[Chem. Pharm. Bull. 34(10)4377—4381(1986)]

Effect of Activation or Blockade of the Phagocytic System on the Antitumor Activity of Grifolan

NAOHITO OHNO,[a] MAMI HAYASHI,[a] IWAO SUZUKI,[a] SHOZO OIKAWA,[b] KICHIRO SATO,[b] YOSHIYUKI SUZUKI,[b] and TOSHIRO YADOMAE*,[a]

Tokyo College of Pharmacy,[a] Horinouchi, Hachioji, Tokyo 192–03, Japan and Nippon Beet Sugar Mfg., Co., Ltd.,[b] Chuo-ku, Tokyo 104, Japan

(Received March 27, 1986)

The effect of activation or blockade of the phagocytic system on the antitumor activity of grifolan was investigated. The activity of grifolan was abrogated by treatment of mice with a blocker of the phagocytic system, *i.e.*, carrageenan, colloidal carbon, or trypan blue. The effect of carrageenan was observed when it was given prior to or with sarcoma 180 tumor cells. On the other hand, the activity of grifolan was slightly increased by treatment of mice with an activator of the phagocytic system, *i.e.*, thioglycollate medium or casein. These results suggested that the antitumor activity of grifolan is affected by modifications of the phagocytic system.

Keywords——antitumor activity; glucan; *Grifola frondosa*; grifolan; carrageenan; phagocytic system

Recently, several glucans have been clinically used as antitumor drugs.[1] The effects of antitumor glucans are known to appear not by directly killing of the tumor cells but by activating the immune systems.[2] During the course of studying the antitumor mechanism of the polysaccharide fraction, GF-1, participation of the phagocytic system in the antitumor activity was suggested by the findings that peritoneal exudate cells were increased and that carbon clearance activity was increased.[3] Further, specific immunity was also involved because rechallenged sarcoma 180 tumor cells could not grow in the cured mice.[3] However, direct evidence that the phagocytic system is involved in the antitumor activity of grifolan has not been obtained.

It has been shown that treatment of the host with agents that diminish the functional capacity of the phagocytic system markedly abrogated the antitumor activity *in vivo*.[4] This paper is concerned with the effect of activation or blockade of the phagocytic system on the antitumor activity of grifolan in mice.

Materials and Methods

Materials——Grifolan (NMF-5N) extracted from the matted mycelium of *Grifola frondosa* was prepared by the procedure described previously.[5a] Carrageenan (Type II, C-1138) and Trypan blue (T-0887) were purchased from Sigma Chem. Co. (St. Louis, MO, U.S.A.). Casein (purified) and Thioglycollate broth were from Difco. Lab. (Detroit, Michigan, U.S.A). Carbon black was from Rotring Co. (Germany).

General Methods——Evaluation of antitumor activity and other methods were performed as described previously.[3,5]

Results

Effect of Phagocytic System Blockade on the Antitumor Activity of Grifolan

In previous papers, it was assumed that the phagocytic system is important for the

antitumor activity of glucans obtained from *G. frondosa*.[3] To clarify the participation of the phagocytic system in the antitumor activity of grifolan, the effect of several phagocytic system blocking reagents, *i.e.*, carrageenan,[4a,b] colloidal carbon,[4c] or trypan blue,[4d] on the antitumor activity of grifolan were examined.

Carrageenan (days −4, −2, 0, 4 mg/mouse × 3, i.p.), colloidal carbon (days −1 (i.v.), 0 (i.p.), 5 mg/mouse × 2) or trypan blue (day 0, 2 mg/mouse, once, i.p.) was given at indicated days (in parenthesis) before tumor transplantation (day 0). Doses of 20, 100 or 500 μg × 5 (days 1, 3, 5, 7, and 9) of grifolan were administered. Antitumor activity was compared at 35 d after tumor transplantation by weighing the tumor. As shown in Table I, the antitumor activity of grifolan was suppressed by these agents. Abrogation of the activity at doses of 20 μg ($p<0.01$) and 100 μg ($p<0.01$) for carrageenan, and 20 μg ($p<0.05$) for colloidal carbon and trypan blue was statistically significant. However, the antitumor activity of higher doses of grifolan were not abrogated by these agents. These results suggest that the phagocytic system is important for the antitumor activity of grifolan, and that other immune systems also participate in the antitumor activity, especially at higher doses of grifolan.

Effect of Timing of Carrageenan Treatment on the Antitumor Activity of Grifolan

To clarify the effective timing of the blockade of the phagocytic system on the antitumor activity of grifolan, carrageenan was administered at various times and the antitumor activities were compared. Carrageenan was administered at (1) days −5, −3, −1 (week −1), (2) days 1, 3, 5 (week 1), (3) days 8, 10, 12 (week 2) or (4) days 15, 17, 19 (week 3), respectively, and grifolan (100 or 500 μg) was administered on days 1, 3, 5, 7, and 9. As shown in Table II, the antitumor activity of grifolan was abrogated when carrageenan was administered at week

TABLE I. Effect of Phagocytic System Blocking Reagents on the Antitumor Activity of Grifolan[a]

Group	Treatment	Grifolan[b] (μg × 5)	Tumor weight[c] (g, mean ± S.D.)	Inhibition ratio (%)	Complete regression[i]
1	Carrageenan[j]	0	8.0 ± 2.7	16	0/10
2		20	7.3 ± 4.8[h]	23	0/8
3		100	6.9 ± 3.7[h]	27	0/8
4		500	0.3 ± 0.7[f]	97	8/10
5	Carbon[k]	0	11.9 ± 5.8	−26	0/10
6		20	4.7 ± 3.6[e,g]	51	2/10
7		100	0.1 ± 0.3[f]	99	5/9
8		500	0.2 ± 0.5[f]	98	4/10
9	Trypan blue[l]	0	7.4 ± 6.8	22	0/10
10		20	3.4 ± 2.8[g]	64	0/10
11		100	0.7 ± 1.3[d]	93	5/10
12		500	0.4 ± 0.7[d]	96	3/10
13	Nil	0	9.4 ± 5.4	—	0/17
14		4	5.8 ± 2.1[d]	39	0/10
15		20	1.2 ± 1.3[f]	87	1/10
16		100	0.2 ± 0.3[f]	98	1/8
17		500	0.2 ± 0.3[f]	98	3/10

a) Sarcoma 180 tumor cells (5×10^6) were inoculated subcutaneously (day 0). *b*) Grifolan at the indicated doses was intraperitoneally administered on days 1, 3, 5, 7, and 9 in 0.2 ml of saline solution. *c*) The significance was evaluated according to Student's *t*-test. Significant difference from the corresponding control group (groups 1, 5, 9, 13) *d*) $p<0.05$, *e*) $p<0.01$, *f*) $p<0.001$, and from the same doses of grifolan (groups 15—17) *g*) $p<0.05$, *h*) $p<0.01$. *i*) Number of cured mice/total mice. *j*) Carrageenan (4 mg/0.8 ml) was intraperitoneally administered on days −6, −4, −2, 0. *k*) Colloidal carbon (×6 diluted (5 mg/0.2 ml)) was intravenously and intraperitoneally administered on day −1 and day 0, respectively. *l*) Trypan blue (2 mg/0.2 ml) was intraperitoneally administered on day 0.

TABLE II. Effect of Timing of Carrageenan Treatment on the Antitumor Activity of Grifolan[a]

Group	Carrageenan[j] (d)	Grifolan[b] (μg × 5)	Tumor weight[c] (g, mean ± S.D.)	Inhibition ratio (%)	Complete regression[i]
1	−5, −3, −1	0	13.9 ± 5.6[d]	−49	0/8
2		100	6.6 ± 7.0[g]	29	0/8
3		500	1.4 ± 3.3[f]	85	2/7
4	1, 3, 5	0	8.8 ± 3.4	6	0/10
5		100	2.1 ± 2.1[f]	78	2/8
6		500	<0.1[f,g]	>99	7/9
7	8, 10, 12	0	10.3 ± 5.5	−11	0/9
8		100	0.9 ± 1.8[f]	90	1/8
9		500	0.6 ± 0.5[f,g]	94	1/10
10	15, 17, 19	0	8.8 ± 3.6	5	0/8
11		100	0.4 ± 0.5[f]	96	1/9
12		500	0.7 ± 1.3[f]	92	3/10
13	Nil	0	9.3 ± 3.8	—	0/20
14		100	0.3 ± 0.8[f]	97	6/10
15		500	0.2 ± 0.2[f]	98	1/10

a, b, i) See Table I. *c*) The significance was evaluated according to Student's *t*-test. Significant difference from the corresponding control group (group 13) *d*) $p<0.05$, *e*) $p<0.01$, *f*) $p<0.001$, and from the same doses of grifolan (groups 13—15) *g*) $p<0.05$, *h*) $p<0.01$. *j*) Carrageenan (4 mg/0.8 ml) was intraperitoneally administered on the indicated days.

TABLE III. Effect of Activators of the Phagocytic System on the Antitumor Activity of Grifolan[a]

Activator	Grifolan[b] (μg × 5)	Tumor weight[c] (g, mean ± S.D.)	Inhibition ratio (%)	Complete regression[i]
Thioglycollate[j]	0	8.1 ± 4.3	34	0/8
	4	8.4 ± 2.5	32	0/8
	20	2.6 ± 3.0[f,g]	79	1/8
	100	2.0 ± 3.4[f]	84	5/8
Casein[k]	0	10.4 ± 5.6	15	0/8
	4	11.2 ± 7.4	9	0/8
	20	0.8 ± 1.5[f,g]	94	1/6
	100	3.0 ± 7.7[d]	76	1/7
Nil	0	12.3 ± 7.5	—	0/20
	4	7.9 ± 4.7	36	0/10
	20	3.9 ± 3.1[e]	68	2/10
	100	0.1 ± 0.1[f]	99	5/9

a, b, i) See Table I. *c*) The significance was evaluated according to Student's *t*-test. Significant difference from the corresponding control group *d*) $p<0.05$, *e*) $p<0.01$, *f*) $p<0.001$, and the same dose groups (in Nil) *g*) $p<0.05$, *h*) $p<0.01$. *j*) Thioglycollate medium (30 mg/ml) was intraperitoneally administered on days 1, 3, 5, 7, and 9. *k*) Casein (20 mg/ml) was intraperitoneally administered on days 1, 3, 5, 7, and 9.

−1. Treatment of mice with carrageenan at week 1 produced about 20% reduction of the antitumor activity of grifolan. However, the antitumor activity of grifolan was not abrogated when carrageenan was administered at week 2 or 3 after tumor transplantation.

These results suggest that the phagocytic system participates in the early stage of tumor recognition. Previously, we showed that the antitumor activity of grifolan was observed at 2 or 3 weeks after the tumor transplantation.[5a] It may be assumed that, under these

experimental conditions, the phagocytic system participates in the recognition of tumor cells but is involved only slightly in the cytocidal or cytostatic reaction against tumor cells.

Effect of Phagocytic System Activation on the Antitumor Activity of Grifolan

Since blocking of the phagocytic system abrogated the antitumor activity of grifolan, it was of interest to know whether activation of the phagocytic system would augment the antitumor activity of grifolan. Many activators of the phagocytic system have been reported, though many of them show antitumor activity themselves.[6] Therefore, we used thioglycollate broth[7] and casein[8] as activators of the phagocytic system. Activators (30 mg/d for thioglycollate broth and 20 mg/d for casein) and grifolan (4, 20, or 100 μg/d) were administered on days 1, 3, 5, 7, and 9. As shown in Table III, the antitumor activity of grifolan was slightly augmented only when 20 μg of grifolan were used. However, the effect was not large. These results suggest that the antitumor activity of grifolan is not readily augmented by drugs affecting the phagocytic system.

Discussion

Grifolan NMF-5N is a 6-branched β-1,3-glucan isolated from the alkali extract of the matted mycelium of *Grifola frondosa*.[5] In previous papers, we suggested that the phagocytic system and specific immunity participated in the antitumor activity of the crude polysaccharide fraction, GF-1.[3] To clarify the participation of the phagocytic system in the antitumor activity of grifolan more precisely, the effects of several modifying reagents of the phagocytic system on the antitumor activity of grifolan were examined. The results obtained in this paper may be summarized as follows. (1) The antitumor activity of grifolan was abrogated by blocking of the phagocytic system (Tables I and II). (2) Abrogation of the antitumor activity by carrageenan was observed when the phagocytic system was blocked before or at an early stage after the tumor transplantation (Table II). These results suggest that, under these experimental conditions, the phagocytic system participates not in the cytocidal or cytostatic phase against tumors, but in the early stage of tumor recognition.

Carrageenan is a sulfated galactan obtained from marine plants, and usually used as a macrophage blocking reagent.[4a,b] The antitumor activity of grifolan was abrogated by carrageenan treatment. A similar effect was also observed in the cases of *Lactobacillus* LC-9018,[9] Bacillus of Calmette and Guerin (BCG) or *Corynebacterium parvum*.[10] Previously, we assumed a requirement of macrophages for the antitumor activity of GF-1.[3] The data presented in this paper also support the participation of macrophages in the antitumor activity of grifolan. Trypan blue has also been used as an inhibitor of lysosomal enzymes in the phagocytic system,[4d] and the antitumor activities of mannan[11] and *Lactobacillus* LC-9018[9] are reduced by this treatment. Further, colloidal carbon has been used as a blocking reagent of the phagocytic system.[4c] The present finding that trypan blue or colloidal carbon diminished the antitumor activity of grifolan also suggests the participation of the phagocytic system in the antitumor activity of grifolan. However, blocking of the phagacytic system was effective only at relatively low doses of grifolan (Tables I and II). This suggests that other immune systems are also important for the antitumor activity of grifolan.

Recently, combination therapy *e.g.*, with lentinan, OK432, lipopolysaccharides (LPS), BCG, *Corynebacterium parvum*, polynucleotide and/or chemotherapeutic agents, has been examined.[12] Cyclophosphamide (CPA) is known to remove suppressor cells,[13] and is also used in cancer chemotherapy as a tumor-alkylating prodrug. On the other hand, CPA may augment or suppress the immune system.[14] Indeed, the immune system is usually suppressed by cancer and/or chemotherapy. Therefore, combination therapy involving CPA with adoptive immune therapy,[15] with vaccine,[16] or with immunomodulator[12,17] is expected to

be useful. As described in this paper, the antitumor activity of grifolan was not greatly augmented by phagocytic system-activating drugs. However, it might be worth investigating the optimum timing for administrations of CPA and grifolan. Combination therapy would also be useful for expanding the spectrum of effective tumor systems.[12)]

GF-1[3)] and grifolan[18)] are effective not only on allogeneic but also on syngeneic tumor systems. As described above, in allogeneic tumors, the phagocytic system appears to participate at an early stage of tumor transplantation. It would be interesting to know whether blocking of the phagocytic system influences the antitumor activity of grifolan on syngeneic tumor systems.

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