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Preclinical report

Tumor cytotoxicity of peritoneal macrophages induced by OK-432

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In the present study we investigated the enhancement of cytotoxicity of peritoneal macrophages induced by OK-432. Rats received an i.p. injection of OK-432 at doses of 0.1, 0.5 or 1.0 KE/rat. Two days later, rats were sacrificed and peritoneal macrophages were isolated. Then the number of macrophages was counted, and the macrophages were analyzed for their lactic dehydrogenase (LDH) activity, acid phosphatase (ACP) activity, phagocytic activity, secretion of nitric oxide (NO) and cytotoxicity. The number of peritoneal macrophages, the activity of LDH and ACP, phagocytic activity, NO secretion, and cytotoxicity were increased with the increasing doses of OK-432. The results suggested that OK-432 enhanced tumor cytotoxicity of peritoneal macrophages by three steps. The first step is to attract a great number of macrophages into the peritoneal cavity. The second step is to enhance the phagocytic and eliminating function of these macrophages. The last step is to increase the non-contact cytotoxicity of macrophages. [© 2001 Lippincott Williams & Wilkins.]

Key words: Macrophages, OK-432, peritoneal, tumor cytotoxicity.

Introduction

OK-432 is a streptococcus agent that acts as a biological response modifier. It has been clinically used for cancer therapy and it was effective in improving the survival of cancer patients.[1]

Macrophages constitute an important part of the first line of defense against microbial invaders and malignancies by the nature of their phagocytic, cytotoxic and intracellular killing capacities. It is reported that, *in vitro*, in the very early phrase of i.p. injection of OK-432, peritoneal macrophages work as effector cells in tumor inhibition, other than peritoneal neutrophils.[2] The cytotoxicity of macrophages is mediated through either continuous contact with target cells[3] or without contact.[4] The cytotoxicity mechanisms involve (i) the phagocytosic activity of macrophages against target cells, and (ii) many kinds of effectors released from the macrophages such as reactive oxygen, arginase, neutral proteases, interleukin-1, tumor necrosis factor and L-arginine-dependent nitric oxide (NO).[5]

The present study investigated the mechanisms of tumor cytotoxicity of peritoneal macrophages (PMϕ) induced by treatment with OK-432.

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Materials and methods

Animals and tumor cells

Male, 150–180 g, SD rats, purchased from the Experimental Animal Center of Xian University of Medicine, were used in the experiment. Forty rats were divided into four groups of 10. One was the control group and the other three were the experimental groups. K562, one of human leukemia cell lines, was obtained from the Medicine and Science Institute of China (Beijing) and was used as the target tumor cell line. OK-432 was produced by culturing a low virulence Su strain of type III, group A *Streptococcus pyogens* of human origin in Bernheimers basal medium supplemented with penicillin G potassium.[6] OK-432 was generously donated by Chugai Pharmaceutical (Tokyo, Japan). The cell content of OK-432 was expressed in Klinische Einheit (KE). One KE contains 0.1 mg dried cells (approximately 10^9 cells) of streptococcal Su strain. OK-432 was dissolved in 0.9% NaCl in the experiment.

Experiment 1: PMϕ number

OK-432 suspension (1 ml), was i.p. injected into experimental groups at doses of 0.1, 0.5 or 1.0 KE/

rat, respectively. The same volume of 0.9% NaCl without OK-432 was i.p. injected into control rats. Two days post-injection, rats were sacrificed by exsanguination under ether anesthesia and then 10 ml of ice-cold RPMI 1640 medium (Gibco, Paisley, UK) was peritoneally injected. After gently massaging the rat's abdomen for 10 min, the RPMI 1640 medium was collected from the peritoneal cavity. Using a modified Neubauer hemocytometer,[7] the number of PMϕ in the collected RPMI 1640 medium was counted twice and the mean number in each rat was used. Then, the RPMI 1640 medium collected from the peritoneal cavity of rats was centrifuged at 2000 r.p.m. for 10 min to isolate PMϕ. The sediment containing the PMϕ was washed twice then re-suspended in 2 ml of RPMI 1640 medium for the following assay.

Experiment 2: enzyme activity assay

The suspension of PMϕ was placed into microplate wells at a cell concentration of 2×10^5/well. Then 1 ml of RPMI 1640 was added to each well. The PMϕ were disrupted by freezing at -60°C and thawing at 30°C. This cycle was repeated 3 times, each for 30 min. Then the suspension of the disrupted PMϕ was assayed for the activity of lactic dehydrogenase (LDH) and acid phosphatase (ACP), by the continuous-flow fluorimetric method using a fully automatic spectrophotometer (COBAS MIRA; Roche, Basel, Switzerland). The activities of LDH and ACP were measured at wavelengths of 340 and 405 nm, respectively.[8]

Experiment 3: phagocytic activity

The phagocytic activity of PMϕ was assayed by measuring the neutral red absorption of PMϕ. The PMϕ were placed in wells at 2×10^5 cells/well and rinsed 3 times in neutral red-free phosphate-buffered saline (PBS). Then the PMϕ were incubated for 2 h at 37°C, 95% air and 5% CO_2, in RPMI 1640 medium containing 0.1% neutral red, supplemented with 10% fetal calf serum (FCS; Gibco). The suspension of PMϕ in the wells was rinsed 3 times with PBS to discard the neutral red that had not been incorporated and 1 ml of 1 M NaOH was added to each well. After 24 h, when PMϕ were entirely disrupted, the suspension became a solution of neutral red which was released from the disrupted PMϕ. The density of neutral red released from the disrupted PMϕ was assayed with a spectrophotometer (U-2000A; Hitachi, Tokyo, Japan) at a wavelength of 540 nm.[9]

Experiment 4: NO secretion

The suspension of PMϕ (2×10^5 cells/ml) in RPMI 1640 medium was used to measure NO secretion by PMϕ cells. The suspension of PMϕ at 100 μl was mixed with 100 μl of Griess (1% sulfanilamide, 2.5% phosphoric acid and 0.1% *N*-naphthyl-ethylenediamine). This mixture was incubated for 10 min at room temperature and then the optical density of NO in the mixture was assayed at a wavelength of 540 nm using a microtiter plate reader (Multiscan, PLUS; Labsystems, Helsinki, Finland).[10]

Experiment 5: cytotoxicity of PMϕ

To determine whether OK-432 therapy influences the antitumor cytotoxicity of PMϕ, a cytotoxicity assay was performed in the present study by *in vitro* coculturing PMϕ of effector cells with target K562 cells. PMϕ, as effector cells, were incubated in 96-well plates[11] at 2×10^6 cells/well in a volume of 1 ml under 37°C for 4 h in order for the macrophages to adhere on walls. After the incubation, the microplates were vigorously rinsed 5 times with RPMI 1640 medium to remove non-adherent cells. K562 cells, as target cells in the exponential growth phase, were labeled with 5 μCi/ml of [methyl-^{3}H]thymidine (NEN, Boston, MA; 147.9 GBq/mmol) for 24 h at 37°C in RPMI 1640 medium supplemented with 10% FCS. The labeled K562 cells were rinsed 3 times with RPMI 1640 medium to remove unbound radiolabel, then the cells were re-suspended in RPMI 1640 at a concentration of 2×10^5 cells/ml. The labeled K562 cells were placed at 2×10^5 cells/well into the wells containing 2×10^6 of PMϕ cells/well. PMϕ cells and K562 cells were coincubated in RPMI 1640 medium supplemented with 10% FCS for 24 h, so that effector cells should disrupt the target cells and the cytotoxicity of macrophages could be measured. Then the K562 cells in the supernatant were harvested onto fiber glass filters, while macrophages remained in the microplate walls. As an additional control, radiolabeled K562 cells alone at 2×10^5 cells/well were incubated similar to the coculture of K562 cells and PMϕ cells. The radioactivity of K562 cells was counted on a Tri-Carb 1500 fluid scintillation analyzer (Packard, Groningen, The Netherlands). Cytotoxicity of macrophages was expressed as the percentage of specific [^{3}H]thymidine release that was calculated using the following formula: cytotoxicity=$(1-E/C) \times 100\%$, in which E is the count per minute (c.p.m.) of target cells in four groups (control group and experimental groups) with the presence of effector cells and C is the c.p.m. of target cells alone in an additional control.

Statistical analysis

The results are presented as means ± SD. One-way analysis of variance was used to evaluate the significance of differences between two groups. It was defined to be significant when $p < 0.05$.

Results

Experiment 1: PMϕ number

The effect of different doses of OK-432 on the number of PMϕ is shown in Figure 1. In rats given i.p. injection with a dose of 0.1 KE/rat, the number of PMϕ significantly ($p < 0.01$) increased compared to that of the control group. The number of PMϕ increased with increasing doses of OK-432 and reached more than 4-fold at 1.0 KE/rat of OK-432 as compared to that in the control group.

Experiment 2: enzyme activity assay

In the experimental groups, levels of both LDH and ACP activity significantly ($p < 0.05$–0.01) increased with increasing doses of OK-432 as compared to that in the control group (Figure 2).

Experiment 3: phagocytic activity

Figure 3 shows the levels of phagocytic activity of PMϕ. The phagocytic activity increased significantly ($p < 0.01$) after stimulation of OK-432 at a dose of 0.1 KE/rat. The activity at the OK-432 dose of 0.5 KE/rat was similar to that at the dose of 0.1 KE/rat. When the dose of OK-432 reached 1.0 KE/rat, the phagocytic

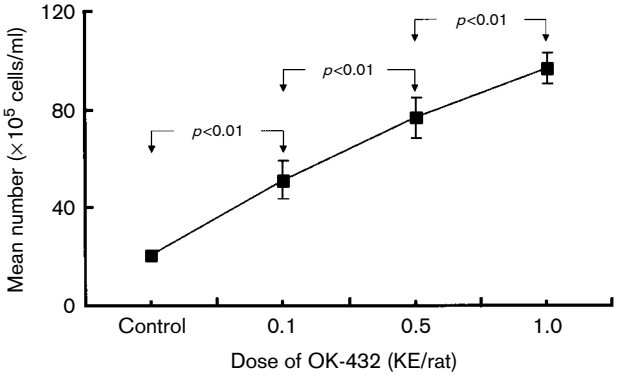

Figure 1. Control indicates the physiological saline group. Vertical bars indicate the SD in 10 rats. The number of PMϕ was counted twice in 10 rats. In the control group, the PMϕ number per rat was $20.6 \pm 1.6 \times 10^5$ cells/ml. In the experimental groups, the PMϕ numbers per rat were 51.4 ± 7.9, 76.8 ± 8.5 and $91.7 \pm 6.1 \times 10^5$ cells/ml, at doses of 0.1, 0.5 and 1.0 KE/rat, respectively. (Control group versus 0.1 KE/rat, $p < 0.01$; 0.1 versus 0.5 KE/rat, $p < 0.01$; 0.5 versus 1.0 KE/rat, $P < 0.01$.)

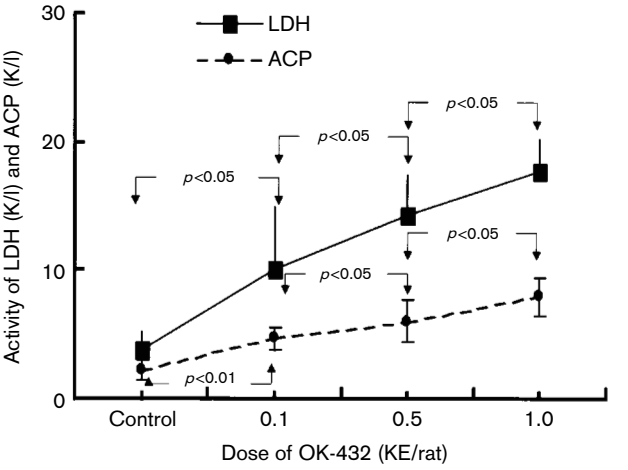

Figure 2. Control indicates the physiological saline group. Vertical bars indicate the SD in 10 rats. The ACP activity and LDH activity were assayed in triplicate in 10 rats, and expressed as Klinische unit per liter (K/l). In the control group, the activity of LDH was 3.7 ± 1.6 K/l. In the experimental groups, the activity of LDH was 10.1 ± 4.8, 14.3 ± 3.2 and 17.8 ± 2.5 K/l, in the groups of 0.1, 0.5 and 1.0 KE/rat, respectively. (Control group versus 0.1 KE/rat, $p < 0.05$; 0.1 versus 0.5 KE/rat, $p < 0.05$; 0.5 versus 1.0 KE/rat, $p < 0.05$.) In the control group, the activity of ACP was 2.1 ± 0.8 K/l. In the experimental groups, the activity of ACP was 4.6 ± 0.8, 6.0 ± 1.6 and 7.9 ± 1.5 K/l in the groups of 0.1, 0.5 and 1.0 KE/rat, respectively. (Control group versus 0.1 KE/rat, $p < 0.01$; 0.1 versus 0.5 KE/rat, $p < 0.05$; 0.5 versus 1.0 KE/rat, $p < 0.05$.)

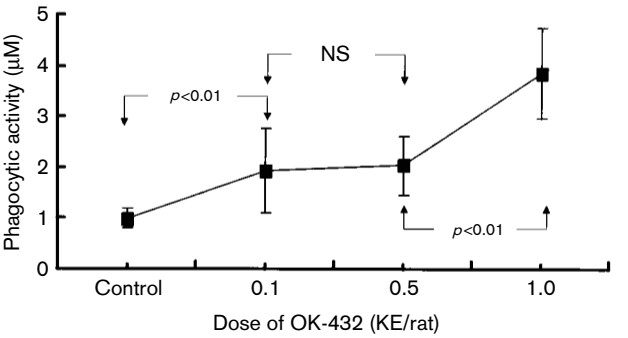

Figure 3. Control indicates the physiological saline group. Vertical bars indicate the SD in 10 rats. The phagocytic activity of macrophages was assayed in triplicate in 10 rats and expressed as the density of neutral red (μM). In the control group the phagocytic activity of the PMϕ was 1.0 ± 0.2 μM. In the experimental groups, the phagocytic activity was 1.8 ± 0.9, 2.0 ± 0.5 and 3.8 ± 1.5 μM in the groups of 0.1, 0.5 and 1.0 KE/rat, respectively. (Control group versus 0.1 KE/rat, $p < 0.01$; 0.1 versus 0.5 KE/rat, p=NS; 0.5 versus 1.0 KE/rat, $p < 0.01$.)

activity increased significantly ($p<0.01$) as compared to that at the OK-432 dose of 0.5 KE/rat.

Experiment 4: NO secretion

The NO secretion is shown in Figure 4. When rats were treated with 0.1 KE/rat of OK-432, NO secretion had a tendency to increase (p=0.06). When 0.5 KE/rat of OK-432 was given, NO secretion increased significantly ($p<0.01$) as compared to that at the OK-432 dose of 0.1 KE/rat. When OK-432 was given at 1.0 KE/rat, NO secretion increased significantly ($p<0.01$) as compared to that at the OK-432 dose of 0.5 KE/rat.

Experiment 5: cytotoxicity of PMϕ

PMϕ showed a low cytotoxicity (10.3%) in the control group in which there was no stimulation by OK-432. When the rats were treated with 0.1 KE/rat of OK-432, the cytotoxicity of PMϕ was 18.7%, which was significantly ($p<0.05$) increased as compared to that in the control group. When treated with 0.5 KE/rat of OK-432, cytotoxicity of PMϕ demonstrated a significantly ($p<0.01$) higher level (42.9%) than that following treatment with 0.1 KE/rat. With stimulation of OK-432 at a dose of 1.0 KE/rat, the cytotoxicity of PMϕ reached 59.8%, which was significantly ($p<0.01$) higher than that at an OK-432 dose of 0.5 KE/rat (Figure 5).

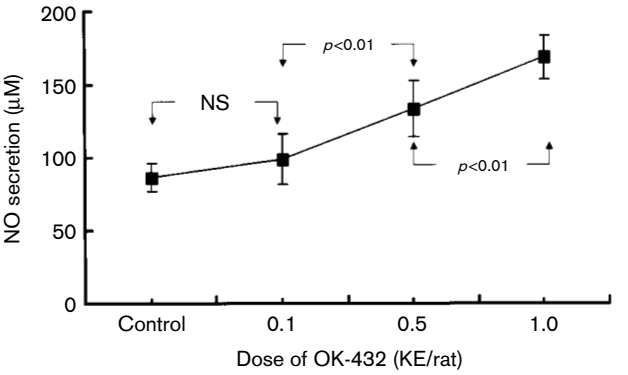

Figure 4. Control indicates the physiological saline group. Vertical bars indicate the SD in 10 rats. The secretion of NO was assayed in triplicate in 10 rats. In the control group, the secretion of NO by PMϕ was 86.4 ± 9.8 μM. In the experimental groups, the secretion of NO was 99.0 ± 17.5, 133.6 ± 19.1 and 168.9 ± 15.1 μM, in the groups of 0.1, 0.5 and 1.0 KE/rat, respectively. (Control group versus 0.1 KE/rat, p=NS; 0.1 versus 0.5 KE/rat, $p<0.01$; 0.5 versus 1.0 KE/rat, $p<0.01$.)

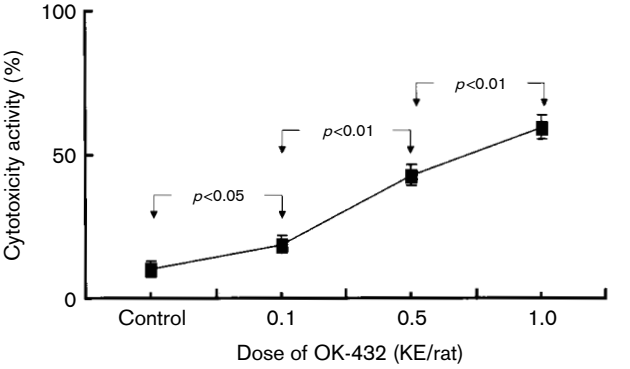

Figure 5. Control indicates the physiological saline group. The additional control in which K562 cells alone was incubated is not shown. Vertical bars indicate the SD in 10 rats. The cytotoxicity of PMϕ was assayed in triplicate in 10 rats. In the control group, the cytotoxicity of PMϕ was 10.3%. In the experimental groups, the cytotoxicity of PMϕ was 18.7, 42.9 and 59.8% in the groups of 0.1, 0.5 and 1.0 KE/rat, respectively. (Control group versus 0.1 KE/rat, $p<0.05$; 0.1 versus 0.5 KE/rat, $p<0.01$; 0.5 versus 1.0 KE/rat, $p<0.01$.)

Discussion

About 70–80% of the peritoneal cell population consists of macrophages. Activated macrophages can distinguish tumor cells, and are capable of phagocytosis and lysis of tumor cells.[12–14] In the present study, using a human leukemia cell line, we investigated the enhanced cytotoxicity of PMϕ induced by OK-432 by using two experiments to examine phagocytic cytotoxicity and effector-mediated cytotoxicity.

In the present study, the number of macrophages increased with increasing doses of OK-432, which should be directly attracted by OK-432 into the peritoneal cavity, or migrated into the peritoneal cavity by other cytokines or chemokines that were induced by OK-432.

The levels of LDH and ACP parallel the macrophages' phagocytic activity and function in eliminating foreign particles, as increased LDH and ACP activity was seen as the result of the activated function of the macrophages.[15] In the process of phagocytosis, a typical function of macrophages, macrophages phagocytoze foreign particles to form phagosomes and then eliminate the foreign particles by lysosomal enzymes including ACP. In the present study, the phagocytic activity was measured by the phagocytosis of neutral red and the activity in eliminating foreign particles was measured with ACP activity as described elsewhere.[16] The results of the remarkably increased LDH and ACP activity and phagocytic activity seen in the present assays suggest that OK-432 markedly enhances the

activities of macrophages in phagocytozing and eliminating foreign particles.

It is reported that NO participated in the non-contact antitumor cytotoxicity of macrophages.[17] Our study showed that NO secretion was increased with the dose of OK-432, accompanied by enhanced cytotoxicity.

Based on our findings, we conclude that i.p. injection of OK-432 enhances the cytotoxicity of PMϕ through three steps. The first step is that a great number of macrophages were attracted and migrated into the peritoneal cavity. When OK-432 was administered i.p., OK-432 activated these macrophages' cytotoxicity through both the second and the third steps. The second is activation of macrophages' phagocytic ability and elimination of foreign particles. The third is the enhancement of the non-contact cytotoxicity of macrophages which is assayed by NO secretion.

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