Characterization of a Cytotoxic Factor Induced in Murine Serum after the Intravenous Administration of Dehydrogenation Polymers of Phenylpropenoids (a Class of Synthetic Lignins)

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A cytotoxic factor (CF) appeared in murine serum after the intravenous injection of the dehydrogenation polymers (DHPs) of *p*-coumaric acid (DHP-*p*CA), caffeic acid (DHP-CA), and ferulic acid (DHP-FA), which are categorized as a class of synthetic lignins. The highest CF activity was observed 15 min after the i.v. injection of DHP-*p*CA. CF is likely to be cytotoxic through an apoptotic mechanism accompanied by nucleosome-sized DNA fragmentation. CF is extractable with aqueous ethanol and highly stable against heat, proteases, and acid/alkali treatments. The ethanol extract showed cytotoxicity toward various cultured cell lines and also ascites carcinoma cells *in vivo*. The parent molecules DHPs did not show any appreciable cytotoxicity. After the induction of CF activity, the activity quickly diminished and completely disappeared from the blood stream within an hour or so. The cytotoxicity was observed only when the target cells were exposed to CF for longer than 10 h.

Key words lignin; dehydrogenation polymer; cytotoxic factor; cytotoxicity; anti-human immunodeficiency virus

Lignins are a major class of constituents in the land-plant kingdom, but little attention has been paid until lately to their biological activities. Recently, many biological activities of lignin-related substances, such as antiviral,^{1–4)} antibacterial,^{5–7)} antitumor,⁸⁾ antimutagenic,^{9–12)} and immunopotentiating^{13–16)} activities, have been reported. As we previously demonstrated, synthetic lignins, *i.e.*, the dehydrogenation polymers (DHPs) of phenylpropenoids, inhibited the growth of human immunodeficiency viruses (HIV-1 and -2) in an *in vitro* cell assay system.³⁾ This activity was further confirmed in a *quasi in vivo* assay system by following the time-course of fluctuation in the anti-HIV activity of the serum prepared from DHP-injected mice.⁴⁾ It was revealed that the anti-HIV activity of the serum reached its highest level immediately after DHP injection, then gradually decreased within 5 h. In the course of study, it was noticed that the serum obtained after DHP injection contained a cytotoxic factor toward cultured mammalian cells and that it reached the highest level 15 min after injection and disappeared within 1 h. In the present study, chemical and cytotoxic properties of this factor (CF) were investigated.

MATERIALS AND METHODS

Animals Female ICR mice (5-week-old) were obtained from Japan SLC, Inc. (Hamamatsu). They were caged with wood-chip bedding and maintained in an air-conditioned animal room at $23 \pm 2^\circ\text{C}$ and $50 \pm 10\%$ humidity for a week before use.

Materials Ferulic acid (FA), caffeic acid (CA), *p*-coumaric acid (*p*CA), and horseradish peroxidase were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo); RPMI 1640 and Eagle's minimal essential medium (MEM) culture medium from Nissui Pharmaceutical Co., Ltd. (Tokyo); fetal bovine serum from Summit Biotechnology Co., Ltd. (Ft. Collins, CO); kanamycin sulfate from Meiji Seika Co., Ltd. (Tokyo); agarose and ethanol from Nakalai Tesque, Inc. (Kyoto); propidium iodide, RNase

A, trypsin, soybean trypsin inhibitor, and chymotrypsin from Sigma Chemical Co., Ltd. (St. Louis, MO); others from Wako Pure Chemical Industries (Osaka). CCRF-HSB-2 cells were a gift from Yamasa Shoyu Co., Ltd. (Chiba). Phosphate buffered saline (PBS) was Dulbecco's PBS unless otherwise noted.

Preparation of DHPs The DHPs of phenylpropenoic acids (FA, CA, *p*CA) were synthesized at pH 8 with 1.5 molar eq of H_2O_2 by the bulk method, as described in a previous paper.¹⁷⁾ Briefly, 0.1% hydrogen peroxide (1.5 molar eq to phenylpropenoic acid) dissolved in 0.05 M Na-phosphate buffer (pH 8) was added dropwise to 600 ml of buffer containing 1 g of phenylpropenoic acid and 10 mg of horseradish peroxidase. The mixture was stirred at 25°C for 1 h. The pH of the mixture was then adjusted to 3 with acetic acid and cooled in a refrigerator for 1 h. For the polymerization of FA and *p*CA, the precipitates thereby formed were collected by centrifugation and washed twice with 400 ml of dil HCl (pH 2). The precipitates were dissolved in 80 ml of MeOH and filtered through a Toyo filter paper (No. 2). The filtrate was dripped into 800 ml of HCl (pH 2) with stirring. The precipitates were collected by centrifugation, dissolved in a small volume of H_2O , and then lyophilized. When caffeic acid was polymerized, the polymer formed was soluble even in the acidic medium. Therefore, after the reaction mixture was acidified to pH 3.0, it was thoroughly dialyzed against distilled water for 72 h, then evaporated under reduced pressure. The residue was dissolved in EtOH and filtered through a Millipore filter. After the filtrate was evaporated to dryness, the residue was dissolved in a small amount of H_2O and lyophilized.

Preparation of the Water Extract of Slash Pine The water extract of wood chips of slash pine SP(H_2O) was prepared by the method described in a previous paper.¹³⁾ Briefly, wood chips of coniferous slash pine (100 g) were ground into small pieces and extracted with 1.0 l ethanol each time for two 20 h periods at room temperature. The residue was then extracted three times with 1.0 l each of

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boiling water for 3 h. The hot water extracts were combined and dialyzed against running water for 12 h and then lyophilized.

Cell Cultures Murine leukemia L1210, human leukemia HL60, and human leukemia CCRF-HSB-2 cells were maintained in a suspension culture with RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 0.1 mg/ml kanamycin at 37°C in a humidified atmosphere containing 5% CO₂. Chinese hamster V79 cells were maintained in a monolayer culture with Eagle's MEM supplemented with 10% heat-inactivated FBS under the same conditions as above.

Injection of DHPs and Isolation of the Serum Each DHP was dissolved in saline containing 0.28% NaHCO₃, and SP(H₂O) was in plain saline, just before administration. The DHP solution was injected into the tail vein of mice at doses from 10 to 100 mg/kg. Then, blood was withdrawn from the heart under anesthesia at appropriate times, and the serum isolated from the blood was subjected to cytotoxicity assays.

Preparation of the Ethanol Extract of the Serum (EtOH-sup) Three volumes of ethanol were added to the serum and mixed. After standing at 4°C for 30 min, the mixture was centrifuged at 12000 rpm (12000 × g) for 15 min at 4°C. The supernatant was isolated and evaporated *in vacuo*. The residue was dissolved in a half volume of PBS compared to the original serum.

Cytotoxicity Assays L1210, CCRF-HSB-2, and V79 cells in the log phase were seeded into culture dishes at a concentration of 1 × 10⁵ cells/ml in 1.8 ml of culture medium. HL60 cells were seeded at a concentration of 1 × 10⁴ cells/ml in 0.5 ml of culture medium. After preincubation for 16 h (for L1210 cells) or 24 h (for the others), 10% murine serum or EtOH-sup was added to the dishes and the cells were incubated at 37°C for 48 h (for L1210 cells) or 72 h (for the others) in a CO₂ incubator. The viability of the cells was determined by counting the cells which excluded trypan blue dye.

For the assay of cytotoxicity toward *Salmonella typhimurium*, the pre-incubation method for mutagenicity assay¹⁸⁾ was used with slight modification. Briefly, a mixture of 0.1 ml of a *Salmonella typhimurium* TA100 culture in the early stationary phase of, 0.2 ml of EtOH-sup, and 0.7 ml of 100 mM phosphate buffer (pH 7.4) was incubated at 37°C for 15 min. The cell suspension was diluted and poured onto a plate of minimal glucose agar medium. The plate was incubated at 37°C for 48 h, and then the colonies were counted to determine the viability.

Antitumor Assays Ehrlich ascites carcinoma cells (5 × 10⁵ cells) were intraperitoneally inoculated into ICR mice on day 0. On day 1, 0.2 ml of EtOH-sup was intraperitoneally injected as a test group (6 mice). Another group (6 mice) was treated with an equal volume of saline as a control. Then the life-spans of the animals were determined.

DNA Electrophoresis DNA fragmentation was monitored by agarose gel electrophoresis.¹⁹⁾ The cells (6 × 10⁵ cells) were washed with 1 ml of PBS and resuspended in 20 µl of 50 mM Tris-HCl (pH 8.0) containing 10 mM EDTA, 0.5% (w/v) sodium lauroyl sarcosinate, and 0.5 mg/ml

proteinase K. After 2 h incubation at 50°C, 10 µl of 0.5 mg/ml RNase A was added. Incubation at 50°C was continued for an additional hour, followed by heating to 70°C. The cell suspension was then mixed with 10 µl of 10 mM EDTA (pH 8.0) containing 1% (w/v) low-melting-temperature agarose, 0.25% (w/v) bromophenol blue, and 40% (w/v) sucrose, and loaded into wells prepared on a 2% (w/v) high-melting-temperature agarose gel plate. Electrophoresis was carried out at a voltage of 100 V in 2 mM EDTA-89 mM Tris-borate (pH 8.0) until the marker dye had migrated a distance of 3 cm. The agarose gel was then stained for 10 min with 0.1 µg/ml ethidium bromide and visualized on a UV-transilluminator.

Flow Cytometric Analysis of Cell Cycle The cells (1 × 10⁶) to be analyzed were washed with 2 ml of PBS and fixed in 80% ethanol at -20°C for 1 h. The cells were then washed with 1 ml of PBS, resuspended in 1 ml of PBS containing 0.1 mg/ml RNase A, and incubated at 37°C for 30 min. After being washed with PBS, the cell suspension was mixed with 250 µl of PBS containing 0.05 mg/ml propidium iodide, and was kept standing in the dark at 25°C for 30 min. The suspension was diluted with 750 µl of PBS and then analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and Consort30 software using an excitation wavelength of 488 nm and a detection wavelength of 585 nm.

Stability of Extracted CF Treatment with Proteases: EtOH-sup was treated with 1 mg/ml trypsin, 1 mg/ml chymotrypsin, or 20 units/ml proteinase K by incubation at 37°C for 1 h. Then, 2 mg/ml soybean trypsin inhibitor (for trypsin) was added, or the mixture was heated at 90°C for 15 min (for the other proteases) in order to inactivate the proteases. The samples were added to culture dishes at a concentration of 10% (v/v) EtOH-sup for cytotoxicity assays.

Acid and Alkaline Treatments: The pH of EtOH-sup was adjusted to 2 or 13 by the addition of HCl or NaOH, respectively. After standing at 4°C for 30 min, the samples were neutralized with NaOH or HCl, and adjusted to pH 7.4. Then, the samples were added to culture dishes at a concentration of 10% (v/v) EtOH-sup for cytotoxicity assays.

RESULTS

Induction of CF by i.v. Injection of Synthetic Lignins (DHPs) DHP-FA, DHP-CA, DHP-*p*CA, and SP(H₂O) were tested for CF-inducing activity. The sera were collected 15 min after i.v. injection and assayed for their cytotoxicity. As shown in Fig. 1, all DHPs showed CF-inducing activity in a dose-dependent manner, and DHP-*p*CA showed the highest activity. SP(H₂O) did not show any cytotoxic activity. Therefore, DHP-*p*CA was used for the characterization of CF throughout the following experiments. It is worth mentioning that all DHPs and SP(H₂O) were not appreciably cytotoxic by themselves when they were added to the culture medium of L1210 cells, IC₅₀ values of these lignins being 250 µg/ml or more.

Time-Course of CF Activity of the Serum after i.v. Injection The time-course of CF activity of the serum

Fig. 1. Dose-Response of CF Induction by Various Lignins

Lignins were intravenously injected into ICR mice and the blood was withdrawn 15 min after the injection. Then, the serum isolated from the blood was subjected to a cytotoxicity assay using L1210 cells. Closed circles, DHP-pCA; closed triangles, DHP-CA; open squares, DHP-FA; open triangles, SP(H₂O).

Fig. 2. Time-Course of CF Induction by Intravenous Injection of DHP-pCA into Mice

DHP-pCA was intravenously injected into ICR mice at a dose of 50 mg/kg, and the blood was withdrawn at the indicated times after the injection. Then, the serum isolated from the blood was subjected to the cytotoxicity assay using L1210 cells.

was traced after i.v. injection of DHP-pCA into mice at a dose of 50 mg/kg. The serum cytotoxicity reached the highest level 15 min after injection, then quickly diminished, and almost completely disappeared within an hour or two (Fig. 2). CF activity was absent in the serum immediately after the injection (at 0 h). It was worth noting that CF activity was induced as potently in rats as in mice. However, the highest activity in rats was shown a little later than in mice, i.e., 30 min after i.v. injection at the same dose (data not shown).

In this paper, the mouse serum prepared 15 min after the injection, which contains the highest level of CF, is termed CF-serum.

Fig. 3. Effect of Exposure Time on the Appearance of Cytotoxicity

CF-Serum from the mice injected with DHP-pCA was added to culture dishes of L1210 cells at a concentration of 10%. The cells were washed with PBS at the indicated times and resuspended in fresh culture medium. The total incubation period was adjusted to 48 h and the growth rate of cells was determined.

Fig. 4. DNA Fragmentation in the L1210 Cells Treated with CF-Serum

Lane A, control; lane B, 3 h; lane C, 14 h; lane D, 20 h; lane E, 22 h; lane F, 26 h treatment.

Lethal Processes of the Cells Exposed to CF After exposure to CF for 1 to 48 h, the cells were resuspended in fresh culture medium containing no CF-serum and incubated for another 48 h. The cell viability depended on the exposure time, as shown in Fig. 3, which indicated that a long exposure time, 10 h or longer, is required for appreciable cytotoxicity. Agarose gel electrophoresis of the cells exposed to CF for 14 h showed nucleosome-sized DNA fragmentation, which is a hallmark of apoptosis (Fig. 4). In addition, condensation of chromatin and apoptotic bodies, which is another hallmark of apoptosis,

was observed in those cells after staining with fluorescent acridine orange (data not shown). Then, the cells treated with CF-serum were subjected to flow cytometric analysis, which indicated that the cells in the G₁-phase increased and those in the S-phase decreased during incubation (Table 1).

Characterization of CF In order to determine whether CF is a metabolically modified DHP-*p*CA, or whether any proteinaceous factor was induced *in vivo*, the serum obtained from the CF-treated mice was mixed with 3 volumes of EtOH and the supernatant (EtOH-sup) was assayed for cytotoxicity, in view of the fact that lignins, such as synthetic DHPs, are generally very soluble in EtOH. The CF activity was almost quantitatively retained in EtOH-sup. Then, since EtOH-sup contained 1.1 ± 0.1 mg/ml of protein, as quantitated by Lowry's method (the original CF-serum contained 54.3 ± 4.5 mg/ml), EtOH-sup was treated with several proteases, but its CF activity did not decrease at all. Since the CF activity of EtOH-sup was not decreased by heat or acid/alkali treatment (Table 2), it is not likely that CF could be a proteinaceous cytotoxic cytokine, such as tumor necrosis factor (TNF), induced by DHP treatment.^{20,21} Neither is it likely that CF could be an active oxygen species released from leukocytes such as neutrophils/macrophages, since the activity was not affected at all by treatment with either catalase, superoxide dismutase, or sodium thiosulfate (data not shown). It is, therefore, highly probable that CF is a partially modified DHP-*p*CA. It is worth noting that the parent molecule DHP-*p*CA shows anti-HIV activity but no appreciable cytotoxicity.³

Cytotoxicity of CF toward Various Cell Lines Several cultured mammalian cell lines were tested for susceptibility

Table 1. Distribution of Cells through the Cell Cycle

Time after treatment ^{a)}	Cell cycle analyzed by flow cytometry		
	G ₁ (%)	S (%)	G ₂ + M (%)
0 h	23 ± 3	65 ± 4	12 ± 7
12 h	36 ± 2	53 ± 1	11 ± 1
24 h	44 ± 1	43 ± 3	13 ± 3

a) L1210 cells were treated with 10% CF-serum and cultured for the indicated hours, then the cell cycle was analyzed by flow cytometry.

Table 2. Stability of EtOH-sup against Various Treatments

Treatment of EtOH-sup ^{a)}	Cell viability (%) ^{c)}
(Control) ^{b)}	100
No treatment	0.8
Heating	
at 60 °C for 1 h	0.9
at 90 °C for 1 h	1.2
Proteases	
Trypsin	1.8
Chymotrypsin	0.5
Proteinase K	0.8
Acidic condition (pH 2)	1.6
Alkaline condition (pH 13)	0.0

a) Cells were treated with the ethanol extract of CF-serum under various conditions as described in Materials and Methods. b) Ethanol extract of control serum and without treatment. c) EtOH-sup was added to the culture dish at a concentration of 10%. After incubation at 37 °C for 48 h, the viability of the cells was determined.

to CF-cytotoxicity. EtOH-sup was added to the cell culture and incubated for 48 h. The survival rates are shown in Table 3. All the mammalian cell lines tested were effectively killed by treatment with EtOH-sup prepared from CF-serum as effectively as was the L1210 cell line. The control cells treated with EtOH-extract obtained from the untreated mice were not affected at all in either cell line.

For the assessment of cytotoxicity *in vivo*, EtOH-sup was intraperitoneally injected into mice which had been intraperitoneally inoculated with Ehrlich ascites carcinoma cells on the preceding day. Slight elongation of the life span (ILS: 15.7%) was observed in the EtOH-sup-treated group, as shown in Fig. 5. This result indicates that CF could inhibit cell growth to some extent in the peritoneal cavity, in spite of its ready inactivation in the blood stream.

The CF activity of EtOH-sup toward *Salmonella typhimurium* TA100, a tester strain of bacteria for mutagenicity, was observable, but much weaker than that toward mammalian cells (Fig. 6). DHP-*p*CA itself did not show any cytotoxicity toward the same strain of bacteria, even at a dose of 1000 µg/ml.

DISCUSSION

We have previously reported that the DHPs of phenylpropenoids, a class of synthetic lignins, had potent anti-

Table 3. Cytotoxicity of EtOH-sup against Cultured Mammalian Cells

Cell line	Cell viability (%)	
	EtOH-sup (DHP) ^{a)}	EtOH-sup (control) ^{b)}
L1210 (mouse leukemia)	1	95
HL60 (human leukemia)	0	100
CCRF-HSB-2 (human leukemia)	0	98
V79 (Chinese hamster lung)	0	100

a) Cells were treated with the ethanol extract of CF serum at a concentration of 10% and cultured for 48 or 72 h, as illustrated in Materials and Methods. b) Cells were treated with the ethanol extract of control serum (without DHP-*p*CA treatment) at a concentration of 10% and cultured for 48 or 72 h.

Fig. 5. Elongation of the Life Span of Ehrlich Ascites Carcinoma-Bearing Mice by Treatment with EtOH-sup

Ehrlich ascites carcinoma cells (5×10^5) were intraperitoneally inoculated into female ICR mice. One day after the inoculation, EtOH-sup (0.2 ml) was injected into the peritoneal cavity, then the life span was observed.

Fig. 6. Cytotoxicity of EtOH-sup in *Salmonella typhimurium* TA100

EtOH-sup, prepared at the indicated times after the injection of DHP-pCA, was added to cell cultures of *Salmonella typhimurium* TA100 at a concentration of 20%. The cultures were incubated at 37°C for 15 min and then poured onto a plate with dilution. After incubation for 48 h, the viability was determined.

HIV activity *in vitro* and also in a *quasi in vivo* assay system.^{3,4)} In those experiments, we were aware of the appearance of cytotoxicity in the serum toward the host cells. It was revealed in the present study that a CF was detected in the serum at the highest level 15 min after the injection, and then it quickly diminished. However, once it was taken out from the host animals, it was highly stable in the isolated serum or aqueous EtOH solution. Thus, CF activity was preserved quantitatively even after the EtOH extract was treated with heat, proteases, and acid/alkali. The cytotoxicity of CF was such that it led the target cells to apoptotic death only when the cells were exposed to CF for longer than 10 h.

As previously reported, DHPs are capable of inducing cytokines such as TNF^{20,21)} and stimulating granulocytes to release active oxygen species.¹⁷⁾ However, it was demonstrated in the present study that CF is not a proteinaceous material serving as a cytotoxic cytokine or a generator of lethal active oxygen species. It can also be safely concluded that CF is different from DHP-pCA itself, which is regarded as the active principle for anti-HIV activity. Although the highest anti-HIV activity was observed in the serum from the blood withdrawn immediately after DHP injection,³⁾ CF activity had not yet been induced in the same serum. In addition, although the anti-HIV activity of DHPs decreases gradually with the lapse of several hours, CF, after reaching the peak quickly, diminished and completely disappeared from the blood stream within an hour or so. Assuming that CF is a metabolite derived from DHPs, it is a transient metabolite serving as a cytotoxic factor, which is readily metabolized to an inactive form within a short period. It may be worth mentioning that some of polyphenols, including tannins and lignans, have apoptosis-inducing activity in cultured mammalian cells.²²⁾ Therefore, one may speculate that, as a possible mechanism, molecular species of CF might be polyphenolic fragment(s) transiently formed by the digestion of DHP molecules. The identification of CF is under way using ³H-labeled

DHP-pCA.

Since the life span of Ehrlich cell-bearing mice was slightly but significantly prolonged by the injection of EtOH-sup, CF might be more stable in the peritoneal cavity than in the blood stream. Therefore, CF might possibly be a high- or medium-sized molecule(s), because high molecular weight substances are retained for a long time in the peritoneal cavity.

The detailed mechanism involved in CF induction are now under investigation.

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