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Novel OK-432-conjugated Tumor Vaccines Induce Tumor-specific Immunity against Murine Tongue Cancer

ABSTRACT

Priming with tumor antigens is one of the most important strategies in cancer immunotherapy. To enhance tumor antigenicity, OK-432, a streptococcal preparation, was coupled to squamous cell carcinoma (KLN-205) by means of a 0.2% glutaraldehyde method. The purpose of this study was to investigate whether OK-432-conjugated tumor vaccines could induce tumor-specific immunity. Our originally developed mouse tongue cancer model was used throughout this work for the analysis of antitumor effects. Prepared OK-432-conjugated KLN-205 vaccines were immunized 3 times to DBA/2 mice. The results showed that the KLN-205 vaccines induced cytolytic activity and strongly suppressed both KLN-205 tumor incidence and growth, and survival of the mice was improved. Moreover, the histological results showed that a greater number of lymphocytes had infiltrated around tumor cells by 24 hours after tumor inoculation in the vaccine group. These results suggest that immunizations with KLN-205 vaccines increase the antitumor effects against tongue cancer.

KEY WORDS: OK-432, tumor vaccine, glutaraldehyde, conjugation, tongue cancer.

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INTRODUCTION

The induction of tumor-specific immunity is currently one of the most important fields in medical research and development. To date, although some kinds of immunotherapy—such as an adoptive immunotherapy and a peptide vaccine from a tumor-associated antigen (TAA) (Rosenberg *et al.*, 1986; Nestle *et al.*, 1998; Shichijo *et al.*, 1998)—have been developed, there remains no appropriate and effective tumor-specific immunotherapy.

The most important strategy for the induction of tumor-specific immunity seems to be the priming of tumor antigens. It is widely accepted that a low level of tumor antigenicity is one of the mechanisms by which a tumor escapes from the host immune system. Cell-based tumor vaccines seem to be more effective on some tumor antigens than single-peptide-based tumor vaccines; however, there are only a few papers describing cell-based tumor vaccine development. Recently, we developed a novel method to increase tumor-specific antigenicity for effective priming. Complexes of tumor cells and other foreign bodies having strong antigenicity for the host immune system may have the potential to increase immunogenicity of the tumor-specific antigen. OK-432, a streptococcal preparation (Okamoto *et al.*, 1966; Saito *et al.*, 1988), was used as the foreign antigen for coupling to the tumor. To evaluate the effects of the newly developed OK-432-conjugated tumor vaccines, we used an experimental murine tongue cancer model (Li *et al.*, 2002). The objective of this study was to examine the feasibility of this vaccine candidate against tongue cancer.

MATERIALS & METHODS

Animals and Tumor Cells

Six-week-old female DBA/2 mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and raised in pathogen-free conditions. Tumor cells derived from the squamous cell carcinoma cell lines of DBA/2, C57BL/6, and C3H mice were KLN-205, B16 melanoma, and Sq1979 cell lines, respectively. They were obtained from Riken Cell Bank (Tsukuba, Japan). The KLN-205 and Sq1979 cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM), and B16 melanoma cell lines were maintained in RPMI 1640. The murine tongue cancer model developed by the authors was used throughout this experiment (Li *et al.*, 2002).

The Institutional Animal Care and Use Committee of Yokohama City University School of Medicine approved all procedures for use and care of the mice.

Preparation of the OK-432-conjugated Tumor Vaccines

OK-432, kindly provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan), was used as a binding antigen. The conjugation method with glutaraldehyde (GA) was performed as previously described (Bukawa *et al.*, 1995a,b). Briefly, GA (20%) was diluted to 2.0% with PBS at pH 7.4. KLN-205 cell lines (5 x 10^5/350 μL) were mixed to OK-432 (0.7 KE/100 μL), followed by

addition to 50 μL of 2% GA. The final concentration of GA was adjusted to 0.2% in 500 μL. Conjugation between tumor cells and OK-432 was slowly performed in small rotating columns. Two hrs after conjugation, the OK-432-conjugated tumor cells were centrifuged (at 4°C) at 800 rpm for 5 min 3 times to eliminate unreacted GA and OK-432. OK-432-conjugated KLN-205 cells (5×10^5) were added to 100 μL of PBS as the KLN-205 vaccines. Both B16 and Sq1979 cell lines were conjugated to OK-432 by the same means, and called B16 vaccine and Sq1979 vaccine, respectively. Coupling formation of each tumor vaccine was morphologically observed and confirmed through a microscope in all experiments.

Cytolytic Assay

DBA/2 mice ($H\text{-}2^d$) received subcutaneous immunizations of 5×10^5/μL KLN-205 vaccine, KLN-205 treated with 0.2% GA, OK-432 treated with 0.2% GA, or OK-432 alone at seven-day intervals over a three-week period. Control groups of the other DBA/2 mice received no treatment (saline alone). Cytolytic assay was performed 7 days after the final immunization. The spleen cells from immunized mice (3×10^6 cells/well) were co-cultured with MMC-treated KLN-205 cells (3:1 responder:tumor cell ratio) in 2 mL of complete T-cell medium (RPMI 1640 containing Con A) at 37°C in a humidified atmosphere containing 5% CO_2. Cytotoxic effector lymphocytes were harvested after 5 days of incubation and subjected to cytolytic assay. Target cells (KLN-205 cells) were labeled with ^{51}Cr and incubated with effector cells for 4 hrs at various E:T ratios. The maximum or spontaneous release was defined as counts from samples incubated with 5% Triton X-100 or medium alone, respectively. Cytolytic activity was calculated according to the following formula:

Percentage of specific ^{51}Cr release = (experimental release - spontaneous release) / (maximum release - spontaneous release) x 100.

Depletion of T-cell Subsets

Rat monoclonal antibodies against the murine CD4 (L3T4) and CD8 (Ly2) were used for depletion of T-cell subpopulations. Anti-CD4 and anti-CD8 antibodies were purchased from PharMingen (San Diego, CA, USA). Depletion of T-cell subsets was carried out as described elsewhere (Mukai *et al.*, 1996). Briefly, effector lymphocytes were suspended at a concentration of 10^7/mL in 1 mL of RPMI-1640 containing 25 μg of monoclonal antibody. Effector cells were incubated for 45 min at 4°C, washed, and re-suspended to the original volume with complement (C) (Guinea Pig Complement, Inter-Cell Technologies, Inc., Jupiter, FL, USA) at a 1/10 dilution for an additional 45 min at 37°C. After treatment with C, effector cells were washed twice.

Tumor Incidence and Growth

DBA/2 mice were divided into 7 groups of 18 to 20 animals each. The mice were anesthetized by diethyl ether and given subcutaneous immunizations with KLN-205 vaccine, B16 vaccine, Sq1979 vaccine, KLN-205 treated with GA, OK-432 treated with GA, or OK-432 alone. Immunizations were administered into the left flank of each mouse once each week for 3 wks. Control group mice were injected with an equal volume of saline in the same manner. One wk after injection, all mice were inoculated, in the tongue, with 2×10^5/40 μL of KLN-205 cell lines by means of a 100-μL syringe and a 30-gauge needle. Tumor incidence and growth were observed. We also investigated whether post-immunization with KLN-205 vaccine elicited an antitumor effect.

Table 1. The Cytolytic Activity[a] Induced by KLN-205 Vaccine

Immunization	Target Cells	% Specific Lysis at E:T 10:1	20:1	40:1
Saline	KLN-205	4.8 ± 1.6	6.5 ± 1.5	7.8 ± 1.2
OK-432	KLN-205	6.0 ± 1.1	7.4 ± 1.2	8.7 ± 1.4
OK-432 treated with GA	KLN-205	5.3 ± 1.8	7.0 ± 0.8	9.3 ± 1.7
KLN-205 treated with GA	KLN-205	7.4 ± 0.8	8.4 ± 1.2	11.8 ± 2.5
KLN-205 vaccine	KLN-205	9.2 ± 1.4	13.7 ± 1.7[b]	21.5 ± 2.7[b]
KLN-205 vaccine	P815	4.4 ± 1.5	5.4 ± 1.3	6.5 ± 2.2

[a] The cytolytic activity induced by the KLN-205 vaccine. Splenocytes were taken from DBA/2 mice 7 days after the 3 injections of KLN-205 vaccine, KLN-205 treated with GA, OK-432 treated with GA, OK-432 alone, or saline. They were co-cultured with MMC-treated KLN-205 cells and then subjected to analysis of cytolytic activity against KLN-205 or P815 cells (syngeneic to DBA/2 mice). Three mice were used in each group. All experiments were carried out in triplicate. The results shown are data from a representative mouse's splenocytes in each group (mean ± SD of triplicate experiments). Similar results were obtained in other mice of each group. The spontaneous release of all assays was < 25% of the maximum release.

[b] Significant difference between KLN-205 vaccine and saline (control) ($P < 0.05$).

Histological Examination

DBA/2 mice were divided into 2 groups, a KLN-205-vaccine experimental group and a saline control group. There were 20 mice in each group. The mice were given subcutaneous immunizations with KLN-205 vaccine in the left flank once a wk for 3 wks. For the control group, saline was injected in the same manner. At the fourth wk, all mice were inoculated with 2×10^5/40 μL of KLN-205 in the tongue. Three mice from each group were killed randomly at 24 hrs, 5, and 20 days after tumor inoculation, respectively. The excised tongue tissue was fixed in 10% buffered formalin, routinely processed, and embedded in paraffin.

RESULTS

The KLN-205 Vaccines Elicit Cytolytic Activity

Table 1 shows that the KLN-205 vaccine elicited a greater cytolytic response than the others. Although KLN-205 treated with 0.2% GA was somewhat effective in this respect, KLN-205 vaccines induced an approximately two-fold greater response when the effector:target ratio was 40:1. OK-432 treated with GA and OK-432 alone induced almost the same level of response as the controls (saline). KLN-205 vaccine failed to induce cytolytic effect for another DBA/2-derived cell (P815 mastocytoma) (Table 1).

To investigate the phenotypes of effector cells, we treated them with the anti-CD4 or anti-CD8 antibodies and C to deplete the respective T-cell subpopulation for cytolytic assay. The cytolytic activity of effector cells was not affected by treatment with anti-CD4 antibodies and C, while effector cells treated with anti-CD8 antibodies and C decreased the cytolytic activity (Table 2).

Table 2. The Cytolytic Activity after Depletion with Each Monoclonal Antibody[a]

Treatment	% Specific Lysis at E:T 10:1	20:1	40:1
None	12.0 ± 1.5	16.4 ± 2.0	28.2 ± 2.2
C	10.2 ± 1.8	17.5 ± 2.1	27.0 ± 2.4
Anti-CD4 + C	9.8 ± 3.8	15.2 ± 2.5	24.8 ± 3.6
Anti-CD8 + C	5.4 ± 1.2[b]	6.5 ± 1.5[b]	9.5 ± 1.6[b]

[a] Rat monoclonal antibodies against the murine CD4 (L3T4) and CD8 (Ly2) were used for depletion of T-cell subpopulations. Three mice were used in each group. All experiments were carried out in triplicate. The results shown are data from a representative mouse's splenocytes in each group (mean ± SD of triplicate experiments). Similar results were obtained in other mice of each group.

[b] Significant difference between None and Anti-CD8 + C ($P < 0.05$).

The KLN-205 Vaccines Suppress Both the Incidence and Growth of KLN-205 Cells

In mice immunized with the KLN-205 vaccine, the incidence of tumors was observed at 5 days after KLN-205 inoculation. Until 30 days after inoculation, 56.6% incidence was observed. In the other groups, the incidence of tumor was 100% 20 days later (Fig. 1A). There were statistically significant differences between the KLN-205-vaccine group and the other groups. When tumor size was measured, the KLN-205-vaccine group revealed greater inhibition of tumor growth than the other groups, and there were statistically significant differences among them (Fig. 1B). The suppression of mortality rate was also studied among the 7 groups. In the KLN-205-vaccine group, only 56.6% of the mice died of tumor growth by 40 days after tumor inoculation. In contrast, all mice in the other groups and the control group died before 40 days after tumor inoculation (Fig. 1C). As shown in Fig. 1D, antitumor effect induced by post-immunization with KLN-205 vaccine also revealed a stronger inhibition for tumor growth than did the control.

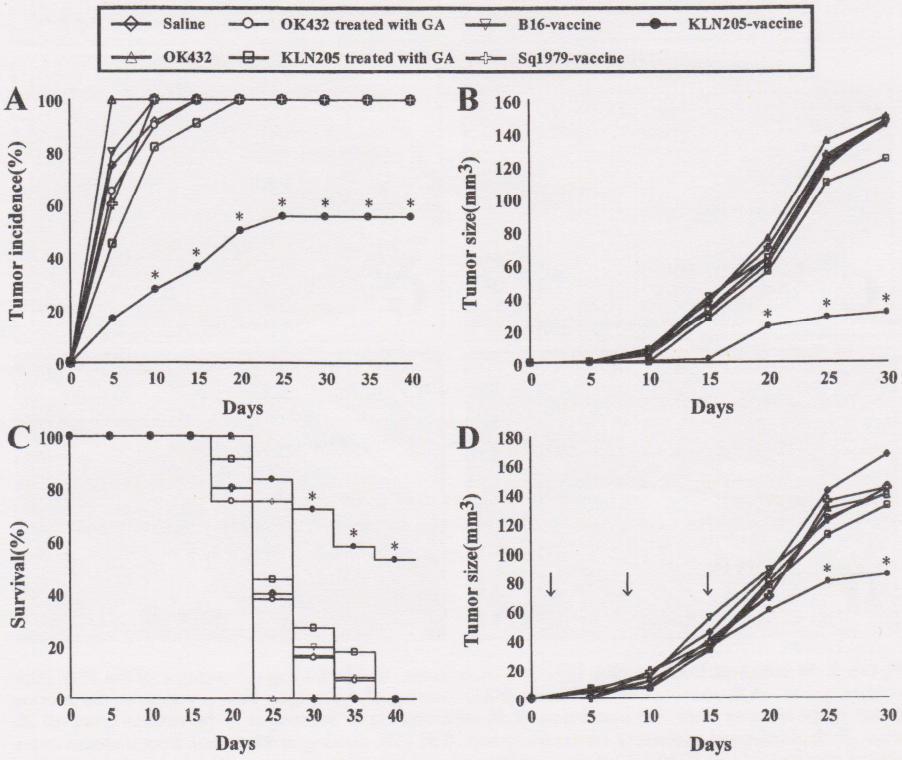

Figure 1. Antitumor effects induced by immunization with KLN-205 vaccine. The mice were immunized with KLN-205 vaccine, B16 vaccine, Sq1979 vaccine, KLN-205 treated with GA, OK-432 treated with GA, or OK-432 alone by means of a 100-μL syringe and a 30-gauge needle, once a wk for 3 wks. Control-group mice were injected with saline in the same manner. *Significant difference between the groups of KLN-205 vaccine and saline (control) ($p < 0.05$). One wk after final injection, all mice were inoculated with KLN-205 cells. **(A)** Suppression of tumor incidence by the KLN-205 vaccine. **(B)** Suppression of tumor growth by the KLN-205 vaccine. We monitored tumor size by measuring the longest diameter (mm) and the perpendicular diameter (mm) of the mass, and scored it by using the formula $0.5 \times a \times b^2$ (mm^3), a being the longer diameter and b being the shorter diameter. **(C)** Suppression of mortality rate by the KLN-205 vaccine. Kaplan-Meier curves showing the probability of survival were constructed for immunized mice groups and control mice groups. **(D)** Suppression of KLN-205 tumor growth by post-immunization with KLN-205 vaccine. Arrows represent each post-immunization. One day after inoculation with KLN-205, mice were post-immunized 3 times with KLN-205 vaccine, B16 vaccine, Sq1979 vaccine, KLN-205 treated with GA, OK-432 treated with GA, or OK-432 alone. Tumor size was then monitored as described above.

Histological Findings

In the control group, 24 hrs after tumor inoculation, KLN-205 cells were surrounded by a small number of lymphocytes (Figs. 2E, 2F). In contrast, a large number of lymphocytes infiltrated around KLN-205 cells in the KLN-205-vaccine group (Figs. 2A, 2B). Five days after tumor inoculation, there was a greater level of lymphocyte infiltration in the vaccine group than in the control group. The former lymphocytes surrounded the KLN-205 cell mass as a barrier, but the latter did not enclose it (data not shown). Fig. 2 (C,D,G,H) shows histological sections of the KLN-205-vaccine group and the control group at 20 days. There were few tumor cells in the KLN-205-vaccine group, while a massive tumor mass was observed in the control group. Moreover, notable fibrosis was observed around the small insular mass. Mitoses were obvious in the control group, but not in the KLN-205-vaccine group.

DISCUSSION

Strengthening of the antigenicity of tumor cells seems to be one of the most important strategies in cancer immunotherapy. We hypothesized that

tumor cells conjugated with foreign antigens failed to escape detection by the host immune system, leading to acquisition of effective priming by the host. OK-432 is a lyophilized biological preparation containing cells of the *Streptococcus pyogenes* Su strain treated with benzylpenicillin (Okamoto *et al.*, 1966; Saito *et al.*, 1988), and is broadly used as a biological response modifier (BRM) for tumor-non-specific immunotherapy. We selected OK-432 as the foreign antigen, since it needed to be both strongly antigenetic and safe for the host. KLN-205 cells were conjugated with OK-432 by 0.2% glutaraldehyde methods (Bukawa *et al.*, 1995a,b).

The antitumor effects elicited by the KLN-205 vaccines were analyzed for cytolytic activity, suppression of tumor growth, and histological study. In the cytolytic assay, splenocytes from mice immunized with the KLN-205 vaccines indicated a greater cytolytic response than those from mice immunized with the others. In contrast, the others showed almost the same levels of percent-specific lysis as the controls. Table 1 shows that cell-mediated immunity against not P815 but KLN-205 cells can be induced by immunizations with the KLN-205 vaccine, indicating that KLN-205 vaccine elicited anti-KLN-205-specific immunity. These findings also suggest that the conjugation of both (tumor cells and OK-432) may be important for cancer immunotherapy. The cytolytic activity induced by immunization with KLN-205 vaccine was decreased by treatment with anti-CD8 antibody and C. It was assumed that the activity was mediated by CD8+ cells (Table 2).

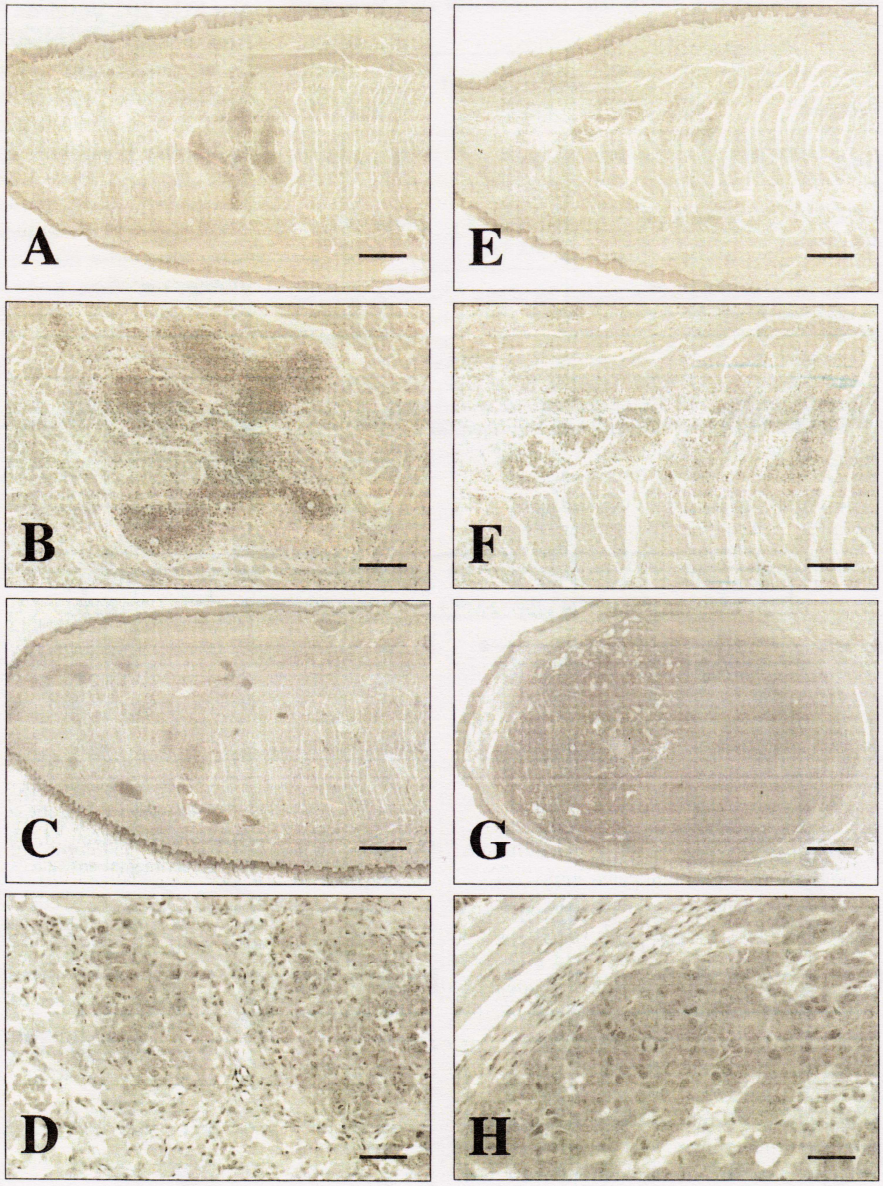

Figure 2. Histological findings after KLN-205 inoculation. **(A-D)** Histological sections of the KLN-205-vaccine group. **(A,B)** Hematoxylin and eosin (H&E) staining of the tongue from a mouse in the vaccine group at 24 hrs after KLN-205 inoculation. **(C,D)** H&E staining of the tongue in the vaccine group at 20 days. **(E-H)** Histological sections of the control group. **(E,F)** H&E staining of the tongue from a mouse in the control group at 24 hrs. **(G,H)** H&E staining of the tongue in the control group at 20 days. Scale bars = 100 μm (A,E,C,G), 50 μm (B,F), and 20 μm (D,H).

In the KLN-205-vaccine group, the tumor incidence rates were 25% at 10 days and 56.6% at 30 days after inoculation. The tumor suppression rate was 43.4% (Fig. 1A). In the control (saline-injected) group, tumors were formed at 3 days after inoculation. The tumor incidence rate reached 100% by 10 days. When the mice were immunized with the others (Bib vaccine, Sq1979 vaccine, KLN-205 treated with GA, OK-432 treated with GA, or OK-432 alone), not only the tumor incidence but also the tumor growth and survival period were almost the same as those of the control group (Figs. 1A-1C). Although OK-432 is broadly used for tumor immunotherapy, the results from this study showed that it failed to induce a strong antitumor effect. This may be explained by the use of a low dose of OK-432. Fig. 1D shows that significant antitumor

effects were also induced in mice post-immunized with KLN-205 vaccine. This finding suggests that our vaccine method will be effective in planning subsequent treatment in human clinical situations, such as micrometastasis or positive surgical margin after surgery.

To analyze the process of antitumor effects after inoculation of KLN-205 cells, we performed histological examination (Figs. 2A-2H). Infiltrating lymphocytes were markedly observed around the tumor cells at 24 hrs after tumor inoculation in the KLN-205-vaccine group. In contrast, scattered lymphocytes were shown and no infiltrating lymphocytes could be observed in the control group (Figs. 2A, 2B, 2E, 2F). These results indicated that immunized mice acquired an immunological memory for KLN-205 cells. At Day 20 (Figs. 2C, 2D, 2G, 2H), there were few tumor cells in the vaccine group. In contrast, large tumor masses and many mitoses were observed in the control group. All results indicate that OK-432-conjugated tumor vaccines led to acquisition of effective priming, induced tumor-specific cell-mediated immunity, and suppressed tumor activity.

Mukai *et al.* (1996) reported that *in vivo* priming of BL6 tumor cells mixed with various doses of OK-432 was effective. They used adoptive immunotherapy of *in vitro*-sensitized lymphocytes with IL-2 injections. Our conjugation methods induced antitumor effects without adoptive transfer and injections of cytokine such as IL-2. Our results suggest that OK-432-conjugated vaccine may be more effective in priming than a mixture with OK-432. Recently, Vermorken *et al.* (1999) reported that surgical resection with an autologous tumor-cell-BCG vaccine was more beneficial than resection alone in stage II and stage III colon cancer. Although they used 10^7 irradiated autologous tumor cells mixed with BCG organisms as a vaccine, the present results suggest that administration of the conjugated tumor cells has the potential to induce stronger antitumor effects than does a simple mixture. Our conjugated tumor vaccines may induce more effective antitumor effects than a mixture of tumor and BCG organisms in clinical trials. The preparation of the OK-432-conjugated tumor vaccines is simple and cost-effective, and many facilities throughout the world might easily be able to construct this type of tumor vaccine candidate. Furthermore, this vaccine candidate seems to be safe for clinical use in humans. It is possible to inject a patient repeatedly from the time of tumor resection to the patient's death, to prolong his lifetime and to improve quality of life, even if the tumor is not completely cured. This new tumor vaccine method may be a strong candidate for future clinical use.

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