Exp Oncol 2009
31, 3, 144–148

Experimental ONCOLOGY

IN VIVO ANTI-TUMOR ACTIVITIES OF GELATIN

Y. Inamura[1], *T. Koide*[2], *T. Kojima*[3, *], *H. Nagata*[4], *N. Ito*[4], *K. Suzumura*[4], *Y. Hashimoto*[5]
[1]*Department of Surgery, Yamaguchi Hospital, Seto-City, Aichi 489-0866, Japan*
[2]*Health Research Center, Aichi-Gakuin University, Nisshin-City, Aichi 470-0195, Japan*
[3]*Department of Surgery, Aichi-Gakuin University School of Dentistry, Chikusa-ku, Nagoya 464-8651, Japan*
[4]*Department of Digestive Surgery, Aichi Medical University, Nagakute-cho, Aichi-gun, Aichi 480-1103, Japan*
[5]*Department of Biochemistry, Aichi-Gakuin University School of Dentistry, Chikusa-ku, Nagoya 464-8650, Japan*

Aim: **As reported previously, porcine skin gelatin exerted direct anti-tumor effect *in vitro* and induced anti-tumor peritoneal macrophages *in vitro*. The present study investigated whether or not the gelatin exerted anti-tumor effect *in vivo*. *Methods: In vitro* anti-tumor activities of peritoneal macrophages and the gelatin were evaluated with tritium thymidine uptake by target tumor cells. *In vivo* anti-tumor activity was evaluated with the survival of tumor-bearing animals and the size of the tumor. *Results:* Intraperitoneal daily administration of 12.5 mg of the gelatin prolonged the survival of mice which had been intraperitoneally inoculated with MH134 (hepatic cell carcinoma cell line) or Colon 26 (colon carcinoma cell line) tumor cells, and there were no tumors in case of MH134 cells inoculation. Intraperitoneal daily administration of 12.5 mg of the gelatin did not affect growth of subcutaneous MH134 tumor. The gelatin administered subcutaneously did not affect the survival of mice with intraperitoneal MH134 tumor. On the other hand, bovine skin gelatin administered subcutaneously achieved statistically significant prolongation of the survival. The contact of MH134 cells with porcine skin gelatin for 5 min was required for the gelatin to exert its anti-tumor activity *in vitro*. Porcine skin gelatin of 12.5 mg injected intraperitoneally was detected as protein in the peritoneal cavity 5 min after the injection. Peritoneal macrophages elicited by intraperitoneal injection with porcine skin gelatin suppressed tritium thymidine uptake by MH134 cells more strongly than those elicited by thioglycollate injection. *Conclusion:* Porcine skin gelatin administered intraperitoneally prolonged the survival of tumor-bearing mice via activation of peritoneal macrophages and involvement of direct anti-tumor activity of porcine skin gelatin.**
Key Words: **porcine skin, gelatin, dissemination.**

Previously we have shown that bovine bone (BB) gelatin stimulated proliferation of murine spleen cells *in vitro* [1]. BB and porcine skin (PS) gelatins stimulated murine peritoneal macrophages (mPMs) to secrete several cyokines, e. g., interleukin (IL)-6, IL-12, TNF-α and MCP-1 [2]. PS gelatin suppressed proliferation of cells of human as well as murine cell lines [1, 3, 4]. Recently we observed that PS gelatin stimulated murine anti-tumor peritoneal macrophages [5]. The ability of PS gelatin to induce anti-tumor peritoneal macrophages was greater than LPS or interferon-γ (IFN-γ). In this study, we investigated whether or not three kinds of gelatin including PS gelatin exerted anti-tumor activity *in vivo*.

MATERIALS AND METHODS

Mice. Five to eight week-old female C3H/HeN mice and Balb/c mice were purchased from Chubu Kagaku Shizai Co. Ltd. (Nagoya, Japan). The care of the animals used in this study was in compliance with the guideline of Aichi-Gakuin University.

Cell lines. A hepatic cell carcinoma cell line of C3H/HeN mice, MH134 has been maintained in suspension culture in our laboratory and a N-nitroso-N-methylurea-induced colon undifferentiated carcinoma cell line of BALB/c mice, Colon 26 has been maintained in monolayer culture.

Reagents. PS (Code No.G-6144) and bovine skin (BS) (Code No.G-6650) gelatin were purchased from Sigma Chemical Co. (St.Louis, MO, USA). BB gelatin (Code No.076-02765) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of peritoneal macrophages. Three mice, each was injected with 3 ml of 3% thioglycollate medium intraperitoneally (i. p.) on day or with 12.5 mg of PS gelatin (0.5 ml) on day 0,1 and 2. On day 3, Peritoneal exudate cells (PECs) were collected by means of peritoneal lavage with phosphate buffered saline (PBS). They were incubated in 10%FCS modified MEM at 37° for 90 min. Modified MEM was composed of 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 25 mM HEPES and Eagle's minimum essential medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan). Approximately 90% of the adherent cells were F4/80 (macrophage marker)-positive [5]. The adherent PECs were used as mPMs.

Anti-tumor activity of peritoneal macrophages or gelatin in vitro. Anti-tumor activity of peritoneal macrophages *in vitro* was estimated as described elsewhere [5]. Briefly, MH134 cells (5×10^3/well) were incubated with 1×10^5 of adherent PECs in wells of a 96-well round-bottomed microtiter plate, each containing 0.2 ml of 5% FCS modified MEM at 37 °C for 24 h followed by an additional 24 h-incubation for ^{3}H-TdR uptake (1 μCi/ml). Radioactivity in the cells per well was counted using a scintillation counter. Anti-tumor activity of peritoneal macrophages was estimated by subtracting the ^{3}H-TdR uptake by peritoneal mac-

Received: February 25, 2009.
*Correspondence: Fax: 052-759-2107
E-mail: taku@dpc.aichi-gakuin.ac.jp
Abbreviation used: ^{3}H-TdR – tritium thymidine; BB – bovine bone; BS – bovine skin; IFN-γ – interferon-γ; IL – interleukin; MCP-1 – monocyte chemoattractant protein-1; mPMs – murine peritoneal macrophages; PECs – peritoneal exudate cells; PS – porcine skin; TNF-α – tumor necrosis factor-α; NS – not significant.

rophages from that by both peritoneal macrophages and MH134 cells. Anti-tumor activity of gelatin was estimated using liquid form gelatin instead of peritoneal macrophages.

Anti-tumor activity of gelatins in vivo. In all experiments, gelatin was administered 2 days after tumor cell challenge. When MH134 or Colon 26 tumor cells (1 x 10^5/mouse) were inoculated i. p., 6.25 or 12.5 mg of PS gelatin was administered i. p. everyday until the mice died. When MH134 tumor cells (1 x 10^4/mouse) were inoculated subcutaneously (s. c.), 12.5 mg of PS gelatin was administered i.p. everyday until the mice died. When MH134 tumor cells (1 x 10^4/mouse) were inoculated i. p., 12.5 mg of PS, BB or BS gelatin was administered s. c. or 2.5 ml of PS gelatin solution (12.5 mg/ml) was done per os (p. o.) everyday until the mice died.

Retention period of PS gelatin injected i. p. Three mice (C3H/HeN), each were injected with 0.5 ml of PBS or 12.5 mg (25 mg/ml) of PS gelatin i. p. After 5 min, 8 ml of PBS was injected into the peritoneal cavity, and the PBS was collected. The concentration of protein in the PBS was determined by staining the protein with Quick Start™ Bradford Dye Reagent (BIO-RAD) and measuring the optical density (OD595) using Ultraspec 3000 (Pharmacia Biotech). Dilution of PS gelatin was used to construct the standard curve for the protein assay.

Statistics. Difference between 2 groups was evaluated using two-tailed Student's *t*-test or Welch's test unless otherwise noted. Difference between 2 groups in tumor sizes was evaluated using Mann — Whitney test because each population of tumor size did not show statistic normal distribution. Difference between 2 groups in survival was evaluated using Log-rank test. *P* values of 5% or less were considered statistically significant.

RESULTS

Effect of gelatin on proliferation of murine tumor cells in vitro. Fig. 1 shows that at least 3.75 mg/ml of PS and BS gelatins suppressed proliferation of MH134 cells (Fig. 1, *a*) or Colon 26 cells (Fig. 1, *c*) *in vitro* and that the anti-tumor effect of PS gelatin was partially cytocidal (Fig. 1, *b*, *d*).

Effect of PS gelatin administered i. p. on the survival of mice i. p. inoculated with MH134 or Colon 26 tumor cells. Fig. 2, *a* shows that intraperitoneal daily administration of 12.5 mg of PS gelatin prolonged the survival of mice inoculated with MH134 tumor cells i. p. and produced tumor-free mice. Fig. 2, *b* shows that i. p. daily administration of 6.25 or 12.5 mg of PS gelatin prolonged the survival of mice inoculated with Colon 26 tumor cells.

Effect of i. p. administered PS gelatin on growth of subcutaneous MH134 tumor and the survival of mice bearing the tumor was not shown since there was no difference between control and experimental groups.

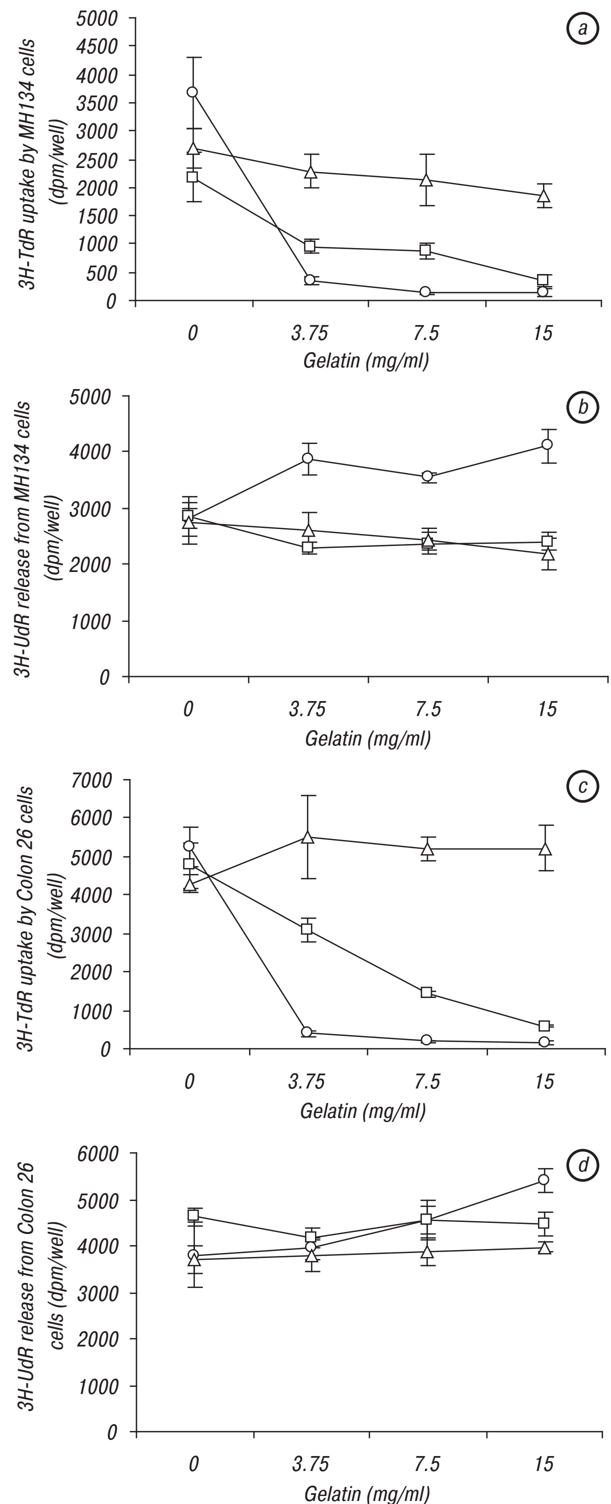

Fig. 1. Anti-tumor activity of three kinds of gelatin *in vitro*. MH134 (*a*) or Colon 26 (*c*) cells (5 x 10^3/well) were incubated with the indicated concentration of PS (○), BS (□) or BB (△) gelatin in wells of a 96-well round-bottomed microtiter plate, each containing 0.2 ml of 5% FBS modified MEM at 37 °C for 48 h. Then 1 µCi/ml of ^{3}H-TdR was added to the wells followed by 7 h-incubation. MH134 (*b*) or Colon 26 (*d*) cells labeled with 3H-UdR (5 x 10^3/well) were incubated with the indicated concentration of PS (○), BS (□) or BB (△) gelatin in wells of a 96-well round-bottomed microtiter plate, each containing 0.2 ml of 5% FBS modified MEM at 37 °C for 24 h. The radioactivity in 0.1 ml of the supernatant was counted

Required duration of contact of tumor cells with PS gelatin to exert its anti-tumor activity. As it could be seen from Table, it required as short

as 5 min for PS gelatin to exert its anti-tumor activity. Fig. 3 shows that after the 5 min-incubation of MH134 cells with or without PS gelatin, ^{3}H-TdR uptake by the cells treated with it decreased and without it increased with the lapse of time.

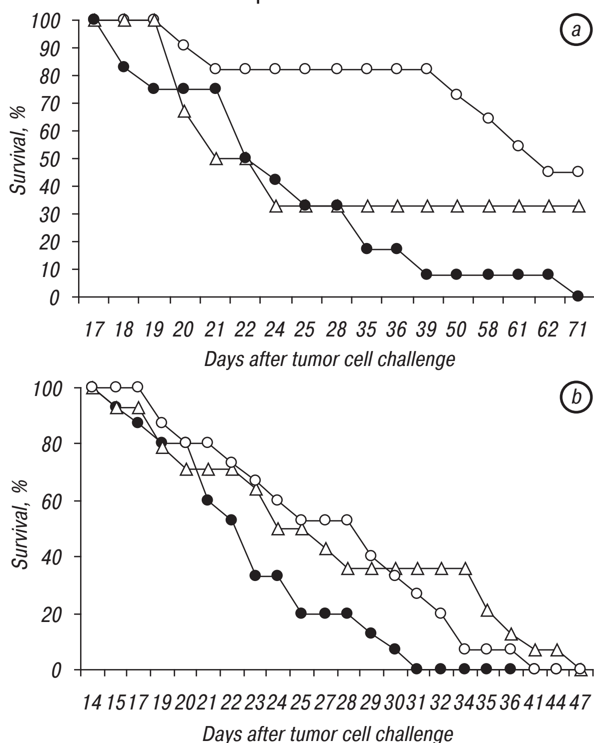

Fig. 2. Effect of intraperitoneal injection of PS gelatin on the survival of mice bearing MH134 and Colon 26 tumors. MH134 (*a*) or Colon 26 (*b*) tumor cells (1 x 10^5) were inoculated i. p. on day 0 into mice. On day 2, 6.25 mg (n = 6) (△) or 12.5 mg (n = 11) (○) of PS gelatin was administered i. p., together with control group without PS gelatin (n =12) (●). Then the same treatment was done everyday until those mice died. *a*, ● *vs* ○: $p < 0.002$, ● *vs* △: not significant (NS). *b*, ● *vs* ○: $p < 0.02$, ● *vs* △: $p < 0.05$

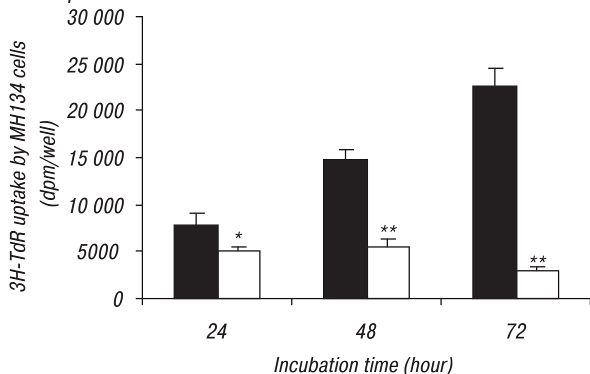

Fig. 3. PS gelatin exclusively suppresses the proliferation of tumor cells. MH 134 cells (5 x 10^3/well) were incubated with (□) or without (■) 15 mg/ml of PS gelatin in a 96-well round-bottomed microtiter plate, each containing 0.2 ml of 5% FBS modified MEM at 37 °C for 5 min, and they were washed with 0.2 ml of PBS twice. Then they were incubated in the same manner without PS gelatin for the indicated period. ^{3}H-TdR (1 µCi/ml) was added to each well 7 h before completion of the incubation. *NS from control (■). ** Significant from control (■) at $p < 0.001$.

Retention of i. p. injected PS gelatin in the peritoneal cavity. Fig. 4 shows that there was an appreciable amount of protein in the peritoneal cavity 5 min after i. p. injection of PBS (0.5 ml) and that a larger amount of protein was detected 5 min after i. p. injection of PS gelatin (12.5 mg) than i. p. injection of PBS (0.5 ml).

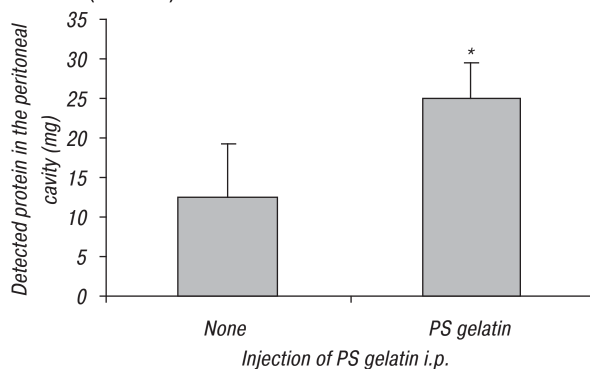

Fig. 4. Retention of PS gelatin injected i. p. in the peritoneal cavity. Three mice, each was injected with or without 12.5 mg/ 0.5 ml of PS gelatin. After 5 min, the peritoneal cavity was lavaged with 8 ml of PBS. The concentration of protein in the collected PBS was measured
*$p = 0.0549$ (two tailed *t*-test), $p = 0.0274$ (one tailed *t*-test).

Anti-tumor activity of peritoneal macrophages which were elicited by intraperitoneal administration of PS gelatin. Fig. 5 shows that mPMs elicited by thioglycollate injection suppressed ^{3}H-TdR uptake by MH134 cells as compared with medium alone. mPMs elicited by PS gelatin injection suppressed ^{3}H-TdR uptake by MH134 cells more strongly than those elicited by thioglycollate injection.

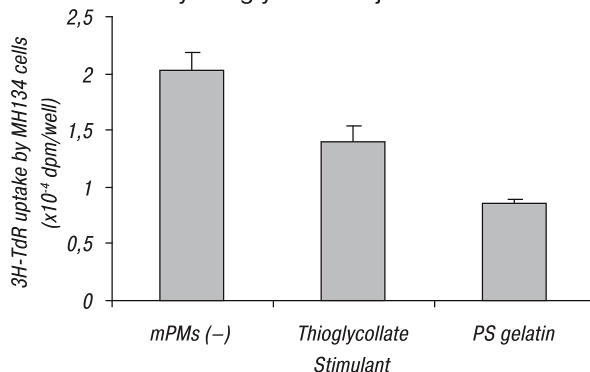

Fig. 5. Anti-tumor activity of mPMs which were elicited by intraperitoneal injection of PS gelatin. Three mice, each were injected with 3 ml of 3%thioglycollate medium i. p. on day or with 12.5 mg of PS gelatin on day 0, 1, 2. On day 3, PECs were collected. MH134 cells (5 x 10^3/well) were incubated with 1 x 10^5 of the indicated mPMs in wells of a 96-well round-bottomed microtiter plate, each containing 0. 2 ml of 5% FCS modified MEM at 37 °C for 24 h followed by additional 24 h-incubation for ^{3}H-TdR uptake (1 µCi/ml)

Effect of subcutaneously administered gelatin on the survival of mice i. p. inoculated with MH134 tumor cells. Fig. 6, *a* shows that subcutaneously administered PS gelatin did not affect the survival of mice with intraperitoneal MH134 tumor. Fig. 6, *b* shows that subcutaneously administered BB gelatin tended to prolong the survival. Fig. 6, *c* shows that subcutaneously administered BS gelatin significantly prolonged the survival.

The data on the effect of orally administered PS gelatin on the survival of mice i. p. inoculated with MH134 tumor cells are not shown since there was no difference between control and experimental groups.

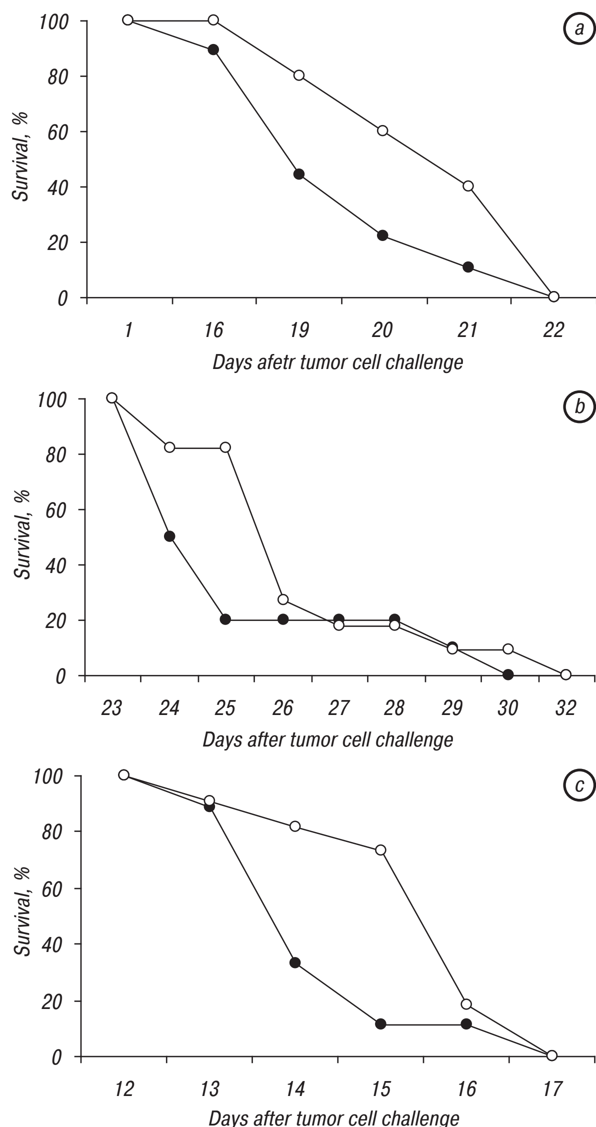

Fig. 6. Effect of s. c. administration of 3 kinds of gelatin on survival of mice inoculated with MH134 tumor cells i. p. MH134 tumor cells (1 x 10^4) were inoculated i.p. into each mouse. PS (*a*, ○) (n = 10), BB (*b*, ○) (n = 11), BS (*c*, ○) (n = 11) gelatin (12.5 mg/0.5 ml of PBS) or 0.5 ml of PBS (*a*, *b*, *c*, ●) (*a*, n = 9, *b*, n = 10, *c*, n = 9) was administered s. c. 2 days after the tumor inoculation. Then the same treatment was done everyday until those mice died. *a:* NS, *b:* NS, *c:* $p < 0.05$

Table. Required period of contact between tumor cells and PS gelatin

Exp	Incubation time (min)	PS gelatin (mg/ml)	^{3}H-TdR uptake by MH 134 cells (dpm/well)
1	15	0	3042 ± 243
		15	715 ± 178
	360	0	2996 ± 326
		15	591 ± 126
2	5	0	1711 ± 214
		15	797 ± 84
3	0	0	8109 ± 683
		15	7636 ± 398

Notes: MH134 cells (5 x 10^3/well) were incubated with 0 or 15 mg/ml of PS gelatin in a 96-well round-bottomed microtiter plate, each containing 0.2 ml of 5% FBS modified MEM at 37 °C for the indicated duration, and they were washed with 0.2 ml of PBS twice. Then they were incubated in the same manner without PS gelatin for 48 h (Exp. 1 & 2) or 72 h (Exp. 3). ^{3}H-TdR (1 μCi/ml) was added to each well 7 h before completion of the incubation.

DISCUSSION

The present study investigated whether or not PS gelatin administered i. p., s. c., or p. o. controlled growth of tumor *in vivo*. PS gelatin exerted a stronger *in vitro* anti-tumor activity than BB or BS gelatin, mainly cytostatic toward 2 kinds of tumor cell lines. It remains to be solved what causes these different anti-tumor activity among such gelatins. It is well known that gelatins have a common amino acid sequence, i. e., glycine-X-Y. At present, we suppose that different anti-tumor activities of gelatins are attributed to different X and Y. PS gelatin administered i. p. prolonged the survival of mice bearing intraperitoneal MH134 or Colon 26 tumor. Colon 26 tumor cells formed a lesion similar to clinical state, what is called dissemination though MH134 tumor cells grew as single cells in the peritoneal cavity (data not shown). Administration of PS gelatin started 2 days after the tumor cell inoculation and was continued everyday until death of the mice. It is not clear whether or not this schedule of administration is appropriate in order to evaluate the anti-tumor activity of this gelatin from a clinical viewpoint. In any case, it has become clear that PS gelatin exerts an anti-tumor activity *in vivo* as well as *in vitro*. Previously, we reported that PS gelatin stimulated peritoneal macrophages *in vitro* to secrete TNF-α [2, 5], and to obtain anti-tumor activity which was exerted *in vivo* as well as *in vitro* [5]. In the present study, peritoneal macrophages which exerted anti-tumor activity *in vitro* had been induced by i. p. injection of PS gelatin. MH134 tumor cells inoculated s. c. was not affected by PS gelatin administered i. p. (data not shown). This result may suggest that anti-tumor peritoneal macrophages induced by i. p. injection of PS gelatin did not accumulate to the cutaneous region and that the amount of TNF-α secreted by peritoneal macrophages stimulated with PS gelatin was not enough to suppress tumor proliferation. The previous study showed that peritoneal macrophages stimulated with PS gelatin secreted an appreciable amount of TNF-α, but the TNF-α did not participate in the anti-tumor activity of the culture supernatant *in vitro* [5].

Alternatively, these *in vivo* experiments suggest the following possibilities. One is that tumor cells inoculated i. p. were attacked directly by PS gelatin administered i. p. Another is that anti-tumor peritoneal macrophages induced by PS gelatin administered i. p. exert anti-tumor activity *in situ*. Retention of PS gelatin administered i. p. in the peritoneal cavity was examined. Protein of approximately 12.5 mg remained in the peritoneal cavity at least 5 min after the administration. If the protein is PS gelatin, this concentration of PS gelatin (approximately 12.5 mg/ml) was enough to suppress tumor proliferation *in vitro*. So there is a little fluid containing proteins, less than 1 ml, in normal peritoneal cavity. However, a possibility cannot be excluded that detected protein is not PS gelatin but proteins in the exudate elicited with i. p. administration of PS gelatin. On the other hand, it was examined how long contact with tumor cells was required for PS gelatin to exert its anti-tumor activity. The contact for several seconds was not enough. It required at least 5 min. These results suggest a possibility

that tumor cells inoculated i. p. are directly attacked by PS gelatin administered i. p. In conclusion, results suggest that the anti-tumor macrophages as well as PS gelatin attack tumor cells inoculated i. p.

In order to examine anti-tumor effect of systemic administration of gelatin, intraperitoneal administration of PS gelatin was conducted. However, s. c. inoculated tumor cells were not affected. Inversely effect of subcutaneous administration of BB, BS or PS gelatin on tumor cells inoculated i. p. was examined. BS gelatin administered s. c. exerted a statistically significant anti-tumor activity but not PS or BB gelatin. However, the anti-tumor activity was weaker than that of PS gelatin administered i. p. to tumor cells inoculated i. p. The present study showed that BS gelatin exerted moderate direct anti-tumor effect *in vitro* among BB, BS and PS gelatins. The previous study, however, showed that BS gelatin did not induce cytokine secretion by peritoneal macrophages [2]. In order to clarify the mechanism of this strange result, it should be investigated how gelatin is digested and how it or its digested products are transferred.

PS gelatin administered orally did not exert anti-tumor activity to intraperitoneal MH134 tumor. PS gelatin administered orally may be digested to be inactive amino acids and/or peptides.

There has been no effective cure for peritoneal dissemination, e. g., gastric cancer or ovarian tumors. Cisplatin, (SP-4-2)-Diamminedichloroplatinum and its derivatives are administered intravenously or i. p. to treat these diseases [6–11]. However, they have exerted partial effects. This study shows that PS gelatin administered i. p. can partially control intraperitoneal tumor. It is well known that gelatin [12–14] as well as collagen [15–17] possesses low immunogenicity. Therefore i. p. application of PS gelatin may be effective for controlling peritoneal dissemination in collaboration with chemotherapeutic agents.

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