Antitumor Effect of Intratumoral Administration of Biological Response Modifiers: Induction of Immunosuppressive Acidic Protein, a Type of α_1 -Acid Glycoprotein, in Mice

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The antitumor effects of biological response modifiers (BRMs) in an experimental mouse model, the "double grafted tumor system" were analyzed. Male BALB/c mice received simultaneous inoculations of Meth-A fibrosarcoma cells on the right flank (10^6 cells) and left flank (2×10^5 cells) on day 0, and BRMs were injected intratumorally into the right tumor on days 3, 4 and 5. PSK (a protein-bound polysaccharide preparation), interleukin-1 (IL-1) and cepharanthin (CR) cured not only the right, but also the left, non-treated tumor in a double grafted tumor system. OK-432 (a *Streptococcus* preparation) and BCG and tumor necrosis factor (TNF) cured the right tumor and inhibited the growth of the left tumor. Lentinan (a polysaccharide preparation) and IL-6 inhibited neither the right nor the left tumor. Immunosuppressive acidic protein (IAP) in serum was increased transiently soon after intradermal injection of PSK, CR, OK-432 and TNF in BALB/c mice. Lentinan, however, did not induce IAP. IAP in serum was gradually increased after intradermal inoculation of Meth-A tumor in BALB/c mice. The biochemical difference between PSK-induced IAP (early, inflammatory IAP) and Meth-A-induced IAP (late, tumor-induced IAP) was investigated by crossed affinity immunoelectrophoresis with concanavalin A. IAP of murine serum was separated into 4 peaks. IAP in normal mouse was rich in high-mannose type sugar chain (Peak 3) and contained no hybrid-type sugar chain (Peak 4), which was present in inflammatory and tumor-induced IAP. Inflammatory IAP was rich in biantennary sugar chain (Peak 2) and tumor-induced IAP was rich in tri-tetraantennary sugar chain (Peak 1).

Key words: Biological response modifier — Intratumoral administration — Immunosuppressive acidic protein — Activated macrophage — Cytokine network

In our previous papers,¹⁻³⁾ the antitumor effects at a distant site of PSK,⁴ a protein-bound polysaccharide preparation, and recombinant human IL-1 β were analyzed by using the double grafted tumor system in which male BALB/c mice received simultaneous intradermal inoculations of Meth-A tumor in the right (10^6 cells) and the left (2×10^5 cells) flanks and were then injected with PSK or IL-1 β in the right-flank tumor on days 3, 4 and 5. Among several BRMs examined, PSK and IL-1 β significantly inhibited the growth of not only the right but also the left (non-treated) tumor.

Our previous studies on the antitumor effect of PSK and IL-1 in the double grafted tumor system showed that the intratumoral administration of PSK and IL-1 first

induces neutrophils in the right tumor via an IL-8-like factor and then macrophages are induced by an MCF produced by tumor cells and neutrophils. Then Lyt-1 (L3T4)-positive cells are induced in the right regional lymph nodes and in the spleen, probably via IL-1, which might be produced from macrophages in contact with tumor cells. Subsequently, Lyt-1-positive and L3T4-positive cells in the spleen reach the left tumor through the blood stream, come into contact with Meth-A cells and then produce MCF. Intratumoral administration of PSK and IL-1 in the right tumor thus induces activated macrophages in the left, non-treated tumor, thereby bringing about the regression of the distant tumor.

α_1 -AG is one of the major acute phase proteins in plasma which increases dramatically in inflammation, pregnancy and cancer.⁹⁾ Many sera and ascitic fluids obtained from cancer patients contained dramatically increased amounts of α_1 -AG.^{10, 11)} Furthermore, α_1 -AG from cancer patients exhibited a different composition of pI subspecies when compared with those derived from healthy or pregnant donors.¹²⁾ We purified the major subspecies of α_1 -AG from cancer ascitic fluids to apparent

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⁴ The abbreviations used are: PSK, polysaccharide Kureha; BRM, biological response modifier; IAP, immunosuppressive acidic protein; CR, cepharanthin; IL-1 β , interleukin-1 β ; TNF, tumor necrosis factor; LNT, lentinan; CAIE, crossed affinity immunoelectrophoresis; Con A, concanavalin A; MCF, macrophage chemotactic factor; α_1 -AG, α_1 -acid glycoprotein.

homogeneity (MW: 50 kDa, pI: 3.0). This $\alpha 1$ -AG was designated IAP,¹³⁾ since a high dose of it (>1 mg/ml) exhibited irreversible immunosuppressive activity towards various immunological responses *in vitro*¹³⁾ and *in vivo*,^{14, 15)} enhanced tumor growth in mice,¹⁶⁾ and induced suppressor macrophages.¹⁴⁾ On the other hand, preliminary experiments showed that an inflammatory drug, carageenan¹⁵⁾ and a Streptococcal preparation, OK-432¹⁷⁾ induced a low level of IAP (<1 mg/ml) transiently soon after an intraperitoneal administration. Such a low dose of IAP did not show immunosuppression.¹⁴⁾ IAP was produced by liver cells, neutrophils and activated macrophages.¹⁵⁾ Therefore, IAP in serum of BRM-treated mice was measured as a marker protein of activated macrophages and neutrophils. The first purpose of the present paper is to show that the induction of inflammatory IAP in serum is an important trigger of antitumor effect caused by the intratumoral injection of BRMs. The second purpose is to clarify the biochemical difference between the PSK-induced IAP (early, inflammatory IAP) and Meth-A-induced IAP (late, tumor-induced IAP). CAIE with Con A was used for fractionation and for structural studies of asparagine-linked sugar chains of IAP.

MATERIALS AND METHODS

Mice and tumor Six-week-old male BALB/c (+/+) and BALB/c (nu/nu) mice were obtained from Shizuoka Laboratory Animal Center, Hamamatsu. Meth-A, a methylcholanthrene-induced fibrosarcoma, was administered to syngeneic BALB/c mice in solid form by intradermal inoculation.

BRMs PSK was purified from a hot water extract of cultured mycelia from *Coliolum versicolor*, which belongs to Basidiomycetes. The average molecular weight is about 100,000 and its protein content is about 38%. The main glycoside portion of PSK is β -D-glucan. PSK was

supplied by Kureha Chemical Industry Ltd., Tokyo. LNT was extracted from fruit bodies of *Lentinus edodes* (Berk) and is a β -1,3-glucan with average molecular weight of 500,000 daltons. LNT is composed of only glucose with no other sugar component and was supplied by Yamanouchi Pharmaceutical Co., Ltd., Tokyo. OK-432 (Chugai Pharmaceutical Co., Tokyo) is a penicillin- and H_2O_2 -killed lyophilized preparation of Su strain of *Streptococcus pyogenes*. Recombinant TNF (2.1×10^6 units/mg protein) was kindly donated by Asahi Chemical Industry Co., Ltd., Tokyo. The stock solution of rTNF was 10^5 units/ml of 5 mM phosphate-buffered saline, pH 7.4, containing 0.1% gelatin. CR is an extract from the root of *Stephania cepharantha* HAYATA, consisting of 6 kinds of biscoclaurine alkaloid and was provided by Kaken Shoyaku Co., Ltd., Tokyo. BCG was supplied with Koseikai BCG Laboratories, Sendai. Recombinant human IL-6 was supplied by Toray Industries, Inc., Kamakura.

Double grafted tumor system As described in previous papers,¹⁻⁸⁾ we devised the double grafted tumor system as a new experimental tumor model. BALB/c mice receive simultaneous intradermal inoculation of Meth-A tumor cells in both the right (1×10^6 cells, primary region) and the left (2×10^5 cells, distant region) flanks. Drugs are injected into the right-flank tumor on days 3, 4 and 5, and the left (non-treated) tumor is observed for 21 days. As there is a possibility that drugs in the right tumor may directly affect the left tumor via the blood stream, we devised as a control the single tumor system in which mice received intradermal inoculations of 2×10^5 Meth-A tumor cells only in the left flank, and then were injected subcutaneously with drugs into the right flank on day 3 and observed for 18 days.

Evaluation of antitumor activity Tumor diameter was measured 3 times a week with calipers and the tumor size was calculated as the square root of the long diameter $\times$ short diameter (mm). After 3 weeks, the animals were

Table I. Anti-Meth-A Effect of Cepharanthin in the Double Grafted Tumor System

Group	Right tumor (1×10^6 cells)		Left tumor (2×10^5 cells)	
	Tumor-free /tested	Tumor weight (g $\pm$ SD)	Tumor-free /tested	Tumor weight (g $\pm$ SD)
Double-tumor				
Control	0/8	5.3 ± 1.34	0/8	3.1 ± 0.50
Cepharanthin 0.5 mg $\times$ 3 d3, 4, 5	3/8	1.2 ± 0.60	2/8	2.5 ± 0.88
Single-tumor				
Control	—	—	0/8	4.2 ± 0.82
Cepharanthin 0.5 mg $\times$ 3 d3, 4, 5	—	—	0/8	2.5 ± 1.19

SD: standard deviation.

killed and each tumor was weighed to obtain the mean value(g) $\pm$ standard deviation. Each experimental and control group consisted of 6 to 9 mice. The difference in tumor growth (tumor size or tumor weight) between the control and experimental groups was tested statistically by using the Student's *t* test. The differences in cure rates were statistically evaluated by means of the chi-square test.

Determination of mouse IAP The serum concentration of IAP was determined with a single radial immunodiffusion method using rabbit anti-mouse IAP serum (Mouse IAP plate, Sanko Junyaku, Tokyo). The calibration curve with purified IAP was linear between 30 and 1,500 μ g/ml.

CAIE with Con A Microheterogeneity of IAP was determined by CAIE.¹⁸⁾ Electrophoresis was performed at 15°C in 1% agarose in Tris/Veronal buffer, pH 8.6. The thickness of the gels was 1.5 mm. First dimension electrophoresis was performed with 3 μ l of sample serum in a gel containing 0.5 mg/ml of Con A (Seikagaku Kogyo, Tokyo) at 10–15 V/cm until a mixture of bromophenol blue and serum had migrated 4.5 cm. In addition to antibodies against mouse IAP (Saikin Kagaku, Sendai), the second dimension gel contained 4% α -D-methylglucopyranoside in 1% agarose. Second dimension electrophoresis was performed for 16–18 h at 2 V/cm. The gels were washed, pressed, dried, and stained with Coomassie brilliant blue R-250. The areas below the immunoprecipitates were measured by semiautomatic planimetry, and each peak was expressed as a percentage of the total area enclosed.

RESULTS

Antitumor effect of four different BRMs in the double grafted tumor system The antitumor effects of PSK,^{1,3,5,6,8)} OK-432,^{1,5)} IL-1,^{4,7)} IFN,^{1,4,5)} TNF,⁴⁾ IL-2,⁴⁾ MMC (mitomycin C)^{1,5)} and NCS (neocarzinostatin)⁵⁾ in the double grafted tumor system have been reported previously. As shown in Table I, a plant alkaloids preparation, CR (0.5 mg $\times$ 3), inhibited the growth of not only the right but also the left, non-treated tumor. However, no tumor-free mice were found in the single tumor system with CR injection. These results show that CR itself does not directly affect the opposite flank and that intratumoral CR immunotherapy in the primary region does affect tumor growth in the distant region. In BALB/c nude mice, CR inhibited the growth of the right tumor but not that of the left tumor (Fig. 1a). Therefore, the antitumor activity of CR on the left tumor in the double grafted tumor system is associated with a sequential immune mechanism in which T cells may play an important role. The antitumor effect of CR on the right tumor is a direct cytotoxic effect. As shown in Table II, BCG

Fig. 1. Effect of several BRMs in the double grafted tumor system. (a) Cepharanthin, BALB/c nude mice. ● control, ○ cepharanthin. Bars show standard deviation. (b) Lentinan, BALB/c mice. ● control, ○ lentinan (0.1 mg $\times$ 3). (c) IL-6, BALB/c mice. ● control, ○ IL-6 (0.2 μ g $\times$ 3), ▲ IL-6 (1 μ g $\times$ 3), × IL-6 (5 μ g $\times$ 3).

(5 mg $\times$ 3) completely cured the right tumor in the double grafted tumor system and inhibited the growth of the left tumor, as was the case with OK-432.⁵⁾ On the

other hand, β -1,3-glucan, LNT ($0.1 \text{ mg} \times 3$) was effective on neither the right nor the left tumor in this system, as shown in Fig. 1b. Moreover, another cytokine, rIL-6 did not show any antitumor effect (Fig. 1c). The present and previous results show that there are notable differences in antitumor effect among BRMs, as summarized in Table III.

Induction of IAP by BRM treatment IAP was found to be produced by liver cells, neutrophils and macrophages.^{14,15} Therefore, IAP in serum of BRM-treated mice was measured as a marker protein of activated macrophages and neutrophils. IAP in serum increased dramatically soon after intradermal injection of PSK (Fig. 2a, $880 \mu\text{g/ml}$), CR (Fig. 2b, $840 \mu\text{g/ml}$), rTNF ($990 \mu\text{g/ml}$) or OK-432 ($520 \mu\text{g/ml}$). An inflammatory drug, carrageenan, also induced IAP in serum after intradermal injection ($650 \mu\text{g/ml}$). LNT did not induce IAP, as shown in Fig. 2c.

On the other hand, IAP in serum gradually increased after intradermal inoculation of Meth-A tumor in BALB/c mice (Fig. 3). At 21 days after tumor inocula-

tion, IAP in serum reached a maximum level ($300 \mu\text{g/ml}$). The serum IAP level of Meth-A bearing mice as well as that of normal mice increased after the intratumoral injection of PSK, as shown in Fig. 3. At 21 days after tumor inoculation, IAP in PSK-treated mice returned to the normal level. Therefore, the biochemical difference between PSK-induced IAP (early, inflammatory IAP) and Meth-A-induced IAP (late, tumor-induced IAP) was next investigated.

Microheterogeneity of sugar chains in IAP Various immobilized lectins can be used for fractionation and for structural studies of asparagine-linked sugar chains of glycoprotein.¹⁹ The biochemical heterogeneity between PSK-induced IAP and Meth-A-induced IAP was investigated by CAIE with Con A. CAIE revealed at least four subfractions (Peaks 1–4) in IAP molecules: a Con A non-reactive fraction, the most anodic (P1), a Con A weakly reactive fraction (P2), a Con A-reactive fraction (P3) and a strongly reactive fraction (P4) (Fig. 4). Areas under the peaks give a semiquantitative measurement of the proportion of each population of IAP molecules

Table II. Antitumor Effect of BCG in the Double Grafted Tumor System

Group	Right tumor			Left tumor		
	Tumor-free /tested	Tumor diameter (mm $\pm$ SD)	Tumor weight (g $\pm$ SD)	Tumor-free /tested	Tumor diameter (mm $\pm$ SD)	Tumor weight (g $\pm$ SD)
Control	0/6	24.5 ± 1.3	4.7 ± 0.3	0/6	18.7 ± 2.9	2.8 ± 0.8
BCG						
0.5 mg $\times$ 3	1/9	9.7 ± 5.0	0.6 ± 0.5	0/9	10.1 ± 3.3	0.7 ± 0.5
5 mg $\times$ 3	9/9	0	0	0/9	10.9 ± 3.7	0.8 ± 0.5

SD: standard deviation.

Table III. Characterization of Various BRMs in the "Double Grafted Tumor System" and "Single Tumor System"

Category	Drug	Dose	Double Tumor		Single Tumor
			Right	Left	Left
Immunopotentiators	PSK	5 mg $\times$ 3	++	++	—
	CR	0.5 mg $\times$ 3	++	++	+
	OK-432	0.1 mg $\times$ 5	++	+	—
	BCG	5 mg $\times$ 3	++	+	ND
	LNT	0.1 mg $\times$ 3	—	—	—
Cytokines	IL-1	0.2 μg $\times$ 3	++	++	—
	IFN	10^5 U $\times$ 8	++	++	+
	TNF	$3 \times 10^3 \text{ U}$ $\times$ 3	++	—	ND
	IL-2	10^4 U $\times$ 10	—	—	—
	IL-6	5 μg $\times$ 3	—	—	—
Chemotherapeutics	MMC	100 μg $\times$ 1	++	+	+
	NCS	10 U $\times$ 1	++	—	—

Scoring: ++, tumor rejection; +, growth inhibition; —, no effect; ND, not done.

Fig. 2. Change of serum IAP levels in BALB/c mice after intradermal injections of several BRMs. (a) PSK, (b) CR, (c) LNT.

(Fig. 5). Agneray²⁰ has concluded that P1 is a glycopeptide with tri- and tetra-antennary complex-type sugar chains, and that P2 is a glycopeptide with biantennary complex-type sugar chain. P3 is a glycopeptide with high-mannose type sugar chain and P4 is one with hybrid-type sugar chain.

Fig. 3. Change of serum IAP levels in PSK-treated (○) and untreated (●) Meth-A-bearing mice after an intratumoral injection of PSK.

As shown in Figs. 4 and 5, IAP in normal mouse contained no P4 (hybrid type). The Con A strongly reactive P4 appeared in PSK-injected mouse and Meth-A-bearing mouse. The proportion of P3 (high-mannose type) decreased progressively in PSK-injected mouse and tumor-bearing mouse. In PSK-injected mouse undergoing an acute inflammatory process, P2 (biantennary complex) type of IAP is predominant. On the other hand, in Meth-A bearing mouse the Con-A nonreactive P1 (tri- and tetra-antennary complex type) is predominant. These results clearly showed that sugar chains of IAP are different among normal, inflammatory and tumor-bearing mice.

DISCUSSION

IAP was first discovered in the sera of cancer patients and found to suppress both phytohemagglutinin-induced lymphocyte blast formation and mixed lymphocyte reaction *in vitro*. Purification and characterization of IAP from the ascitic fluid revealed that this acidic protein belonged to the α -1 group of glycoproteins usually found in healthy individuals, but this type differed in its gel isoelectric focusing behavior, molecular weight, and carbohydrate content.¹³ *In vivo* analysis reconfirmed that IAP was increased in tumor-bearing mice and a close correlation between IAP level and the induction of suppressor macrophages was reported.¹⁴ In humans, IAP was also purified from ascitic fluids developed from cancer of the lung, colon, and breast.²¹ High serum IAP levels were observed in patients with head and neck cancer,²² ovarian cancer,¹¹ and gastric cancer¹² and it is speculated that serum IAP values are closely correlated with the growth of the tumor and changes in the host immune responses. Sakamoto *et al.*²³ examined the prognostic value of IAP in a prospective randomized trial

Fig. 4. Con A-CAIE patterns of murine serum IAP. (N) Normal BALB/c, 8-week male mice. (T) Meth-A tumor-bearing mice, 24 days after tumor inoculation. (P) PSK-treated mice, one day after PSK injection.

of advanced gastric cancer patients, designed to evaluate the effect of PSK. Preoperative serum IAP levels were determined in patients who received radical gastric resection. All patients were followed up for 24 months or more. There was an overall significant difference in disease-free survival time in favor of the PSK-treated group compared with the control group. Preoperative IAP values were strongly associated with disease-free survival time. The most significant difference was observed at the threshold value of 580 $\mu\text{g}/\text{ml}$. Patients in the PSK-treated group with a pre-operative IAP of lower

Fig. 5. Relative amount of IAP subfractions in normal (N), tumor-bearing (T) and PSK-treated (P) mice. Proportion of each peak is given as percent of total amount.

than 580 $\mu\text{g}/\text{ml}$ showed improved disease-free survival ($P=0.029$), whereas no significant difference was seen between the two groups when the pre-operative IAP exceeded the threshold level. From these results, 580 $\mu\text{g}/\text{ml}$ is postulated to be the most appropriate threshold value for predicting the prognosis of advanced gastric cancer patients, and it is suggested that PSK would be most effective in patients whose preoperative IAP level is lower than the threshold level.

The present report clearly shows that a tumor-induced IAP differs biochemically from an inflammatory IAP in the mouse (Figs. 4 and 5). Therefore, it may be suggested that preoperative serum IAP should be different immunologically between a tumor-induced IAP and an inflammatory IAP. In the present study, the correlation between the induction of inflammatory IAP in serum of mice and the antitumor effect of BRMs in these mice was

compared. PSK (a protein-bound polysaccharide preparation) and cepharanthin (a plant alkaloids preparation, Table I) cured not only the right, but also the left, non-treated tumor in a double grafted tumor system. OK-432 (a *Streptococcus* preparation) and BCG (Table II) cured the right tumor and inhibited the growth of the left tumor. TNF cured the right tumor only. Lentinan (a polysaccharide preparation, Fig. 1b) and IL-6 (Fig. 1c) inhibited neither the right nor the left tumor. Several BRMs were injected intradermally three times and the inflammatory IAP serum level was measured. Clear IAP induction was observed in serum of PSK- (Fig. 2a), CR- (Fig. 2b), TNF- and OK-432-treated mice. However, IAP induction was not observed in serum of LNT-

treated mice (Fig. 2c). These results show that the anti-tumor effect of BRM is correlated with the induction of an inflammatory IAP in serum and that the activation of macrophages and neutrophils in tumor tissue is an important trigger of the antitumor cascade reaction caused by intratumoral BRM treatment in the double grafted tumor system.

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