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Local Delivery System of Immune Modulating Drug for Unresectable Adenocarcinoma: *In Vitro* Experimental Study and *In Vivo* Animal Study

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Abstract

The purpose of the study was to evaluate the efficacy and safety of a developed drug delivery system containing OK-432 through *in vitro* and animal study. An OK-432-impregnated polycarbonate/polyurethane stent membrane was used to develop a drug delivery system (DDS) enabling the locoregional release of OK-432. Polyethyleneglycol was used as a detergent and porosity generator. The stability of OK-432 in solvent, releasing kinetics of drug, and cytotoxicity of the DDS were evaluated. OK-432-impregnated DDS was implanted in mice in which a human adenocarcinoma cell line was injected and grown in their back. Flow cytometry and enzyme-linked immunosorbent assay were used for quantifying the amount of drug. OK-432 exposed to phosphate-buffered saline and OK-432 exposed to *N*,*N*-dimethylacetamide showed similar results on dot graphs and histograms. However, OK-432 exposed to tetrahydrofurane showed different dot graphs and histograms, which means that the antigenicity of the drug was changed. The release rate of OK-432 was maintained at a constant level for 6 weeks. The local delivery of OK-432 was found to have an antitumor effect on a human adenocarcinoma cell line in an animal study, but no effect on this cell line in *in vitro* cell culture. Histologic examination showed minimal inflammatory reaction in surrounding tissue. Our study shows that local treatment using this OK-432 release system is safe and effective in reducing adenocarcinoma in a mouse model.

Key words: Drug delivery—OK-432—Sustainable release—Adenocarcinoma—Gastrointestinal tract

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Many cases of gastrointestinal tract malignancy are already at an unresectable stage by the time they are discovered, despite significant advances in diagnostic modality. Many of these patients with obstructive symptoms are often treated using metallic stents to relieve obstructive symptoms. However, the stent patency period is as short as 2 weeks to 2 months, due to tumor ingrowth or overgrowth, and mean survival is at most 6–12 months. Therefore, both continuous relief from obstructive symptoms and local tumor control are required in the treatment of advanced gastrointestinal tract malignancies, including cholangiocarcinoma [1].

Local stent-based drug delivery [drug-eluting stent (DES)] is a new technology aimed to prevent the development of neointimal hyperplasia and restenosis following percutaneous coronary interventions. A number of DESs have been developed using different carrier stents, different kinds of coating, and different drugs.

OK-432 has been widely used in Japan as an adjuvant chemotherapy agent. It is a biological response modifier derived from the weakly virulent Su strain of *Streptococcus pyogenes*. OK-432 augments the activities of macrophages, lymphocytes, and natural killer (NK) cells and induces multiple cytokines, such as interleukin (IL)-1, IL-2, IL-6, tumor necrosis factor (TNF)-α(5), and interferon (IFN)-γ. In a few clinical studies and in animal experiments, OK-432 has been found to augment the activities of peripheral blood lymphocytes (PBLs), pleural effusion lymphocytes, splenic lymphocytes, and hepatic macrophages [2–9]. The purpose of this study was to develop a method of utilizing locally controlled OK-432 release for the treatment of inoperable gastrointestinal malignancies, including cholangiocarcinoma, and the

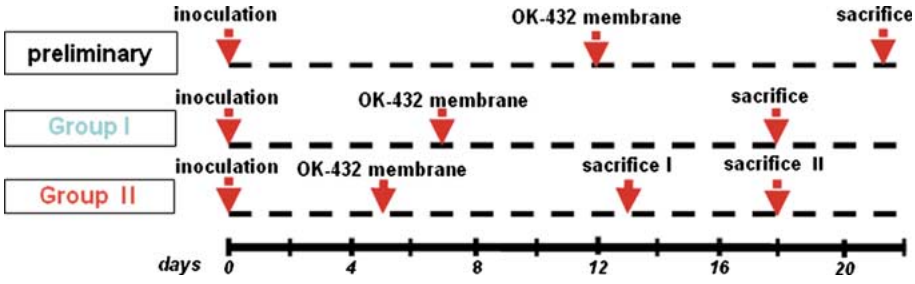

Fig. 1. Protocol of the animal study.

investigation of its feasibility and the direct antitumor effects on adenocarcinoma cells in the mouse model. Finally, we examined the stability of OK-432 in the presence of organic solvents for safety reasons.

Materials and Methods

Preparation of the OK-432 Releasing Membrane

We developed a drug delivery system (DDS) using a nonbiodegradable polymer, polyurethane, as a matrix containing various Polyethyleneglycols (PEGs) (MW 4000, 8000, 10,000) as detergents and porosity generators, as drug release control agents. PEG was dissolved in *N*,*N*-dimethylacetamide (DMAc) at 50°C with stirring. After mixing OK-432 with the PEG-DMAc solution, polyurethane was added. The solution was then emulsified using a magnetic stirrer. DMAc was then removed in a vacuum oven for 5 days. OK-432-releasing membranes were finished by punching dried membranes. (Fig. 1)

Stability of OK-432 in Various Solvents

We chose polyurethane as a matrix for the OK-432 releasing system. DMAc or tetrahydrofurane (THF) were used as solvents for making the polyurethane membranes. Because OK-432 is a biological drug, we should deliberate the safety of OK-432 in various organic solvents. After dissolving the OK-432 (picibanylR; Chugai Pharmaceuticals, Tokyo, Japan) in phosphate-buffered saline (PBS) (control solvent), DMAc, or THF, primary and fluorescein isothiocyanate (FITC)-labeled secondary antibody was added to each OK-432 dissolved solution. Flow cytometry (FACScan; Becton Dickinson, Heidelberg, Germany) dot graphs and histograms of OK-432 were obtained to evaluate the safety of OK-432.

Releasing Kinetics of OK-432

An *in vitro* drug release test using three different types of OK-432-containing membranes (20.4 μg/membrane) was to evaluate the drug release profile of the membranes. Three types of DDS incorporating different molecular-weight PEGs (MW 4000, 8000, 10,000) were used. Each membrane was placed in a centrifuge tube (Falcon Labware) containing 10 mL of PBS, and the tubes were placed in a shaking incubator. A modified enzyme-linked immunosorbent assay (ELISA) was used to quantify the amounts of OK-432 released into the buffer. We prepared curves representing cumulative drug release over 35 days (i.e., 1, 2, 4, 8, 12, and 24 h, and after 2, 4, 7, 14, 21, 28, and 35 days). The modified ELISA technique used was developed by combining fluorescene activated cell scan (FACS) and ELISA. At first, we centrifuged conical tubes containing released OK-432 to obtain OK-432 pellets. Specific OK-432 antibody (provided by Chugi Medical) diluted 1:50 was added at 100 μl per tubes. Tubes were then incubated at 37°C for 1 h and then recentrifuged. Secondary antibody (AP-Goat Anti-Rabbit IgG Conjugate; Zymed Laboratories) diluted 1:100 was added at 100 μl per tube, and the tubes were then incubated at room temperature for 30 min and centrifuged twice. After diluting the OK-432 pellet by 2000:1, 100 μl was transfered to an ELISA plate (Nunc), and pNPP substrate solution (100 μl) was added to each tube. The reaction was stopped by adding 100 μl of 0.1 *N* NaOH. Absorbance measurements were taken at 405 nm, using an ELISA plate reader.

Cytotoxicity Assay

Cytotoxicity of the OK-432-containing membrane was determined using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) viability assays. No membrane (blank), OK-432 membranes, and non-OK-432 membranes (control membrane) were added to CT-26 (a colon metastatic carcinoma cell line) containing media for evaluating the cytotoxicity of DDS. CT-26 was cultured using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were counted to 2 × 104/ml and seeded at 2 ml to a six-well plate and incubated for 5 days. At 1, 2, and 5 days, 100-μl aliquots of the cultures were added to a 96-well plate followed by 100 μl of MTT reagent.

Plates were incubated at room temperature until a suitable color development, and optical densities were measured at 570 nm using a microtiter plate reader. Percent viability was calculated from the determined optical densities. The formula used to determined percent viability was as follows:

$$\frac{\text{The value of O.D. at OK-432 membrane or control membrane}}{\text{The value of O.D at no membrane}} \times 100$$

The direct cytotoxicity of OK-432 and of materials (polyurethane, DMAc, PEG) to CT-26 was determined by MTT assay.

Animal Study

In the animal study, we inoculated CT-26 (a murine colonic metastatic cell line) cultured in DMEM supplemented with 10% FBS into the BALB/c (Daehanbio Co., Seoul. Korea). Mice were divided into two groups based on the number of cells inoculated. In Group I, mice were injected with 0.1 ml of 8 × 105/ml of cell-line, and in group II, mice were injected with 0.1 ml of 1.5 × 107/ml. OK-432 releasing membrane and non OK-432 releasing membrane (control) were implanted in the mouse when the volumes of the two tumors

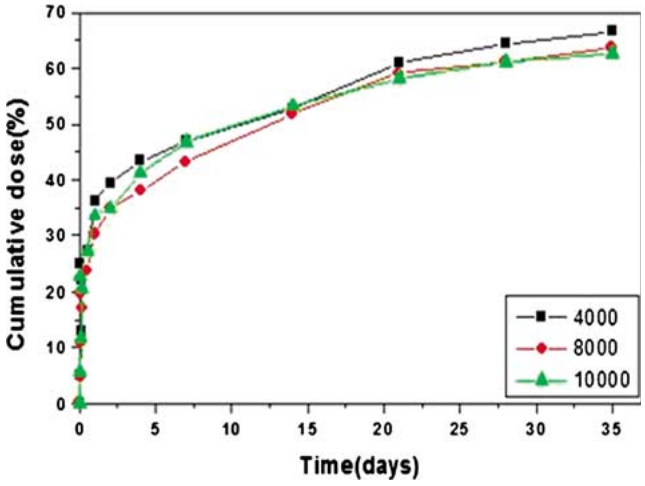

Fig. 2. The results of *in vitro* release tests with OK-432-containing membrane.

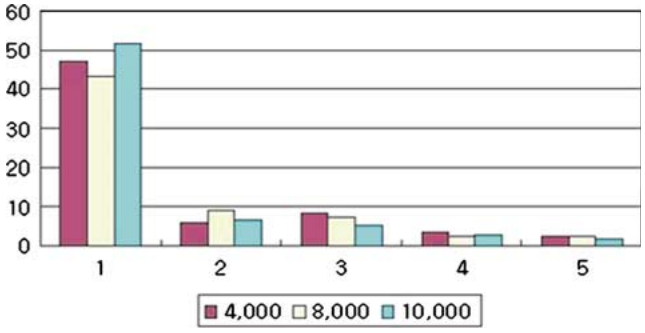

Fig. 3. The results of *in vitro* release tests, weekly excretion of OK-432 from a membrane (μg/week).

increased significantly and simultaneously. In group I, tumor volume was increased gradually, and 7 days after cell inoculation, the membranes were implanted. In group II, membrane implantation was performed 5 days after inoculation. Group I mice were sacrificed at 11 days after implantation, whereas group II mice were sacrificed at 8 and 13 days after membrane implantation. Following the surgical exploration of both tumors, tumor volumes were calculated using (major axis) × (the minor axis) (2 × 100). Removed tumors were dipped in 10% neutral formalin solution, and fixed tissues were processed for histology. Hematoxylin and eosin (H&E) and TUNEL staining (ApoTag® peroxidase kit) were performed to examine the effects of OK-432. TUNEL stain showed apoptotic single-strand DNA (ssDNA). H&E stain showed inflammatory cell infiltration in the tumor and surrounding tissue. The animal study protocols are described in Figure 1.

Results

Morphologic Findings of OK-432

The streptococcal preparation of OK-432 is a lyophilized powder of a virulent Su strain of *Streptococcus pyogenes* treated with penicillin. Bluish violet streptococci were observed by Gram staining, which scanning electron microscopy (SEM) showed were 0.5 μm in size.

Stability of OK-432 in Various Solvents

In dot graphs, low-angle forward scatter intensity was approximately proportional to the cell diameter and orthogonal (90°) scatter intensity was approximately proportional to the quantity of granular structure within the cell. OK-432 that was exposed to DMAc showed a right shift of peak formation, compared with the control. This means that OK-432 in DMAc maintains its antigenicity. However, OK-432 that was exposed to THF showed a change in the dot distribution of the dot graph and no right shift of peak formation. Therefore, the antigenicity of the OK-432 was changed in THF.

Amount of OK-432 Released by the Releasing System

The average thickness of the membrane was 200 μm. The average amount of OK-432 in each membrane was about 20.4 μg. By *in vitro* drug release testing, the OK-432-containing membrane released 50% of the drug within the first week, with low-dosage drug release continuing during the entire period. During the 35-day experiment, OK-432 was released continuously. (Fig. 2). The amount of OK-432 released during the first and sixth days were 33.4% (6.2 μg) and 13.95% (2.59 μg), respectively. The amount of OK-432 released during the second, third, fourth, and fifth weeks were 7.0% (1.3 μg), 6.8% (1.26 μg), 2.8% (0.52 μg), and 1.2% (0.22 μg), respectively (Fig. 3). The total amount of OK-432 released during the 35-day period was 65.15% (12.09 μg). However, no difference was observed among the membranes containing PEG with different molecular weights.

Cytotoxicity Assay

Cellular density was similar between control membrane and DDS, which is different from that of the blank. The optical density values of two membranes (control membrane and DDS) were lower than that of the blank (Fig. 4).

Animal Study

In the animal study, OK-432-releasing membranes were shown to reliably inhibit tumor growth in all six cases of study group versus the control group (Fig. 5). Gross findings showed that apoptotic cells were stained brown and that the tumor volume changed. Treatment with OK-432-releasing membranes inhibited RAG adenocarcinoma growth. Temporal changes in tumor dimensions are shown in Figure 6. Histological results showed that more necrotic and apoptotic cells were observed in the OK-432-releasing membrane-treated tumor (Fig. 7). No inflammatory cell infiltration in the tumor or surrounding tissue was detected.

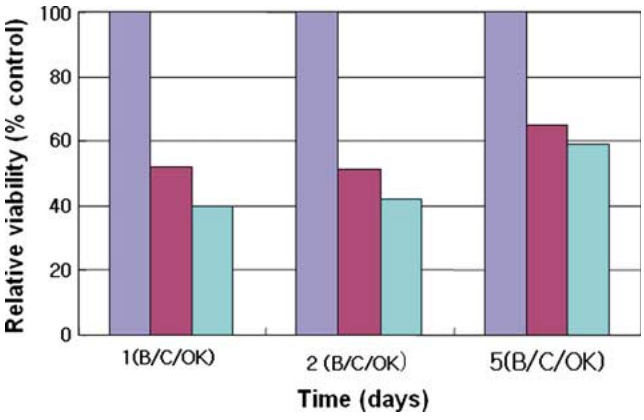

Fig. 4. Cytotoxicity by MTT assay. Viabilities of the blank were higher than for the control or the OK-432 membrane. (B: blank; C: control; OK: OK-432 membrane).

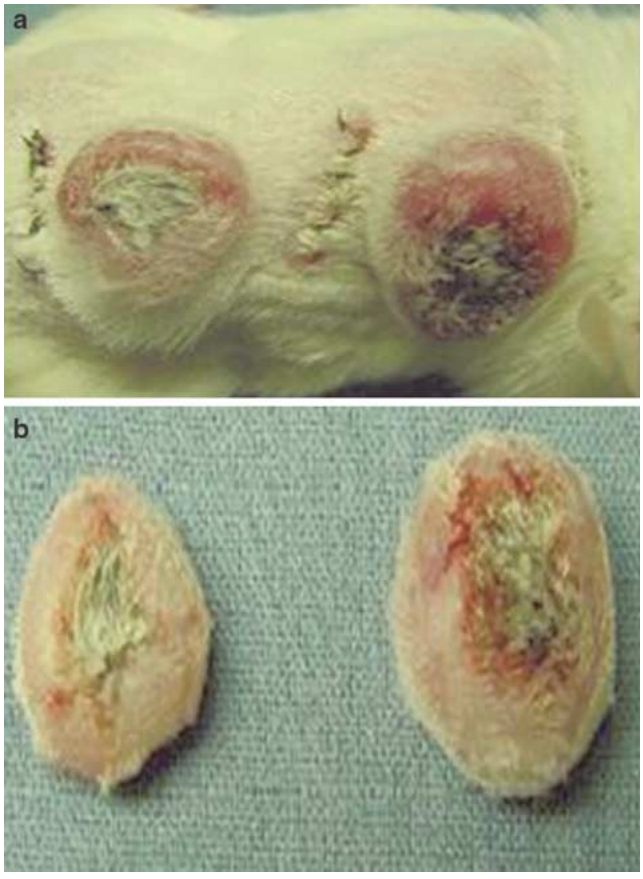

Fig. 5. Eight days after membrane implantation **(a)**. These removed tumors show the size discrepancy **(b)**.

Discussion

The drug-eluting stent is considered a great breakthrough in the history of percutaneous intervention. Although the stent-coating technology is considered a proprietary process, several coating techniques have been developed in which, most commonly, a pure drug or a polymer–drug matrix coating is applied to the surface of the stent struts. To achieve local delivery, a drug must be loaded, retained, and released in a controlled manner. Recent studies have demonstrated significant reductions in the development of in-stent thrombosis and intimal hyperplasia with drug-coated stents [10, 11]. According to our review, reports of a DDS for malignant tumor are very limited, but coating endoluminal stents with a chemotherapeutic-polymer blend could reduce the incidence of malignant overgrowth and increase the efficacy and duration of clinical effectiveness of the device [12].

Malignant obstructions of the stomach, duodenum, colon, rectum, or bile duct are preterminal events and lead to a progressive deterioration of a patient's quality of life. Although surgical palliation is an available option in such patients, morbidity and mortality are high, and adequate symptomatic control is achieved in only about half of the patients treated [13–16]. Self-expandable metallic stents have been used recently for palliative treatment of malignant gastrointestinal tract obstruction, including biliary obstruction [17]. However, recurrent stenosis of the stent because of progressive tumor ingrowth is a problem because metal stents are usually used [17–21]. Overall, recurrent stenosis rates of 8–46% after intervals of 2–21 weeks (mean: 7.5 weeks) have been reported [17–21].

OK-432 is widely used in adjuvant chemotherapy for lung cancer, esophageal cancer, gastrointestinal cancer, bladder cancer, and intraperitoneal metastasis [2–9]. Although many effective anticancer chemotherapeutic agents, such as Pactitaxol and Cisplatin, are available, theoretically these drugs cannot be used as local delivery agents for antitumor treatment. In anticancer chemotherapy, the dose of a chemotherapeutic agent is important, but the local DDSs cannot contain enough dose of the drug for effective cancer treatment. From this viewpoint, OK-432 is an ideal drug for local delivery because of its actions on cancer by a different mechanism.

The streptococcal preparation, OK-432, has been shown be a biological response modifier and has been applied clinically against various types of cancer. It has been proposed that its immunomodulating mechanism relies on the activation of NK cells, macrophages, polymononuclear cells, and CD4 T-cells. In addition, OK-432 has been reported to induce many cytokines, including IL-1, IL-2, IL-6, TNF-α, IFN-γ, and IL-12. Local injections of OK-432 can significantly enhance the immunotherapeutic potential of tumor-draining lymph node (dLN) cells by augmenting their Th1-type cytokines from OK-432-reactive CD4 T-cells. Moreover, OK-432 has been reported to help the infiltration of T-cells into injection sites to improve the survival rate of cancer patients when concomitantly used with chemotherapy [22]. Moreover, in the present study, we have found that local delivery of OK-432 from a polyurethane membrane induces more apoptosis of adenocarcinoma cells in mice. We observed more apoptotic cells by the TUNEL staining

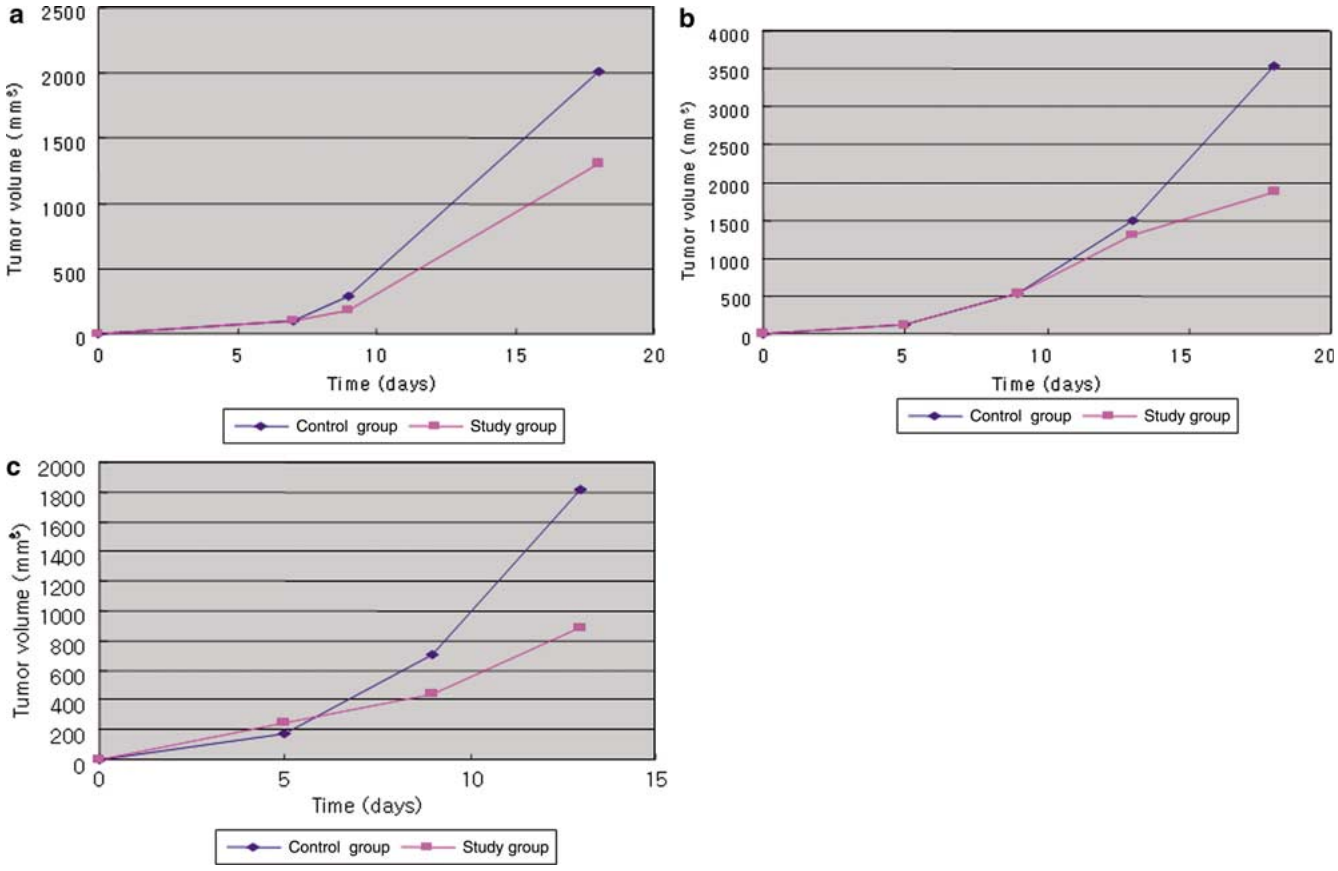

Fig. 6. The results of tumor volume in group I [**(a)** 8 × 104/100 μL inoculation] and group II [**(b, c)** 1.5 × 106/100 μL inoculation]. Tumors treated with DDS were larger than the control in all groups.

of a tumor specimen, which had been treated with the developed DDS.

The developed DDS incorporating a stent covered with a polyurethane membrane could be applied in the local treatment of gastrointestinal tract cancers, including bile duct cancer, tracheo-bronchial malignancies, and urinary tract malignancies. Moreover, the developed DDS had no suppressive effect on tumor cells *in vitro* but suppressed tumor growth remarkably in an animal model, thus indicating that its activity is mediated by immune activation by OK-432, which has already been reported by many authors [2–9].

The effect of OK-432 is the result of its antigenicity, and this antigenicity is not affected by the solvent used for making the DDS. In the cytotoxicity test, cellular density is higher in the blank than the DDS and control membrane, which could have been caused by the cytotoxicity of the solvent.

The limitations of our experimental study are as follows: First, measurements of immunological parameters, such as NK activity and lymphocyte subsets in the tissue, were not performed. Second, the drug release profiles of different membranes containing different molecular-weight PEGs were almost the same. We postulate that the amount of drug used was too low to allow the release profiles to be differentiated. Measurements of the drug release profile when larger amounts of drug are incorporated into DDS are warranted.

In conclusion, our study shows that local treatment using the OK-432-releasing system is feasible and effective at reducing adenocarcinoma in a mouse model. Therefore, the described OK-432-releasing system could be useful for patients who need local and sustained immunotherapy. Further experiments with various drug-eluting membranes containing other anticancer drugs and to clarify the mechanism of the antitumor effect of the developed DDS are needed to confirm our experimental results.

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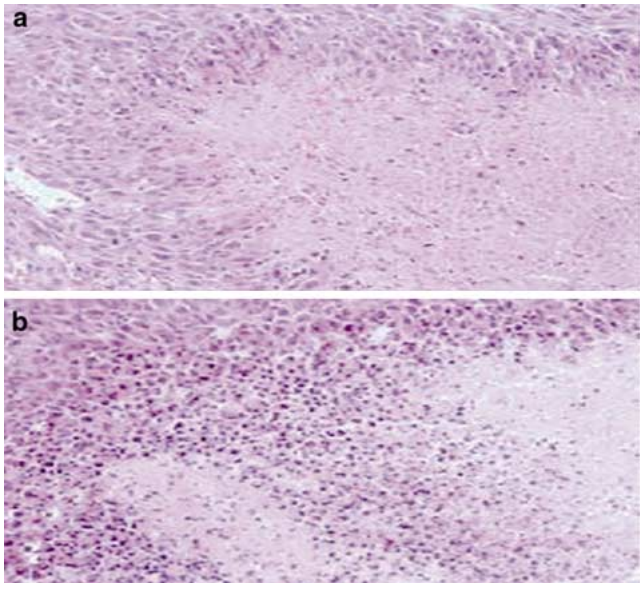

Fig. 7. In a microscopic examination, solid and local necrosis was noted in the tumor treated with the control membrane. Very extensive necrosis was observed in the tumor treated with DDS. At high magnification, cheesy necrosis and a small number of apoptotic cells were observed in the blank treated tumor **(a)**. On the other hand, extensive cheesy necrosis and an abundance of apoptotic cells were noted in the tumor treated with DDS **(b)**.

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