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Short report

Prevention of peritoneal metastasis of cancer with dextran sulfate—an experimental study in mice

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Peritoneal metastases occur most often in the greater omentum, where tumor implantation sites are densely distributed. We used dextran sulfate (S-Dex) as an anti-cell-adherence agent to prevent i.p. seeded malignant cells from causing peritoneal metastases. S-Dex was tested for its anti-adherent activity against B-16 melanoma cells on plastic, and was examined for its ability to prevent implantation in the omentum and to improve survival in mice after B-16 melanoma was inoculated i.p. S-Dex prevented B-16 melanoma cells from adhering to the plastic wall. S-Dex reduced the number of B-16 melanoma cells implanted into the greater omentum and improved the survival of mice inoculated with B-16 melanoma cells. We conclude that S-Dex attenuated peritoneal metastases when B-16 melanoma cells were seeded i.p.

Key words: Animal experiment, anti-adherence therapy, B-16 melanoma, cell adherence, dextran sulfate, peritoneal metastasis.

Introduction

Peritoneal metastasis is one of the most important modes of disease transmission in abdominal malignancies. We have found that some of the acid polysaccharides, e.g. dextran sulfate (S-Dex), prevented malignant cells from attaching to fibrin, collagen and to the peritoneal wall.[1] In this study, we tested whether S-Dex could prevent malignant cells from adhering onto plastic *in vitro* and whether S-Dex could prevent i.p. seeded malignant cells from causing peritoneal metastases in mice *in vivo*.

Materials and methods

The B-16 melanoma cell line was used as the experimental tumor, because B-16 melanoma cells can be identified easily by their intrinsic melanin on microscopy, without any special histopathologic staining. Tumor tissue from B-16 melanoma (Sasaki Institute, Tokyo, Japan) was taken from C57/BL mice (males, 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 medium, and were incubated as a primary culture under standard incubation conditions (5% CO_2 atmosphere at 37°C). The melanoma cells were collected from the primary culture and were then incubated as a second culture so that fibroblasts would be excluded. The adherent melanoma cells were detached with EDTA-trypsin and were prepared as a single cell suspension.

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

S-Dex (mean molecular weight 5×10^5; Sigma, St Louis, MO) was used as the anti-adherence agent. S-Dex was dissolved at a range of 6.25–400 μg/ml in medium for cell culture and was filtered through a 200 nm filter to remove bacteria before use.

Experiment 1: cell adherence to plastic *in vitro*

B-16 melanoma cells were suspended at 10^4 cells/ml in normal medium for cell culture (control) or medium containing S-Dex at 6.25, 25, 100 or 400 μg/ml. Plastic dishes coated with fibronectin, collagen or without coating were then incubated with the melanoma cells in normal medium or in S-Dex-containing medium under standard incubation conditions for 4 days. The medium

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containing the free cells was then removed and an equal volume of medium was added. The adhering cells were detached with EDTA-trypsin, and the cell concentration was determined by a cell counter.

Experiment 2: cancer implantation into the greater omentum

Forty mice received an i.p. inoculation with B-16 melanoma cells suspended in normal medium for cell culture at 3×10^6 cells/mouse. The 40 mice were then divided at random into two groups of 20 each. Thirty minutes after the inoculation, 20 mice received an i.p. injection of 1 ml of medium containing 200 μg/ml of S-Dex and the other 20 mice received 1 ml of normal medium. One day after the inoculation, all of the mice in each group were sacrificed to assay for the amount of cancer cells implanted into the greater omentum, because the greater omentum is the location where i.p. seeded malignant cells tend to implant most densely.[2] The greater omentum was then taken from the 20 mice and was used as a specimen for the bioassay of viable cancer cells.

The amount of viable cancer growth was compared between the two treatment groups as follows: the greater omentum was minced into a tissue fraction and the tissue fractions were suspended in 1 ml of normal medium under aseptic conditions. Another 40 normal mice were then prepared as assay mice. Tissue suspensions made from each specimen were individually injected i.p. into one assay mouse each. Of the 40 assay mice, 20 received tissue suspensions taken from mice given medium containing S-Dex, whereas the other 20 received tissue suspensions taken from mice given normal medium. The assay mice were then observed for survival. The dead mice received an autopsy and the cause of death was confirmed. The amount of viable cancer growing on the greater omentum was compared using the survival curves from the assay mice groups, because survival shortens with an increased number 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 and sixty mice were divided into eight groups of 20 mice each. These groups received an i.p. inoculation of 10^3, 3×10^3, 10^4, 3×10^4, 10^5, 3×10^5, 10^6 or 3×10^6 B-16 melanoma cells/mouse and were then observed for their survival curves.

Experiment 3: survival experiments in mice

In the same manner as experiment 2, 40 mice received an i.p. inoculation with B-16 melanoma cells suspended in normal medium for cell culture at 3×10^6 cells/mouse. The mice were then divided into two equal groups. Thirty minutes after the i.p. inoculation with B-16 melanoma cells, one group received i.p. S-Dex-containing medium (S-Dex at 200 μg/ml in medium for cell culture) at 1 ml/mouse and the other group received 1 ml/mouse of normal medium for cell culture.

The mice were observed for their survival and an autopsy confirmed the cause of death. The survival curves were compared between the two groups.

Statistical analyses

The number of cancer cells was compared by ANOVA and the survival curves were compared using the generalized Wilcoxon test. When the probability value (p) was less than 0.05, the difference was considered to be statistically significant.

Results

Experiment 1: cell adherence to plastic *in vitro*

The number of adherent B-16 melanoma cells was decreased by S-Dex-containing medium (Figure 1). B-16 melanoma cells were seen in the form of cell clusters or single cells in the S-Dex-containing medium. In contrast, cells in the normal medium adhered well to the plastic walls and were not

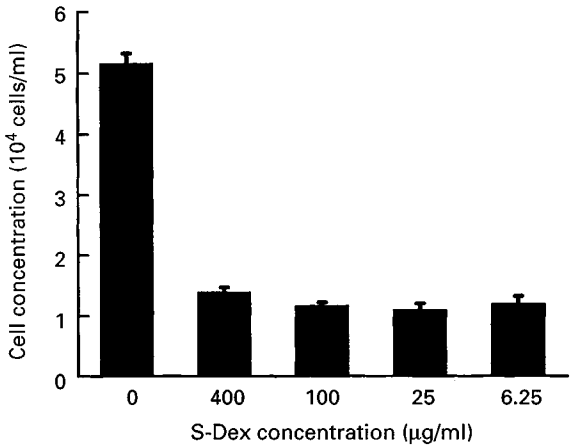

Figure 1. Influence of the number of adherent cells by S-Dex *in vitro*. B-16 melanoma cells were prevented from adhering to the plastic dish by S-Dex. Bars represent the 95% confidence intervals.

detached when the culture dish was shaken. These phenomena were equivalent in all three culture dishes with the different coating preparations.

Experiment 2: cancer implantation in the greater omentum

The survival was significantly better ($p<0.01$) in the assay mice who received a tissue fraction suspension of the greater omentum taken from mice treated with S-Dex, as compared to the assay mice who received a tissue fraction suspension of the greater omentum taken from mice given normal medium (Figure 2).

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

Experiment 3: survival experiments

The survival curve of mice given S-Dex-containing medium was significantly better ($p<0.01$) than mice given normal medium (Figure 4).

Discussion

It is known that anti-coagulants such as S-Dex prevent hematogenous metastases[3,4] and that S-Dex has no direct cytotoxic effects on cancer cells.[4] It has not been previously reported that S-Dex can prevent the adherence of cancer cells to the peritoneum. We have found that some acid polysaccharides, including S-Dex as one of their derivatives, can prevent cancer cells from adhering to the peritoneum.[1]

In experiment 1, the anti-adherence effect was obviously seen even in low concentrations of S-Dex *in vitro*.

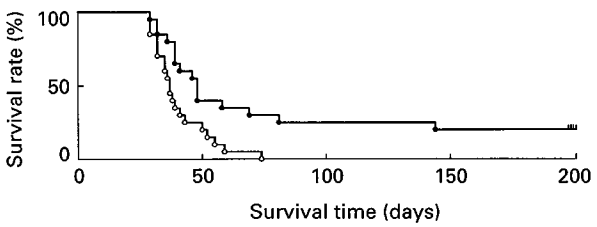

Figure 2. Survival in assay mice. The survival was significantly ($p<0.01$) improved in the assay mice (closed circles) who were i.p. inoculated with tissue fractions of the greater omentum taken from mice given S-Dex-containing medium, as compared to assay mice (open circles) who were i.p. inoculated with tissue fractions taken from mice given normal medium.

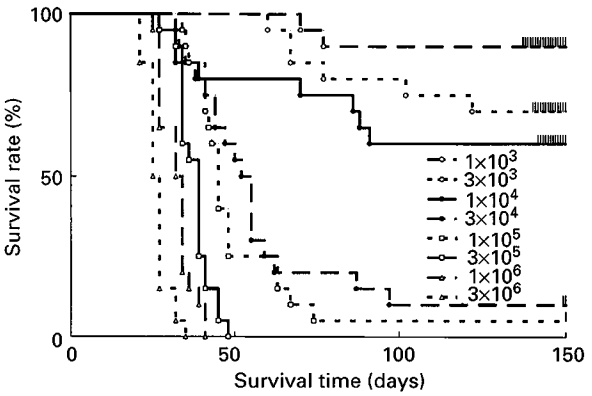

Figure 3. Relationship of survival to the inoculation dose of B-16 melanoma cells. The survival of the mice improved with a decreasing number of B-16 melanoma cells inoculated i.p.

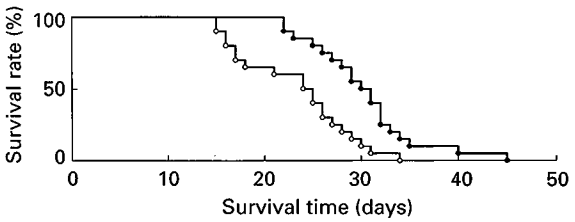

Figure 4. Survival in mice after an i.p. inoculation with B-16 melanoma cells followed by S-Dex or normal medium. Mice (closed circles) receiving S-Dex after an i.p. inoculation of B-16 melanoma cells survived significantly longer ($p<0.01$) than those (open circles) receiving normal medium.

During the initial stages of peritoneal metastasis, i.p. seeded malignant cells implant selectively in milky spots, which are a kind of small lymphoid apparatus located on the serosa that are distributed most densely on the greater omentum.[2] Therefore, the greater omentum was used when the number of malignant cells implanted was compared between S-Dex-treated mice and control mice in experiment 2. In experiments 2 and 3, we examined whether a positive effect of S-Dex could be observed. S-Dex successfully prevented cancer cells from implanting into the greater omentum and also prolonged survival. Our results suggest that i.p. S-Dex prevents i.p. seeded cancer cells from implanting into the peritoneum and from subsequently causing peritoneal metastases.

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