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Inhibition of Hepatocellular Carcinoma Cell Growth by the Extract of *Symphytum officinale* L. and the Possible Mechanisms for this Inhibition

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Abstract

A crude extract of *Symphytum officinale* L.(comfrey) was examined for its ability to inhibit the growth of hepatocellular carcinoma cells and expression of the insulin-like growth factor II(IGF-II) gene. The DNA synthesis of hepatocellular carcinoma cell lines, Hep G2, Hep 3B, and PLC/PRF/5 was inhibited by a crude extract of *Symphytum officinale* in both a time- and a dose-dependent manners. This plant extract also inhibited expression of the IGF-II gene. Since IGF-II exerts a mitogenic effect on Hep G2 cells, these results suggest that the growth inhibition by *Symphytum officinale* extract is, in part, mediated through the inhibition of IGF-II gene expression.

Key words: *Symphytum officinale* L. extract, insulin-like growth factor II, growth inhibition, gene expression

INTRODUCTION

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 necessity to develop basic research and experimental methods in order to estimate more accurately their curative properties and/or side effects is required. *Symphytum officinale* L. (comfrey), a member of the Boraginaceae family(1), has long been known for its medicinal properties. *Symphytum officinale* contains allantoin, which is considered to be responsible for the healing effect of the plant, essential oils, amino acids such as asparagine and choline, pyrolisidinic alkaloids, carbohydrates(2), etc. It has been used to treat external injuries, gastrointestinal diseases, hepatic cirrhosis, and type 2 diabetes(3,4). Recently, it has been demonstrated that a crude water extract of *Symphytum officinale* exerts an antimitotic effect on neoplastic cells(5). In this study, we examined the effect of *Symphytum officinale* extract on the growth of hepatocellular carcinoma cell lines. We found that an extract from the leaves of this plant inhibited the growth of these cell lines and that *Symphytum officinale* extract specifically suppressed expression of the human insulin like growth factor-II gene(IGF-II), known to have a mitogenic effect on these cells. High level of IGF-II mRNA has been reported in human tumors including hepatocellular carcinoma(6). It has been shown that IGF-II is involved in the pathogenesis of cirrhotic/noncirrhotic liver disease to hepatocellular carcinoma. The increased levels of IGF-II mRNAs, especially of the 6.0 and 4.8-kb fetal type transcripts in the tissues and hepatocellular carcinoma cell lines, suggest that the expression of the fetal forms of the RNA transcripts is formed in hepatocellular carcinogenesis(7).

MATERIALS AND METHODS

Preparation of extracts

Freeze-dried leaves(100g) of *Symphytum officinale* were extracted with 10% acetic acid or ethanol, concentrated

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under reduced pressure, and then lyophilized. The dried extract for using was dissolved in a small amount of dimethyl sulfoxide(DMSO).

Cell culture

Hep G2, PLC/PRF/5, and Hep 3B cells were grown in minimal essential medium(MEM) supplemented with 10% fetal bovine serum(FBS). For the growth inhibition assays, the cells were trypsinized, then washed once with growth medium and once with assay medium(growth medium with only 0.2% calf serum). For growth inhibition assays, the cells were plated in either 24-well dishes at 0.5×10^5 cells per 0.5ml per well for [^{3}H]-thymidine incorporation or 6-well dishes at 2.5×10^5 cells per 3ml per well for cell count. For RNA extraction, the cells were plated at 3×10^5cells per 15ml per 150cm^2 dish. In either assay the cells were allowed to attach to the plate overnight before the test substances were added. Human recombinant insulin-like growth factor II and its antibody were purchased from Upstate Biotechnology, Inc(Lake Placid, NY, USA) and a mouse IgG from Sigma(St. Louis, Mo, USA)

Growth inhibition assay

The test substances and carriers were added in 0.5ml of fresh medium and then incubated for the appropriate times as indicated in the figure. The cells were pulse-labeled for two hours with 0.5μCi of tritiated thymidine (Amersham) in 50μl assay medium. The cells were fixed for one hour in a solution of 75% methanol and 25% acetic acid at room temperaure, washed twice with 2ml of 80% methanol, incubated at 37°C with 0.5ml trypsin for 30 minutes, fully solubilized with 0.5ml 1% SDS for 5 minutes and finally transferred to scintillation vials for counting. To determine the cell number, after incubation for three days in the presence of test substances, the cells were trypsinized and counted. Anti-rat IGF-II monoclonal antibody was used to examine whether IGF-II synthesis by the Hep G2 cells was inhibited by the extract.

RNA extraction and northern blots

Total RNA was prepared from Hep G2 cells as previously described(8). 10μg of each sample of total RNA was run on a 1.0% agarose, 1.1M formaldehyde gel, and blotted onto Duralon membrane(Stratagene) in 10×SSC. RNA was cross-linked by exposure to ultraviolet light and then air-dried. Prehybridization, hybridization, and washing protocols were performed as described previously(9). The probes [human IGF-II and glyceraldehyde 3-phosphate dehydrogenase(GAPDH)] were prepared by random-primed labeling of excised insert DNAs

RESULTS AND DISCUSSION

Despite the fact that *Symphytum officinale* has been used in the therapy of various disease for a long time, its real pharmacological properties are yet incompletely known. The recent report(5) that a crude extract of *Symphytum officinale* inhibits proliferation of neoplastic cells prompted us to examine the effects of a crude extract of *Symphytum officinale* on growth of the hepatocellular carcinoma cell lines, Hep G2, Hep 3B, and PLC/PRF/5. DNA synthesis of Hep G2, PLC/PRF/5, and Hep 3B, cells was inhibited by a crude extract obtained from *Symphytum officinale* in both a time- and a dose-dependent manners (Fig. 1, 2, and 3).

The degree of inhibition against Hep G2 was maximal (about 90% at 72h) at a concentration of 100μg/ml of the

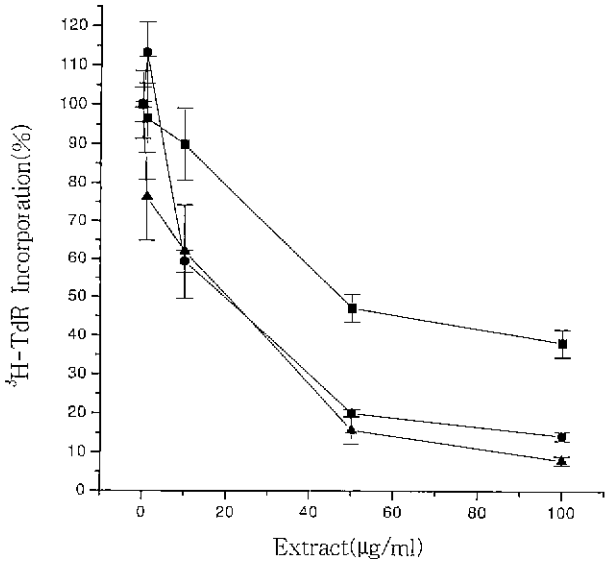

Fig. 1. Tritiated thymidine uptake by Hep G2, Hep 3B, and PLC/PRF/5 cells after treatment with increasing concentrations of *Symphytum officinale* extract.
Hep G2(closed triangle), Hep 3B(closed circle), and PLC/PRF(closed square) cells were plated in 24-well dishes in MEM supplemented with 5% FBS. After 12hr incubation, increasing concentration of *Symphytum officinale* extract were added(A). DNA synthesis was measured by a 2hr pulse of [^{3}H]-thymidine after incubation for 72hr(3 days). Percent growth is defined as the percent of tritium taken up by the treated cells compared to cells receiving only carrier solutions. Triplicate wells were tested.

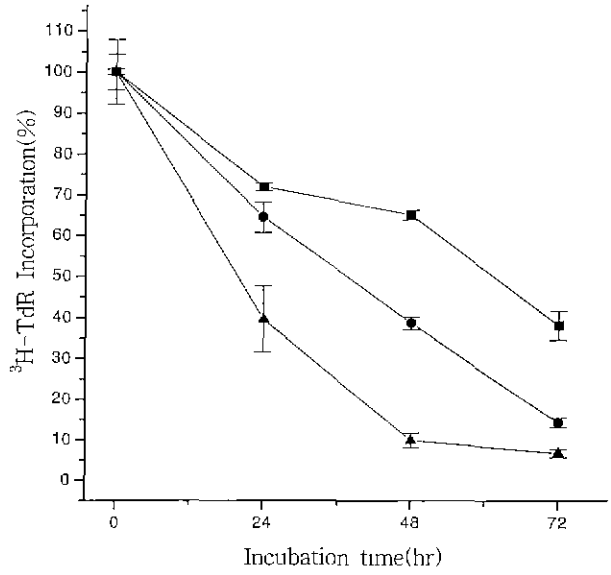

Fig. 2. Time-dependent tritiated thymidine uptake by Hep G2, Hep 3B, and PLC/PRF/5 cells after treatment with *Symphytum officinale* extract.
Values are expressed as % [^{3}H]-thymidine incorporation of extract-treated cells(100μg/ml) versus untreated control(100%). DNA synthesis was measured by a 2hr pulse of [^{3}H]-thymidine after incubation for 24hr, 48hr, 72hr. Hep G2(▲-▲), Hep 3B(○-○), and PLC/PRF/5(□-□). Triplicate wells were tested.

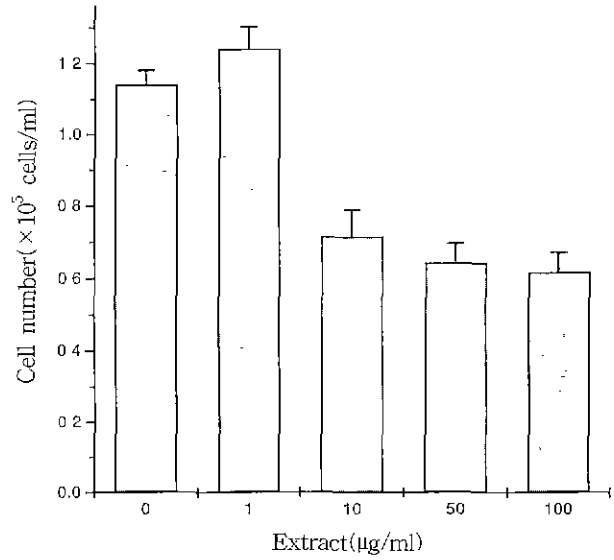

Fig. 3. Determination of Hep G2 cell numbers after treatment with increasing concentrations of *Symphytum officinale* extract.
Hep G2 cells were plated in 6-well dishes and after 12hr incubation increasing concentrations of *Symphytum officinale* extract were added and then three days later the cell number was counted. Triplicate wells were tested.

crude extract(Fig. 1). The inhibition was evident at 48hrs and 72hrs(3 days) after the addition of the crude extract of *Symphytum officinale* in all three cell lines(Fig. 2). Treatment of Hep G2 cells with the crude extract of *Symphytum officinale* resulted in marked decrease in cell number (Fig. 3).

Since IGF-II plays an impotant role in hepatocellular carcinoma, we examined the effects of a crude extract of *Symphytum officinale* on the expression of human IGF-II mRNAs in Hep G2 cells. Incubation of Hep G2 cells with the crude extract resulted in a decrease in human IGF-II mRNAs within 24hrs after treatment(Fig. 4B). Down-regulation of the expression of the human IGF-II gene in Hep G2 cells by the crude extract of *Symphytum officinale* was dose-dependent(Fig. 4A). At a concentration of 1μg/ml, the *Symphytum officinale* extract showed little effect on expression of the human IGF-II gene, but at a concentration of 50 or 100μg/ml, the *Symphytum officinale* extract significantly suppressd expression of the human IGF-II mRNAs.

We next examined whether IGF-II exerts a mitogenic effect on Hep G2 cells. Incubation of Hep G2 cells with human recombinant IGF-II induced DNA synthesis in a dose-dependent manner(Fig. 5). To determine the functional relevance of the production of IGF-II by Hep G2 cells, we assessed the effect of an antibody to IGF-II on the DNA synthesis. Fig. 6 showed that incubation with this antibody resulted in a 30% inhibition of [^{3}H]-thymidine incorporation by Hep G2 cells. Thus, IGF-II appears to play a role in the mitogenic activity of Hep G2 cell.

In this paper, we have shown that *Symphytum offici-*

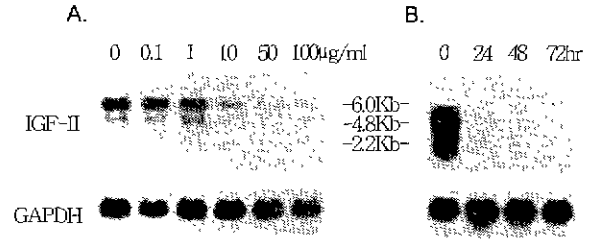

Fig. 4. Down-regulation of steady state human IGF-II gene expression by the extract of *Symphytum officinale*.
Hep G2 cells were treated with different concentrations of the extract of *Symphytum officinale*(0, 0.1, 1, 10, 50, 100μg/ml) for 48hr(A), and Hep G2 cells were treated with its extract(100μg/ml) for 24, 48, and 72hr, respectively(B). The 6.0-, 4.8-, and 2.2-kb transcripts encoded by the third and fourth promoters which are active at fetal stage in the liver cell are expressed in Hep G2 cell at high level. 10μg of total RNA was hybridized to a radiolabeled human IGF-II probe or glyceroaldehyde-3-phosphate dehydrogenase(GAPDH) probe.

nale extract inhibited the growth of hepatocellular carcinoma cell lines. This extract also inhibited the expression of the human IGF-II gene. In the liver, IGF-II is believed to function by autocrine or paracrine mechanisms of hepatocyte growth during malignant transformation (10). IGF-II transcripts are overexpressed in certain embryonic tumors(11) and in many liver neoplasia(12). Increased levels of the fetal form of IGF-II transcripts have been reported in hepatocellular carcinomas. *Symphytum officinale* extract specifically inhibited expression of these fetal forms of IGF-II transcripts, suggesting that growth inhibition by *Symphytum officinale* extract is in part mediated through the inhibition of IGF-II gene expression.

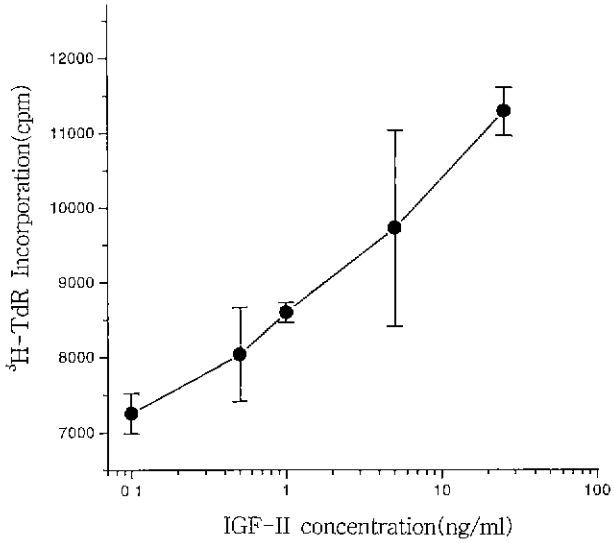

Fig. 5. Stimulation of DNA synthesis by the recombinant human IGF-II.
Hep G2 cells were plated in 24-well dishes for 12hr. After washing with serum-free medium, fresh serum-free medium containing various concentrations of IGF-II was added. DNA synthesis was measured by a 2hr pulse of [^{3}H]-thymidine after incubation for 24 hr. Triplicate wells were tested.

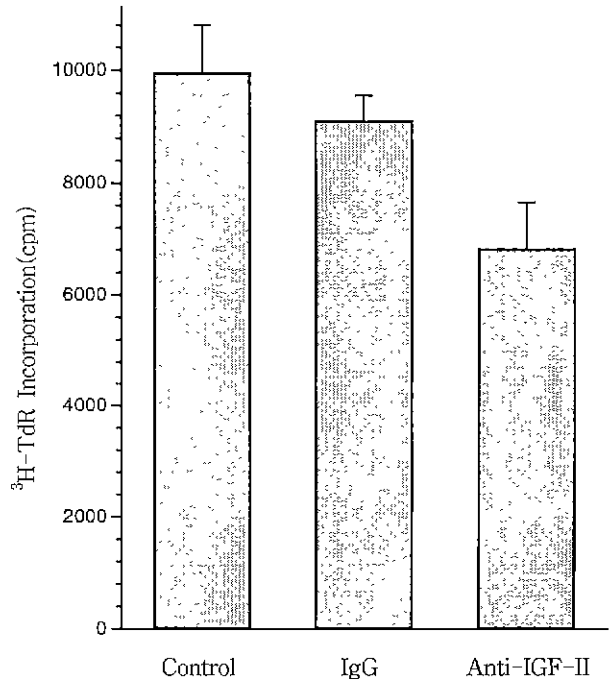

Fig. 6. Antibody to IGF-II partially reverse the DNA synthesis of Hep G2 cell.
Hep G2 cells were plated in 24 well dishes in MEM supplemented with 10% FBS in the presence or absence of IGF-II monoclonal antibody(100μg/ml) or mouse control IgG(100μg/ml) for 24hr. Triplicate wells were tested.

Hepatocellular carcinoma(HCC) is one of the most common and devastating malignant tumors. The major risk factors for the development of hepatocellular carcinoma are now well recognized. Aberrant expression of proto-oncogenes or the expression of mutant forms of these genes may lead to neoplastic transformation. HCC occurs frequently in patients with chronic hepatitis B virus(HBV). The product of the HBV X gene, HBX, was suspected to be a main cause for the carcinogenicity of HBV, which was supported further by reports that HBX gene induced HCC in transgenic mice(13).

Results obtained from IGF-II transgenic mice suggest that IGF-II transgenic animals not only develop HCC but also tumors in other organs that do not express the transgene, indicating that IGF-II may function in oncogenesis by both autocrine and endocrine mechanism(14).

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