Effective therapeutic approaches for liver cancer are expected to be the prevention of chronic inflammation, progression of chronic liver injury to liver cancer and/or tumor cell growth by activating various oncogenes. Activation of the c-jun oncogene occurs in many cases at the early stage of transformation of chronic hepatitis into liver cancer. Accordingly, inhibition of c-jun gene function is thought to be important for the therapy of liver cancer. Although the junB gene has been identified as a c-jun-related gene, it acts as a tumor suppressor gene through competitive binding of JUNB with c-JUN. Therefore, alteration in junB gene expression in chronic hepatitis or liver cancer is an interesting target for the development of both therapeutic treatment and medicines.

Monoammonium glycyrrhizinate (MAG) is used for the treatment of viral hepatitis or the prevention of chronic liver diseases. However, the mechanism by which MAG is involved in the suppression of oncogene function has not yet been characterized. In the present study, we first found that MAG highly stimulated JUNB expression in a human hepatoma cell line, HepG2. We examined the mechanism by which MAG increases junB gene expression by considering the previously published effects of MAG on the onset or development of chronic hepatitis or liver injury. The present data suggest that marked activation of junB expression leads to a pivotal role for MAG in multiple medical applications.

Introduction

Effective therapeutic approaches for hepatocellular carcinoma (HCC) are expected to be the prevention of chronic inflammation of virus-infected liver cells, malignant transformation of chronic liver injury and/or tumor cell growth by various oncogenes. Activation of the c-jun oncogene occurs in many cases at the early stage of transformation of chronic hepatitis into HCC. Accordingly, it has been suggested that the inhibition of c-jun gene function is important for the prevention and therapy of virus-caused liver cancer (1).

The junB gene has been identified as c-jun-related, but the junB gene behaves competitively in function among the jun family genes (2). The JUNB protein is a product of the junB gene and is assembled with other proteins, like FOS or RAF, to form the AP1 complex (activation protein 1). JUNB protein has a similar structure and function to that of the c-JUN protein (c-jun gene product) (3), but it lacks the JNK (c-jun N-terminal kinase) binding site, thus could not be activated by JNK. Because of this property, JUNB hampers malignant cell growth by competitive binding with c-JUN (4), whereby junB gene acts as a tumor suppressor gene.

It has been reported that when the JUNB protein was increased in the cell, inflammation, virus infection and tumor cell growth were reduced (4-9). In another case, a junB gene-defective knock-out mouse became lethal at 12.5-13.5 days of fetal development by hypoplastic placentation caused by anangioplastia (10). Thus, alteration in junB gene expression as well as its regulatory mechanism in chronic hepatitis or HCC could be an important target in developing both therapeutic treatment and medicines.

Glycyrrhizin (glycyrrhizic acid) extracted from the plant, Glycyrrhiza (11), and monoammonium glycyrrhizinate (MAG) are used as anti-inflammatory drugs for viral hepatitis or for the prevention of chronic liver diseases. Immunoregulatory activity of MAG has been reported in the regulation of T cell activation, induction of interferon, activation of NK cells, and the activation of extrathymic T lymphocyte differentiation (12,13). MAG was also found to inhibit or inactivate viral growth and the survival time of mice infected with hepatitis virus was found to be elongated by administration of MAG. In another study, repression activity of MAG was reported in herpes virus replication and inactivation (14,15).

MAG and anti-oxidization chemicals, such as N-acetyl cysteine (NAC) and glutathione (GSH), are widely used for therapeutic treatment of liver toxicity or the prevention of chronic liver damage. However, in contrast to the case of NAC or GSH, at the molecular level, the mechanism by which MAG acts to suppress inflammation or is involved in the regulation of proto-oncogene and/or tumor suppressor gene remains obscure.
In this study, we focused on the drug-induced activation of the junB gene as a tumor suppressor gene. Interestingly, we first found that MAG highly stimulated the expression of both junB mRNA and JUNB protein in a human hepatoma cell line, HepG2. Next, we analyzed the possible mechanism by which MAG increases junB gene expression by considering the previously published therapeutic effects of MAG on the onset or development of chronic hepatitis and liver cancer development. The present data strongly suggest that the activation of junB expression explains multiple roles of MAG in reducing inflammation, virus growth and liver carcinogenesis, as has been previously reported in the clinical literature (11-15).

Materials and methods

Therapeutic drugs and transcription inhibitors. Therapeutic drugs used in the interaction study with the junB gene were MAG and N-acetylcysteine (NAC) (both from Wako Pure Chemicals, Osaka Japan) at each concentration indicated in the figure legends. Transcription inhibitors used were: U0126 (ERK inhibitor) (16), SB203580 (p38 inhibitor) (both from Wako Pure Chemicals), JNKII (JNK inhibitor) (17), AG490 (JAK inhibitor) (18), and PKC inhibitor peptide (PKC inhibitor) (all from Calbiochem-Merch Biosciences, Tokyo Japan) in each indicated concentration, which was determined based on the previous reports of their IC50 values (50% inhibitory concentration).

Cell line and culture conditions. Cell line HepG2 was derived from a human hepatoblastoma. Cells were cultured in the Dulbecco's modified Eagle's medium (DMEM: Nissui Pharmaceutical Co., Tokyo Japan) supplemented with 10% fetal bovine serum, 10% sodium bicarbonate, 60 μg/ml kanamycin and 8 mM glutamine, using a 60-mm culture dish or fetal bovine serum, 10% sodium bicarbonate, 60 μg/ml kanamycin and 8 mM glutamine, using a 60-mm culture dish or

Western blot analysis. Nuclear proteins were extracted from harvested cells using the Qproteome nuclear protein kit (Qiagen Inc.) and the protein concentration was measured by the BioRad protein assay (Bio-Rad Laboratories, Hercules CA, USA) (20). Next, 6X loading buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 10% DTT, 20% glycerol, 0.25% BPB) was added to each nuclear protein extract of 30 μg. Samples were denatured for 5 min at 100˚C, and subjected to SDS-PAGE (94˚C for 15 sec; 60˚C for 25 sec; and 72˚C for 12 sec), and melting curve analysis of the amplified sample was performed under the conditions of 95˚C for 0 sec; 58˚C for 15 sec, and 95˚C for 0 sec, by increasing the temperature at intervals of 0.5˚C from 58˚C. The same experiments as for junB were carried out with c-jun using the same cDNA sample with c-jun real-time PCR primer sets (Fig. 1; Takara Bio Inc.).

Quantification and normalization of amplified samples were performed using human GAPDH gene mRNA as the control with the LightCycler Faststart DNA Master SYBR-Green 1 (Roche Diagnostics K.K.) and the LightCycler-Primer set human GAPDH (Roche Diagnostics K.K.). The human GAPDH (glyceraldehyde-3-phosphate dehydrogenase, acc. no. M33197.1) primer set consisted of, forward: AGCC ACATCGCTTCAGAC, and reverse: GCCCAATAGC CAAATCC.

 Luciferase assay. Cells were plated in 24-well dishes at the density of 5x10^4 cells/well, incubated for 18 h in 5% CO₂ atmosphere and subjected to transfection using the vector mixture, which was prepared as indicated below for 1 h in a CO₂ incubator. Cells were then incubated for 48 h after the addition of serum containing DMEM medium. Vector mixture was prepared from serum-free DMEM medium containing 2.5 μg/ml test vector and 50 ng/ml of the internal control vector pLR-TK and three times its volume of Ribofection Tfx-20 Reagent (Promega KK).

After incubation, the drug was added to the wells and the cells were incubated for 6 h, and then washed with PBS. Luciferase activity in the lysate was monitored using the Dual Luciferase assay system (Promega KK) following the manufacturer's protocol. Both luciferase activities in the cell lysates were measured by adding two luminescence reagents to the cell lysates, one for the test vector and another for the internal control vector.

Real-time RT-PCR. junB DNA was amplified from cDNA by the Quantitect SYBR-Green PCR kit (Qiagen Inc.) and the amplified junB copy number was quantified using the junB primer set (Fig. 1; Takara Bio Inc.) prepared specifically for a real-time RT-PCR reaction. Amplified conditions used were 95˚C for 15 min for the initial activation, followed by 30 cycles (94˚C for 15 sec; 60˚C for 25 sec; and 72˚C for 12 sec), and melting curve analysis of the amplified sample was performed under the conditions of 95˚C for 0 sec; 58˚C for 15 sec, and 95˚C for 0 sec, by increasing the temperature at intervals of 0.5˚C from 58˚C. The same experiments as for junB were carried out with c-jun using the same cDNA sample with c-jun real-time PCR primer sets (Fig. 1; Takara Bio Inc.).

Expression of junB mRNA is highly increased by MAG treatment. To analyze how junB mRNA expression is affected
by MAG. HepG2 cells were treated with 10 mg MAG in a DMEM/60-mm dish for 24 h and mRNA was extracted from the treated cells or untreated control cells, and then subjected to real-time RT-PCR analysis for copy number detection. A >5-fold increase in junB mRNA expression was observed in MAG-treated cells as compared to the untreated cells. When the same samples were subjected to c-jun mRNA expression analysis, the level of c-jun mRNA in MAG-treated cells exhibited a roughly 2-fold increase (Fig. 2A).

As previous literature has indicated that NAC activates the ERK kinase cascade (21) and that junB gene expression is activated by ERK (22), the effect of NAC on junB mRNA expression was examined as a reference. A roughly 2-fold increase in junB mRNA expression was observed in MAG-treated cells as compared to the untreated cells. When the samples were subjected to c-jun mRNA expression analysis, the level of c-jun mRNA in MAG-treated cells exhibited a roughly 2-fold increase (Fig. 2A).

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**JUNB protein is highly increased in the presence of MAG.** Nuclear proteins were extracted from cells treated with MAG and the expression of both JUNB and c-JUN proteins was studied by Western blot analysis. Data clearly showed a marked increase in JUNB expression in the presence of MAG, while c-JUN expression exhibited no increase as compared to the control. There also was no increase in SPI protein expression, as shown in Fig. 3A. Some other proteins, FOS, NF-xB, JUND etc., were examined, but no significant increase was observed in their expression (data not shown). In addition, the effect of NAC on JUNB protein expression was also examined in the presence of 10 mM NAC. Nuclear extracts from 10 mM NAC-treated and untreated cells were analyzed, however, an increase in JUNB protein expression was not detected in NAC-treated cells as compared to the untreated cells. There was no increase in c-JUN or SPI protein expression as compared to the control (Fig. 3B).

**Signal transduction cascade encourages MAG activation of junB gene expression.** To identify which signal transduction cascade is targeted by MAG in the case of junB gene expression, junB mRNA expression was monitored in the presence of a specific inhibitor of the signal transduction cascade after
the addition of MAG. Inhibitors used were U0126 for ERK, SB102538 for p38, JUK11 for JNK, AG490 for JAK and peptide inhibitor for PKC.

Expression of junB mRNA was investigated in the presence or absence of each inhibitor in the MAG-treated or control HepG2 cells. Results indicated that MAG-induced expression of junB mRNA was not blocked by the addition of any inhibitor (Fig. 4). The results suggested that MAG activation of junB gene expression occurs through a different pathway from that of the signal transduction cascades described above.

As a control experiment, analysis of junB mRNA expression was carried out in the presence or absence of each signal transduction cascade inhibitor using NAC-treated or -untreated cells. When the extracted mRNA was subjected to real-time PCR analysis, the ERK inhibitor, UO126 suppressed NAC-induced increase of junB mRNA (Fig. 5A). However, no other inhibitors exhibited their inhibitory effects on NAC-induced junB mRNA (Fig. 5B and C). The present data are consistent with the previous observation, in which NAC activation of junB gene expression was due to involvement of the ERK kinase cascade (23,24).

Analysis of the human junB gene promoter region. To understand the mechanism of human junB gene regulation by MAG, we focused on the 5-kb upstream region containing the enhancer and core promoter sequences of the mouse junB gene, which are well-characterized. The 5-kb upstream sequences of the mouse junB gene were obtained from Ensemble Genome Browser (25) and their homology with the human junB gene was searched using VISTA (26).
3-kb upstream region of the human junB gene, but there was no significant homology in the 3-5-kb upstream sequences, as was previously described (Fig. 6A) (27,28). This 3-kb upstream region was therefore amplified by PCR and introduced into the pGL4 luciferase vector. The fusion vector (named 3kbUP vector) was then transfected to HepG2 cells at concentrations of 0.25-2 μg of 3kbUP vector DNA/ml, and promoter activity of the transfected DNA was assayed. Strong luciferase activity was detected in a concentration-dependent manner, indicating that the promoter-enhancer activity was located within the 3-kb upstream region of the human junB gene (Fig. 6A), similar to the mouse junB gene (27,28).

In addition to the 3kbUP vector, four mutant vectors that contained upstream 2.5-kb (del1), 1-kb (del2), 0.5-kb (del3) and 150-bp (del4) sequences were constructed and transfected to cells at the concentration of 0.5 μg DNA/well, and the promoter activities were examined using a dual luciferase assay. Results indicated that del1 and del2 mutants showed a slightly higher activity than that of the 3kbUP vector and that luciferase activity was then decreased according to reduction of the enhancer region (Fig. 6B). The present data are consistent with previous observations, in which SRE2 was found to be present between an upstream 3.0-2.5-kb region and exhibited a negative effect on promoter activity (27,28).

Furthermore, it is clear that the del4 mutant containing a 150-bp upstream region showed significant promoter activity as compared to that of the control vector without a 150-bp upstream region (Fig. 6C), which corresponds to the core promoter region of the junB gene (27,28), where the IL6-responsive element and TATA box are located (29).

To investigate whether MAG-induced junB mRNA expression was dependent on the junB promoter region or not,
luciferase activity of 3kbUP or the deletion vector-transfected cells was examined in the presence of MAG. Results indicated that luciferase activity was not increased by MAG in the case of 3kbUP and luciferase activity was rather decreased by MAG, when compared to the control (Fig. 6D). Activity assay was also carried out using del2- or del3- vector, but the promoter activity was not changed by MAG treatment (data not shown). The present observations indicated that expression of the endogenous junB gene was increased by MAG, but it was not the case in the exogenously introduced luciferase system. One explanation may be an absence of the chromatin structure in the enhancer region in the 3.0-2.5-kb upstream region of the junB gene in the exogenously transfected DNA vector (30). The chromatin structure of the upstream 3.0-2.5-kb region containing the SRE2-responsive element may play an important role in the activation of endogenous junB gene expression by MAG.

On the other hand, we examined the NAC effect on the promoter activity using 3-kb UP or the deletion mutant vector. Luciferase activity of 3-kb UP was found to be increased by NAC (Fig. 6E). Luciferase activity of the deletion mutant, except for the del4 mutant, was also increased by NAC. The present data are consistent with the previous observations indicating that the NAC-responsive element was present in a 500-150-bp region, where the Ets-binding and CRE-like sites were located (27,28).

Figure 5. Effect of inhibitor on junB mRNA expression in the presence of NAC. Cells were plated at the density of 1x10^6 cells/60-mm dish and incubated for 18 h, and further cultured in the presence or absence of 10 mM NAC with or without MAPK inhibitors (A), JAK inhibitor (B) or PKC inhibitor (C). Expression of junB mRNA was examined, as described in the legend of Fig. 2. The data represent the mean ± SD. *P<0.05 versus control.
Discussion

The junB gene exhibits multiple functions; for instance, the anti-inflammatory effect of the junB gene product has been reported in psoriatic erythroderma, a form of dermatopathy, as junB gene expression was found to be decreased in the affected part (31). In the case of mouse skin, inflammation was induced by down-regulation of junB gene expression. 

Figure 6. Promoter activity analysis of the 3-kb upstream region in the junB gene. (A) Schematic presentation of the 3kbUP vector and deletion mutants in the junB gene promoter region. Shaded region indicates homology between mouse and human junB genes. For construction of the plasmid vector DNA containing the promoter region of the junB gene, a 3-kb upstream region of junB DNA was amplified by a PCR reaction using the junB primer set (Fig. 1A; Operon Biotechnologies) that was designed to introduce the EcoRV site at the 5’ end and HindIII site at the 3’ end of the target DNA. Amplified DNA was purified with the QIAquick gel extraction kit after separation by agarose gel electrophoresis. Purified DNA and luciferase vector pGL4.12 (luc2CP; Promega KK) were subjected to restriction enzyme digestion with EcoRV and HindIII, followed by purification with the QIAquick PCR purification kit (Qiagen Inc.) and the plasmid vector DNA was constructed using the DNA ligation kit (Takara Bio Inc.). Plasmid vector DNAs were then introduced into DH5α competent cells and cultivated in LB Broth (Difco BD) before purification with Plasmid maxi kit (Qiagen Inc.). Mutant vector that contained upstream 2.5-kb (del1), 1-kb (del2), 0.5-kb (del3) or 150-bp (del4) was constructed, as described above, and transfected to cells at the concentration of 0.5 μg DNA/well. (B) Relative promoter activity of deletion mutants. (C) Promoter activity of the del4 mutant or a vector without the upstream region of the junB gene. Vertical axis of (B) or (C) shows the relative promoter activity of the 3kbUP or the del4 mutant as 1.0, respectively, after calibration of promoter activity using the pLR-TK control vector. Data represent the mean ± SD of at least three independent experiments. (D) Promoter activity of the 3-kb upstream region in the presence of 1 mg/well MAG. Vertical axis shows the relative promoter activity, as described in (D). The data represent the mean ± SD of five independent experiments. *P<0.05 versus control.
JUNB forms the AP1 complex with a transcription factor like FOS and is involved in cell cycle regulation as a regulatory factor. The AP1 complex with JUNB activates p16 and down-regulates cyclin D1, thereby causing suppression of G1-to-S transition and the inhibition of cell growth (34-35). So far, TAK1, SRE1 binding protein, c-Ets and IL6 have been reported as activators of junB gene expression. These factors are involved in junB gene expression via MAP kinase pathways like p38, and ERK or via the JAK/STAT pathway (22,27,28,36-44). A previous report verified that p38 was activated by various stress conditions, such as osmotic stimulus or cytokine, suggesting its relationship to cell death (45-49). ERK induced cell growth and differentiation and regulated cell cycle transition (50-52). JAK/STAT was activated by a free radical and/or IL6 (53-55).

As it has not yet been reported how MAG interacts with junB activating factors, we firstly focused on the reported result that MAG amplified the IL12 molecule, which is known to have anticancer action via the NF-kappa B transcription factor and/or SRE2(27) of the junB gene exist within an upstream 2.5-kb region (28). Although NF-kB is activated in cancer cells, improvement of the immune system and enhancement of junB gene expression have been reported (57). However, the therapeutic effect of drugs on the activation of junB gene expression in liver carcinogenesis has not been reported previously. In this study, we first demonstrated that MAG had a specific stimulatory effect on junB gene expression in HepG2 cells. We then examined a possible mechanism of MAG action using inhibitors for signal transduction cascades (58).

Based on the observation that MAG-induced expression of mRNA was much higher for the junB gene than the c-jun gene and that the functional level of JUNB protein was much higher than that of c-JUN, the role of MAG would most likely be the suppression of AP-1 function. Studies with five inhibitors of different signaling cascades revealed no influence on the MAG-induced activation of junB mRNA expression. From these observations, MAG activation of endogenous junB gene expression is predicted to be dependent on other signaling pathways than signaling cascades, such as MAP kinase, JAK/STAT and PKC cascades (56-58).

Promoter-enhancer activity assay using the 3-kb UP region of the junB gene or its deletion mutants, revealed that del1 and del2 deletion mutants showed higher activity than that of 3kbUP-transfected cells. Mutant del4 showed lower activity because of the length of the inserted enhancer region. The present results suggested that binding site(s) for an inhibitory factor and/or SRE2(27) of the junB gene exist within an upstream 3-2.5-kb region (27) and that binding sites for other factors, such as CRE, CRE-like and STAT family proteins etc., are situated within an upstream 2.5-kb region (28).

Expression of endogenous junB gene is increased by MAG, but not in an exogenously introduced expression system. Activity of the del2 mutant was found to be unchanged in the presence of MAG. Furthermore, the core promoter activity of del4, del3 or del2 was not changed at all by MAG. Considering the result that MAG activated endogenous junB mRNA expression, it is less probable that MAG affects the 2.5-kb region and increases junB gene expression. According to the previous and present observations, the NAC responsive site is located within a 500-bp upstream region, in which the level of promoter activity and mRNA expression were parallel, and the MAG responsive region may be located further upstream in the 2.5-3-kb region. The structure of exogenously introduced junB DNA fused to the luciferase gene was different from the chromatin structure of the endogenous gene (30). Transfected 3-kb UP DNA in the junB gene would not sustain the chromatin structure of MAG interaction site in cells.

Lastly, it should be emphasized that MAG clearly activates junB gene expression as seen in both the mRNA and protein levels in the present study, although the mechanism by which MAG increases the amount of mRNA has not yet been clarified. Marked stimulation of junB gene expression may explain the multiple pharmacological effects of MAG described in the previous clinical literature (11-15). Further experiments are needed to clarify the mechanism of stimulation by MAG of cellular junB gene expression.

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