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Original Article

Inhibitory Effect of *Scutellaria barbata* Don Water-extracts on Growth and DNA Incorporation of Human Cancer Cells

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The water-extracts of Scutellaria barbata Don (SBDE) were isolated from Chinese medicinal plant sources. The extracts showed strong growth-inhibitory activity and cancer chemopreventive activity on the growth and DNA incorporation of MG63 human osteosarcoma and K562 human leukemia cell lines.

The growth of human cancer cells was inhibited in the presence of the extracts (20, 50 and 100 ㎍/㎖), and the effects were concentration-dependent and incubation time-dependent up to 8 days. When 50 ㎍/㎖ of the extracts was added to the media of MG63 and K562, cell growth after 8 days or 6 days of incubation was retarded by 93.2 to 97.3% of the control group. Morphological changes of MG63 and K562 cell lines were observed. As the concentration of the extracts increased up to 50 ㎍/㎖, degree of cell aggregation decreased.

Moreover, the DNA incorporation of the cells which were labeled with [3H] thymidine was significantly reduced after 3 days of incubation at 37℃ with the extract. Therefore, it is suggested that the extract is highly effective on inhibition of cancer cell growth.

The extract also inhibited gene expression of IGF-Ⅱ in transcriptional level. Since IGF-Ⅱ works as a mitogenic effector on MG63 and K562 cell lines, these results suggest that the growth inhibition is in part mediated through the inhibition of IGF-Ⅱ gene expression.

Key Words : *Scutellaria barbata* Don, MG63, K562, cancer

Introduction

Inrecent years, many efforts to seek naturally nontoxic antimutagenic and anticancer compounds have been carried out through various anticancer medicines and foods[1]. Thus, many plants have been empirically used as therapeutic agents with beneficial results in various diseases containing incurability. However, some components of the plants induce harmful side effects. Hence the development of experimental methods in order to estimate more accurately their curative properties and/or side effects is required.

Cancer is the largest single cause of death in both men and women, claiming over 6 million lives each year worldwide. Chemoprevention, the prevention of cancer by ingestion of chemical agents that reduce the risk of carcinogenesis[2], is one of the most direct ways to reduce morbidity and mortality. Cancer chemopreventive agents include nonsteroidal anti-inflammatory drugs (NSAIDS) such as indomethacin, aspirin, piroxicam and sulindac, all of which inhibit cyclooxygenase (COX)[3,4,5,6]. This inhibitory activity is

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relevant to cancer chemoprevention because COX catalyzes the conversion of arachidonic acid to pro-inflammatory substances such as prostaglandins, which can stimulate tumor cell growth and suppress immune surveillance[7,8,9]. In addition, COX can activate carcinogens to forms that damage genetic materials[9,10].

In searches for new cancer chemopreventive agents over the past several years, hundreds of plant extracts have been evaluated for their potential to inhibit COX. SBDE, collected in China, was identified as a potent inhibitor. This traditional medicinal plant belongs to febrifugal and detoxicant drugs. In China, it has been used in local area as a anti-tumor drug with other traditional medicinal plants, e.g., *Lobelia radicans* Thunb, *Oldenlandia diffusa* Roxb, *Solanum nigrum* L., etc.[11].

In previous studies[12], the inhibitory effect of the SBDE on the growth of gynecological cancer cell lines such as HeLa and human ovary cancer (HOC) cell were also examined. When HeLa and HOC cells were treated with SBDE, expression and production of transforming growth factor-β, a tumor growth factor, was inhibited, suggesting that SBDE merits investigation as a potential cancer chemopreventive agent in humans, especially for gynecological cancers[12].

In this study, the effect of the extract on the growth of MG63 and K562 cell lines was examined. It was found that the extract inhibited the growth of these cell lines, and that the extract specifically suppressed expression of the human insulin-like growth factor-Ⅱ (IGF-Ⅱ) gene, known to have a mitogenic effect on hepatocarcinoma cells. High level expression of IGF-Ⅱ mRNA has been reported in human tumors, including hepatocarcinoma[13].

Materials and Methods

1. Materials

Scutellaria barbata Don was obtained from an herbal supplier in China. After the sample was selected, washed and blended by a mixer, the portion of *Scutellaria barbata* Don juice was obtained by centrifugation at 9,000 rpm for 30 min. The supernatant was sterilized through a millipore filter (0.45 μ m) or through heat boiling before addition to the experiment.

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), 0.05% trypsin-0.02% ethylenediamine tetraacetic acid (EDTA), and 100 units/㎖ penicillin-streptomycin were purchased from Gibco BRL (Grand Island, NY). A CO2 incubator (Nua) was used for cell culture. An inverted microscope (Nikon, Japan) was used to see the morphological changes of cancer cells. A liquid scintillation counter (Beckman LS250) was used for DNA incorporation experiments. Lipopolysaccharide (LPS) and carrageenan were purchased from Sigma.

Human recombinant insulin-like growth factor-Ⅱ (IGF-Ⅱ) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

2. Cells and cell cultures

MG63 and K562 cell lines were from the cell bank of the Korea Research Institute of Bioscience and Biotechnology, KIST (Daejeon, Korea). The cells were cultured routinely at 37℃ in DMEM or RPMI 1640, supplemented with 100 units/㎖ penicillin-streptomycin and 10% FCS in 5% CO2 incubator. Media were changed two or three times every week. After six or seven days, cultured cancer cells were washed with phosphate buffered saline (PBS). Cells were harvested after trypsin-0.02% EDTA treatment

followed by centrifugation. Media were added to integrated cells and it was evenly dispersed via pipette. Five milliliters of medium containing cancer cells were transferred to the cell culture flask, and it was cultured for further experiments. For long term storage, the harvested cells in DMEM containing 10% FCS and 10% dimethylsulfoxide were stored in liquid nitrogen. K562, suspension cells, were cultured routinely at 37℃ in DMEM, supplemented with 100 units/㎖ of penicillin-streptomycin and 10% FCS. The cells were maintained as described above.

For RNA extraction, the cells were plated at 4 × 106 cells per 15 ㎖ per 150cm2 dish (Nunc). In either assay the cells were allowed to attach to the plate overnight before the test substances were added.

3. Cell growth experiments

MG63 were plated in 24-well plates and cultured for 24 hours in 10% FCS-supplemented DMEM at a plating density of 10,000 cells/㎖. After cancer cells were attached to the plate, they were cultured in 5% CO2 incubator at 37℃, changing culture medium with SBDE every other day. After 6 days, the cells were treated with 0.05% trypsin-0.02% EDTA, inhibitory effects of SBDE to that of control on the growth of cancer cells were observed by counting the cell numbers.

The suspended K562 cell lines were also seeded in 24-well plates at a density of 10,000 cells/㎖, and SBDE at desired concentrations was added directly in medium. After 4 days, the inhibitory effect on the growth of K562 lines to that of control was observed. The outline to describe the cell growth experiment is shown in Scheme 1.

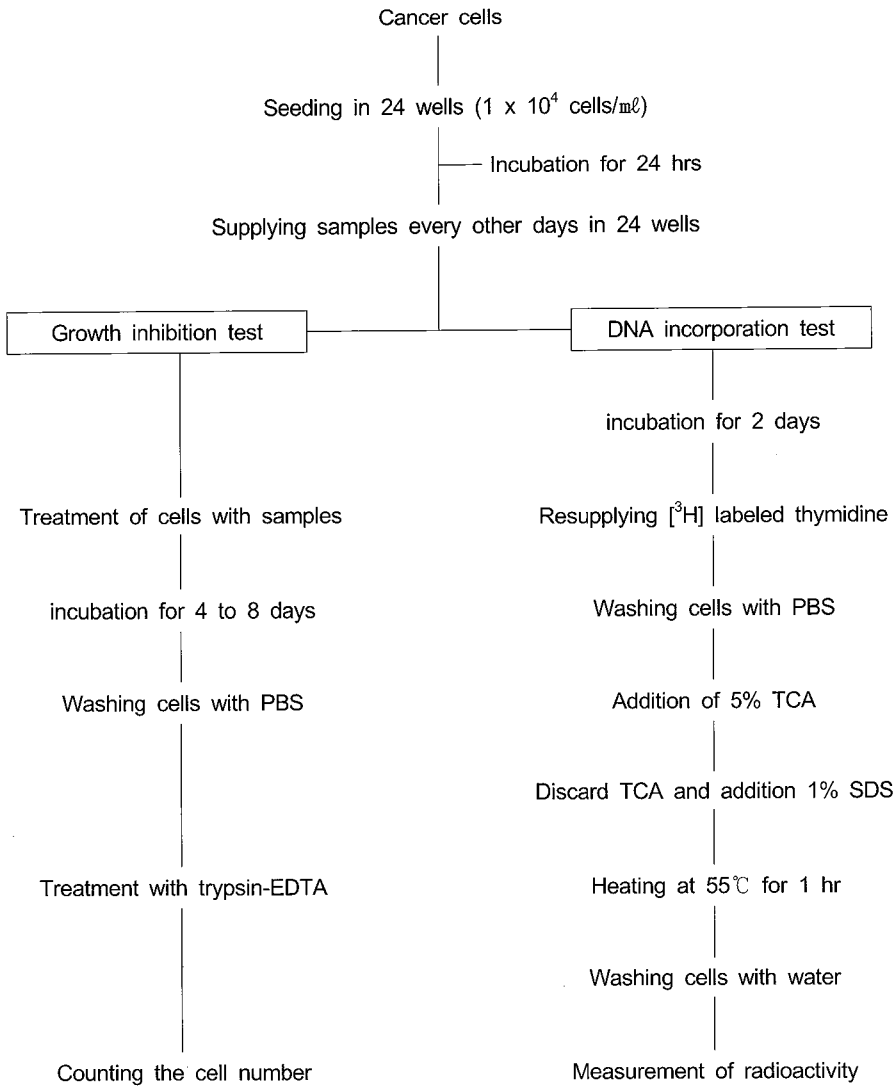
In order to see the morphological change of the cancer cells by SBDE, cancer cells were cultured for 24 hrs in 10% FCS supplemented DMEM at plating density of 10,000 cells/㎖. After cancer cells were attached to the plate, they were cultured with medium containing SBDE in 5% CO2 incubator at 37℃. The shape of cancer cells was observed under inverted microscope.

4. DNA incorporation

MG63 were plated in 24-well plates and cultured for 24 hrs in 10% FCS supplemented DMEM at a plating density of 10,000 cells/㎖. After cells were attached to the plate, cultured medium was replaced with new medium containing 10% FCS and SBDE. They were cultured in 5% CO2 incubator at 37℃. After 48 hrs, medium was replaced with the indicated medium labeled with 3 ㎕ Ci/㎖ of [3H] thymidine. After 2 hrs of incubation, the indicated medium was discarded and the solid component was washed twice with PBS. Cells were kept refrigerated at 4℃ with 1 ㎖ of 5% cold trichloroacetic acid (TCA). After 1 hr, TCA was removed and 250 ㎕ of 1% sodium dodecyl sulfate (SDS) was added. The cells were heated to separate from 24-well plates for 1 hr at 55℃. After cells were transferred into scintillation vials, they were washed twice with 150 ㎕ of water. The radioactivity was measured with a Beckman LS250 scintillation counter after adding 3.5 ㎖ of scintillation cocktail[14]. The outline is described in Scheme 1.

5. RNA extraction and northern hybridization

Total RNA was prepared from cancer cells as described by Sacchi and Waddell WR. et al.[5]. 10 ㎍ of each sample of total RNA was run on a 1.0% agarose, 1.1 M formaldehyde gel, and blotted onto hybondTM membrane (Amersham) in 10 × SSC. RNA was cross-linked by UV

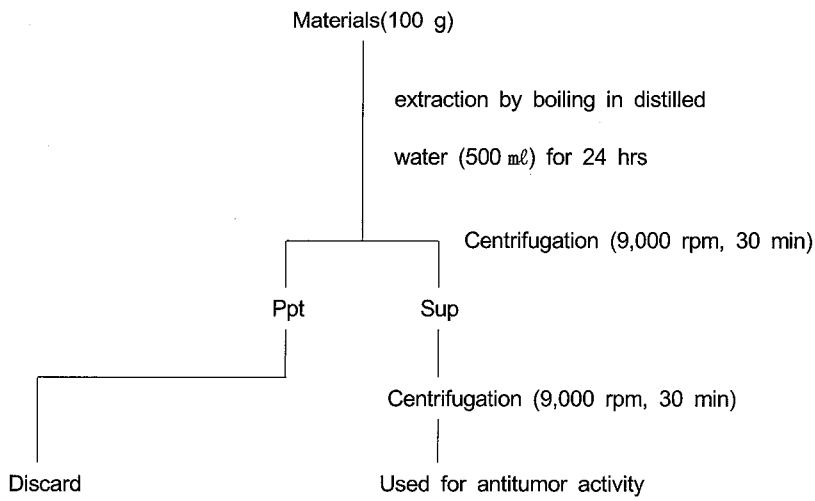


Scheme 1. The experimental scheme for inhibitory effect of the *Scutellaria barbata* Don water-extracts on the growth and DNA incorporation of human cancer cells.

illuminator. Prehybridization, hybridization and washing were carried out as described[18]. The probes IGF-Ⅱ and β -actin were prepared by random-primed multi-labeling kit (Boehringer Mannheim Co.).

6. General analytical methods and statistical analysis

Protein was determined by the procedure of Lowry et al.[16] using bovine serum albumin (BSA) as a standard. The statistical significance



Scheme 2. Preparation of the water-extracts.

of difference among groups was evaluated by Student's t-test or Duncan's new multiple range test; $p<0.01$ was considered significant, reflecting data obtained from control and sample groups.

7. Extraction, fractionation, and purification

The dried samples were homogenized using a mechanical disintegrator with a tissue homogenizer (Tekmar Co., Cincinnati, OH) in distilled water, and the crude fraction was collected by centrifugation (15,000 × g, for 20 min) at 4℃. The supernatant solution was concentrated to about 120 mℓ, and used for the experiments (Scheme 2).

8. Assay of antitumor activity

Cell cytotoxicity of SBDE was carried out using MTT assay[17].

Results

Many polysaccharides of plant and microbial origins show growth-inhibitory activity against mouse-implanted tumors, e.g., Sarcoma 180, Sarcoma 37, adenocarcinoma, etc. [9,10,19]. However, the chemical structure of the active site of antitumor activity in these polysaccharides has not been elucidated. Despite the fact that *Scutellaria barbata* Don has been used in therapy for various diseases for a long time, its real pharmacological properties are yet incompletely known.

1. Antitumor properties of SBDE

The results of antitumor assay indicate that the extracts show growth-inhibitory activity against MG63 and K562 cell lines. Thus, it is suggested that the extracts could be further assayed for a potent antitumor activity against MG63 and K562 cell lines. When survival rates of MG63 and K562 cell lines treated with the extracts were examined, they decreased in a dose-dependent manner (Table 1 and Table 2), somewhat more on MG63. When MG63 were treated with 20, 30, 50, 100 and 120 μg/mℓ of the extracts, the survival rates were 90%, 88%, 81%, 72% and 68%, respectively. The K562 cell lines showed 92%, 88%, 86%, 74% and 67% survival rates, respectively.

Table 1. Inhibitory effect of the extracts on MG63.

Cell lines	Dose(㎍/㎖)	Survival Rate (%)
Control	0	100
	5	99 ± 7
	10	93 ± 6
	20	90 ± 5
	30	88 ± 4
	50	81 ± 4
	80	76 ± 6
	100	72 ± 5
	120	68 ± 5

Each point represents the mean±S.E of the three experiments.

Table 2. Inhibitory effect of the extracts on K562.

Cell lines	Dose(㎍/㎖)	Survival Rate (%)
Control	0	100
	5	96 ± 8
	10	96 ± 4
	20	92 ± 3
	30	88 ± 7
	50	86 ± 3
	80	81 ± 4
	100	74 ± 8
	120	67 ± 5

Each point represents the mean±S.E of the three experiments.

2. Inhibitory effect of SBDE on the growth of MG63 and K562 cell lines

The inhibitory effects of the extracts on the growth of the two cancer cells of MG63 and K562 cell lines were studied (Fig. 1 and Fig. 2). When the SBDE was added to the medium of MG63 in the concentration of 10, 20, 30, 50, 100 and 200 ㎍/㎖, 9.8%, 92.8%, 96.4%, 97.3%, 99.7% and 98.5% of growth inhibition to that of control was observed, respectively ($P<0.01$).

In the case of K562 cell lines, 58.6%, 83.4%, 93.5%, 93.2%, 94.7% and 95.3% of growth were inhibited in the presence of 10, 20, 30, 50, 100 and 200 ㎍/㎖, respectively ($P<0.01$). Furthermore,

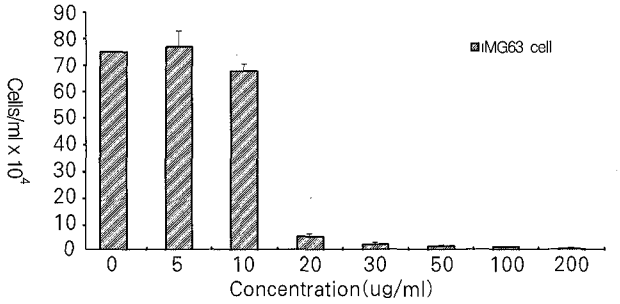


Fig. 1. Inhibitory effects of the extracts on the growth of MG63 cell lines. Cell numbers were counted after 7 days of incubation. The assay procedure is described in the experimental methods. Values are mean S.E for three experiments. *Significantly different from the control at the $p<0.01$ level.

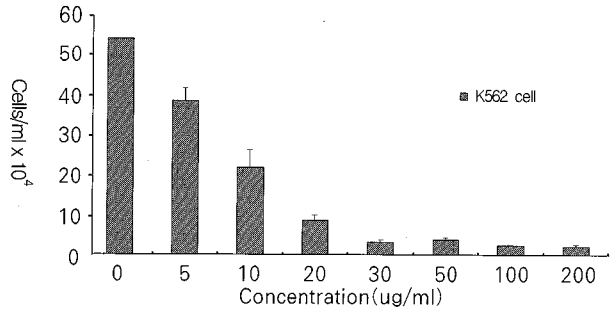


Fig. 2. Inhibitory effects of the extracts on the growth of K562 cell lines. Cell numbers were counted after 7 days of incubation. The assay procedure is described in the experimental methods. Values are mean S.E for three experiments. *Significantly different from the control at the p<0.01 level.

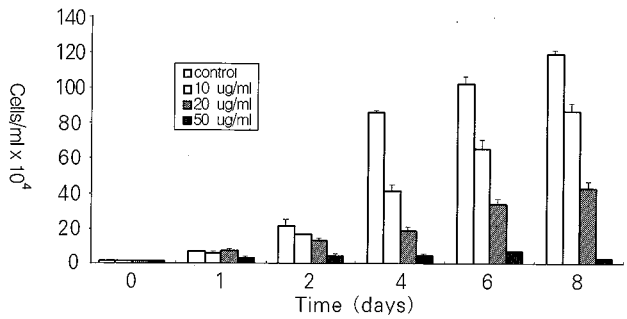


Fig. 3. Inhibitory effects of the extracts on the growth of MG63. The assay procedure isdescribed in the experimental methods. Values are mean S.E for three experiments.
*Significantly different from the control at the p<0.01 level.

when the extract concentrations were increased up to 200 ㎍/㎖, 95.3% inhibitory effect was observed (P<0.01), indicating that the cancer cells were retarded in concentration-dependent manner.

The inhibitory effect of the extracts on the growth of K562 cell lines was also carried out according to the culture time courses (Fig. 4).

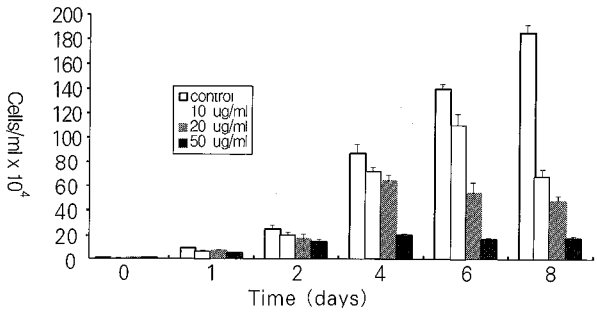


Fig. 4. Inhibitory effects of the extracts on the growth of K562 cell lines. The assay procedure isdescribed in the experimental methods Values are mean S.E for three experiments.
*Significantly different from the control at the p<0.01 level.

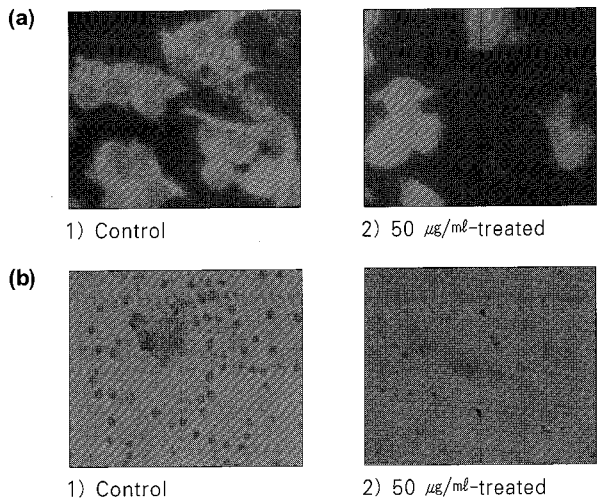


Fig. 5. Microscopical observation of the growth inhibition of MG63(a) and K562(b) treated with or without the filtered extracts for 8 days.

When the extracts were added to the cultured medium in the concentration of 50 ㎍/㎖ for 1, 2, 4, 6 and 8 days of incubation, 39.3%, 39.5%, 76.8%, 87.6% and 90.3% of inhibitory effect were found, respectively.

When 10 ㎍/㎖ of the extracts were added, the inhibitory effect on the growth was not significantly changed after 2 days of incubation, however, those effects were significant after 8 days of incubation. Also, when 20 ㎍/㎖ of the extracts were added, although the inhibitory effect on the growth was not significantly changed after 2 days of incubation, the effects were significant after 6 days of incubation.

3. Microscopical observation of the growth inhibition

Microscopical observations of growth inhibitory effects on MG63and K562 cell lines are shown in Fig. 5. The cells were incubated for 8 days

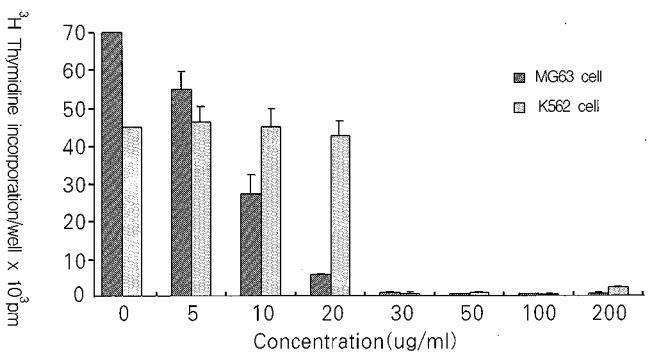


Fig. 6. Inhibitory effect of the extracts on MG63 and K562 cell lines.
*Significantly different from the control at the $p<0.01$ level.

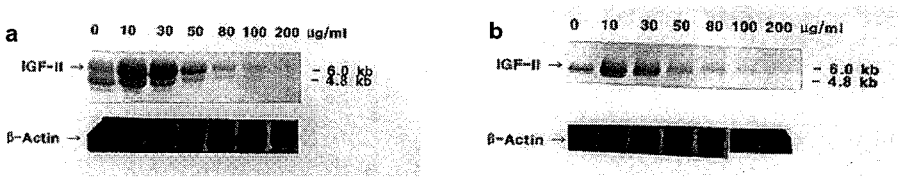


Fig. 7. Northern hybridization and down-regulation of steady state human IGF-II gene expression by the *Scutellaria barbata* Don water-extracts .
a) MG63 and K562 cell lines were treated with different concentrations of the extracts (0, 5, 10, 30, 50, 80, 100 and 200 ㎍/㎖) for 2 - 8 days, respectively.
b) The 6.0-, 4.8- and 2.2-kb transcripts encoded by the third and fourth promoters which are active in the liver cell are expressed in the cells at high level. 10 ㎍ of total RNA hybridized to a IGF-II probe or β-actin probe.

with or without the filtered extracts and then observed by inverted microscope. The more extracts were added into the medium, the less aggregation of cell mass was detected, compared to the control group. Morphological change was observed, showing that the flat forms in both of MG63 and K562 cell lines were being necrotized when 60 ㎍/㎖ concentration of the extracts was treated (Fig. 5).

4. Inhibitory effect of SBDE on DNA incorporation in cancer cells

The inhibitory effects of DNA incorporation within [3H] thymidine-labeled cells after 2 days of incubation with the extract using MG63 and K562 cell lines were examined by liquid scintillation counter (Fig. 6). Adding 10 ㎍/㎖ of the extract to MG63 showed significant inhibitory effect on DNA incorporation. When the concentration of the extracts was increased to 20 ㎍/㎖ and 50 ㎍/㎖, 91.3% and 98.5% of inhibitory effects were observed, respectively ($P<0.01$). Furthermore, adding the extracts up to 20 ㎍/㎖ in K562 lines had few inhibitory effects of DNA incorporation, however, when 50 ㎍/㎖ of the extracts was added, 97.3% of inhibition on DNA incorporation was observed. Therefore, DNA incorporation in MG63 was much more reduced than that in K562 cell lines, indicating that the MG63 is more sensitive against SBDE.

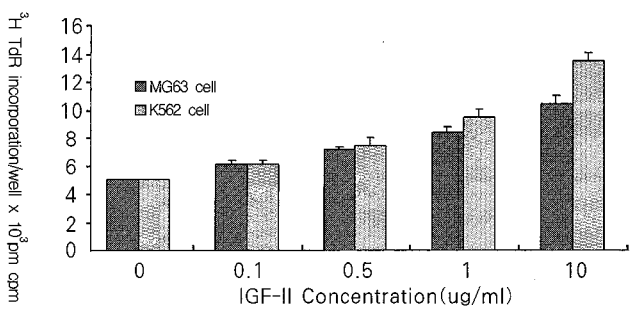


Fig. 8. Stimulation of DNA synthesis by the recombinant IGF-II on MG63 and K562 cell lines. Cells were plated in 24-well plates for 24 hrs. After washing serum-free medium, fresh serum-free medium containing various concentrations of IGF-II was added. DNA synthesis was measured by a 2 hr pulse of [3H]-thymidine after incubation for 24 hrs. Triplicate wells were examined.

5. Northern hybridization

Since IGF-Ⅱ plays an important role in hepatocarcinoma, the effects of crude SBDE on the expression of human IGF-Ⅱ mRNAs in MG63 and K562 cell lines were studied. Incubation of the MG63 and K562 cell lines with the crude extract resulted in a decrease in IGF-Ⅱ mRNA expression within 2 to 4 days after treatment (Fig. 7). Down-regulation of the expression of the IGF-Ⅱ gene in MG63 and K562 cell lines by the extracts was highly dose-dependent in manner. At a concentration of 5 ㎍/㎖, the extract showed little effect on expression of the IGF-Ⅱ gene, however, and at a concentration of 50 to 200 ㎍/㎖, the extract significantly inhibited expression of the gene.

Next, in order to examine whether IGF-Ⅱ gives a mitogenic effects on the cells, incubation of MG63 and K562 cell lines in a dose-dependent manner (Fig. 8). These results indicated that IGF-Ⅱ shows the mitogenic activity of the cells.

Discussion

Many oriental medicines have been shown to be growth-inhibitory against mouse-implanted and allogeneic tumors[18,19]. The extracts of several yeasts have also been shown to manifest similar antitumor activity[20]. The National Research Council in the US has recommended consumption of more plant sources as a means of decreasing the growth of human cancer, based on results of animal experiments and epidemiological researches[21]. Lawson et al.[13] have reported that some indole compounds in plants are effective against cell growth of gastric cancer which is induced by 3,4-benzo[a]pyrene (Bp) and the breast cancer that is induced by 7, 12-dimethyl-benz[a]anthracene (DMBA).

For understanding of the mechanism of antitumor activity of the polysaccharides, which was designated by Nakahara et al.[22] as the "host-mediated defense", and also in order to analyze the active sites of the antitumor activity in polysaccharide, analytical study of the polysaccharide has long been performed. Those interests were because the polysaccharide possesses an extremely large proportion of D-N-acetylglucosamine.

In a previous paper[23], *Phellodendri cortex sheneid* (PCS) was extracted with water and alkali and it was confirmed that the water and alkali extract of PCS showed anti-tumor activity against mouse-implanted Ehrlich-carcinoma solid tumor. The tumor-inhibiting effect was considered to be indirect and host-mediated, and not due to their cytocidal action on tumor cells[23].

The process of chemical carcinogenesis can be divided into three general stages, and chemopreventive agents have been categorized according to the stage that they inhibit[24]. Thus the main feature of models derived from an experimental system is of a discrete, ordered series of changes to which terms such as initiation, promotion, progression and immortality can be applied. Progression, invasion and metastasis, fundamental features of human cancer, occur infrequently in primary animal tumors. Our extract inhibits cellular events associated with tumor initiation, promotion, and progression. In previous studies[12], SBDE showed strong growth-inhibitory activity and cancer chemopreventive activity in assays representing three major stages of carcinogenesis. The extracts was found to act as an antioxidant and antimutagen and to introduce phase Ⅱ drug-metabolizing enzymes (anti-initiation activity); it mediated anti-inflammatory effects and inhibited cyclooxygenase and hydroperoxidase functions (antipromotion activity); and it induced human promyelocytic

leukemia cell differentiation (antiprogression activity). In addition, it inhibited the development of preneoplastic lesions in carcinogen-treated mouse mammary glands in culture and inhibited tumorigenesis in a mouse skin cancer model.

From the results of this study, SBDE showed strong inhibitory effects on the growth and DNA incorporation of MG63 and K562 cell lines. These results are in good agreement with the epidemiological results that some plant extracts exhibit high anticancer effects[25]. Even though the compounds allowing the anti-cancer effects are not identified in this study, some flavonoid compounds such as quercetin and kaempferol are known as anticancer molecules. For further examination of the effects of the extracts on human cancer cells, identification of the active compounds, the mechanisms of the anticancer effect, and in vitro experiments are in progress.

In this study, SBDE also inhibited the expression of IGF-Ⅱ gene. In the liver, it is well known that IGF-Ⅱ functions as autocrine or paracrine mechanisms of hepatocyte growth during malignant transformation[26]. IGF-Ⅱ transcripts are over-expressed in certain embryogenic tumors[27] and many liver neoplasia[28]. SBDE specifically inhibited expression of these IGF-Ⅱ transcripts, suggesting that growth inhibition by the extract is in part mediated through the inhibition of IGF-Ⅱ gene expression.

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