multiskan, Flow Laboratories Inc).

Adhesion and Cell Viability Assays

Freshly prepared spermine, spermidine, or putrescine at various concentrations was added to human PBMCs cultured in 96-well. PBMCs were cultured for 24 to 80 hours and were gently washed 3 times with PBS(-) using a multi-pipetter, and non-adherent cells were removed. To estimate the cell viability and activities of whole cultured cells, the culture plate was centrifuged, and culture medium was gently aspirated. 0.35 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma Chem. Co.) was added to the culture media and cultured for 3 to 4 hours. After color change was confirmed, supernatant was removed, and 100 µl isopropyl alcohol containing 12 M HCl was added to the wells. The absorption at 570 nm and 690 nm was determined with an automated EIA analyzer (Titertek Multiskan, Flow Laboratories Inc). Trypan blue exclusion was also used to evaluate cytotoxicity of polyamines.

Cytotoxicity Assay

The cytolytic activity of Lymphokine-Activated-Killer (LAK) cells was measured in a standard 3.5-hour 51Cr-release assay using Daudi cells as target cells. After removing the extra-cellular polyamines by washing, PBMCs were re-suspended in RPMI-1640 with 10% fetal bovine serum (FBS) at 1 x 10⁶ cells/ml. Recombinant human interleukin-2 (IL-2) was added to the PBMCs at a concentration of 25 units/ml, and the cells were cultured for 72 hours. Daudi cells labeled with 51Cr were co-cultured with PBMCs for 3.5 hours (E/T ratio = 1), then radioactivity in the supernatant was measured.

Statistical analysis

The data from the analyses of TNF production in culture medium, adhesion to plastic plate, and cytotoxic activities were expressed as a percentage 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 less than 0.05 was considered significant.
Results

Spermine and spermidine, but not putrescine, suppressed TNF production upon LPS stimulation

To study the effect of spermine, spermidine, and putrescine on TNF production, human PBMCs were stimulated with LPS (100ng/ml). Pretreatment (60 min before LPS challenge) with spermine and spermidine decreased TNF production of PBMCs in a dose-dependent manner. Fifty μM of spermine and spermidine effectively suppressed TNF synthesis (p<0.05). However, putrescine did not decrease TNF production even when cells were cultured with 500 μM of putrescine (Figure 1). Only the highest concentration of putrescine (1 mM) slightly decreased TNF production (p=0.041), however, the inhibitory effects of putrescine on TNF synthesis seemed not apparent. Spermine and spermidine did not interfere with ELISA detection of TNF as evidenced by the fact that TNF standard curves measured in the presence of spermine and spermidine were comparable to TNF standard curve generated in their absence.

Spermine and spermidine, but not putrescine decreased adhesion characteristics

Three days culture with spermine effectively weakened the capacity of human PBMCs to adhere to plastic plate, in a dose-dependent manner (Figure 2). The decrease in adhesion characteristics by spermine was not due to decreased cell viability or activity. When a 96-well culture plate, in which PBMCs were cultured for 72 hours with polyamines, was centrifuged to adhere all cultured cells to the culture plate, the experiments of the metabolism of MTT showed no decrease in cell activities on PBMCs (Figure 2). Separate experiments using trypan blue exclusion also confirmed that spermine concentrations of up to 1mM were not toxic for up to 80 hours (data not shown).

Seventy-two hours culture with spermidine also weakened the capacity of human PBMCs to adhere to plastic plate, in a dose-dependent manner (Figure 3). The effects of spermine on the decrease of adhesion characteristics seemed more potent than those of spermidine. However, no such effect was observed with putrescine, even when PBMCs were cultured with 1 mM of putrescine for up to 80 hours (Figure 3).
Figure 1. (LEFT) Spermine and spermidine inhibit TNF synthesis from LPS-stimulated human PBMCs. Human PBMCs were exposed to the concentration of polyamine as indicated for 4 h after LPS stimulation. Data shown are mean ± SD.

Figure 2. (RIGHT) Spermine weakened the capacity of human PBMCs to adhere to plastic plate. Human PBMCs were cultured in 96-well plates for 72 h with various concentrations of spermine. Each well was gently washed 3 times with PBS(−), using a multi-pipette, and non-adherent cells were removed. To determine the cell viabilities of whole cultured PBMCs, the culture plate was centrifuged and whole cultured cells adhered to the bottom of the culture plate. The numbers of PBMCs in each well were estimated by the metabolism of MTT, and results were expressed as percentage of control (no polyamine). Each point represents mean ± SD.
Figure 3. (LEFT) Spermine and spermidine, but not putrescine, weakened the capacity of human PBMCs to plastic plate. Human PBMCs were cultured in 96-well plates for 72 h with various concentrations of spermine, spermidine, or putrescine. Non-adherent cells were removed as described in Materials and Methods. The numbers of adherent cells were estimated by the metabolism of MTT, and results were expressed as percentage of control (no polyamine). Data are mean ± SD.

Figure 4. (RIGHT) Human PBMCs cultured overnight with spermine and spermidine decreased the cytotoxicities of lymphokine-activated killer cells. PBMCs cultured overnight with polyamine were washed thoroughly by abundant PBS(-) to remove the extra-cellular polyamine. Cells re-suspended in PRMI-1640 supplemented with 10 % FBS were cultured with 25 units/ml of human interleukin-2 for 72 h. Cytotoxic activities were estimated as described in Materials and Methods. The results were expressed as percentage of control (no polyamine). Data shown are mean ± SD.
Activated killer cells generated by the culture of human PBMCs with IL-2, namely LAK cells, are able to lyse autologous tumor cells that cannot be lyzed by untreated natural killer cells. LAK cytotoxicities of PBMCs cultured overnight with 100 μM or 500 μM spermine were lower ($p<0.05$) than those of cells cultured with no polyamine. The decrease of LAK activities by spermine was observed in a spermine dose-dependent manner. LAK activities of PBMCs cultured overnight with 500 μM of spermidine were lower ($p<0.05$) than those of control cells (PBMCs cultured overnight with no polyamine). However, even a 500 μM of putrescine did not decrease LAK activities (Figure 4).

**Discussion**

We showed that spermine and spermidine inhibited some of the cellular functions closely related to the anti-cancer immunity such as the production of cytotoxic cytokine, cell adhesion, and cytotoxic activities of lymphokine activated killer (LAK) cells. These effects were not due to decreased cell viability or activity as the experiment using whole cultured cells confirmed that spermine and spermidine were not toxic. In earlier studies regarding the effects of polyamines on immune functions, many immunomodulatory effects were found to be likely caused by cytotoxic substances derived from the polyamines oxidation by amine oxidases contained in the culture medium (Byrd, Jacobs et al., 1977). However, in our study, we examine the cellular function by adding polyamines directly, in the absence of enzymes that oxidize polyamines and produce cytotoxic substances.

The production of TNF upon stimulation is important for the killing of tumor cells and microbial organisms. Some reports have shown that, in cancer patients, the ability to produce several cytokines that are needed for cytotoxic activities of immune cells is impaired (Hersey, Edwards et al., 1982; Rey, Klein et al., 1983; Monson, Ramsden et al., 1986). As shown in our previous report and by others, the decrease of TNF production became profound as the cancer progression. (Soda, Satake et al., 1994; Heriot, Marriott et
Spermine and spermidine have been shown to decrease the production of TNF and other pro-inflammatory cytokines from cultured human PBMCs, while they do not suppress the production of anti-inflammatory cytokine, TGF-β (Zhang, Caragine et al., 1997). Quite interestingly, in cancer patients, the productions of anti-inflammatory cytokines are reported to be higher than those of non-cancer patients (Twardzik, Sherwin et al., 1982).

Many previous reports have shown that leukocyte from cancer patients lack adhesion capacity to tumor cells as well as to culture plates (Blindar' and Zubrikhina 1998; Hersh, Gschwind et al., 1982). The first step of the mechanism of tumor cell cytolysis by killer cells is target cell binding. Therefore, the characteristics of decreased adhesion were considered as one of the mechanisms that hinder the anti-tumor immunity in cancer patients. In fact, augmentation of the expression of molecules required for cell adhesion successfully enhanced cytotoxicity of killer cells, and vise versa (Aramburu, Balboa et al., 1993; Timonen, Gahmberg et al., 1990; Webb, Mostowski et al., 1991). The culture of human PBMCs with IL-2 results in the generation of cytotoxic cells, namely LAK cells, which are able to lyse autologous tumor cells that cannot be lysed by untreated natural killer cells. However, the killer cell activities of activated lymphocytes from cancer patients are sometimes decreased (Pisani, Krco et al., 1989, Shirai, Watanabe et al., 1990). Overnight culture with spermine and spermidine successfully suppressed LAK cell cytotoxicities without decreasing cell activities or viabilities.

In the present study, we showed that some of the changes in immune functions in cancer patients could be observed by adding polyamines to cultured human PBMCs. In addition, previous studies have shown that polyamines decrease IL-2 production from monocytes and macrophages. The decrease in IL-2 production in cancer patients is documented in many previous reports (Hersey, Bindon et al., 1983; Monson, Ramsden et al., 1986; Rey, Klein et al., 1983). Therefore, the increase in polyamine synthesis in cancer tissues may be responsible for the suppression of cellular immunity in cancer patients.

References
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