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## Preclinical report

# Dextran sulfate inhibits injured abdominal wall-specific tumor implantation in mice

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**Tumor recurrence is often seen at sites where the peritoneum has been injured during surgery for gastrointestinal malignancies. It is thought that malignant cells released from the tumor during surgery implant in the sites of injury in the abdominal wall and cause tumor recurrence. Here we use dextran sulfate (DS) as an antagonist to cell adhesion for preventing implantation of i.p. seeded malignant cells, thus suppressing the recurrent tumor formation often observed at the site of injury in postoperative abdominal walls. DS was tested for anti-adherent activity against B-16 melanoma cells to injured abdominal wall specimens *ex vivo* and showed the capacity to significantly impair B-16 melanoma cell adherence compared to controls without DS. DS was also tested for the activity to prevent i.p. seeded B-16 melanoma cells from implanting in the site of injury in the abdominal wall *in vivo* and DS prevented B-16 melanoma cells from implanting in the sites of injury in the abdominal wall. In the test for the activity to improve survival in mice after B-16 melanoma was inoculated i.p., DS improved the survival of mice as compared to the controls without DS. We conclude that DS may be useful in preventing surgically promoting tumor implantation at sites of injury in post-operative abdominal wall treated for gastrointestinal malignancies. [© 2000 Lippincott Williams & Wilkins.]**

*Key words*: Abdominal wall recurrence, B-16 melanoma, dextran sulfate, peritoneal metastasis.

## Introduction

Tumor cell implantation at sites of injury in postoperative abdominal walls is an important causative aspect of relapse and tumor regeneration seen after surgical treatments of gastrointestinal malignancies. Animal experimentation has shown that malignant cells can implant in the abdominal wall where the peritoneum is injured or surgically removed.[1] Malignant cells released from a tumor during surgery may attach and implant at sites of injury in the abdominal wall, and produce new tumors and disease relapse. However, there is currently no efficacious method to prevent this kind of tumor recurrence after surgery. We have developed a new method that uses i.p. administered dextran sulfate (DS; an anti-adherence agent) to block the implantation of tumor cells released during surgery and prevent re-emergence of recurrent tumors. We have previously reported that DS efficaciously prevents cancer cells from attaching to plastic dishes coated with fibronectin, collagen or without coating,[2] and from implanting in the greater omentum, where i.p. seeded malignant cells implant in the initial stage of peritoneal carcinomatosis.[3] In the present study, we examine using mice if i.p. injected DS may be useful for prevention of tumor cell implantation and associated tumor re-emergence at sites in injury in the abdominal wall.

## Materials and methods

The B-16 melanoma cell line was used as an experimental tumor, because the B-16 melanoma cell line is identified easily by its intrinsic melanin under a microscope without any special histopathologic staining. Tumor tissue from B-16 melanoma (Sasaki Institute, Tokyo, Japan) was taken from C57/BL mice (male, 5 weeks old; Shimizu Laboratory Animal Center, Kyoto, Japan) and minced with scissors into a suspension in cell culture medium. Single cells were prepared by centrifugation and filtration, diluted with standard medium for cell culture, and incubated as a primary culture under the standard incubation condi-

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tions (under an atmosphere with 5% $CO_2$ at 37°C). Melanoma cells were collected from the primary culture and incubated as a second culture so that fibroblasts would be excluded. Adherent melanoma cells were detached with EDTA–trypsin and prepared as a single-cell suspension.

BDF1 mice (male, 5 weeks old; Shimizu Laboratory Animal Center) were used as the experimental animal. Animals were bred under standard conditions (specific pathogen-free, day–night cycle of 12 h, relative humidity 60%, temperature 22°C).

DS (mean molecular weight of $5 \times 10^5$; Sigma, St Louis, MO), the cell adhesive antagonist, was dissolved at 0.2 mg/ml in standard medium for cell culture (DS-containing medium). DS-containing medium was filtered through 200 nm pores to remove bacteria before use.

### Experiment 1: cell adherence to the abdominal wall *ex vivo*

Under general anesthesia mice received laparotomy, and parietal peritoneum and subperitoneal tissues were removed with scissors at the right or left flank (injured abdominal wall). The peritoneum of the opposite served as an intact (uninjured) abdominal wall. After the operation abdominal walls, both injured and intact, were resected at full thickness, extended and fixed on plastic dishes. DS-containing medium or normal medium was put into the dishes and then the dishes were incubated at 37°C for 30 min under standard incubation conditions. Then, the medium was removed and B-16 melanoma cells suspended in normal medium ($3 \times 10^6$ cells/ml) were added. Dishes were shaken gently for 30 min and adherence of B-16 melanoma cells to the abdominal wall was observed under a 20-fold stereo microscope.

### Experiment 2: observation of cancer growth on the abdominal wall

Under general anesthesia, 40 mice received laparotomy and random resection of the right or left parietal peritoneum and subperitoneum. Thirty minutes after operation, all mice received i.p. inoculation with B-16 melanoma cells suspended in normal medium at $3 \times 10^6$ cells/mouse. The 40 mice were then divided at random into two groups of 20 each. One hour after inoculation (90 min after operation), 20 mice received i.p. administration of 1 ml of DS-containing medium and the other 20 mice received i.p. administration of 1 ml of normal medium.

At 1, 2, 4 and 7 days after the operation, five mice from each group were sacrificed to examine the macroscopic and microscopic findings in the injured abdominal wall. After macroscopic observation, the abdominal walls were fixed with 10% formalin, embedded in paraffin, sliced into microscopic specimens (4 $\mu$m thickness) and stained using the hematoxylin & eosin method. These microscopic specimens were observed microscopically.

### Experiment 3: assay for cancer growth in the abdominal wall *in vivo*

In the same manner as experiment 2, 40 mice underwent operation. Thirty minutes after operation, all mice received i.p. inoculation with B-16 melanoma cells suspended in normal medium at $3 \times 10^6$ cells/mouse. The 40 mice were then divided at random into two groups of 20 each. One hour after inoculation (90 min after operation), 20 mice received i.p. administration of 1 ml of DS-containing medium and the remaining 20 mice received 1 ml of normal medium.

One day after the operation, the mice of each group were sacrificed to assay the amount of cancer cells implanted onto the injured abdominal wall. The injured abdominal wall was taken from each of the 20 mice and used as a specimen for a bioassay of viable cancer cells growing on the abdominal wall. The amount of viable cancer cells was assayed as follows. A portion of the injured abdominal wall was minced into tissue fractions and the tissue fractions were suspended in 1 ml of normal medium under aseptic conditions. Another 40 normal mice were prepared as assay mice. The tissue suspension made from each specimen taken from each mouse was individually injected i.p. into a corresponding assay mouse. Of the 40 assay mice, 20 received tissue suspensions taken from mice given DS-containing medium, while the other 20 received tissue suspensions taken from mice given normal medium. The assay mice were observed for deaths. Dead mice received an autopsy and the cause of death was confirmed.

The amount of cancer tissue growing on the abdominal wall was compared using the survival curves of the assay mice group, because survival shortens with increased numbers of i.p. inoculated B-16 melanoma cells. We confirmed the relationship between survival and the number of viable B-16 melanoma cells as follows. One hundred sixty mice were divided into eight groups of 20 mice each. Groups received an i.p. inoculation of $10^3$, $3 \times 10^3$, $10^4$, $3 \times 10^4$, $10^5$, $3 \times 10^5$, $10^6$ or $3 \times 10^6$ B-16 melanoma cells/mouse and were then observed for survival to create survival curves.

**Table 1**. B-16 melanoma cell adherence to abdominal wall *ex vivo*

| | B-16 melanoma cells adhering to the abdominal wall in five visual fields per experiment [mean (95% confidence interval in five experiments)] | |
|---|---|---|
| Injured abdominal wall in normal medium | 45 (22–68) | $p<0.01$ |
| Injured abdominal wall in DS-containing medium | 8.2 (0–16) | |
| Normal abdominal wall in normal medium | 5.4 (0–12) | |
| Normal abdominal wall in DS-containing medium | 4.1 (0–10) | |

Experiment 4: survival experiments in mice

In the same manner as experiment 2, 40 mice underwent operation. Thirty minutes after the operation, B-16 melanoma cells suspended in normal medium were inoculated at $3 \times 10^6$ cells/mouse i.p. in all 40 mice. The mice were divided into two equal groups. One hour after inoculation (90 min after operation), one group (the DS group) received i.p. injection of DS-containing medium at 1 ml/mouse and the other group received 1 ml/mouse of normal medium (the normal medium group).

Mice were observed for death. An autopsy confirmed the cause of death. Survival curves were compared between the two groups.

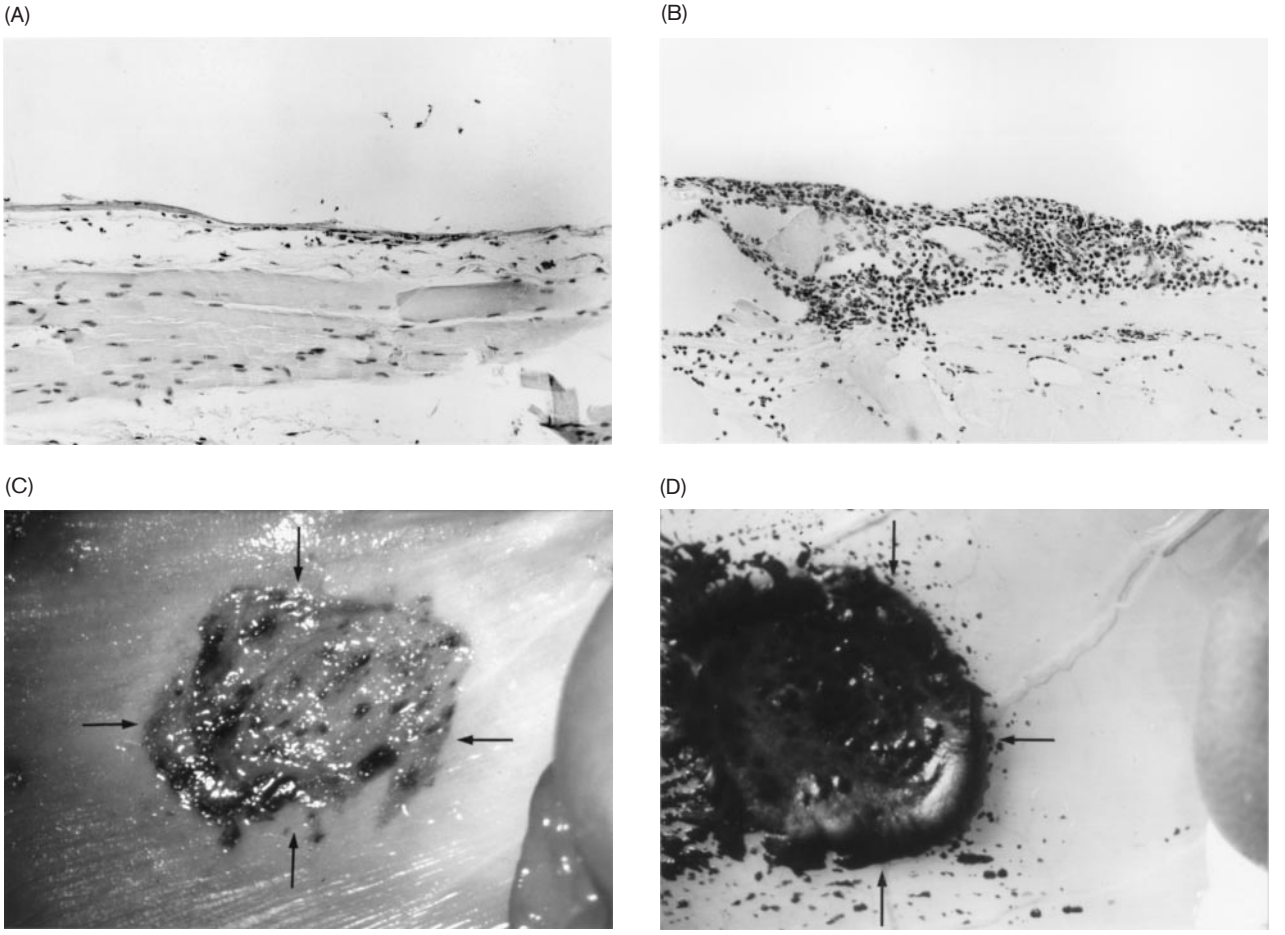


**Figure 1.** Observation of cancer growth on the abdominal wall. In mice given DS, at 2 days after tumor inoculation, the injured abdominal wall was covered by a thin fibrin-like membrane with scattered lymphatic cells (A) and at 7 days after tumor inoculation cancer lesions grew poorly on the injured portion of the abdominal wall (C, arrow). In mice given normal medium at 2 days after tumor inoculation many lymphatic cells accumulated on the surface, especially the peripheral margin, of the injured abdominal wall and formed an appearance of milky spots (B), and at 7 days after tumor inoculation, large tumors appeared on the injured wall (D).

### Statistical significance

Cell numbers were compared by analysis of variance. Survival curves were compared using the generalized Wilcoxon test. When the probability value ($p$) was less than 0.05, the difference was defined to be statistically significant.

## Results

### Experiment 1: cell adherence to the abdominal wall *ex vivo*

The results are shown in Table 1. In the abdominal wall specimen prepared in normal medium, a large number of B-16 melanoma cells adhered to the injured abdominal wall. When abdominal walls were prepared in DS-containing medium, a significantly ($p<0.01$) lower number of B-16 melanoma cells adhered to the injured portion of the abdominal wall, as compared to the injured portion of the abdominal wall specimen prepared in normal medium. On the intact (uninjured) portion of the abdominal wall, melanoma cells rarely adhered in either the abdominal wall prepared by normal medium or DS-containing medium.

### Experiment 2: observation of cancer growth on the abdominal wall

In all five mice given DS-containing medium, at 2 days after tumor inoculation, the injured portion of the abdominal wall was covered by a thin fibrin-like membrane with scattered lymphatic cells (Figure 1A) and at 7 days after tumor inoculation cancer lesions grew poorly on the injured portion of the abdominal wall (Figure 1C, arrow).

On the other hand, in all five mice given normal medium, at 2 days after tumor inoculation many lymphatic cells accumulated on the surface of the injured abdominal wall and formed milky spot-like tissue (Figure 1B). Milky spots are known as a kind of lymphoid tissue that serves as an implanting site for malignant cells in the initial stage of peritoneal carcinomatosis.[3] Seven days after tumor inoculation, large tumors appeared on the injured portion of the abdominal wall (Figure 1D, arrow).

On the uninjured portion of the abdominal wall, cancer lesions were rarely found in either mice treated with DS-containing or normal medium (Figure 1C and D).

### Experiment 3: assay of cancer growth on the abdominal wall *in vivo*

Survival was significantly ($p<0.01$) improved in assay mice who received tissue fractions from the injured abdominal wall taken from mice treated with DS, as compared with assay mice who received tissue fractions from injured abdominal walls taken from mice given normal medium (Figure 2).

Survival of the mice was inversely proportional to the number of B-16 melanoma cells inoculated (Figure 3).

### Experiment 4: survival experiments

The survival curve in mice given DS-containing medium was significantly ($p<0.01$) improved over mice given normal medium (Figure 4).

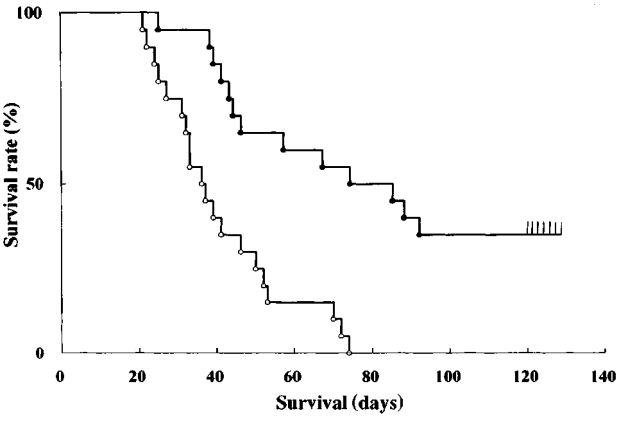


**Figure 2.** Survival in assay mice. Survival was significantly improved in assay mice (●) who received i.p. injection of tissue fractions of injured abdominal wall taken from mice given DS, as compared to assay mice (○) who received i.p. injection of tissue fractions of injured abdominal wall taken from mice given normal medium.

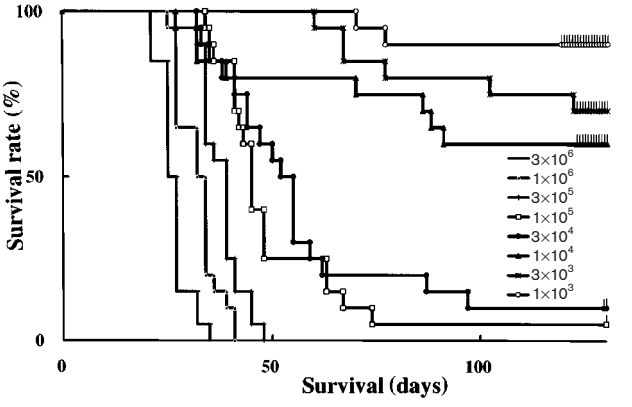


**Figure 3.** Relationship of survival to the inoculation dose of B-16 melanoma cells. The survival of mice became significantly better with a decrease in the number of B-16 melanoma cells inoculated i.p.

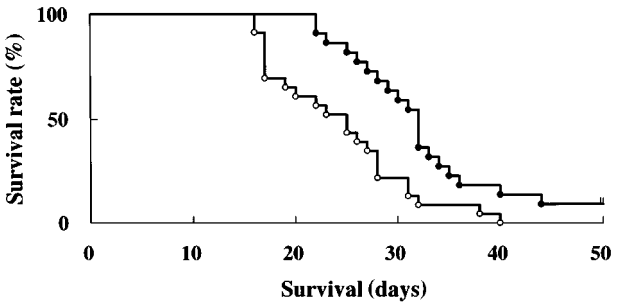


**Figure 4.** Survival in mice after melanoma inoculation followed by DS or normal medium. Mice (●) receiving DS after i.p. inoculation of B-16 melanoma cells survived significantly longer than those (○) receiving normal medium.

## Discussion

It has been shown that port-site recurrence occurs in 4% of 'curative' surgeries for colorectal cancer.[4] Recurrences appear at the sites of ports where the specimen was retrieved as well as at the sites of ports where the specimen was not retrieved. Port-site recurrence has been seen when the specimen was placed in a specimen bag (L Newman, unpublished results). It has been reported that malignant cells can implant in the abdominal wall where the peritoneum is injured or surgically removed.[1] During second-look operations for recurrent tumors on the peritoneal surface after previous surgeries, we found that peritoneal recurrence of tumors sometimes occurs selectively in the portions where the peritoneum had been injured by the previous surgery. These reports and findings suggest that port-site recurrence and local recurrence at the injured abdominal wall are explained by selective implantation of i.p. free cancer cells in the abdominal wounds such as port sites and injured peritoneum. With this in mind, we examined the ability of prophylactic DS to prevent implantation of cancer cells in the injured abdominal wound when DS was administered shortly after i.p. inoculation of cancer cells. DS successfully prevented cancer cells from adhering to the abdominal wall and DS prolonged survival even when administered 1 h after inoculation of B-16 melanoma. Our results suggest that DS will be efficacious for reducing the number of cancer cells implanting in the injured abdominal wall, even after free cancer cells have seeded during surgery, although further study is necessary to clarify how to best use DS.

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