Carbohydrate Research, 181 (1988) 135–142
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

THE SULFATED POLYSACCHARIDE–PEPTIDOGLYCAN COMPLEX POTENTLY INHIBITS EMBRYONIC ANGIOGENESIS AND TUMOR GROWTH IN THE PRESENCE OF CORTISONE ACETATE

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(Received September 1st, 1987; accepted for publication, October 6th, 1987)

ABSTRACT

In combination with cortisone acetate, the sulfated polysaccharide (SP)–peptidoglycan (PG) complex produced by an *Arthrobacter* species was found to inhibit both embryonic angiogenesis of chick chorioallantoic membrane (CAM) and growth of solid Sarcoma 180 tumor in mice. Three fractions obtained from the SP–PG complex by gel filtration showed a great difference in the inhibitory effect on angiogenesis of the CAM, whereas in *ex vivo* study such a difference was reduced. Of the three fractions, SP–PG-L having the lowest molecular weight and peptidoglycan content exhibited the highest antiangiogenic and antitumor activities, even higher than those of heparin. Neither desulfation nor sulfation of the SP–PG complex greatly affected the antiangiogenic activity, and dextran sulfates and mannoglucan sulfates were lacking the activity. This suggested that glycosyl sequences of sulfated polysaccharides are more important for the activity, rather than content or intramolecular distribution of sulfate groups.

INTRODUCTION

Several groups of investigators have reported naturally occurring antiangiogenic substances, such as the extracts from cartilage[1,2], the cell-wall extract from aorta[3], and protamine sulfate[4]. Recently, Folkman *et al.*[5] developed a new way to inhibit both angiogenesis and tumor growth by combination of heparin and cortisone. They showed that anticoagulant activity of heparin was not responsible for antiangiogenesis[5], and also that neither the glucocorticoid nor the mineralocorticoid activity of cortisone was necessary for antiangiogenesis[6]. They also indicated[5] that the antiangiogenic activity of heparin in the presence of cortisone varied markedly among the heparins from ten suppliers. The synergistic inhibitory effects of heparin and cortisone acetate were confirmed by Sakamoto *et al.*[7].

These results prompted us to examine the antiangiogenic and antitumor activities of some sulfated polysaccharides in combination with cortisone acetate. We isolated[8,9] a series of complexes composed of sulfated polysaccharide (SP)

0008-6215/88/$ 03.50

chains and peptidoglycan (PG) fragments in the culture supernatant of an *Arthrobacter* sp., and report herein the synergistic antiangiogenic and antitumor effects of the SP–PG complex and cortisone acetate, in comparison with those of heparin and other sulfated polysaccharides.

EXPERIMENTAL

Analytical methods. — Optical rotation was measured with a Perkin–Elmer 141 polarimeter. Molecular weights were estimated by gel-permeation chromatography (g.p.c.) in 0.1M potassium acetate buffer (pH 6.5) at a flow rate of 0.8–1.0 mL/min, at 40° in a column (7.5 × 600 mm) of G3000 SW or G5000 PW with a Toyo Soda model 803D liquid chromatograph, equipped with a refractive-index detector; the standards were Dextran T-500, T-70, T-40, and T-10 (Pharmacia Fine Chemicals). The contents of carbohydrate, protein, sulfur, and phosphorus were determined as described previously[8]. Sugars were analyzed as the alditol acetates by g.l.c.[8].

Preparation of SP–PG complex and desulfated SP–PG complex. — The complex (sodium salt) was prepared[8] from the culture supernatant of an *Arthrobacter* sp. and desulfated by solvolysis according to the method of Nagasawa *et al.*[9] The complex (1.0 g) was converted into the pyridinium salt (1.1 g) by passing through a Dowex 50-X2 (H^+) column, followed by neutralization with pyridine, and treatment for 5 h at 80° with 9:1 dimethyl sulfoxide–methanol, to give the desulfated material (SP–PG-D; 0.39 g).

Fractionation of SP–PG complex. — The complex (2.0 g) was dissolved in 0.1M NaCl (10 mL) and applied to a column (5 × 80 cm) of Sephacryl S-300 preequilibrated with 0.1M NaCl. The column was eluted with the same solvent and fractions (12 mL) were collected. After monitoring of the elution profiles of the

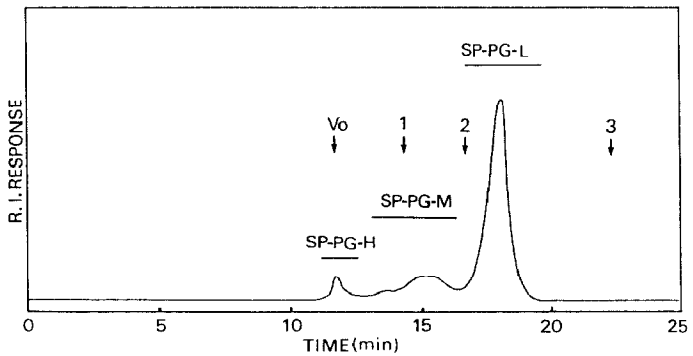

Fig. 1. Gel-permeation chromatogram of SP–PG complex on G3000 SW, with 0.1M potassium acetate buffer (pH 6.5) as the carrier (0.9 mL/min). The SP–PG complex was fractionated by gel filtration on Sephacryl S-300 into SP–PG-H, SP–PG-M, and SP–PG-L with the respective elution profiles indicated. Arrows indicate the elution positions of the standard dextrans: 1 (T-70), 2 (T-40), and 3 (T-10).

fractions by g.p.c., the respective fractions corresponding to SP–PG-H, SP–PG-M, and SP–PG-L (see Fig. 1) were pooled and desalted by dialysis against de-ionized water. Each of the three nondialyzable solutions was concentrated *in vacuo* and poured into ethanol (8 vol.). The respective precipitates were collected by centrifugation, washed successively with 8:1 ethanol–water, ethanol, and acetone, and dried *in vacuo*, to give SP–PG-H (0.06 g), SP–PG-M (0.32 g), and SP–PG-L (1.31 g).

In another experiment, the SP–PG complex (5.0 g) was chromatographed on Sephacryl S-300, as just described, to remove the SP–PG-H fraction from the complex, and SP–PG-LM (3.84 g) was obtained.

Sulfation of SP–PG complex. — To a solution of the complex (1.0 g) in formamide (10 mL) was added a sulfating reagent prepared by dropwise addition of chlorosulfonic acid (2.0 mL) into pyridine (10 mL) <0° with vigorous stirring. The mixture was stirred for 4 h at 40° and poured into methanol (100 mL) at 0°. The precipitate was washed with methanol and dissolved in water (20 mL) at 0°, and the pH of the solution was adjusted to 9 with 2M NaOH. The solution was poured into ethanol (160 mL) and the precipitate was collected. The precipitation with ethanol was repeated once, and the precipitate was washed and dried, as described above, to give SP–PG-S (1.24 g).

Other materials. — D-Manno-D-glucan sulfates, MG-SH, MG-SL, DMG-3-SH, and DMG-3-SL, were prepared as described previously[10]. Dextran sulfates, DS-G and DS-I, were extracted with cetylpyridinium chloride[11], from the commercial granule and injection (Kowa Shinyaku Co. Ltd.), respectively. Bovine intestinal heparin was obtained from Daiichi Pure Chem. Co. Ltd. Sephacryl S-300 was purchased from Pharmacia Fine Chemicals.

Assay for antiangiogenic activity. — Chick chorioallantoic membrane (CAM) was used for this assay as described previously[12]. To examine directly the activity, a solution (5 μL) containing a test sample or cortisone acetate (0.5 μg), or both, and 1% (w/v) saline solution of methylcellulose (5 μL) was added to the CAM of fertilized Norin-cross chicken eggs (Funabashi Farm). In the *ex vivo* study, blood (10 μL) obtained from mice 6 h after subcutaneous (s.c.) or oral (p.o.) administration of a test sample (300 mg/kg) or cortisone acetate (5 mg/kg), or both, was added to the CAM. Two days after the addition of agents or blood, antiangiogenic activity was determined. The dose required to inhibit 50% of CAM vascularization (ID_{50} values) was calculated by Probit analysis on the basis of (T/C) · 100 [(vascularization of test CAM/vascularization of control CAM) × 100].

Assay for antitumor activity. — Sarcoma 180 (S-180) cells (1×10^6) were inoculated s.c. into male ICR mice (Shizuoka Lab. Animal Center). Three days after cell inoculation, a test sample (300 mg/kg) was administered s.c. or p.o. in combination with cortisone acetate at a tapering dose of 250 mg/kg/day for 3 days and 100 mg/kg/day for 1 day. Seven days after inoculation, the tumors were removed and weighed. Statistical significance was determined by Student's t-test.

RESULTS

Chemical properties of sulfated polysaccharides. — The SP–PG complex is composed of SP chains and PG fragments in various ratios[8], where the SP chains are probably linked to the PG fragments through phosphoric diester linkages[9]. The complex contains[8] D-galactose (molar ratio to glutamic acid, 56), D-glucose (9.0), sulfate (68), phosphorus (6.4), 2-amino-2-deoxy-D-glucose (2.0), muramic acid (1.1), alanine (2.1), glutamic acid (1.0), glycine (1.2), and LL-diaminopimelic acid (1.2). It was fractionated into three fractions (SP–PG-H, SP–PG-M and SP–PG-L) by gel filtration on Sephacryl S-300, indicating mol. wts. of 15×10^4 or more, 6×10^4, and 3×10^4, respectively, as estimated by g.p.c. on G3000 SW (Fig. 1). The protein content of these fractions was proportional to the molecular weight, and the content of SP–PG-H was significantly high, compared with that of the other fractions (Table I). On the other hand, in these fractions, the components other than those of the peptidoglycan had almost the same molar ratios to neutral sugar (Table I). These results indicate that any of the fractions possesses SP chains having essentially the same chemical composition, although their peptidoglycan contents were different.

SP–PG-LM was obtained from the SP–PG complex by removal of pyrogen-active SP–PG-H. The SP–PG complex was also desulfated with partial elimination of D-glucose, phosphorus, and peptidoglycan, and the sulfur content and molecular weight were reduced to 0.82% and 6000, respectively (Table I). SP–PG-S was obtained by sulfation of the SP–PG complex, and the sulfur content was increased to 16.5%. The chemical properties of SP–PG-S, dextran sulfates, and D-manno-D-glucan sulfates are summarized in Table II. The D-manno-D-glucan used for sulfation has a tetrasaccharide repeating-unit[13] with a single α-D-mannosyl group located at both O-3 and O-6 of every other (1→4)-β-D-glucosyl residue.

TABLE I

ANALYSES OF SP–PG COMPLEX AND ITS DERIVATIVES

Sample	*Carbohydrate*		*Sulfur*		*Phosphorus*		*Protein*[c] *(%)*	$[\alpha]_D$[d] *(degrees)*
	(%)[a]	*Gal/ Glc*[b]	*(%)*	*Molar ratio to neutral sugar residue*	*(%)*	*Molar ratio to neutral sugar residue*		
SP–PG	54	6.2	10.8	1.0	0.86	0.08	1.3	−36
SP–PG-H	42	6.2	7.9	0.95	0.72	0.09	7.6	−34
SP–PG-M	53	6.1	10.4	0.99	0.85	0.08	1.8	−36
SP–PG-L	58	6.2	11.3	0.99	0.92	0.08	0.9	−37
SP–PG-LM	56	6.1	11.1	1.0	0.88	0.08	1.1	−37
SP–PG-D	92	6.9	0.82		0.78		1.6	−37

[a]D-Galactose was used as standard. [b]Molar ratio of D-galactose to D-glucose. [c]Bovine serum albumin was used as standard. [d]*c* 0.5, water.

Inhibition of angiogenesis with or without cortisone acetate. — Antiangiogenic activities of the sulfated polysaccharides with or without cortisone acetate were determined by the CAM assay (Table III). The SP–PG complex and its fractions had direct inhibitory effects on angiogenesis much higher than that of heparin. Of the fractions, SP–PG-L was the most potent, and SP–PG-LM containing SP–PG-L as its major component showed a high activity, comparable to that of SP–PG-L. The direct inhibitory effects were greatly increased in combination with cortisone acetate. Heparin used alone had no effect on angiogenesis even at a dose of 100 μg/chick embryo.

In the *ex vivo* study, SP–PG-L and SP–PG-LM exhibited antiangiogenic effects higher than those of SP–PG-H, SP–PG-M, or heparin by either route of administration, s.c. or p.o., in combination with cortisone acetate (Table III).

TABLE II

PHYSICOCHEMICAL PROPERTIES OF SULFATED POLYSACCHARIDES

Sample	*Mol. wt.* $\cdot 10^{-4}$ [a]	*Sulfur (%)*	$[\alpha]_D^b$ *(degrees)*
SP–PG-S		16.5	−29
DS-G	0.85	18.0	+90
DS-I	0.77	14.5	+110
MG-SH	140	14.2	+32
MG-SL	115	5.8	+51
DMG-3-SH	4.8	16.5	+20
DMG-3-SL	3.4	6.5	+38
Heparin	3.6	12.0	+52

[a]Determined by g.p.c. with dextran as standard. [b]*c* 0.5, water. [c]SP–PG-S gave an elution profile similar to that of the SP–PG complex on G3000 SW.

TABLE III

INHIBITORY EFFECT OF SP–PG COMPLEX AND ITS FRACTIONS ON EMBRYONIC ANGIOGENESIS

Sample	*Direct effect*[a], ID_{50} *(ng/chick embryo)*		Ex vivo *effect*[b], *T/C*[c] *(%)*	
	With cortisone	*Without cortisone*	*s.c.*	*p.o.*
SP–PG	30	900	16.7	11.2
SP–PG-H	530	24800	13.2	17.2
SP–PG-M	770	25100	22.8	16.8
SP–PG-L	2	140	9.3	9.7
SP–PG-LM	3	160	7.8	7.3
Heparin	9810	[d]	23.8	25.4

[a]In combination with cortisone acetate (0.5 μg/chick embryo). [b]Each of the samples (300 mg/kg) was administered s.c. or p.o. with cortisone acetate (5 mg/kg, p.o.), and blood taken after 6 h of treatment was used for CAM assay. [c]Mean angiogenesis-index of treated group/mean angiogenesis-index of control group. [d]No effect below 100 μg/chick embryo.

These *ex vivo* inhibitory effects on angiogenesis were parallel to their direct effects, although the great differences in the ID_{50} values among the agents did not exactly reflect their *ex vivo* activities.

The desulfated and sulfated derivatives from the SP–PG complex, SP–PG-D and SP–PG-S, had direct inhibitory effects (ID_{50}, 80 and 60 ng/chick embryo, respectively) on angiogenesis, similar to that (ID_{50}, 40 ng/chick embryo) of the original SP–PG complex in the presence of cortisone acetate (0.5 μg/chick embryo). SP–PG-D and SP–PG-S (300 mg/kg, p.o.) also showed *ex vivo* antiangiogenic activities (T/C 24.0 and 33.2%, respectively) similar to that (T/C 30.4%) of the original SP–PG complex in combination with cortisone acetate (5 mg/kg, p.o.). Thus, desulfation or sulfation of the SP–PG complex did not essentially affect the antiangiogenic activity.

Other sulfated polysaccharides (1.5–1000 μg/chick embryo) used for the CAM assay had no effect on angiogenesis in the presence of cortisone acetate (0.5 μg/chick embryo). Dextran sulfates, DS-G and DS-I (15 μg/chick embryo), caused an early embryonic death, as previously observed[12] with nonspecific DNA synthesis inhibitors such as mitomycin C and 5-fluorouracil.

Synergistic antitumor activity with cortisone acetate. — In combination with cortisone acetate at a dose level not affecting the tumor growth by itself, the antitumor activities of SP–PG-L and SP–PG-LM against solid S-180 tumor in mice were compared with those of the SP–PG complex and heparin. SP–PG-L potently inhibited the tumor growth by either route of administration, s.c. or p.o., and its activity was similar to that of SP–PG-LM, but higher than that of the SP–PG complex or heparin (Table IV). Neither of these fractions nor heparin used alone exhibited a significant antitumor activity against the solid tumor, although SP–PG-L

TABLE IV

ANTITUMOR EFFECT OF SP–PG COMPLEX AND ITS FRACTIONS ON SOLID SARCOMA 180 TUMOR[a]

Sample[b]	*Route s.c.*		*Route p.o.*	
	Tumor weight[c] *(g) (mean ± sd)*	*T/C*[d] *(%)*	*Tumor weight*[c] *(g) (mean ± sd)*	*T/C*[d] *(%)*
SP–PG	0.064 ±0.020[e]	16.4	0.074 ±0.026[f]	20.6
SP–PG-L	0.035 ±0.006[e]	8.9	0.039 ±0.016[f]	10.8
SP–PG-LM	0.035 ±0.015[e]	6.9	0.028 ±0.011[f]	7.4
Heparin	[g]		0.064 ±0.021[f]	17.6
Cortisone acetate[h]	[g]		0.340 ±0.162	94.2
Control	0.391 ±0.122	100	0.361 ±0.191	100

[a]Sarcoma 180 cells (1×10^6) were inoculated s.c. into male ICR mice on day 0. [b]Each sample (300 mg/kg) was administered s.c. or p.o. daily from day 3 to day 6, with cortisone acetate administered p.o. daily from day 3 to day 5 (250 mg/kg) and on day 6 (100 mg/kg). [c]Weighed on day 7. [d]Mean tumor weight of treated group/mean tumor weight of control group. [e]Significantly differed from control group by Student's t-test ($p < 0.01$). [f]Significantly differed from control group by Student's t-test ($p < 0.05$). [g]Not tested. [h]Administered alone p.o. daily from day 3 to day 5 (250 mg/kg) and on day 6 (100 mg/kg).

and SP–PG-LM tended to inhibit the tumor growth without cortisone acetate (data not shown).

DISCUSSION

The SP–PG complex, which is probably derived from the bacterial cell wall[8,9], was found to have potent antiangiogenic and antitumor activities in combination with cortisone acetate. The homogeneity of the SP component was confirmed by analysis of the fractions (Table I). Therefore, the SP–PG complex may be represented by the formula, $(\text{SP chain})_m$–$(\text{PG fragment})_n$. The SP–PG-H fraction, which had the highest molecular size and peptidoglycan content, was characterized by the smallest ratio of m to n and by the largest value of m plus n of the three fractions.

It is interesting that marked difference in direct inhibitory effect on angiogenesis with or without cortisone acetate was observed between SP–PG-L and SP–PG-M, the latter showing an activity similar to that of SP–PG-H (Table III). As these fractions have almost the same chemical compositions for the SP chains, their molecular sizes or structures, which depend on the numbers of SP chain (m) and PG fragment (n), may play an important role in the antiangiogenic effect.

However, the lack of influence of desulfation or sulfation of the SP–PG complex on the antiangiogenic activity suggested that the glycosyl sequences of sulfated polysaccharides are more important for activity rather than the content or the intramolecular distribution of sulfate groups. In fact, in the presence of cortisone acetate, the SP–PG complex and heparin were active, and dextran sulfates and D-manno-D-glucan sulfates inactive. Also, the SP–PG complex was still active without cortisone acetate, but heparin was inactive. The significant differences in antiangiogenic effect with cortisone among commercial heparins[5] might be attributed to differences in glycosyl sequences.

The results of the *ex vivo* test for antiangiogenesis indicated that active substance(s) from the SP–PG complex or any of its fractions appear(s) in blood by either administration route, s.c. or p.o. There was a good relationship between antitumor activity and *ex vivo* antiangiogenic effect. The previous study[7] had shown that heparin, in combination with cortisone acetate, inhibited the growth of solid S-180 tumor which requires new blood vessels for its growth, but not of the ascitic tumor whose growth does not require angiogenesis. Furthermore, this combination had been shown not to affect tumor cells directly, but to specifically inhibit DNA synthesis of vascular endothelial cells. Therefore, the antitumor activities against solid S-180 tumor of the agents in the present study are also ascribable to their antiangiogenic activities.

The low antitumor activities of SP–PG-L and SP–PG-LM against solid S-180 tumor without cortisone acetate may be ascribed to antiangiogenesis, based on the observation that these fractions used alone showed high antiangiogenic activities but lower than their synergistic activities with cortisone acetate.

It is probable that the PG component of the SP–PG complex or its fractions does not greatly contribute to antiangiogenic activity, because a sulfated polysaccharide from the cell wall of an *Arthrobacter* sp. having a chemical composition similar to that of the SP moiety of the SP–PG complex but lacking the PG components showed an antiangiogenic activity[14].

REFERENCES

1 R. EISENSTEIN, N. SORGENTE, L. W. SOBLE, A. MILLER, AND K. E. KUETTNER, *Am. J. Pathol.*, 73 (1973) 765–774.
2 R. LANGER, H. CONN, J. VACANTI, C. HAUDENSCHILD, AND J. FOLKMAN, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 4331–4335.
3 R. EISENSTEIN, S. B. GOREN, B. SHUMACKER, AND E. CHOROMOKOS, *Am. J. Pathol.*, 88 (1979) 1005–1012.
4 S. TAYLOR AND J. FOLKMAN, *Nature (London)*, 297 (1982) 307–312.
5 J. FOLKMAN, R. LANCER, R. J. LINHARDT, C. HAUDENSCHILD, AND S. TAYLOR, *Science*, 221 (1983) 719–725.
6 R. CRUM AND J. FOLKMAN, *J. Cell. Biol.*, 199 (1984) 158.
7 N. SAKAMOTO, N. G. TANAKA, A. TOHGO, AND H. OGAWA, *Cancer J.*, 1 (1986) 55–58.
8 K. INOUE, H. KORENAGA, AND S. KADOYA, *J. Biochem. (Tokyo)*, 92 (1982) 1775–1784.
9 K. INOUE AND S. KADOYA, *J. Biochem. (Tokyo)*, 94 (1983) 189–197.
10 K. INOUE, K. KAWAMOTO, H. NAKAJIMA, M. KOHNO, S. KADOYA, AND D. MIZUNO, *Carbohydr. Res.*, 115 (1983) 199–208.
11 J. E. SCOTT, *Methods Carbohydr. Chem.*, 5 (1965) 38–44.
12 N. G. TANAKA, N. SAKAMOTO, A. TOHGO, Y. NISHIYAMA, AND H. OGAWA, *Exp. Pathol.*, 30 (1986) 143–150.
13 K. INOUE, K. KAWAMOTO, AND S. KADOYA, *Carbohydr. Res.*, 114 (1983) 245–256.
14 K. INOUE AND H. KORENAGA, unpublished results.