PROFLAMIN, A NEW ANTITUMOR AGENT: PREPARATION, PHYSICOCHEMICAL PROPERTIES AND ANTITUMOR ACTIVITY

Tetsuro IKEKAWA,*1 Hirofumi MARUYAMA,*1 Tetsuji MIYANO,*2 Akira OKURA,*3 Yoshio SAWASAKI,*3 Kyozo NAITO,*3 Kenji KAWAMURA*2 and Kenji SHIRATORI*2

*1National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104, *2Okazaki Research Laboratories, 3-9-1, Kamimutsuna, Okazaki, Aichi 444 and *3Meguro Research Laboratories, Banyu Pharmaceutical Co. Ltd., 2-9-3, Shironeguro, Meguro-ku, Tokyo 153

Proflamin is a new biological response-modifying antitumor agent. It was isolated from the culture mycelium of Flammulina velutipes (Curt. ex Fr.) Sing. by means of ion exchange column chromatography and molecular sieving. It is a weakly acidic glycoprotein containing more than 90% protein and less than 10% carbohydrate, and its molecular weight is 13,000±4,000. The antitumor effect of proflamin was studied with murine tumors. It was markedly effective against the syngeneic tumors, B-16 melanoma (B-16) and adenocarcinoma 755 (Ca-755). At a dose of 10 mg/kg po, the increases in median survival time of mice with B-16 and Ca-755 were 86 and 84%, respectively. Proflamin exhibited no cytocidal effect against the cultured cell lines in vitro. Oral administration of proflamin produced no lethal or any other apparent adverse effect in mice.

Key words: Proflamin — Antitumor activity — Glycoprotein — Syngeneic tumor — Oral administration

In previous studies, it has been shown that aqueous extracts of some edible mushrooms inhibit the growth of murine tumors, and several antitumor polysaccharides have been isolated. For example, we isolated an antitumor polysaccharide from Lentinus edodes (Berk.) Sing. and reported that it was a glucan. These so-called “antitumor polysaccharides” were markedly effective against the solid form of sarcoma 180 (S-180) when given by ip, iv or sc injections. However, they were not effective against the tumors when given orally. Although many drugs with potent antitumor effects have been developed, in clinical applications their adverse effects are serious in general. Therefore, since research on and development of less toxic and orally administrable antitumor drugs are of great value, we carried out screening with syngeneic tumor systems in order to find new antitumor agents that are effective on oral administration and do not produce any adverse effects. As a result, a weakly acidic glycoprotein fraction obtained from the mycelium of Flammulina velutipes (Curt. ex Fr.) Sing. was found to be effective when given po to B-16 and Ca-755 bearing mice.

In this study, the fraction was purified, and the purified agent, named proflamin (PRF), was confirmed to be markedly effective in mice bearing B-16 or Ca-755 on po administration. The preparation, physicochemical properties and antitumor activities of proflamin are presented in this paper.

MATERIALS AND METHODS

Preparation of Proflamin A flow diagram for the preparation of proflamin is shown in Fig. 1. Flammulina velutipes (Curt. ex Fr.) Sing. IFO 4901 was grown in 1600 liters of medium containing glucose 1.0%, corn steep liquor 5.0%, corn starch 3.2%, peptone 0.5%, KH2PO4 0.03%, K2HPO4 0.03% and MgSO4 7H2O 0.02%, pH 5.8, in a jar fermenter at 27° for 4 days. By filtration, 36 kg (dry cell weight) of mycelial cake was obtained. Proflamin was extracted from the cake by boiling with 1300 liters of water for 4 hr and with 1200 liters of 0.1N NaOH aqueous solution for a further 4 hr. The two extracts were combined and passed through a column of Diaion PA 306 (400 liters, OH− form, Mitsubishi Chemical Industries, Tokyo). After the column had been washed with 800 liters of deionized water, the proflamin fraction was eluted with 900 liters of 1.5N NaCl. After neutralization with 3N HCl,
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the proflamin fraction was desalted and the low molecular substances were eliminated by the use of a hollow-fiber membrane (4.7 m², nominal molecular weight limit, 6000, SIP-3013, Asahi Chemical Industries, Tokyo). The residual solution was applied to a column of Diaion WK 10S (70 liters, H⁺ form, Mitsubishi Chemical Industries) and was eluted with 300 liters of 3N NH₄OH after sufficient washing. Purified proflamin fraction was neutralized with 3N HCl, and was again loaded on the same kind of membrane as described above for the purpose of desalination and concentration. After lyophilization, proflamin (415 g) was obtained.

Mice Specific pathogen-free C57BL/6, (C57BL/6 x DBA/2)F₁ (hereafter called BDF₁), and ICR mice were purchased from Charles River Japan, Inc. (Kanagawa) and were maintained in a barrier system. They were all females and were 6 to 7 weeks old at the beginning of the experiments.

Tumors Experiments were performed with three transplantable murine tumors, sarcoma 180 (S-180), B-16 malignant melanoma (B-16), and adenocarcinoma 755 (Ca-755). S-180 was used as an allogeneic tumor, and B-16 and Ca-755 were used as syngeneic tumors. S-180 ascites cells were maintained ip in ICR mice by weekly passage. B-16 cells were maintained sc in C57BL/6 mice by biweekly passage. Single cell suspensions from B-16 were prepared by mechanical disruption; nonnecrotic tumor nodules were thoroughly minced with sterile scissors and passed successively through 80- and 150-mesh wire sieves. The single cell suspensions thus obtained were isolated by centrifugation and resuspended in an adequate volume of Eagle’s minimum essential medium. The viability as determined by trypan blue exclusion was approximately 60%. Ca-755 cells were maintained in BDF₁ instead of C57BL/6 by the same procedures as used for B-16 cells. Ca-755 cells were dispersed by the same techniques as described above for B-16.

Antitumor Activity Evaluation The antitumor activity of proflamin was evaluated in terms of lifespan for B-16 and Ca-755 and S-180 ascites tumors, and in terms of tumor weight in the case of S-180 solid tumor. The numbers of mice used in the test and control groups were usually 6 and 10, respectively, unless otherwise noted. T/C (%) was calculated based on median survival time. The significance of differences was evaluated by means of Student's t-test. A difference between groups was considered significant if the P-value of the comparison was 0.05 or less.

In vitro Cytotoxicity Test⁹) L-1210 and L-5178Y cells were used as cultured cell lines. L-1210 (5 x 10⁴ cells/ml) or L-5178Y (1.0 x 10⁵ cells/ml) cells were cultured in RPMI-1640 medium supplemented with 10% newborn calf serum, and proflamin was added to make the final concentration 250, 500 or 1000 μg/ml. The cells were inoculated into culture tubes and cultured in a humid 5% CO₂ atmosphere at 37° in the presence or absence of proflamin. After a 72 hr cultivation, viable cell counting was performed by means of the trypan blue exclusion test to determine cytotoxicity.

Results

Physicochemical Properties of Proflamin Proflamin was isolated by the method illustrated in Fig. 1. It gave a single band on polyacrylamide gel electrophoresis (pH 8) as shown in Fig. 2, and a single peak on Sephadex G50 column chromatography. It was readily soluble in water, soluble in acetic acid, formic acid and ammonia water, and insoluble in organic solvents (alcohol, acetone, pyridine, ethyl acetate and chloroform).

Cultivation of Flammulina velutipes (Curt. ex Fr.) Sing.

Culture broth

filtration

Filtrate

Mycelial cake

extracted with hot water

Extract

Residue

extracted with 0.1 NaOH

Extract

PA 306 column (OH⁻)

washed with water

eluted with 1.5M NaCl

Molecular sieving

mol. wt. <6,000

mol. wt. >6,000

WK 10S column (H⁺)

washed with water

eluted with 3N NH₄OH

Molecular sieving

mol. wt. >6,000

lyophilization

Proflamin

Fig. 1. Isolation of proflamin.
It was positive in the Rydon-Smith, biuret, Folin-Ciocalteu, phenol-sulfuric acid, anthrone and Molisch color reactions. The acid hydrolysate was positive in the ninhydrin reaction.

Proflamin was found to be a weakly acidic glycoprotein (pI 3.8±0.2) with a molecular weight of 13,000±4,000, based on determination by SDS-polyacrylamide gel electrophoresis and Sephadex G50 column chromatography. It contained more than 90% protein (Folin-Lowry method, with bovine serum albumin as a control) and less than 10% carbohydrate (phenol-sulfuric acid method, with glucose as a control). Specific rotation ([α]D, c=0.1, 0.1N NaOH) was −52° to −57°. Amino acid analysis of the acid hydrolysate (6N HCl, 16 hr, 105°C) revealed that the major amino acid components were glutamic acid, aspartic acid, ala-

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Table I. Effect of Oral Administration of Proflamin on the Survival Time of B-16 Melanoma implanted BDF1 Mice

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>N</th>
<th>Survival time (day)</th>
<th>T/C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>Median: 28.0, Mean±SE: 34.9±4.6, Range: 21-59</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>40.5</td>
<td>170</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>52.0</td>
<td>182</td>
</tr>
<tr>
<td>30</td>
<td>6</td>
<td>50.5</td>
<td>177</td>
</tr>
<tr>
<td>100</td>
<td>6</td>
<td>45.5</td>
<td>160</td>
</tr>
<tr>
<td>MMC*1</td>
<td>6</td>
<td>46.0</td>
<td>161</td>
</tr>
</tbody>
</table>

*P<0.1, **P<0.05 compared with the control.

Table II. Effect of Oral Administration of Proflamin on the Survival Time of B-16 Melanoma implanted C57BL/6 Mice

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>N</th>
<th>Survival time (day)</th>
<th>T/C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>Median: 28.5, Mean±SE: 32.6±3.8, Range: 16-55</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>27.0</td>
<td>144</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>41.0</td>
<td>144</td>
</tr>
<tr>
<td>50</td>
<td>6</td>
<td>41.0</td>
<td>144</td>
</tr>
</tbody>
</table>

*P<0.01 compared with the control.
nine, leucine, and glycine. Trimethylsilyl derivatives of the methanolysate were analyzed by gas chromatography, and the carbohydrates were identified as glucose, galactose and mannose.

**Antitumor Effect of Proflamin against Murine Tumors**

**Antitumor effect against B-16 melanoma**: BDF1 mice were inoculated sc with 5 x 10^6 B-16 cells into the right flank. They received po administration of graded doses of proflamin once a day for 10 consecutive days, beginning one day after the tumor implantation.

Proflamin administration at 1, 3, 10, 30, and 100 mg/kg/day prolonged the survival time in a dose-dependent manner (Table I). Treatment with between 3 and 10 mg/kg seemed to be the optimum to prolong the lifespan. Although there were marked individual variations, the group given 10 mg/kg of proflamin showed statistically significant prolongation of survival time relative to the control (P<0.05). As shown in Table II, a positive effect was also observed in C57BL/6 mice implanted sc with 5 x 10^5 B-16 cells by the same administration route and schedule as used for the BDF1 mice.

**Antitumor effect against adenocarcinoma 755**: Solid tumors of S-180 were induced by sc transplantation of 2 x 10^6 ascites cells into the right groin of ICR mice, and the mice were treated with graded doses of proflamin given po once a day for 10 consecutive days beginning one day after the tumor implantation.

In accordance with the so-called “host-mediated” assay method,^12^ the S-180 solid tumor induced was extirpated and weighed 28 days after the tumor implantation. The oral administration of 10 or 30 mg/kg of proflamin inhibited the growth of S-180 solid tumors, as shown in Table IV. Though the difference between the test group and the control group was not statistically significant (0.05 < P<0.1) in the case of S-180 solid tumor, the optimal dose seemed to be a little higher than that for B-16 and Ca-755. On the other hand, proflamin scarcely prolonged the survival time of mice inoculated ip with S-180 cells (Table V). It has no prolonging effect on the lifespan of mice implanted ip with L-1210 leukemia (BDF1 mice) or Meth A fibrosarcoma (BALB/c mice) when given by oral administration.

**Cytotoxicity of Proflamin towards Cultured Cell Lines** In addition to the experiment on the cytoidal effect of proflamin in mice inoculated with the ascites tumor ip, the cytotoxicity of proflamin towards cultured cell lines (L-1210 and L-5178Y) was

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**Table III. Effect of Oral Administration of Proflamin on the Survival Time of Adenocarcinoma 755^a) - implanted BDF1 Mice**

<table>
<thead>
<tr>
<th>Dose(b) (mg/kg)</th>
<th>N(c)</th>
<th>Survival time (day)</th>
<th>T/C(d) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median</td>
<td>Mean±SE</td>
</tr>
<tr>
<td>Control</td>
<td>13</td>
<td>25.0</td>
<td>29.9±2.9</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>23.0</td>
<td>32.9±5.3</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>46.0</td>
<td>39.8±4.1*</td>
</tr>
<tr>
<td>30</td>
<td>9</td>
<td>37.0</td>
<td>35.0±3.4</td>
</tr>
</tbody>
</table>

a) sc 2 x 10^6 cells/mouse.
b) Mice were given po proflamin once daily on days 1-10.
c) No. of mice.
d) Calculated from median survival time.
* P<0.05 compared with the control.
tested in order to determine whether or not proflamin had a cytocidal effect. Proflamin had no inhibitory effect on the growth of the cultured L-1210 and L-5178Y cell lines at a drug concentration of 1,000 pg/ml. These findings suggest that the inhibitory effect of proflamin on tumor growth in vivo is not due to a cytocidal action.

**DISCUSSION**

A number of biological response modifiers (BRM) effective against tumors have been studied. Some of them are low molecular substances such as levamisole,11 bestatin11 and azimexone.4) Others are high molecular substances such as lipopolysaccharides,18) polysaccharides,5, 7, 8, 10, 12, 19, 20) bacterial cell wall skeletons2,3) and killed whole microbial cells.5,15) Proflamin is a new biological response-modifying antitumor agent effective against syngeneic tumors. It was highly purified, giving a single band on polyacrylamide gel electrophoresis, and it was found to be a weakly acidic glycoprotein, containing more than 90% protein and less than 10% carbohydrate. Its molecular weight is 13,000+4,000, which is substantially quite different from those of other antitumor agents, particularly the so-called “antitumor polysaccharides”5, 7, 8, 10, 12) or protein-binding polysaccharides19) obtained from other kinds of Basidiomycetes.

Antitumor polysaccharides were isolated from the extract of Flammulina velutipes, a proflamin-producing strain,14, 16) and their antitumor activity was found to be identical with that of the other antitumor agents, particularly the so-called “antitumor polysaccharides”5, 7, 8, 10, 12) or protein-binding polysaccharides19) obtained from other kinds of Basidiomycetes.

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oral administration, our efforts were directed towards the purification of proflamin.

A characteristic of proflamin is that, upon oral administration it is effective in prolonging the lifespan of mice bearing slow-growing syngeneic solid tumors. The life-prolonging effect of proflamin in mice with several slow-growing solid tumors was comparable to that of mitomycin C. This result is noteworthy, because proflamin is effective when given orally, and furthermore, in contrast to the antitumor antibiotics, proflamin produced no detectable adverse effects.

The antitumor effect of proflamin did show inter-individual fluctuations in mice, and sometimes showed fluctuations between experiments; the variability was a little greater than that in the effect of mitomycin C. The reason for this is not clear, but the magnitude of immunological response of each mouse may depend on the individual immune status. As described above, proflamin did not show cytotoxicity towards cultured L-1210 and L-5178Y cells, and moreover, in our animal experiments it showed no detectable toxicity in vivo. Toxicological and pharmacological results will be reported elsewhere, and the mechanism of the antitumor effects of proflamin will also be reported.

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