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Cytokine-mediated antitumor effect of OK-432 on urinary bladder tumor cells in vitro

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Abstract Fatal complications from the intravesical instillation of bacillus Calmette-Guérin (BCG) for the treatment of superficial urinary bladder tumors have been reported. OK-432, an immunomodulating agent like BCG, may be an effective and safe agent for the treatment of urinary bladder tumors. We investigated the cytokine-mediated antitumor effect of OK-432 on established human bladder cancer cell lines (T24 and KK-47) in vitro. Peripheral blood mononuclear cells (PBMCs) from a healthy volunteer were cultured with OK-432 for various periods, and the culture supernatants were used as conditioned media. Cytokines in the culture supernatants were quantified. The antitumor effect of OK-432 was evaluated by colony-forming assays, using the conditioned media as the culture media. The colony survival of T24 and KK-47 cells was significantly inhibited by conditioned media from 24-h cultures of PBMCs incubated with OK-432 at concentrations of 0.05 and 0.1 Klinische Einheit (KE)/ml. Conditioned media from PBMCs cultivated with OK-432 for 7 days at 0.01 and 0.05 KE/ml also significantly inhibited the colony survival of both cell lines. Higher concentrations of interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) were detected in conditioned media cultivated with OK-432 for 24 h than in media from PBMCs alone. However, higher concentrations of interferon γ (IFNγ) were detected in conditioned media cultivated with OK-432 for 7 days. Approximately 90% of the inhibition of KK-47 cells by the 24-h conditioned media was neutralized by an anti-TNF monoclonal antibody. The inhibition of T24 cells was neutralized approximately 50% by the same antibody. The inhibition of T24 and KK-47 cells by 7-day conditioned media was completely neutralized by an anti-IFNγ monoclonal antibody. The cultivation of PBMCs with OK-432 inhibited the production of granulocyte-colony-stimulating factor (G-CSF) by PBMCs. The inhibition may play a role in the mechanism of the antitumor effect of OK-432. Urinary bladder tumor cell lines have different sensitivities to cytokines. The cytokines induced by OK-432 vary with the concentration of OK-432 and the culture period. It is suggested that in intravesical instillation of OK-432 for treatment of urinary bladder tumor, the optimal dose and interval of instillation should be considered.

Key words Cytokine · OK-432 · Human bladder cancer cells · In vitro

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Introduction

The intravesical instillation of bacillus Calmette-Guérin (BCG) is currently considered the most effective treatment for refractory superficial urinary bladder tumors [6, 14]. It has been demonstrated that BCG has both direct and indirect effects on urinary bladder tumor cells, related to immunologic mechanisms [10, 12]. Cytokines are believed to play an important role in the indirect effects of BCG. Severe or fatal side effects and complications from the intravesical instillation of BCG, including BCG pneumonitis and hepatitis, have been reported [11].

OK-432 is a penicillin-treated and heat-treated lyophilized powder of the Su substrain of *Streptococcus pyogenes* A3. It has been used as an immunomodulating agent in patients with lung or gastrointestinal cancer [27, 28]. It also has direct effects on tumor cells in vitro [15]. It has been more beneficial in local treatment, such as intrapleural, intraperitoneal, or intratumoral administration, than in systemic therapy [3, 7, 27, 28].

OK-432 also has been used in the treatment of urinary bladder tumors [3, 7]. OK-432 may be a safe and effective agent for the treatment of urinary bladder tu-

mors, because OK-432 is not a living organism, and because urinary bladder tumors could be treated intravesically or intratumorally by topical OK-432.

Although OK-432 has been reported to be a potent immunomodulator of macrophages, natural killer (NK) cell activity, and lymphokine-activated killer (LAK) cell activity [9, 20, 28, 29], the mechanism of its antitumor action on urinary bladder tumor cells, especially mediated by cytokines, has not yet been fully elucidated. We therefore examined the in vitro antitumor effects of cytokines produced by peripheral blood mononuclear cells (PBMCs) stimulated with OK-432 against urinary bladder tumor cells. Whether the intravesical instillation of OK-432 could take the place of BCG for the treatment of superficial urinary bladder tumors was investigated from aspects of an induction and an antitumor effect of cytokines.

Materials and methods

Human urinary bladder tumor cell lines

Two human urinary bladder tumor cell lines were used in this study as target cells in the colony-forming assay. Both cell lines, T24 and KK-47, are derived from human transitional cell carcinomas of the urinary bladder, representing histopathologic grades G3 and G1, respectively [2, 26]. These cell lines were maintained as monolayer cultures in RPMI-1640 medium (Flow Laboratories, McLean, V.A.) supplemented with 10% heat-inactivated fetal calf serum (FCS, Flow Laboratories), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% carbondioxide (CO_2). Subconfluent tumor cells were used throughout the experiments. The monolayer cultures were dispersed by trypsinization using a trypsin/ethylenediaminetetraacetic acid (EDTA) solution: 0.5 g/l trypsin and 0.2 g/l NaEDTA dissolved in phosphate-buffered saline (PBS) without calcium or magnesium (DPBS, Whittaker, Walkerville). Single-cell suspensions were achieved by mixing the retrieved cells thoroughly with a pipette. In rare instances, passage through a 22-gauge spinal needle was done to disaggregate clumps of cells. These cells were free of mycoplasma.

Preparation of PBMCs

Heparinized human peripheral blood was obtained from a healthy volunteer. The blood samples were diluted by addition of an equal volume of 0.9% NaCl solution. The diluted blood was layered on one-half volume of lymphocyte isolation solution (Lymphoprep, Nycomed, Oslo, Norway), and centrifuged at 800 *g* for 20 min at room temperature. The mononuclear cell layer was collected by pipette. After washing with DPBS, the cells were suspended in RPMI-1640 medium. More than 95% of this cell population consisted of mononuclear cells, as determined by morphologic examination using Giemsa-stained preparations.

Reagents

OK-432 (Picibanil), a lyophilized preparation of the penicillin-treated and heat-treated Su substrain of *Streptococcus pyogenes* group A3, was kindly supplied by Chugai Pharmaceutical (Tokyo, Japan). One Klinische Einheit (KE) unit of OK-432 is equivalent to 0.1 mg/ml of the lyophilized preparation.

Anticytokine monoclonal antibodies

The anti-interleukin-1β (IL-1β, Genzyme, Cambridge, Mass.), tumor necrosis factor a (TNFα, Hayashibara Biochemical Laboratories, Japan), and interferon γ (IFNγ, Genzyme) monoclonal antibodies were used at concentrations high enough to neutralize each cytokine.

Preparation of conditioned media

The PBMCs, at a concentration of 5×10^5/ml, were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FCS and OK-432 at concentrations of 0.01, 0.05, or 0.1 KE/ml at 37°C in a humidified atmosphere of 5% CO_2. After 24 h or 7 days, culture supernatants were collected by centrifugation at 800 *g* for 5 min to remove the OK-432, followed by filtration through a 0.20-μm pore membrane (Toyo Roshi, Japan). The supernatants were stored at −20°C until use as conditioned media. Supernatants from plates covered with RPMI-1640 alone (no PBMCs) with and without OK-432 were also collected. In all experiments, supernatants from PBMCs cultures unstimulated with OK-432 were used as a control. No differences were observed in the PBMC viability during either the 24-h or 7-day cultures, irrespective of the presence of the OK-432. Significant differences were also not observed in the pH of the culture supernatants in the presence or absence of OK-432, and adjustments to the pH of the samples were not needed.

Colony-forming assay

The method described by Ham[5] was used in this study with slight modifications. Briefly, 2 h prior to the experiment, 2 ml of RPMI-1640 medium supplemented with 10% heat-inactivated FCS were placed in each of several 35-mm tissue culture Petri dishes (Falcon 3801, Becton Dickinson, Sunnyvale, Calif.). The dishes were then placed in the tissue culture incubator. A single-cell suspension of tumor cells was obtained by trypsinization. The single-cell suspension was diluted to a plating density of 1×10^2 cells per dish. The final cell suspension was added to each previously prepared Petri dish. All the dishes were incubated and left undisturbed for 24 h to facilitate attachment. Following the attachment of the tumor cells, the media in the dishes were replaced by conditioned media, and the dishes were incubated under the same conditions. Each experiment was conducted in triplicate. After 6–7 days, colonies were fixed with methanol and stained with Giemsa. Colonies defined as aggregates of 30 or more cells were counted under a microscope. The results are expressed as a percentage of the control value. The entire procedure was repeated three times for each cell line.

Measurement of cytokines in the supernatant

The PBMCs were suspended at a concentration of 5×10^5/ml in RPMI-1640 medium supplemented with heat-inactivated FCS and cultured in the presence of OK-432 at concentrations of 0, 0.01, 0.05, or 0.1 KE/ml for 7 days. IL-1β, TNFα, IFNγ, and granulocyte-colony-stimulating factor (G-CSF) in the culture supernatants were quantified with sandwich enzyme immunoassays using solid-phase reaction methods and coated monoclonal antibodies to each cytokine [25]. The minimum measurable level of each cytokine was 20 pg/ml.

Statistical analysis

All data are expressed as the mean ± the standard deviation (SD) of each experiment. The data obtained were subjected to statistical analysis using the two-sample Wilcoxon test. A P value of less than 0.05 was regarded as statistically significant.

Results

Effects of conditioned media from cultures of OK-432 alone

OK-432 at concentrations of 0.01, 0.05, or 0.1 KE/ml without PBMCs were incubated in RPMI-1640 medium supplemented with 10% heat-inactivated FCS for 24 h or 7 days, respectively, and then the supernatants were collected by centrifugation and were filtered through 0.20-μm pore. The conditioned media were put into dishes for colony-forming assay of T24 or KK-47 cells. RPMI-1640 medium supplemented with 10% heat-inactivated FCS incubated for 24 h or 7 days, respectively, which were also centrifuged and filtered in the same way as the conditioned media, were used as controls. There were no differences between the conditioned media and the controls in the colony-forming efficiency of either T24 or KK-47 cells (Table 1).

Effects of conditioned media from cultures of PBMCs with OK-432

When the 24-h conditioned media were used, the colony survival of both the T24 and KK-47 cells was significantly inhibited at OK-432 concentrations of 0.05 and 0.1 KE/ml, compared with that using the conditioned media from PBMCs alone (Fig. 1). OK-432 at a concentration of 0.01 KE/ml did not significantly affect colony survival.

When the 7-day conditioned media were used, the colony survival of both the T24 and KK-47 cells was significantly inhibited at OK-432 concentrations of 0.01 and 0.05 KE/ml (Fig. 1). OK-432 at a concentration of 0.1 KE/ml did not significantly affect colony survival.

Time course of cytokine production by PBMCs cultivated with OK-432

PBMCs were cultured with OK-432 at various concentrations for 7 days. High concentrations of IL-1β, TNFα, and IFNγ were observed in the supernatants (Fig. 2). The maximum values were found at 24 h for IL-1β and TNFα, and 7 days for IFNγ. Although the concentration of IFNγ was high at OK-432 concentrations of 0.01 and 0.05 KE/ml, it was lower at 0.1 KE/ml than it was with no OK-432 (Fig. 2).

Effects of anticytokine monoclonal antibodies on the antiproliferative activity of the conditioned media

To examine which cytokine reduced the colony survival of the T24 and KK-47 cells, anti-TNFα monoclonal antibody, anti-IFNγ monoclonal antibody, or anti-IL-1β monoclonal antibody was added to the conditioned

Table 1 Colony-forming efficiency of two bladder carcinoma cell lines in conditioned media from cultures of OK-432 alone. Media from cultures of RPMI-1640 medium alone are used as controls. Values are given as percentages of controls. All data are expressed as means ± SD of three separate experiments done in triplicate. *KE* Klinische Einheit

Cell line	Culture period	Concentration of OK-432 in culture media			
		0 KE/ml	0.01 KE/ml	0.05 KE/ml	0.1 KE/ml
T24	24 h	100 ± 10.5	99.9 ± 13.6	100.5 ± 10.3	100.5 ± 12.0
	7 days	100 ± 12.0	97.4 ± 14.2	97.1 ± 14.2	96.3 ± 9.3
KK-47	24 h	100 ± 14.8	101.0 ± 13.5	98.7 ± 16.5	99.8 ± 12.7
	7 days	100 ± 13.8	99.5 ± 13.5	104.1 ± 11.4	98.7 ± 8.9

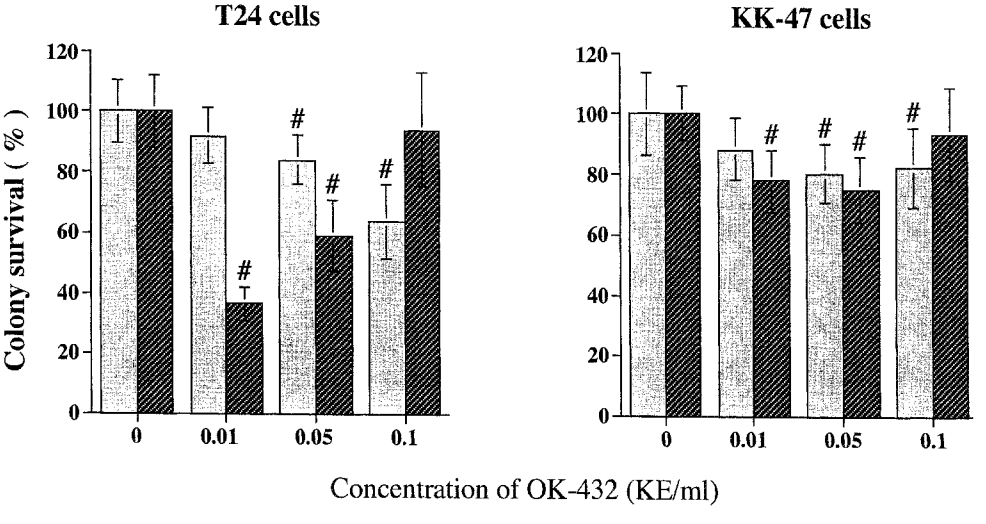

Fig. 1 Colony survival of T24 and KK-47 cells in conditioned media from peripheral blood mononuclear cells (PBMCs) and OK-432 for 24 h or 7 days. Conditioned media from PBMCs alone are used as controls. The values are given as percentages of controls. All data are expressed as means ± standard deviation (SD) of three separate experiments done in triplicate. *PBMCs are applied at a density of 5×10^5/ml. #Significant inhibition ($P < 0.05$) of colony survival compared with controls. *KE* Klinische Einheit, □ Conditioned media from PBMCs* cultivated with OK-432 for 24 h, ▨ conditioned media from PBMCs* cultivated with OK-432 for 7 days

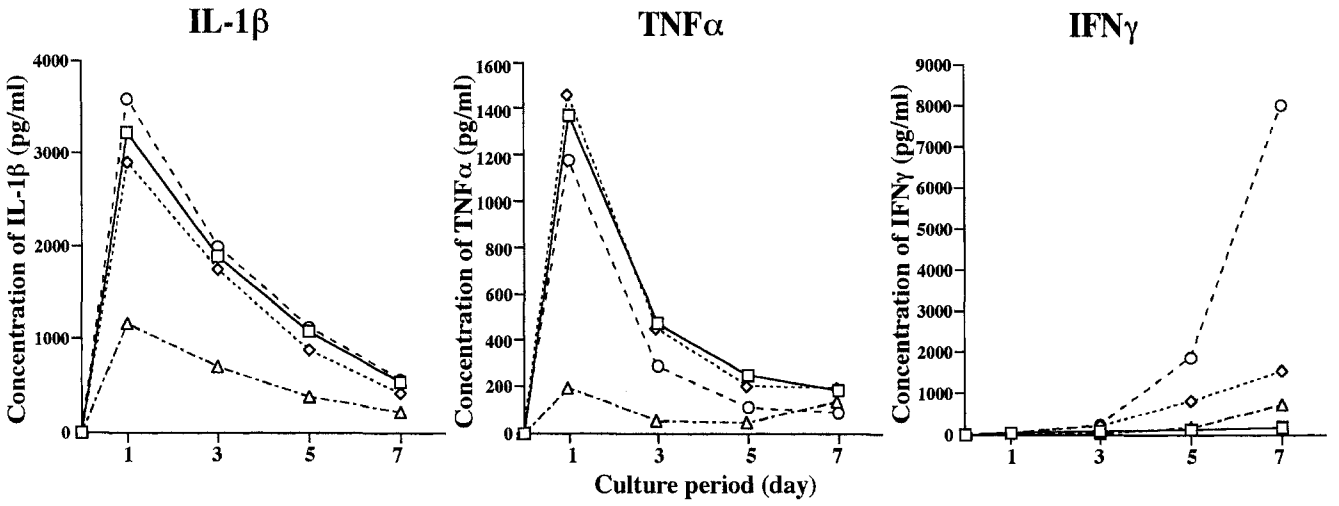

Fig. 2 Time course of cytokine production by PBMCs cultivated with various concentrations of OK-432. PBMCs are applied at a density of 5×10^5/ml. *Open squares* OK-432 concentration of 0.1 KE/ml, *Open diamonds* OK-432 concentration of 0.05 KE/ml, *Open circles* OK-432 concentration of 0.01 KE/ml, *Open triangles* PBMCs alone. *IL-1β* interleukin-1β, *TNFα* tumor necrosis factor α, *IFNγ* interferon γ

culture media. Against T24 cells, when the conditioned media from PBMCs cultivated with OK-432 at 0.1 KE/ml for 24 h were used, approximately 46% of the antiproliferative activity of the conditioned media was neutralized by adding anti-TNFα monoclonal antibody (Fig. 3). No neutralization of the antiproliferative activity, however, was observed with the anti-IL-1β monoclonal antibody. In addition, the neutralization of the antiproliferative activity was not increased by adding anti-IL-1β monoclonal and anti-TNFα monoclonal antibodies together, compared with anti-TNFα monoclonal antibody alone. When the conditioned media from PBMCs cultivated with OK-432 at 0.01 KE/ml for 7 days were used, complete neutralization of the antiproliferative activity of the conditioned media was observed with the anti-IFNγ monoclonal antibody (Fig. 3).

Against KK-47 cells, when the conditioned media from PBMCs cultivated with OK-432 at 0.05 KE/ml for 24 h were used, approximately 86% of the antiproliferative activity of the conditioned media was neutralized by adding anti-TNFα monoclonal antibody (Fig. 4). The anti-IL-1β monoclonal antibody had no effect. In the study using the conditioned media from PBMCs cultivated with OK-432 at 0.05 KE/ml for 7 days, although approximately 91% of the antiproliferative activity of the conditioned media was neutralized by adding anti-IFNγ monoclonal antibody, about 67% of that of the conditioned media was neutralized by adding anti-TNFα monoclonal antibody (Fig. 4).

G-CSF production by PBMCs cultivated with or without OK-432 for 24 h and 7 days

When PBMCs were cultivated with OK-432, the constitutive production of G-CSF by the PBMCs was inhibited. The inhibition of G-CSF secretion was dependent on the concentration of the OK-432 (Table 2).

Discussion

There have been numerous reports on the effects of OK-432 on human tumors, including urinary bladder tumors [3, 7, 13, 15, 27, 28]. According to these studies, OK-432 exerts its antitumor effect by activating effector cells and by inhibiting tumor cell growth directly, and activated effector cells have antitumor activity and produce antiproliferative cytokines. However, few studies have investigated the effects of the cytokines induced by OK-432 on urinary bladder tumor cells. The mechanism of the antitumor effects of OK-432 in vivo are very complicated. In this study, the antitumor effects of cytokines produced by PBMCs stimulated with OK-432 on urinary bladder tumor cells were studied in vitro. Furthermore, from the aspects of an induction and an antitumor effect of cytokines, whether an intravesical instillation of OK-432 could be used instead of BCG for the treatment of refractory superficial urinary bladder tumor was investigated.

OK-432 itself at relatively high doses has direct antitumor effects on urinary bladder tumor cells [8, 13]. It also has been reported that soluble factors derived from OK-432 have direct antitumor effects on some kinds of tumor cells [15]. It was considered that OK-432 co-sedimented with cells after centrifugation [15] and that the streptococcal bacteria were removed by filtering through a membrane [29]. Our results show that the concentrations of OK-432 used in this study had no direct effect on the colony-forming efficiency of the T24 and KK-47 cells (Table 1).

We did not identify subpopulations of lymphocytes/monocytes stimulated with OK-432. In this study, PBMCs were obtained from one healthy volunteer and it was thought that the subpopulations were almost invariable and resembled those in the clinical setting.

The inhibition of the colony survival of KK-47 cells by the 24-h conditioned media appeared to be caused by

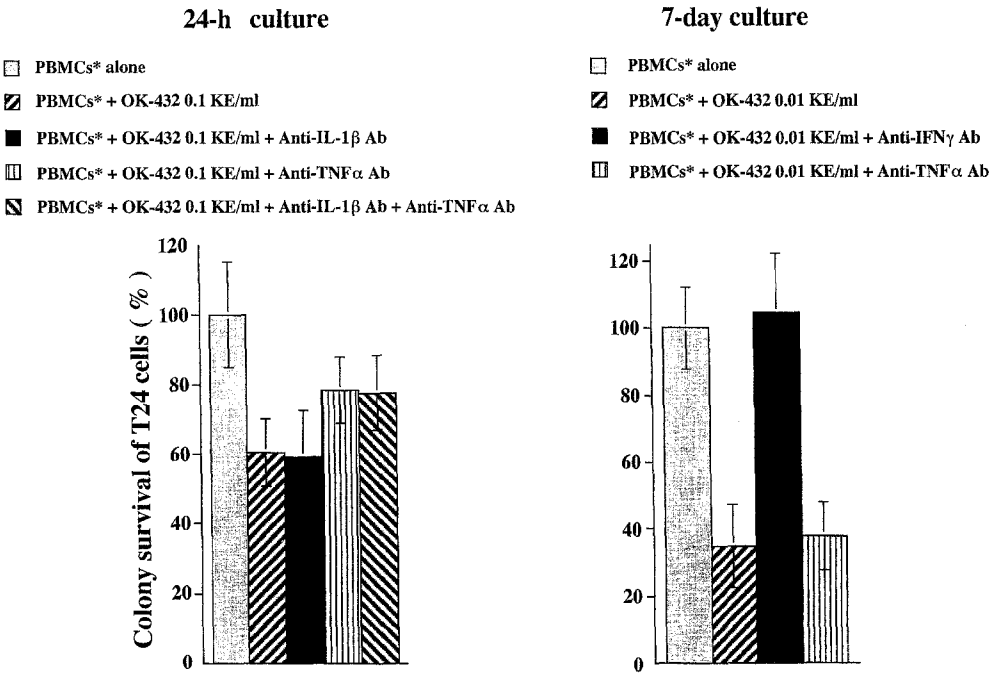

Fig. 3 Effects of anticytokine monoclonal antibodies (*Ab*) on the antiproliferative activity of conditioned media from cultures of PBMCs with OK-432 against T24 cells. The values are given as percentages of controls. All data are means ± SD of four separate experiments done in triplicate. *PBMCs are applied at a density of 5×10^5/ml

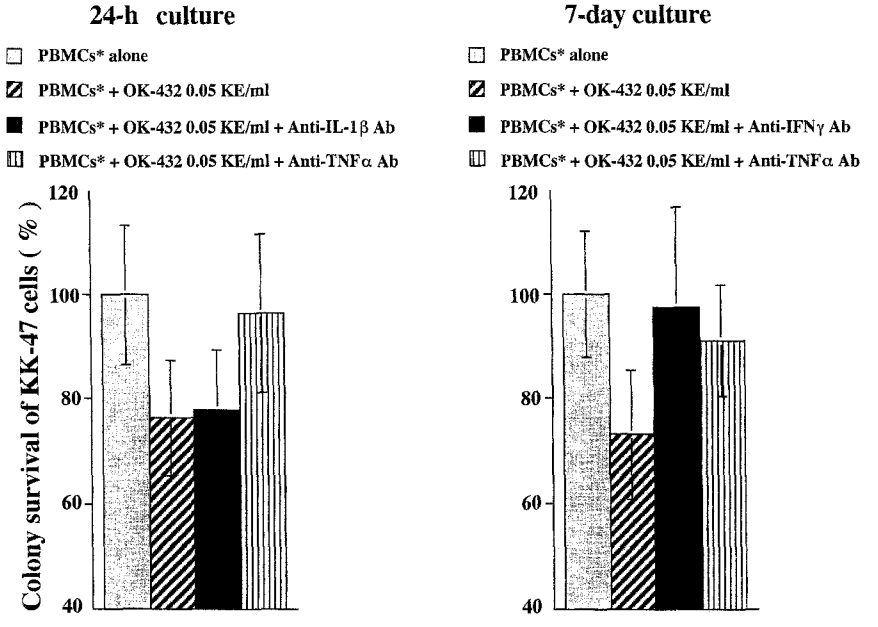

Fig. 4 Effects of anticytokine monoclonal antibodies (*Ab*) on the antiproliferative activity of conditioned media from cultures of PBMCs with OK-432 against KK-47 cells. The values are given as percentages of controls. All data are means ± SD of four separate experiments done in triplicate. *PBMCs are applied at a density of 5×10^5/ml

Table 2 Production of granulocyte-colony-stimulaing factor (G-CSF) by peripheral blood mononuclear cells (PBMCs) cultured with OK-432. PBMCs are applied at a density of 5×10^5/ml. All data are expressed as means ± SD in triplicate. G-CSF concentrations are expressed as pg/ml

Culture period	Concentration of OK-432 in culture media			
	0 KE/ml	0.01 KE/ml	0.05 KE/ml	0.1 KE/ml
24 h	240.3 ± 11.7	61.3 ± 9.5	27.0 ± 6.1	22.0 ± 3.5
7 days	297.0 ± 22.6	91.3 ± 18.2	35.0 ± 3.6	39.7 ± 9.6

TNFα alone. However, the inhibition of T24 cells by the 24-h conditioned media appeared to be caused by TNFα and other factor(s). Although the experimental condition was different from ours, it has been reported that T24 cells are not sensitive to several concentrations of recombinant human TNFα [24]. This seems to suggest that the conditioned medium from PBMCs cultivated with OK-432 contains other, additional inhibitory products. The inhibition of the colony survival of the T24 cells by the 7-day conditioned media appeared to be caused by IFNγ. The IFNγ results are compatible with previous reports [10, 24]. The inhibition of KK-47 cells by the 7-day conditioned media appeared to be caused by the synergistic effect of IFNγ and TNFα [24]. The colony survival of either T24 or KK-47 cells was not inhibited by the 7-day conditioned media from PBMCs cultivated with OK-432 at 0.1 KE/ml. Apparently at the high OK-432 (0.1 KE/ml) concentration no IFNγ was

synthesized by PBMCs. Although no differences were observed in the PBMC viability during the 7-day cultures, irrespective of the concentration of OK-432, the function of IFNγ production of PBMCs might be disordered by the high concentration of OK-432 for 7 days.

It has been shown that G-CSF enhances the growth of urinary bladder tumor cells in vitro [16, 22]. G-CSF has both direct and indirect effects on the growth of tumor cells [22]. In the current study, incubation with conditioned media from PBMCs alone did not stimulate the growth of the bladder carcinoma cells, compared with incubation with the RPMI-1640 alone-conditioned media (data not shown), because there was not only G-CSF but also low concentrations of antitumor cytokine(s), TNFα and/or IFNγ, in the conditioned media from PBMCs alone. We demonstrated that OK-432 inhibits the production of G-CSF by PBMCs in vitro. Although we could not demonstrate that the inhibition of the production of G-CSF directly reduced colony survival, it may be related to the inhibition of the T24 cells by the 24-h conditioned media. Additionally, in vivo, G-CSF or granulocytes induced by G-CSF may suppress host antitumor immunity, or granulocytes induced by G-CSF may stimulate tumor growth [21]. Thus, the inhibition of the production of G-CSF may play a role in the antitumor effects of OK-432 in this study.

Although a high concentration of IL-1β was found in the supernatants of PBMCs cultured with OK-432 for 24 h, IL-1β had no direct effects on the colony survival of either the T24 or KK-47 cells. It is well documented, however, that IL-1β has antitumor effects against some kinds of tumor cells [17], and that the effects are synergistically enhanced by the presence of TNF [19]. IL-1β affects both the induction and the activation of effector cells, and the production of TNFα and IFNγ by PBMCs. Thus, IL-1β also may play an important role in the mechanism of the antitumor effects of OK-432 against urinary bladder tumor cells.

Clinically, intravesical interferon instillation against bladder cancer was less effective than might be expected [4], while biologic response modifiers (BRMs), BCG, and OK-432 induce IL-1β, TNFα and IFNγ serially (Fig. 2) [10] and activate effector cells [9, 12, 13, 20, 28, 29]. The antitumor effects of BRMs are caused by these serial cytokines and effector cells. The mechanism of the reaction may be similar to that of common immunologic reaction in vivo. Therefore, it is thought that intravesical instillation of BRMs against bladder cancer is more effective than that of interferon alone.

The pattern of cytokine levels produced by PBMCs stimulated with OK-432 was similar to that produced by PBMCs stimulated with BCG [10]. It has been reported that IL-1, TNFα and IFNγ are detected in the urine after intravesical BCG therapy [1, 18]. Similarly cytokines may be expected to be detected in the urine after intravesical OK-432 therapy as a result of inflammatory response in the bladder wall.

It has been demonstrated that when OK-432 is incubated with urinary bladder tumor cells, and the OK-432 is rapidly and tightly bound to these cells [13]. If OK-432 is instilled intravesically in a patient with bladder carcinoma, it may bind to the urinary bladder tumor cells, and a definite concentration of OK-432 may be maintained around the tumor and infiltrating cells, even after voiding urine. Therefore, it may be possible to maintain an optimal concentration of OK-432 topically by an intravesical instillation for a brief period, such as 24 h. In addition, it may be possible to maintain the same conditions for a long period, such as 7 days, by instilling intravesically every day. Studies are needed to determine the optimal dose of intravesicular OK-432. Another way to maintain an optimal concentration of OK-432 at tumor sites may be to perfuse OK-432 continuously into the bladder, as is done with BCG for the treatment of superficial transitional cell carcinoma of the upper urinary tract [23].

Urinary bladder tumor cells have different sensitivities to cytokines, and cytokines induced by OK-432 vary with the concentration of OK-432 and the incubation period. This suggests that both the optimal dose and instillation time of intravesicular OK-432 will need to be determined. OK-432 may become more effective against many kinds of urinary bladder tumors.

In summary, the pattern of cytokine production induced by OK-432 is similar to that induced by BCG [10]. The sensitivities of urinary bladder tumor cells to the cytokines induced by OK-432 are also similar to those induced by BCG [10]. Therefore, from aspects of an induction and an antitumor effect of cytokines, the intravesical instillation of OK-432 may be able to replace BCG for the treatment of refractory superficial urinary bladder tumors.

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