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Antitumorigenic Effects of Tannin from Persimmon Leaves on Sarcoma 180-induced Tumor in Mice

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Abstract

The changes of morphology and protein pattern of sarcoma 180 cells treated with or without tannins extracted from persimmon leaves were evaluated by light microscopy, electrophoresis and Western blotting. The sarcoma 180 cells treated with tannins increased the amount of proteins which presumably were intermediate filament cytokeratins detected by electrophoresis and Western blot. Tannins was indirectly cytotoxic to the sarcoma 180 cells and increased the intermediate filament protein level in the cells.

Key words: tannin, sarcoma 180-cells, intermediate filament cytokeratin

INTRODUCTION

Tannins are complex phenolic polymers with molecular masses ranging from 500 to 20,000 Da. and are widely distributed in the plant kingdom as natural products(1,2). Tannins can be classified into three groups based on their structures: (1) hydrolyzable tannins which include gallotannins and ellagitannins(esters of sugar or cyclitol with gallic acid); (2) condensed tannins(condensates of flavan units); (3) complex tannins(3-5).

Traditionally, various plants that contain tannins have been used against cancers and tumors(6-8). The anti-carcinogenicity and antitumor activity of various tannins isolated from plants have been studied in China, Japan, and Korea(9-13). Fifty-seven tannins and related compounds including gallotannins have been reported to have cytotoxicities against human tumor cell lines including malignant melanoma, lung carcinoma, ileocecal adenocarcinoma, epidermal carcinoma, and medulloblastoma cell lines(11). It was also reported that sixty three tannins and related polyphenols showed antitumor activities by increasing the life span when they were injected into mice at 4 days before intraperitoneal inoculation of sarcoma 180 cells(14). Moreover, a dimeric ellagitannin from *Agrimonia pilosa* LEDEB inhibited the growth of sarcoma 180-induced solid tumor, prolonged the life span of mice inoculated with sarcoma 180, increased the weight of spleen of mice, and increased the number of peripheral white blood cells as well as the ratio of monocytes to other leukocytes(7,8,12,15). Woodfordins, hydrolyzable tannin dimers from *Woodfordia fruticosa* KURZ (Lythraceae) exhibited a potent host-mediated antitumor activity against sarcoma 180 in mice(16). It was also reported that the condensed tannins extracted from the leaves of persimmon prolonged life-span of mice inoculated with sarcoma 180 cells, inhibited the growth of sarcoma 180 solid tumor and increased the ratio of spleen weight to body weight *in vivo*(17).

However, it was difficult to speculate the mechanism involved in the antitumor activity of the material because the chemical structures of them were extremely complex and unstable. Recently, the development in purification techniques and analytical methods made it possible to determine the chemical structures of many tannins and to test the antitumor activities of tannins of known structures(3,9,12,16).

In recent years, many researchers actually tried to elucidate the mechanism of various tannin actions on the basis of their structures. Agrimonnin, a dimeric ellagitannin, was confirmed to exhibit its antitumor activity through potentiation of the host-immunity via stimulation of white blood cells including natural killer cells and macrophages. Its primary action was the induction of interleukin-1 from macrophages *in vitro*(13). Kashiwada

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et al.(11) suggested another possibility of mechanism involved in the antitumor activity in which fifty-six tannins showed selective inhibitory effects against protein kinase C(PKC) by interacting with regulatory site of the enzyme *in vitro*.

In the current study, to clarify the antitumor activity of tannins extracted from the leaves of persimmon (*Diospyros kaki* Thunbery) on the cancer cell line, sarcoma 180, the changes of cell morphology and their protein pattern was investigated.

MATERIALS AND METHODS

Animals and tumors

Seven-week old male ICR mice weighing from 35g to 40g were gifts from Dr. Jung, H.Y.(Pusan national university, Korea). The animals were divided into two groups of 10 animals each(Table 1). The animals were housed in plastic cages, exposed to a 12 hour light/dark cycle in a dark room, and fed a commercial diet(Jeil Food Co., Seoul, Korea) *ad libitum* with water.

Sarcoma 180 cells(1.0×10^6/0.1ml) were inoculated into the left groin of the mice on day 0, and tannin(10 mg/kg) was treated intraperitoneally on day -1, 0, 1, 2, 3 for pre-and post-treatment. On the 22nd day of the tumor cell inoculation, mice were sacrificed.

Preparation of tannin from persimmon leaves

Tannin was extracted from the leaves of persimmon according to the methods of Okonogi et al.(8).

Light microscopy

Tumor tissues excised from the left groins of mice were fixed in 10% neutral formalin in phosphate buffered saline(PBS) and embeded paraplast at 60°C. Sections measuring 4-5μm were cut and dewaxed in xylene. The slides were then dehydrated with graded alcohols, washed in distilled water, and stained with hematoxylin and eosin(H&E).

Table 1. Antitumor effects of tannin from persimmon leaves in sarcoma-180 bearing ICR mice

	Dose (mg/kg)	Days	Body weight	Tumor weight
Control		22	32.63	1.56
Tannin	0.1	22	32.49	1.09

Preparation of polyclonal antibody

Sarcoma 180 cells(1×10^7cells/ml PBS) were suspended in 0.1M sodium azide and 0.4% formalin, and stand overnight at 4°C. After spinning at 800×g for 5 minutes, the cells were washed three times with 0.1M sodium phosphate buffer, pH 7.8 and resuspended in the same buffer solutions containing 0.2% sodium azide to make the concentration of 5×10^6 cells/ml at 4°C. A 0.1 ml of the cell suspension(5×10^5 cells) was injected into the abdomen of a mouse once in every two weeks for two months. On the day after 12 days of injection, the mice were killed, and the blood was collected. The blood was kept overnight at room temperature. On the next day, a layer of serum was collected and stored at -80°C until use.

Immunohistochmistry

The paraffin sections of the mouse tissue at 4μm thickness were placed on a slide glass and washed in PBS once. The tissue samples were reacted at room temperature with the primary antibody which was diluted to 1:200 in 20% BSA. After rinsing with PBS three times, an anti-mouse IgG alkaline phosphatase conjugate(Sigma Chem Co.) diluted 200 times was added. The samples were kept to stand at room temperature further for one hour and rinsed again with PBS three times, and then they were immersed in 20 mg 5-bromo-4-chloro-3-indolyl phosphate(BCIP)/ml of N,N-dimethyl formamide(DMFA), 150 mM Tris buffer, pH 9.6 with 2 mg of nitroblue tetrazolium(NBT), and kept to stand at room temperature for 15 minutes.

SDS-Polyacrylamide gel electrophoresis

To isolate proteins of the tissue samples, the tissues were washed three times in ice-cold Tris buffer(50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 150 mM NaCl and 0.5 mM DTT), transfered into the homogenizing buffer(50 mM Tris-HCl, pH 7.5, 10% glycerol, 5 mM magnesium acetate, 0.2 mM EDTA, 0.5 mM DTT, and 1.0 mM PMSF), and homogenized by a polytron homogenizer. The homogenates were centrifuged at 100,000×g for 60 minutes at 4°C, and the supernatant was retained.

The protein content was measured by the Bradford dye binding method(19, 20). The pellet was resuspended in SDS sample buffer(0.065 M Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue) and then boiled for 5 minutes. The

boiled sample was loaded onto a 10% polyacrylamide gel, and then SDS-PAGE was carried out by the method of Laemmli(21).

After the gel was stained for a minimun 4 hours in fixing solution(50% methanol, 5% acetic acid and 45% H_2O) with 0.1% Coomassie brilliant blue, it was destained by diffusion in fixing solution without the dye and incubated until a desired contrast between background and protein was achieved.

Western blot

Western blotting was carried out by the method described by Blake et al.(29). The proteins separated by gel electrophoresis were transferred to a nitrocellulose (NC) membrane for 12 hours using a transfer buffer of 20 mM Tris, pH 8.3, 120 mM glycin, and 20% methanol in a Trans-Blot cell(Bio-Rad). The NC membrane was probed by an incubation at room temperature in a solution containing the anti-sarcoma 180 polyclonal antibody diluted to 1:500 with 1× basic blotting buffer(10 mM Tris, pH 7.5, 150 mM NaCl, 0.01% NaN_3, and 1 mM $CaCl_2$). Following the incubation, the NC membrane was washed in the 1× basic blotting buffer for 5 minutes ten times to remove all antibodies except those associated with antigenic molecules. A second incubation was carried out in a solution containing anti-mouse IgG alkaline phosphatase conjugate diluted to 1:500 with the 1×basic blotting buffer in 5% BSA. The incubation was followed by a second wash sequence with the same solution. Finally, the immunologically identified antigens were visualized by placing the processed NC membrane into a precipitating substrate solution(NBT, BCIP) which reacts specifically with alkaline phosphatase. The reaction was terminated by a washing with water.

RESULTS

Light microscopy

The sarcoma 180 cells mostly showed little or moderate variations in size and shape. They had a monotonously spindled appearance and also showed cellular pleomorphism (Fig. 1A). Nuclei were large and round with chromatin which were dispersed, distinct and occasionally multiple (Fig. 1C). On the contrary to the control sarcoma 180 cells, tannin-treated cells showed more differentiated appearance e.g., degradation and cell death. Typical undifferentiated appearance of malignant tumor cells were rarely discovered in the sarcoma 180 cells treated with tannin; however, most of the cells showed more differentiated appearance like benign tumor cells(Fig. 1 B and D).

In the tannin treated group, the cell adhesion was increased, therefore, tannin inhibited the migration of sarcoma 180 cells compared to the control group(Fig. 1D). Degradation of the tannin-treated tumor cells was shown on the area indicated with arrows, and many cells around the area showed degeneracy in progress(Fig. 1B).

Immunohistochemistry

According to the immunohistochemical analysis, the control cells were diffused throughout the tissue(Fig. 2A). However, the tannin-treated tissue showed a sparse distribution of sarcoma 180 cells(Fig. 2B).

Protein detection by SDS-PAGE

Proteins of the sarcoma 180 solid tumors generated by subcutaneous injection of sarcoma 180 ascitic cells with or without tannin were analyzed in the same quantities by one-dimensional gel electrophoresis. The protein patterns of sarcoma 180 induced solid tumors with and without tannin administeration were shown in the Fig. 3.

The differences of the protein pattern were distinct among the three major proteins; protein A(mw. 66,000), B(mw. 58,000), and C(mw. 55,000). Moreover, the protein A was largely different in quantity compare to the others. Among the bands, band C was assumed as the band of intermediate filament protein.

Western blot

The identification of each protein was ascertained through western immunoblot analysis utilizing polyclonal antibodies specific for sarcoma 180 ascites cells. Results showed that the protein C(55 kDa) revealed distinct difference between the control and the treated group(Fig. 4).

DISCUSSION

Condensed tannins include monomers, dimers, trimers and tetramers and their representative constituents are flavin-3-ols containing(+)-catechin(CAT), (-)-epicatechin (EPI), (+)-gallocatechin(GAL) and (-)-epigallocatechin

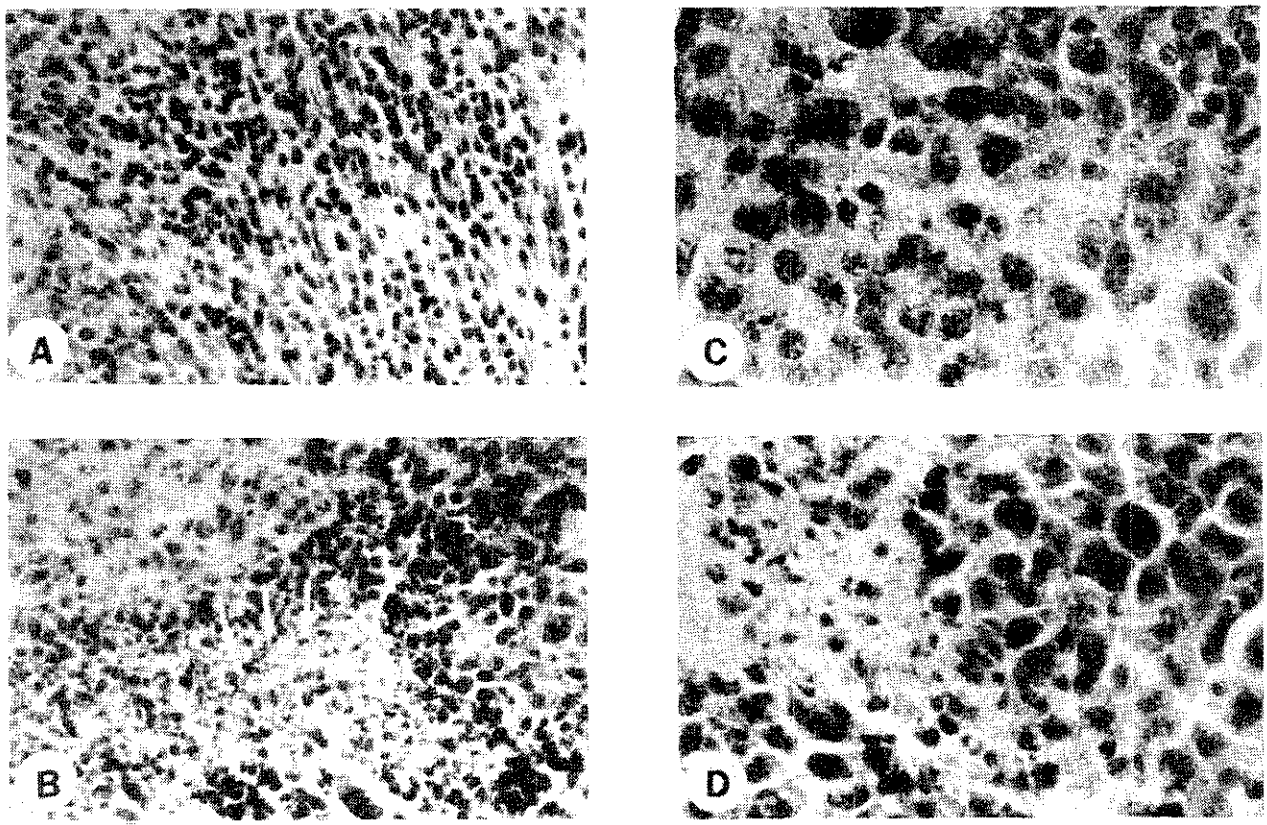

Fig. 1. Morphological alterations of sarcoma 180 cells excised from left groin of mice inoculated sarcoma 180 ascitic tumor cells with or without i.p. administration of tannin.
A: Both cytoplasmic and nuclear reactivity is present in spindle-and oval-shaped sarcoma 180 cells. H&E, ×150
B: Sarcoma 180 cells treated with tannin shown degradation of most sarcoma 180 cells. H&E, ×150
C: Monotonous proliferation of sarcoma 180 cells show large and round nuclei with dispersed chromatin, H&E, ×300
D: Tannin treated to sarcoma 180 cells show an increased cell-cell adhesions compared to the control. H&E, ×300

-3-D-gallate(EGG), procyanidin B-1(B-1) and procyanidin C-1(C-1), and so forth(22). Condensed tannins among species of plants are chemically akin, and their different biological effects may be ascribed to the differences in the degree of polymerization. It was also reported that the activities of tannin were dependent on the degree of condensation(23)

A suggested possible mechanism of the antitumor activity of tannin might be some host-mediated actions and direct cytotoxicity(8). The antitumor activity of tannin might be ascribed to the enhancement of immune response by inducing a IL-1(a cytokine) followed by an incease in the number of peripheral white blood cells, especially monocytes(1,13,14). On the other hand, there are a few reports which suggested that the antitumor activity of tannin is a direct cytotoxic action against tumor cells in the *in vitro* or the *in vivo* tests(5,12,24)

The degradation of the sarcoma 180 tumor cells in this study implied that tannin enhanced immune system through binding it to the tumor cells, which means an indirect cytotoxic effects. There were reports supporting this suggestion that condensed tannins, epicathechin gallate oligomers showed antitumor activity by enhancing the immunity of host animals by stimulating monocytes activities and iodination of melperoxidase-positive polymornuclear leukocytes(8,13,14,25)

Though there were a few reports that the antitumor activity came from only the direct cytotoxic acitivity, since tannin binds to many components(proteins) rapidly in the host cells, its direct cytotoxicity to tumor cells seems to be very weak. This argument was the reason that high cytotoxicity of tannin was markedly diminished by the addition of serum to the culture(8,15)

The results of protein pattern analyses showed that an intermediate filament, presumably cytokeratin was increased in the tannin-treated groups(Fig. 3). There were reports suggesting that sarcoma 180 cells grown in ascitic fluid showed a simultaneous expression of

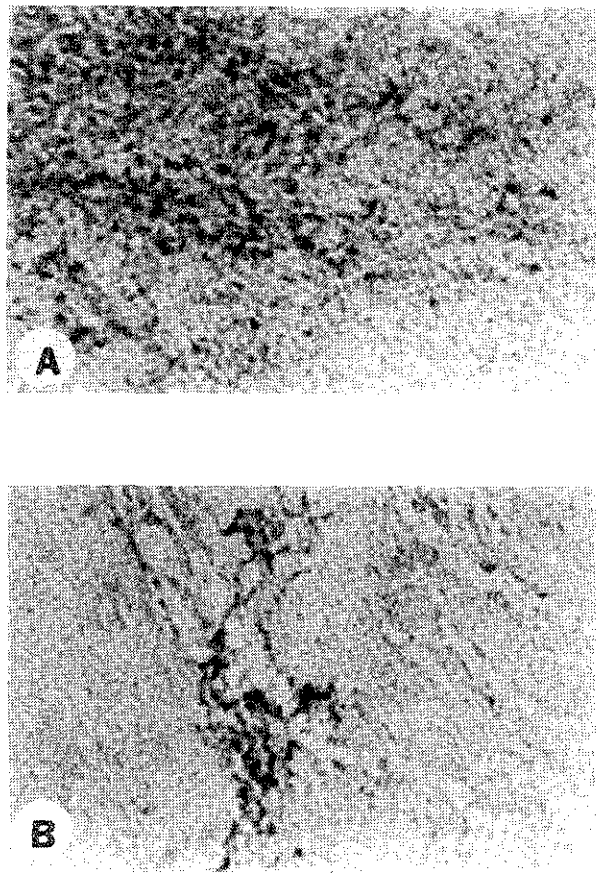

Fig. 2. Immunohistochemical distribution of sarcoma 180 cells with or without i.p. administration of tannin *in vivo*.
A: Control sarcoma 180 cell distribution
B: Tannin-treated sarcoma 180 cell distribution

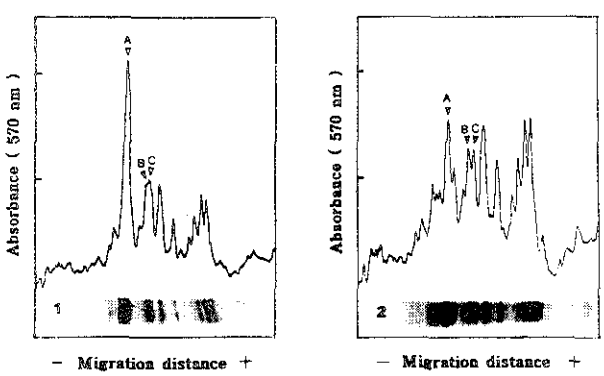

Fig. 3. One-dimensional gel electrophoresis of sarcoma 180 solid tumors administrated with(Lane 2) or without tannin(Lane 1) *in vivo*.
The densitometer tracing corresponds to the protein bands in lane 1 and 2. The arrowheads indicate the migration positions of 66 kDa., 58 kDa. and 55 kDa. proteins.

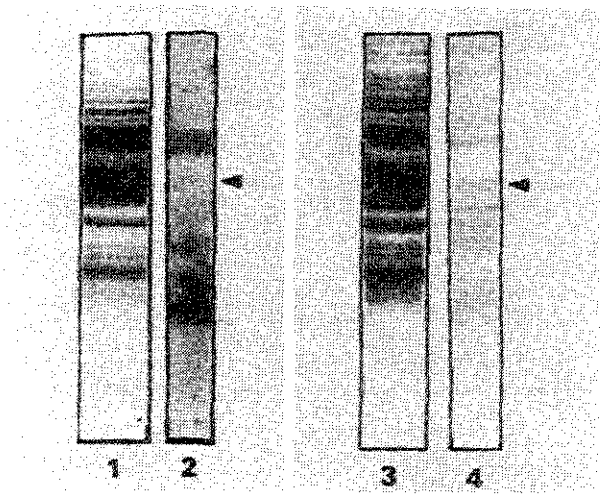

Fig. 4. Protein patterns of sarcoma 180 tumors admin-istrated with(Lane 3, 4) or without(Lane 1, 2) tannin *in vivo*.
Lane 1, 3: SDS-PAGE gel, Lane 2, 4: Western blot. Arrow heads indicate protein C(mw. 55,000) of Fig. 3.

keratin and vimentin(25); however, the corresponding solid tumors synthesized only one type of intermediate filament proteins(keratin) which was characteristic for the epithelial carcinoma. The vimentin cytoskeleton most likely occurred in the epithelial cells only during the process of metastasis, influenced mitotic as well as motile activity of the cells. The decreased expression of vimentin might represent a more differentiated cell state. Epithelial or epidermal cell differentiation might proceed with differential expression of keratin genes. Some cytokeratins in malignant cells differ significantly from their normal tissue counterparts and thus, reflect at least phenotypic differences between the two cell states; therefore, it might be useful as diagnostic indicators of the origin and progression of malignancy. The alterations in the individual intermediate filament polypeptides were more related to the differentiated state of the individual cell lines rather than to the transformation phenotype(25-28). Therefore, the increased intermediate filament in the tannin-treated cells was related to the differentiated state of the tumor cells which was an important phenomenon in the progress of tumor. However, the expression of extracellular matrix proteins and intermediate filament polypeptides should be further investigated to clarify the detail mechanism involved in the tannin action on the malignant tumor cells in the progression of tumor.

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