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Effect of Selected Persimmon Leaf Components against Sarcoma 180 Induced Tumor in Mice

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Abstract

Antitumor activities of tannin extract and chloroform fraction extract from the persimmon leaves, and 2, 4-decadienal identified as an antimutagenic compound in persimmon leaves were examined in sarcoma 180 implanted tumor in mice by using both light and transmission electron microscopes. Among them, tannin extracted from the persimmon leaves delayed the progression of malignant tumor but the other two did not show any noticeable effect. The antitumorigenic activity of tannin extract might not come from the selective cytotoxicity against tumor cells, but might be, in an anaerobic environment, from the inhibitory action against oncogenic protein synthesis or from the proteolysis of the pre-formed oncogenic proteins by autophagocytic granules. Therefore, the tannin from persimmon leaves might protect cells from fast progression of malignant tumorigenesis.

Key words : antitumor, sarcoma 180, tannin, persimmon leaves, TEM

INTRODUCTION

Many antitumor agents were reported after since the anti-proliferative effects of nitrogen mustard were found. However, its selectivity against cancerous cells only, without any harmful effect on normal cells, is the most important aspect to be a successful chemotherapeutic agent. Among antitumor agents with selectivity, synthetic amino acid derivatives were known to have both carrier domain and antitumorigenic domain[1,2]. The carrier domain was composed of amino acids and believed to function to facilitate the membrane transport of the agents. The strategy of the most synthetic antitumor agents was based on the chemical modifications of the preexisting compounds to increase their antitumor activities. Therefore, it seemed not to be promising to develop a brand new drug with high selectivity at this moment.

Because of the poor selectivity and productivity of the synthetic antitumor compound, there is an increasing trend of developing new antitumor agents from the natural compounds[3,4]. Tannin is one of the natural compounds which are known to facilitate the body's immune defense mechanism[5]. In the present research, an extracted or identified compounds include tannin from the persimmon leaves[6~8] that showed antimutagenic and anticanceric effects *in vitro*[9] were studied for their antitumor activities, based on the change of cellular morphology of the tumor induced with sarcoma 180 *in vivo*.

MATERIALS AND METHODS

Tumor cells

After cervical dislocation, ascites were drawn from the ICR mice which were inoculated with sarcoma 180 tumor cells intraperitoneally. To isolate tumor cells, the ascites were adjusted to 0.83% NH_4Cl and set for 2min at room temperature to remove red blood cells. Broken cells were then removed by centrifugation for 3min at 400×g. Pelleted sarcoma 180 tumor cells were washed once with sterile ice-cold PBS, and resuspended again in the concentration of

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1.0×10^6 cells/ml of PBS. The above cell suspension was used to inoculate into mouse.

Implantation of tumor cells

The implantation of sarcoma 180 tumor cells were conducted by inoculating 0.1ml of cell suspension, intradermally, into the left groin of ICR mouse (20~25g).

Preparation of tannin from persimmon leaves

The dried persimmon leaves were boiled in distilled water for 1hr at 120° C. Tannin was extracted according to the method of Okonogi et al.[10], dialyzed and then freeze-dried. Lyophilized powder was dissolved in 10% DMSO and injected peritoneally into mouse for 10days continuously (10mg/kg/day). Reference group was prepared in the same manner with 10% DMSO only.

Preparation of chloroform fraction and 2, 4-decadienal from persimmon leaf

Methanol extraction was done by the method of Moon[9]. Dried and powdered persimmon leaves were treated with 20 vol(w/w) of methanol and extracted for three time, 3hrs for each. After concentration of the extract, it was fractionated into hexane, chloroform, ethyl acetate, butanol, and water fractions. Among the above fractions, chloroform fraction was further purified through different column chromatographies and thin layer chromatography (TLC). The TLC fraction number 5 that contained antimutagenic compounds[9] was identified using GC-MS. One of the major compounds was 2, 4-decadienal[9].

Chloroform fraction was dissolved in 10% DMSO and injected peritoneally into mouse 10days continuously (30mg/kg/day). 2, 4-decadienal which was purchased from Sigma Chemical Co. (St. Louis, USA) was also dissolved in DMSO and injected peritoneally into mouse for 10days continuously (10mg/kg/day). Reference group was prepared in the same manner with DMSO only.

Light and transmission electron microscopy

At the day of 28th of tumor cell inoculation, solid tumor was removed from the left groin of each mouse. The preparation for light microscopy was done by the paraffin embedding method[11] and H & E staining. For the electron microscopy, isolated solid tumor was cut into $1mm^3$ and pre-fixed with 2% glutaraldehyde at 4° C and post-fixed with OsO_4. After the fixation, sample blocks were treated with 0.1M PBS, pH 7.4, overnight and dehydrated by the ethanol treatment, then embedded with epon mixture. Samples were cut into semi-thin section (1μm) by ultramicrotome (LKB Ultrotome NOVA, Sweden), then stained with toluidine blue. The final ultra-thin section was mounted on 200mesh copper grid and stained for 40min with 3% uranyl acetate, 4min with lead citrate, and observed under the transmission electron microscope (JEM 100 SX, Jeol, Japan).

RESULTS AND DISCUSSION

In the light microscopy, sarcoma 180 induced solid tumor tissue showed pleomorphic cells with severe anisonucleosis (Fig. 1-A). The individual cells showed karyolysis and piknosis. The shapes of nuclei varied (mainly reticular shape). Some tumor cells of pale staining nuclei and oval shaped nuclei showed the typical cancerous morphological characteristics with the high ratio of nuclear to cytoplasmic volume[12], and with high incidence of multipolar mitosis. The implanted tumors also contained anuclear dead cell mass by the necrosis processed in the succession of karyolysis, piknosis, and karyorrhexis in some areas.

In the electron microscopy, untreated reference group as shown in Fig. 1-B, revealed cells without clear nuclear boundary and without prominent intercellular junctions. The high incidence of intercellular matrix might be the indication of the ongoing necrosis. Therefore, according to the microscopic study, the implanted malignant tumor by sarcoma 180 showed very similar morphology of osteosarcoma[12], and had also the following typical morphological characteristics of tumor cells : cells with atypical nucleus, giant nucleus, variously condensed nucleolus, highly dispersed granular chromatin, and poorly developed cellular organelles. Especially, atypical nucleolus was the most promi-

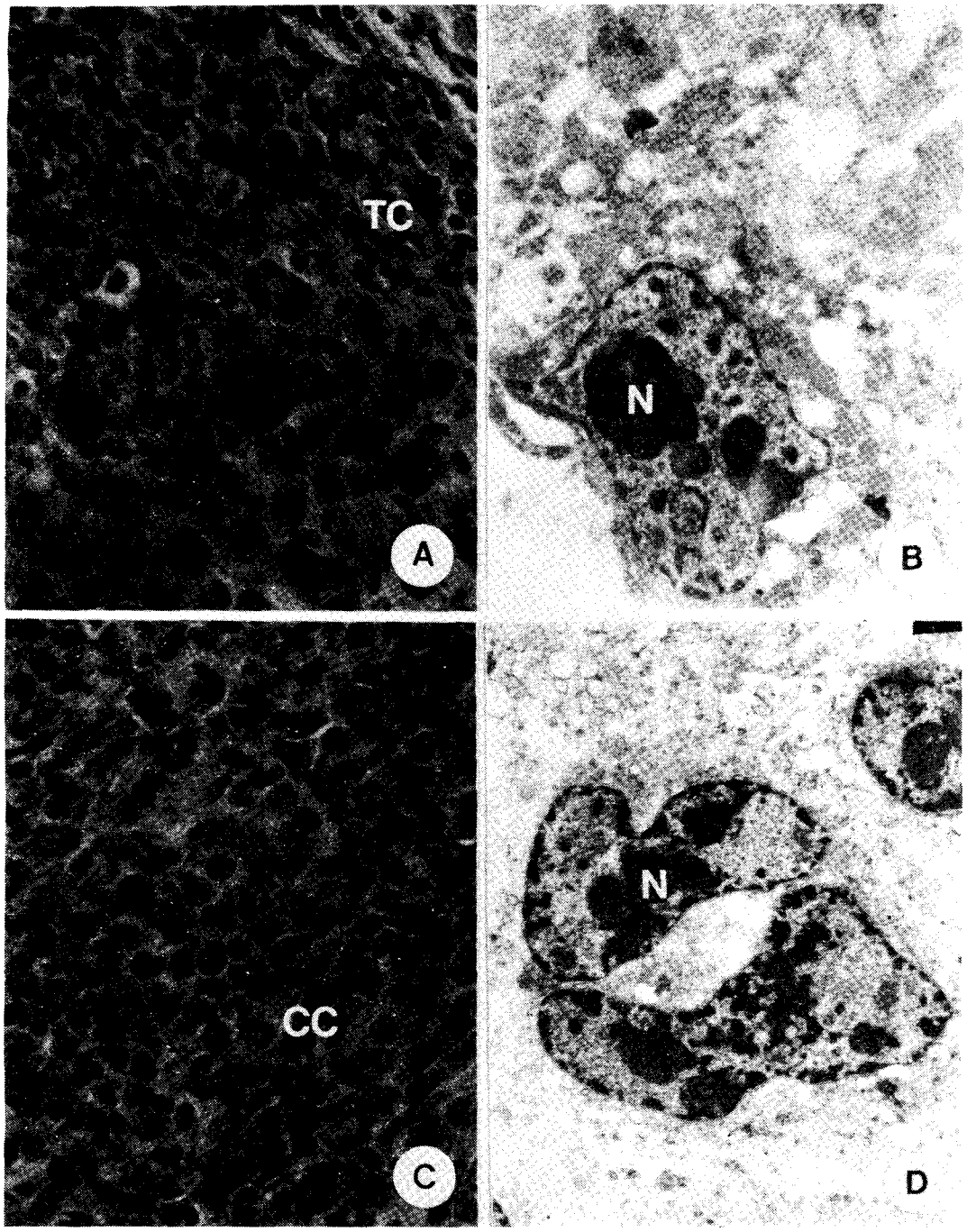

Fig. 1. A : Sarcoma 180 tumor in the left groin of the untreated mice. Note the tumor cells (TC), multipolar mitosis of a giant cell (arrowhead), and piknoisis (doublet arrowhead). H&E, Original Magnification ; ×200. B : Transmission electron micrograph of sarcoma 180 tumor cells in the untreated mice. Note vacuoles in anucleated cells, and nuclolus (N). Poststaining with uranyl acetate and lead citrate, Original Magnification ; ×6,000. C : Tumor treated with tannin extract showing karyolysis, piknosis, and pleomorphic cell pattern. CC ; cigar-shaped cells. H&E, Original Magnification ; ×200. D : Transmission electron micrograph of tumor cells treated with tannin extract from persimmon leaves. Note lipid droplets. N ; Nucleolus. Poststaining with uranyl acetate and lead citrate, Original Magnification ; ×6,000.

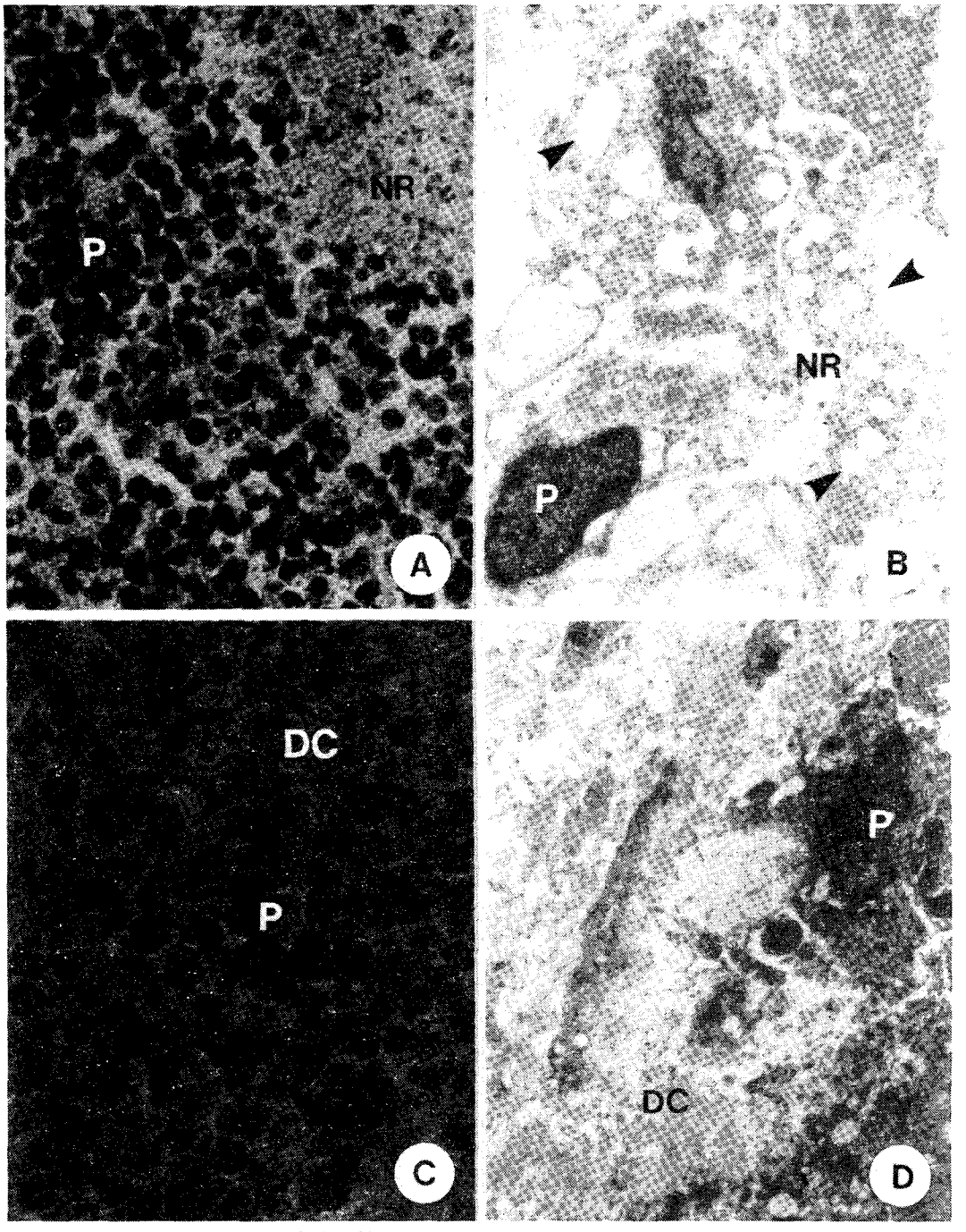

Fig. 2. A : Sarcoma 180 tumor treated with the chloroform fraction extract from the persimmon leaves showing abundant necrotizing region(NR), and many nuclei particles by karyorrhexis. P : Piknosis. H&E, Original Magnification ; ×200. B : Transmission electron micrograph of tumor cells treated with the chloroform fraction extract showing dark cells, and vacoles (arrowheads). P ; Piknosis. Poststaining with uranyl acetate and lead citrate, Original Magnification ; ×4,000. C : Tumor treated 2, 4-decadienal showing dead cell mass(DC), and anucleated cells. P ; Piknosis. H&E, Original Magnification ; ×200. D : Transmission electron micrograph of tumor cells treated with 2, 4-decadienal. Note the abundant dead cell mass(DC), necrotizing vacuoles, and piknosis(P). Poststaining with uranyl acetate and lead citrate, Original Magnification ; ×4,000.

nent character. Tumor induced by sarcoma 180 also showed similar patterns of cellular arrangement as was shown by rhabdomyosarcoma and leiomyosarcoma[13]. As in Ewing tumor, light cells and dark cells were also found but endoplasmic reticulum and Golgi complex were not identified. The above observation might indicate that highly dispersed chromatin and variously condensed nucleolus which were known to contain high amount of mRNA and rRNA, respectively, were responsible for the transformation of tumor cells for growth and differentiation. The destruction of nucleolus also showed the coincidence of the formation of intracellular vacuoles and necrosis followed. The cell further stimulated destruction of muscles in groin and bone marrow of femur in the hind limb of the mouse.

In the tumor treated with the tannin extract, cells were in cigar and spindle shape. Even some small cell groups, probably transformed from lymphocytes, were found in the tumor tissue[12]. However, anisonucleosis and high ratio of nuclear to cytoplasmic volume in the tumor treated with tannin extract were not prominent compared to the untreated reference group. Moreover, the incidence of actively dividing cell by mitosis could be the indication of the delayed progress of tumorigenesis. In the electron microscopic observation, tumor cells treated with tannin extract showed nucleus moulding and still had condensed nuclei and grandular chromatin as in the cells of untreated reference group, but showed Golgi complex and secretory granules, even though vestigial(Fig. 1-D). Therefore, it could be concluded that the tumor treated with tannin extract was slow in the progression of tumorigenesis and was in the prenecrosis stage compared to the untreated tumor.

Tumor treated with the chloroform fraction extract showed high degree of necrosis by condensation and destruction, thus revealed large anucleated dead cell mass and vacuoles as was shown in Fig. 2-A, B. These results indicated that tumorigenesis in chloroform fraction extract treatment was even faster than that in the treatment of tannin extract, or that in reference group.

In the treatment of 2, 4-decadienal, wide appearance of anucleated tumor cells indicated high degree of necrosis (Fig. 2-C, D).

Though the chloroform fraction extract and 2, 4-decadienal from the persimmon leaves showed antimutagenic activities and inhibitory effect on the growth of cancer cells *in vitro*[9], these compounds revealed less antitumoric effect *in vivo*. However, the tannin extract from the persimmon leaves showed not only antimutagenic and anticanceric effects *in vitro*[9] but also antitumoric activity *in vivo* (Fig. 1-C, D).

According to the above results, the facts that tumor cells treated with tannin extract had Golgi complex and secretory granules and had quite a number of lipid droplets[14], might explain that the tannin extract might inhibit the synthesis of oncogenic proteins and change the lipid metabolism. Those metabolic alterations led the tumor cells to change in physiological status including tumorigenic activity, and as a result the cells treated with tannin extract showed the delayed tumorigenesis. Golgi complex was responsible for the formation of lipoproteins and glycoproteins of plasma membrane[15]. The phenomenon that tannin extract from the persimmon leaves inhibited lipid peroxidation[9] was not unrelated to its antitumor activity.

To summarize, the tannin extract might slow the progression of tumor by inhibiting oncogenic protein synthesis or stimulating autophagocytosis to destroy pre-formed oncogenic proteins in an anaerobic environment. Those intracellular changes made cells relatively free from necrosis, kept them less self-destructive and delayed tumorigenesis.

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생쥐 육종에 대한 감잎 성분의 암 성장 억제효과

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요 약

Sarcoma 180 복수형 종양세포를 생쥐의 왼쪽 서혜부에 이식한 후 감잎에서 분리한 tannin, chloroform fraction 추출물 및 2, 4-decadienal을 복강 내에 각각 단독투여하여 그 효과를 형태학적으로 검토하였다. 이식된 sarcoma 180 육종에서 암화의 진행을 지연시키는데는 tannin 투여시에 가장 효과적이었고 chloroform fraction 추출물 및 2, 4-decadienal 투여시에는 형태적으로 간주할 수 있는 지연효과는 없는 것으로 나타났다. 감잎 tannin은 종양세포를 선택적으로 파괴하여 종양의 증식을 억제하는 것이 아니라 일종의 협기상태의 세포 환경에서 종양을 괴사과정으로 유도하지않고, 발암성을 가지는 단백질과 같은 물질의 합성을 저해하거나 또는 자식작용을 가진 과립들로 하여금 이들을 소화시켜 이식된 종양세포를 악성종양으로 유도하는 과정을 지연시키는 효과를 나타내었다.