Antineoplastic Effects in Rats of 5-Fluorocytosine in Combination with Cytosine Deaminase Capsules

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ABSTRACT

5-Fluorocytosine (5-FC) lacks antineoplastic activity in human subjects because of the absence of cytosine deaminase (CDase) in mammalian cells. Intratumoral conversion of 5-FC into 5-fluorouracil (5-FUra) by locally implanted capsules containing CDase followed by systemic administration of 5-FC can be expected to induce antineoplastic activity at a local site with minimal systemic toxicity. In vitro and in vivo experiments were performed to evaluate this hypothesis. Spectrophotometric analysis confirmed the deamination of 5-FC to 5-FUra by CDase extracted from cultivated Escherichia coli. In vitro studies showed that 5-FC combined with CDase induced significant growth-inhibitory effects on the cultured glioma cells. An active CDase capsule, made of cellulose tubing, was newly designed for local implantation. 5-FC concentrations in the s.c. tumors of the rats given these CDase capsules, followed by 5-FC administration, showed a sufficient amount of delivery of 5-FC to the tumor tissue. 5-FUra appearing in the tumor reached the level of 8.0 μg/g at 2 h and stayed at more than 1.0 μg/g at between 1 and 6 h. Significant reduction of the tumor growth and cytotoxic changes were observed. The passive cutaneous anaphylaxis reaction demonstrated no allergic reaction to the host due to the capsule. These results suggest that this chemotherapeutic method is effective for human brain tumors.

INTRODUCTION

The successful treatment of malignant brain tumors remains among the most challenging problems for neurological surgeons. Surgery followed by radiation therapy has been the standard treatment. It must be admitted, however, that the results have been disappointing. Since the introduction of cancer chemotherapy, many agents have been developed and are widely used. Numerous modes of administration of the drugs have been attempted. Nevertheless, the clinical results have generally been poor. 5-FUra 2 is used in cancer chemotherapy, but it has serious side effects, and substitutes for it have been widely sought (45).

Currently, 5-FC, an analogue of 5-FUra, is clinically used for fungal infections with minimal toxicity (1, 9, 13, 42, 43). 5-FC itself lacks antineoplastic activity in humans. The antifungal activity of this drug is explained by deamination of 5-FC into 5-FUa by intrinsic CDase in the fungal cells. The authors attempted local generation of 5-FUa as a new approach for antitumor therapy. If 5-FC is delivered to the tumor bed, in which CDase in immobilized form has previously been implanted surgically, it can be expected that the agent would be converted into 5-FUra by the action of the enzyme, and then antineoplastic activity would develop at the local site with minimal systemic toxicity.

In the present work, basic experiments on the effects of combined therapy with 5-FC and CDase on tissue-cultured glioma cells were first performed. Following this, the antineoplastic activity induced by local implantation of a newly designed CDase capsule followed by systemic administration of 5-FC to rats bearing s.c. tumors was studied by means of determination of 5-FC and formed 5-FUra levels in the tissues as well as tumor growth. Finally, immunological responses (PCA reaction) against the implanted CDase capsule were studied.

MATERIALS AND METHODS

Chemical Agents. 5-FC (Lot A 32274) was made available by Nippon Roche K. K. (Tokyo, Japan), and 5-FUra (Lot 90323-01) was supplied by Kyowa Hakko Co., Ltd. (Tokyo, Japan).

Enzyme Preparation and Its Activity. CDase was extracted from the cells of cultivated Escherichia coli, K-12 3301, and partially purified. CDase activity was measured by spectrophotometric analysis with a Hitachi 340 recording spectrophotometer. The reaction mixture of the assay system contained 6 μmol of substrate 5-FC in PBS, the composition of which was as follows: NaCl, 8.0 g; KCl, 0.2 g; Na2HPO4, 1.15 g in 1000 ml of distilled water (adjusted to pH 7.4); and enzyme solution in a final volume of 2.0 ml. The reaction was conducted at 37°C C. After incubation, at intervals, 0.2-ml samples were withdrawn and transferred into 9.8 ml of 0.1 n HCl to terminate the reaction. Aliquots were then subjected to spectrophotometric analysis. One IU of CDase activity was defined as the amount of enzyme decomposing 1 μmol of cytosine per min.

Cells and Culture Condition. EA-285 glioma cells (52) derived from methyl nitrosourea-induced CDF rat glioma were grown in Eagle’s minimal essential medium supplemented with 10% fetal calf serum (Grand Island Biological Co.) in monolayer culture. Cultured cells were maintained at 37°C C in a humidified 5% CO2 atmosphere.

In Vitro Assay of Cytotoxicity. The assay of the cytotoxicity of the combined therapy with 5-FC and CDase consisted of evaluation of cell proliferation on the cultured EA-285 glioma cells. Monolayered cells were treated with 0.25% trypsin, and cell suspensions, having a count of 2×106 cells/ml, were prepared and seeded in 25-cm Falcon tissue culture bottles. After confirmation of the formation of monolayered culture on the following day, the cells were treated with 1, 5, 10, 25, and 50 μg of 5-FC per ml (each bottle contained 4 ml) and 0.2 IU of CDase for 24 h. The cells were then trypsinized at 0, 48, and 96 h after incubation with the drugs, and the mean cell number was counted using the trypan blue dye exclusion method (26).

In order to evaluate the toxicity of 5-FC or CDase per se, the cells were exposed to 200 μg of 5-FC per ml or 0.4 IU of CDase for 24 h, respectively. The growth curves of the treated cells were then evaluated, compared with that of the control.

Experimental Animals. The animals used were male Fischer (F344, DUCRJ) rats weighing about 100 g.

Tumor Cell Suspension and s.c. Implantation. Cultured EA-285 glioma cells were harvested by trypsinization to obtain a suspension of tumor cells. Tumor cell suspension containing 5 x 10⁷ cells was injected s.c. into the right lower back of the rats. When they grew to approximately 2 cm³ by about 30 days after implantation, these tumors were used for in vivo studies.

Preparation of CDase Capsules. The capsules were prepared from a Spectra/Por 6 dialysis tube (Part 132634; Spectrum Medical Industries, Los Angeles, CA) with a molecular-weight cutoff of 1000 and measuring 10.2 mm in diameter and 20 mm long. After one end was sealed with medical Aron a A "Sanyo" (Sanyko Co., Ltd., Tokyo, Japan), one IU of CDase (0.2 ml) was injected into the capsule using sterile technique. In order to determine the conversion of 5-FC into 5-FUra by the CDase capsule, the capsule was soaked in 5-FC solution, and the sample was taken serially for spectrophotometric analysis.

Determination of Tissue Concentration of 5-FC and Converted 5-FUra. When the s.c. tumor grew to approximately 2 cm³ in the rats, a CDase capsule was implanted in the tumor bed through a skin incision without removing the tumor. On the 14th day after implantation, 150 mg of 5-FC per kg were administered i.p., and the animals were then serially sacrificed to obtain the tumor tissues adjacent to the capsule and sera (one group consisted of 10 animals). Tumor tissues were separately homogenized to make 25% tissue homogenates. The concentrations of 5-FC and converted 5-FUra were determined by microbiological assay with the thin-layer cup method (49).

Test organisms used were Saccharomyces cerevisiae, strain AKU 4100, for 5-FC and Micrococcus luteus, American Type Culture Collection strain 10240, for 5-FUra. For the bioassay, standard solutions of 5-FC and 5-FUra were prepared, and the standard dose-response curves for the drugs were obtained by measurement of the inhibitory zones. Concentrations of the drugs in the samples being assayed were then obtained by determining the concentration on the standard curves corresponding to the specimen zone diameter.

Local Chemotherapeutic Studies. In the treated group, a CDase capsule was implanted in each animal according to the procedure described above. In the control group, a capsule containing PBS instead of CDase was implanted. Each group consisted of 10 animals.

Chemotherapeutic treatment was started on the third day following implantation of the capsule. Both groups were given 150 mg of 5-FC per kg i.p. every day for 30 days. For evaluation of the effects of the treatment on the tumor growth, tumor volume (cm³) was calculated (½ length x width x height), measuring the length, the width, and the height every 3 days. When the treatment with 5-FC ended, the experimental animals were sacrificed, and the s.c. tumors were extirpated. The tumors were weighed, and their histological changes were examined by light microscopy. The histological stain used was hematoxylin:eosin. CDase capsules extirpated were also examined for their enzyme activities.

PCA Reaction (32). Female albino guinea pigs weighing 250 g were used. Twenty animals (one group consisting of 10 animals) were used for the production of antisera. In one group, CDase solution containing 0.1 mg of protein (one IU of CDase activity) was injected s.c. In another group, a CDase capsule was implanted in the animal's back s.c. At 10 and 30 days after sensitization, antisera were obtained. The i.d. injection of the antiserum (0.1 ml) was made on both sides of the animal's back. PBS and nonsensitized sera were also injected as the control. At 6 h after passive sensitization, the antigen, consisting of 0.5 ml of 1% Evans blue in 0.15 M NaCl mixed with 0.5 ml of CDase solution (protein, 0.18 mg), was challenged i.v. At 30 min after challenge, the animals were sacrificed and skinned. PCA reactions were read on the inner side of the skin.

RESULTS

CDase Activity. Estimation of CDase activity is presented in Chart 1. The UV absorption spectra of 5-FC and 5-FUra have the maximum absorbance at 284 nm and 285 nm, respectively. When CDase is added to the substrate 5-FC, the peak wavelength gradually shifts from 284 nm toward 265 nm, suggesting that a part of the 5-FC was converted into 5-FUra. When the deamination was completed, the absorption spectrum obtained from the final sample showed the maximum at 265 nm.

Effects of 5-FC and CDase In Vitro. Growth curves of EA-285 glioma cells following 24-h exposure to 5-FC and CDase at various concentrations are presented in Chart 2. Significant growth inhibition was noted at more than 1 μg of 5-FC per ml. 5-FC or CDase per se had no cytostatic effects (Chart 3).

CDase Capsule. A schematic representation of the CDase capsule is shown in Chart 4. The membrane of this capsule allows permeation of substances with a molecular weight of less than 1,000, through micro pores. The molecular weight of CDase

CANCER RESEARCH VOL. 45 APRIL 1985
1754
LOCAL ANTINEOPLASTIC CHEMOTHERAPY

Chart 3. Growth curves of EA-285 glioma cells treated with a single administration of 5-FC and CDase. O, control; △, 5-FC (200 µg/ml); ×, CDase (0.4 IU).

Chart 4. Schematic representation of the CDase capsule.

is approximately 210,000, and that of 5-FC is 129. Therefore, 5-FC passes freely through the membrane into the capsule and is converted into 5-FUra, which is then diffused out of the capsule, while CDase is entrapped. Spectrophotometric analysis confirmed conversion of 5-FC out of the capsule.

Tissue Concentration of 5-FC and 5-FUra. Preliminary study of the microbiological assay using standard solution of the drugs showed that the minimum inhibitory concentration of 5-FC was 0.5 µg/ml with S. cerevisiae, and that of 5-FUra was 0.02 µg/ml with M. luteus. M. luteus was found to be sensitive to 5-FUra but resistant to 5-FC at less than 100 µg/ml (Chart 5). On the contrary, S. cerevisiae showed the same sensitivity to both 5-FC and 5-FUra (Chart 6).

The 5-FC concentration (A) in the sample mixed with 5-FC and 5-FUra, therefore, could be determined by the difference between the value (B) obtained by S. cerevisiae and that (C) obtained by M. luteus (A = B - C). The 5-FUra concentration was calculated as C. 5-FC concentration curves of the serum and the tumor tissue showed close similarity, although 5-FC in the serum maintained higher levels (Chart 7). Average concentration in the serum reached a peak of 98 µg/ml at 1 h, while that in the tumor rose to 60 µg/ml after 30 min and reached a peak of 88 µg/ml after 1 h. It gradually decreased thereafter. The half-life of 5-FC in the tumor was 4.5 h. The average concentration of 5-FUra formed in the tumor was detected within 30 min and reached a peak of 8.0 µg/g after 2 h (Chart 8). It rapidly decreased for 4 h and then gradually declined. 5-FUra (0.6 µg/g) was detected even after 8 h. The half-life of 5-FUra in the tumor was 3.2 h. On the other hand, 5-FUra in the serum reached a peak of 0.3 µg/ml after 2 h and showed less than 0.1 µg/ml at 4 h or more.

Local Chemotherapeutic Studies. Tumor growth curves are shown in Chart 9. There was neither death nor wound infection of the animals. In the control group, the growth rate of the s.c. tumors exponentially increased with an increase of tumor volume. In the treated group, regression of the tumor volume was observed at the sixth day after the treatment, and the difference of the tumor volume between the control and the treated group was significant (P < 0.001). At the sixth day, the median tumor volume of the treated group was 78% of that of the control group and, at the 30th day, it decreased to 19% of that of the control. The median tumor weight of the treated group at the 30th day was 26% of that of the control (Table 1).

Histological alteration of the tumor was examined after the
LOCAL ANTIMICROBIAL CHEMOTHERAPY

Chart 7. Tissue concentration of 5-FC in the s.c. tumor of the rats treated with 5-FC (150 mg/kg) and locally implanted CDase capsules (1 IU). O, serum; •, tumor. Bars, SD.

Chart 8. Tissue concentration of the converted 5-FUra in the s.c. tumor of the rats treated with 5-FC (150 mg/kg) and locally implanted CDase capsules (1 IU). O, serum; •, tumor. Bars, SD.

Treatment was finished. The implanted capsule was found to be covered with fibrous connective tissues in both groups. In the control, a compact arrangement of the tumor cells with rich vascular proliferation was observed (Fig. 1). On the contrary, in the treated group, tumor tissue surrounding the CDase capsule showed a decrease of cellularity, loss of cytoplasm, and nuclear fragmentation of the tumor cells (Fig. 2). However, there were no definite cytotoxic changes at sites far removed from the capsule. The greater the distance, the less were the changes. A CDase capsule implanted for 1 month was neither contaminated nor injured and possessed sufficient enzyme activity for conversion of 5-FC.

PCA Reaction. The PCA reaction with extravasation of i.v. injected Evans blue is shown in Fig. 3. Antisera taken at the tenth day from the animals that were sensitized with s.c. injection of CDase produced a PCA reaction with dye extravasation. The PCA reaction became pronounced when antisera taken at the 30th day were used. On the contrary, antisera taken at the tenth and 30th days from the animals treated with implantation of CDase capsule did not induce a PCA reaction.

DISCUSSION

Most chemotherapeutic agents lack tumor specificity, thereby resulting in inevitable systemic toxicity to the host. Furthermore, most agents do not readily pass the blood-brain barrier (33). These difficulties do not allow the maintenance of an effective concentration of the drugs at the tumor site for a long period and thus restrict clinical trials in a patient. Although there have been numerous attempts by intraneoplastic or local application of the drugs to a brain tumor (10, 11, 31, 34, 44, 47) to avoid these difficulties, beneficial responses have been rare. Recently, the administration of antitumor drugs with slow diffusion from silicone capsule implants has been documented (29, 30, 35, 38, 39, 48).

5-FC was first synthesized by Duschinsky et al. (6) and has been reported to be effective against several fungal diseases with minimal side effects (1, 9, 13, 42, 43). Although the mode of action of 5-FC has not been clarified, the selective toxicity of this drug against fungal cells is explained by the selective uptake through the membrane and the deamination of 5-FC into 5-FUra by CDase in the fungal cells (5, 20, 22, 23).

5-FC, unlike other related fluorinated pyrimidines such as 5-FUra, is probably not metabolized in the mammalian cells and.
LOCAL ANTINEOPLASTIC CHEMOTHERAPY

has no antineoplastic activity because of the absence of nucleosidase phosphorylase for cytosine and CDase (5, 20, 22, 23). In this experiment, 5-FC per se was found to have no cytostatic effect on the proliferation of cultured glioma cells.

CDase was first observed by Hahn and Schäfer (14) in yeast, and E. coli and has been identified in other organisms (16, 19, 36, 37) mainly belonging to enterobacteriaceae and pseudomonaceae. It is believed to serve as an important enzyme in the salvage synthesis of pyrimidine nucleotide and catalyzes deamination of only cytosine. The first purification of the enzyme from bakers’ yeast was partially performed (340-fold) by Ipata et al. (19), and the final purification to a homogeneous state was done by Yergatian et al. (51). Sakai et al. purified it to a homogeneous state from Serratia marcescens (36) and Pseudomonas aureofaciens (37). The enzyme used in this study was partially purified from the cell extract from cultivated E. coli (36), the enzyme activity of which is relatively heat stable at the range of pH 7 to 9 and is little inactivated at 40°C at least several months later.

In vitro study showed that the enzyme per se had no cytotoxic effect. Once 5-FC was combined with CDase, it was converted into 5-Flur, and remarkable antineoplastic activity developed. In various types of cultured human brain tumor cells, growth-inhibitory effects by the combined treatment were also demonstrated (27).

Encouraged by the in vitro study, the authors performed experiments with rat brain tumor models treated with the intra-tumoral injection of CDase followed by systemic administration of 5-FC. The results showed delivery of sufficient amounts of 5-FC to the brain tumor tissue, intratumoral conversion of 5-FC into 5-Flur, and significant prolongation of survival time (28).

For the purpose of immobilizing CDase in vivo, an active CDase capsule made of Spectra/Por 6 cellulose tubing was devised. Spectrophotometric analysis demonstrated the passive diffusion of the formed 5-Flur out of this capsule.

Stability of CDase activity is required for long-term treatment. The preliminary study demonstrated that the enzyme activity in the capsule implanted in the animals for 3 months preserved about 25% of its initial level. Although this is not sufficient for the purpose of therapy, the problem concerning the stabilization of the enzyme activity will be overcome by purification and immobilization techniques.

The methods available to determine the concentration of 5-FC and 5-Flur in the tissue have been microbiological (21, 40, 50) or gas chromatographic assays (15). Whereas they have proved reliable, these assays have been limited in so far as the needs for differential quantitation of the drugs in the material are concerned. Recently developed high-performance liquid chromatography has facilitated rapid, quantitative determination of the drugs (4). In this study, the thin-layer cup method was used, and differential quantitation of the drugs in the material was successfully performed (49).

The patterns of the concentration curves of 5-FC in the serum and the tumor showed close similarity. They were characterized by rapid rises reaching a maximum after 1 h, followed by gradual declines. The average maximum levels of 5-FC achieved were 98 μg/ml in the serum and 88 μg/g in the tumor. Koestlin et al. (22) reported that, after the administration of 2 g of 5-FC to their patients, serum 5-FC rapidly rose to 30 to 40 μg/ml within 2 h, followed by a gradual decline, remaining at more than 20 μg/ml at between 2 and 8 h. They also stated that the average 5-FC

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<th>Group and drug</th>
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<th>Tumor weight (g)</th>
<th>Median tumor weight at the 20th day after the treatment</th>
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<td>Day 3</td>
<td>2.5 ± 0.7</td>
<td>3.2 ± 0.6</td>
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<td>Day 6</td>
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<td>Day 9</td>
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CANCER RESEARCH VOL. 45 APRIL 1985

1757
levels in the tissues and in the blood are of the same order of magnitude. In the previous study (28), however, the serial determination of 5-FC levels in rats bearing intracerebral tumors and receiving 150 mg of 5-FC per kg i.p. demonstrated that the average maximum levels reached 96 μg/g in the serum and 85 μg/g in the tumor, respectively, after 1 h, although 5-FC in the contralateral normal brain was 46 μg/g, approximately 54% of that in the tumor.

In a recent study, the authors determined the tissue concentrations of 5-FC in brain tumor patients who received 2 g of 5-FC p.o. prior to surgery. The results showed that the average concentrations in the serum reached 40 μg/ml at the maximum level within 4 h, and 35 μg/g were delivered to the tumor tissue at 4 to 5 h. These clinical and experimental data supported the fact that a sufficient amount of 5-FC could be delivered to the human brain tumor for the local generation of 5-FUra with the aid of the CDase capsule.

Although the CDase capsule, implanted 2 weeks prior to the determination of the drugs, was covered with connective tissues, a sufficient amount of 5-FUra could be demonstrated in the tumor tissue adjacent to the capsule. The average maximum concentration was 8.0 μg/g at 2 h and stayed at more than 1.0 μg/g at between 1 and 6 h. For the local antineoplastic chemotherapy, it would be of interest to ascertain how far away the agent diffuses in the tumor. The preliminary study of 5-FUra concentrations at different parts of the s.c. tumor which was extirpated when grown to 7 cm in diameter demonstrated that 5-FUra reached approximately 3.0 μg/g at 2 h after administration in areas 2 cm away from the capsule, although it was unmeasurable at more than 4 cm away.

The effective concentration level of 5-FUra to exert antineoplastic activity has not been established clearly. Some investigators suggest that it is desirable to maintain 5-FUra in the tumor tissue at a level of more than 0.05 μg/g for a long period (8). In this investigation, the intratumoral concentration of 5-FUra reached rather higher levels than the above value. 5-FUra found in the serum was thought to be due to the passage of 5-FUra formed in the tumor into the blood stream. It was only 3.8% of the 5-FUra found in the tumor at the maximum level. Very low passage of 5-FUra into the blood stream might, therefore, considerably reduce the systemic toxicity.

It would be difficult to introduce the present mode of therapy into a small animal model because the capsule was too bulky to apply, although in vivo study would be more meaningful in the intracerebral tumor model.

The combined treatment, however, developed antitumor activity in the s.c. tumor model of the rat and resulted in significant reduction of the tumor growth. Median tumor volume and weight in the treated group were 19% and 26% of those in the control group, respectively. Histological study demonstrated remarkable cytotoxic changes, although no definite changes were observed in areas far removed from the capsule.

5-FUra crosses the blood-brain barrier (2, 3) and is concentrated in the brain tumor (24), but most clinical experiences with 5-FUra alone have indicated that it was of no value for the treatment of patients with malignant gliomas (7, 34, 41, 46). This agent fails into the category of cell cycle-specific ones, which primarily exert their oncolytic effect against cells undergoing active DNA synthesis.

The major problem with solid tumors is that a considerably smaller proportion of the cells is likely to be in active DNA synthesis at a time. Hoshino et al. (17, 18) reported that only 5 to 15% of the cells in a malignant glioma were proliferating and that the cell cycle time was 75.6 ± 45.7 (SE) h. In this respect, long-term courses of the agents, such as 5-FUra, must be required to achieve significant reduction in the tumor mass and clinical improvement.

Contrary to the recently accepted oncological concept, 5-FUra, which is clinically ineffective in the case of its single administration, demonstrated antitumor activity when given in combination with 1,3-bis(2-chloroethyl)-1-nitrosourea (12, 25).

The present method might be useful as a postoperative adjunctive therapy in that it allows continuous delivery of 5-FUra with a potentially higher concentration to the infiltrating edge of the tumor by implanting CDase capsules there at the time of its partial removal, followed by postoperative administration of 5-FC p.o. every 6 to 9 h.

The PCA reaction affords the advantage of investigating the fundamentals of immediate allergic reactions, namely the liberation of vasoactive substances and their action on the minute vessels of the skin induced by antibodies of different immunological classes (32). The results of this PCA reaction against an implanted CDase capsule in vivo suggested that there should be neither leakage of the enzyme out of the capsule nor induction of any unfavorable responses to the host.

Further studies are in progress, aiming at the development of a safer capsule and more stabilized enzyme for permanent implantation in vivo.

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Fig. 1. Histology of the s.c. tumor (EA-285 glioma) at the 30th day after the treatment with 5-FC and locally implanted PBS capsules (control group). Compactly arranged tumor cells with rich vascular proliferations are seen adjacent to the PBS capsule. H & E, x 350.

Fig. 3. Photograph of the PCA reaction on the inner side of the skin of a guinea pig with the extravasation of i.v.-injected Evans blue. The i.d. injections of the antisera, PBS, and control sera (nonsensitized) were made on both sides of the animal's back. A, control sera; B, PBS; C and D, antisera taken at 10 and 30 days from the animals provided with s.c. injection of CDase. The PCA reactions with the dye extravasation are observed. E and F, antisera taken at 10 and 30 days from the animals provided with the implantation of CDase capsules. No dye extravasations are observed.
Fig. 2. Histology of the s.c. tumor (EA-285 glioma) at the 30th day after the combined therapy with 5-FC and locally implanted CDase capsule (treated group). In A, the membrane of the CDase capsule is seen on the right. The capsule is covered with thick, fibrous connective tissues. H & E, x 170. In B, tumor tissue surrounding the capsule shows the cytotoxic changes with the decrease of cellularity, loss of cytoplasm, and nuclear fragmentation. H & E, x 350.
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