Tumorigenesis and Neoplastic Progression

The Induction of Growth Arrest DNA Damage-Inducible Gene 45 β in Human Hepatoma Cell Lines by S-Adenosylmethionine

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Down-regulation of GADD45 β , which is known to influence cell growth control, apoptosis, and cellular response to DNA damage, has been verified to be specific in hepatocellular carcinoma and consistent with the degree of malignancy. Here, we identified promoter elements for several transcriptional factors in the proximal promoter of GADD45 β using the luciferase assay. As a methyl donor for biological transmethylation reactions, S-adenosylmethionine (SAdMe) could restore GADD45 β expression in HepG2 in Northern blot analyses and quantitative real-time polymerase chain reaction. Activity and binding capacity of nuclear factor (NF)- κ B were confirmed to be specifically induced by SAdMe, as evidenced by electrophoretic mobility shift assay, enzyme-linked immunosorbent assay, and a decrease of I κ B α in Western blot analyses. The most upstream NF- κ B-binding site was crucial for transcriptional activation. In contrast to NF- κ B, although there is an E2F-1-binding site adjacent to the NF- κ B sites, treatment with SAdMe could not induce E2F-1-binding activity. Despite showing a similar GADD45 β promoter regulatory pattern as HepG2 (p53 wild type), Hep3B (p53-null) did not exhibit GADD45 β induction by SAdMe, and the induction could be partially recovered on reconstituting p53 in Hep3B. Thus, our results suggest that GADD45 β induction by SAdMe via NF- κ B may represent a novel mechanism of SAdMe-mediated hepatoprotection, with p53 playing an important role. (*Am J Pathol* 2007, 171:287–296; DOI: 10.2353/ajpath.2007.070121)

cancer in some geographic areas, particularly in the Far East, South Sahara, and southern Europe. Recent epidemiological data suggest that the incidence of HCC is increasing in Western countries.¹ In many cases, HCC is known to result from environmental exposures such as hepatitis virus, aflatoxin, alcohol, or other *in vivo* or *in vitro* genotoxins. Human HCC-prone disorders, such as liver cirrhosis, share a very close relationship to genotoxic DNA damage and mutations of known DNA repair genes.² However, the data to support a role for DNA damage in hepatocarcinogenesis are still quite limited.

Growth arrest DNA damage-inducible gene 45 β (GADD45 β , also named MyD118) gene was first identified as a myeloid differentiation primary response gene activated by interleukin-6 in the mouse myeloid leukemia cell line M1 on induction of terminal differentiation, which has been implicated in regulating cell growth, apoptotic cell death, and cellular responses to DNA damage.³ In our previous study, we have demonstrated that GADD45 β was underexpressed in HCC specifically and significantly. More importantly, we observed that down-regulation of GADD45 β was strongly correlated with HCC-poor differentiation and advanced nuclear grade.⁴ Our results suggested that the specific lack of GADD45 β expression might play an important role in hepatocarcinogenesis. Although hypermethylation in proximal promoter of GADD45 β was confirmed in our previous study, the molecular basis of GADD45 β down-regulation in HCC was far from clear. Several transcriptional regulatory regions containing nuclear factor (NF)- κ B- and E2F-1-binding areas were also identified by means of luciferase assay, but functional evidence and transcriptional regulation mechanism need further elucidation.⁵

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Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and the most common

S-Adenosylmethionine (SAME) is an essential compound in cellular transmethylation reactions and serves as a methyl donor in numerous metabolic reactions. SAME is an important precursor in the synthesis of polyamines and glutathione, the main cellular antioxidants in the liver.⁶ In liver injury or chronic liver diseases, the synthesis of SAME is impaired, which might lead to dedifferentiation of hepatocytes with increased regeneration and malignant transformation.⁷ SAME administration attenuates experimental liver damage, improves survival of patients with alcoholic cirrhosis, and prevents experimental hepatocarcinogenesis.^{8–10} Although there is accumulating evidence on the protective potential of SAME in the preservation of liver function, the molecular mechanism of SAME's hepatoprotection is primarily unidentified and needs further exploration.

In this study, based on the significant induction of GADD45 β by SAME, binding capacity and activity of transcriptional regulators in proximal promoter were investigated using luciferase assay, electrophoretic mobility shift assay (EMSA), Western blot, and enzyme-linked immunosorbent assay (ELISA). With the help of a special set of research models, HepG2 (p53 wild type) and Hep3B (p53-null), the role of p53 in transcriptional regulation was also analyzed by p53 transfection.

Materials and Methods

Cell Culture, SAME Treatment, and RNA Extractions

The human hepatoma cell lines HepG2 and Hep3B and normal human embryonic liver cell line CL-48 were purchased from American Type Culture Collection (Rockville, MD) and cultured in high-glucose Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% P/S (100 U/ml penicillin and 100 μ g/ml streptomycin) at 37°C and 5% CO₂. The day before SAME (Knoll Farmaceutici S.P.A., Milan, Italy) treatment, logarithmically growing cells were seeded at a density of 1×10^6 cells per 25-cm cell culture dish. On the 2nd day, cells were treated with SAME (0, 0.5, and 1.0 mmol/L). Forty-eight hours after treatment, media were removed, and cells were washed with phosphate-buffered saline (PBS). Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA). RNA quality was tested by running on a 1.2% diethyl pyrocarbonate/3-(*N*-morpholino)propane-sulfonic acid agarose gel, and the concentration was measured by UV spectroscopy. RNA was stored in diethyl pyrocarbonate water with 10 mmol/L dithiothreitol and RNasin (1 U/ml) at –70°C.

Induction of GADD45 β by SAME

We have previously reported that the low-expression of GADD45 β was specific to HCC and was consistent with the degree of malignancy of HCC. In this study, Northern blot and quantitative real-time polymerase chain reaction (PCR) were used to examine GADD45 β expression change after SAME treatment. In Northern blot, prepara-

tion of ³²P-labeled GADD45 β probe and blot conditions were the same as previously described.⁴ In brief, the 222-bp probe, including exon 3 of GADD45 β , was generated by reverse transcriptase-PCR with the following primers: 5'-GGACCCAGACAGCGTGGTCCTCTG-3' (sense primer, GADD45 β +247) and 5'-GTGACCAGGAGACAATGCAGGTCT-3' (anti-sense primer, GADD45 β +445). The probe was purified using a gel extract and purification kit (Qiagen) and labeled with ³²P using the random priming probe kit from Roche (Indianapolis, IN). After isolation from HepG2 and Hep3B with or without treatment, total RNA was electrophoresed in a 1.2% formaldehyde-agarose gel, blotted to a Hybond-N membrane (Amersham, Arlington, IL) and UV cross-linked. The blots were hybridized for 1 hour at 68°C and washed with 2 $\times$ standard saline citrate/0.1% sodium dodecyl sulfate and 0.1 $\times$ standard saline citrate/0.1% sodium dodecyl sulfate at different temperature. After hybridization, membranes were exposed to phosphorimager screen for 18 hours and then read by phosphorimager scanner. Quantitative analysis was performed using ImageQuant version 5.0 (Molecular Dynamics, Sunnyvale, CA) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. All experiments were performed in triplicates.

In quantitative real-time PCR analyses, total RNA was treated with RNA-free DNase I (Promega, Madison, WI) and reversely transcribed to cDNA using AMV-RT and Oligo(dT)12-18 primer. GADD45 β was amplified and detected using the following TaqMan probe and primers: 5'-GGGTGTACGAGTCGGCCAA-3' (forward), 5'-TGGC-CAAGAGGCAGAGGA-3' (reverse), and 5'-FAM-TTGATGAATGTGGACCCAGACAGCGTG-TAMRA-3' (probe). The predeveloped TaqMan assay reagents control kit (Perkin-Elmer Applied Biosystems, Foster City, CA) was used to detect GAPDH as an internal control. GADD45 β -pEGFP and GAPDH-pT7T3D-PAC plasmids were used as positive controls to generate the standard curve. PCR reaction system was as described previously (Ref. 5): included cDNA, 5 pmol of each primer, 10 pmol of probe, and TaqMan Universal PCR Master Mix. The PCR conditions were as follows: one cycle of 50°C for 2 minutes, 95°C for 10 minutes, and 35 cycles of 95°C for 15 seconds, 60°C for 60 seconds. Each data point was performed in duplicates.

Influence on GADD45 β Proximal Promoter Activity by SAME

In our previous study, we identified the active promoter region of GADD45 β and the possible regulation mechanism. Based on marked induction of GADD45 β by SAME, we further examined the change of proximal promoter activity to provide functional evidence for promoter regulation hypotheses. Luciferase reporter GADD45 β proximal promoter deletion plasmids were constructed as previously described.⁵ In brief, CL-48 genomic DNA was used as a PCR template. Two GADD45 β promoter deletion fragments, spanning –618 to +6, were generated by PCR with sense primers: 5'-GGGAAAGCTTCGGTCCG-

GGACT-3' (−618), 5'-TTTAAAGCTTTTCTGGCATTG-C-3' (−470), and anti-sense primer 5'-TATCTCGCCA-AGGACTTTGC-3' (+6). PCR products were purified and cleaned up using a gel extract and purification kit (Qia-gen) and then cloned into the pDrive cloning vector (Qia-gen) via U-A nucleotide matching. After digestion from pDrive plasmids using *Hind*III, those sequences with another seven fragments were cloned upstream of the luciferase gene in the pGL3 basic luciferase expression plasmid (Promega) via corresponding enzyme digestion sites. Another seven detailed proximal promoter fragments covering active promoter region from −618 to −273 were generated by PCR with the following primers: 5'-CGGAGGTACCGGGGATTCCAGGCCCGCCCGA-3' (−591), 5'-CTCGGGTACCGGAAATCCCGCGCGCGCCCGA-3' (−547), 5'-CCCCGGTACCGCGGCTCGGCTGCGGGAA-3' (−520), 5'-CGGCGGTACCGCGCCCTCCTCCCGGT-3' (−436), 5'-GCCCGGTACCGCGCTCCTCCCGTCCCTCCG-3' (−391), 5'-CGCAGGTACCGCTGACTCGCCCTT-3' (−348), 5'-CAATGGTACCGGCGAATGACTCCA-3' (−314), and anti-sense primer to +6 (5'-CTTCTCGAGCATGTTGCAATTATAATCCAC-3'). Because *Kpn*I site and *Xho*I enzyme digestion sites were incorporated into sense primers and anti-sense primer, respectively, promoter fragments were obtained by *Kpn*I and *Xho*I digestion. After cleaning by phenol-chloroform extraction and ethanol precipitation, seven fragments (−591/+6, −547/+6, −520/+6, −436/+6, −391/+6, −348/+6, and −314/+6) were cloned into the corresponding sites of the pGL3 basic plasmid. DNA sequencings were confirmed in the City of Hope National Medical Center Sequence Laboratory.

The transfection of reporter plasmids was performed as described before.⁵ In brief, 15 μ g of pGL3 promoter luciferase reporter plasmids were transfected into HepG2 and Hep3B with 7.5 μ g of pSV- β -galactosidase control vector (Promega). Cells were transfected by electroporation in a 4-mm gap cuvette (Eppendorf, Hamburg, Germany). The electroporation parameter was 50 μ s at 600 V for HepG2 and 80 μ s at 650 V for Hep3B. SAME was administrated to cells 48 hours after transfection. After an additional 48 hours of culture, cells were harvested by scraping directly into 0.9 ml of reporter lysis buffer (Promega). With standardized protein concentration, the luciferase activity in 20- μ l aliquots of cell lysates was measured then by luminometry using luciferase reagent (Promega). β -Galactosidase activity was determined using a β -galactosidase assay system (Promega). Promoter activation was determined as the luciferase activity relative to the control after normalizing to β -galactosidase activity. HepG2 and Hep3B without SAME treatment were included as reaction control. pGL3 basic plasmid, pGL3 enhancer plasmid, and pGL3 promoter plasmids were also included for system controls.

Isolation of Nuclear Protein

HepG2 and Hep3B cells (1×10^7) with or without SAME were washed in ice-cold PBS and suspended in 800 μ l of solution A (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1

mmol/L ethylenediamine tetraacetic acid, 0.1 mmol/L ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 1 mmol/L dithiothreitol, and 0.5 mmol/L phenylmethyl sulfonyl fluoride) for 15 minutes. Fifty μ l of Nonidet P-40 was added, and cell pellets were harvested and vortexed for 10 seconds. After centrifugation at 10,000 rpm for 30 seconds at 4°C, the cell pellets were resuspended in 100 μ l of solution B (20 mmol/L HEPES, pH 7.9, 0.4 mol/L NaCl, 1 mmol/L ethylenediamine tetraacetic acid, 1 mmol/L ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 1 mmol/L dithiothreitol, and 1 mmol/L phenylmethyl sulfonyl fluoride) and vigorously rocked for 15 minutes at 4°C. Cell lysates were centrifuged (10,000 rpm, 15 minutes, 4°C), and then the supernatant was collected (~55 μ l) and stored at −80°C. Protein concentration was measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Human oropharyngeal carcinoma KB cells and human prostate cancer PC3 cells (both were purchased from American Type Culture Collection) were cultured in RPMI 1640 medium with 10% fetal bovine serum and 1% P/S used for non-HCC cell line control. The treatment of SAME and nuclear protein extraction were performed in the same way as mentioned above.

Electrophoretic Mobility Shift Assay

Three NF- κ B- and one E2F-1 9-binding sites were found in the GADD45 β proximal promoter (positions −602/−593, −581/−572, −537/−528, and −452/−444). Because identification of NF- κ B and E2F-1 were based on consensus sequences and high score in TRANSFAC database alignment search, electrophoretic mobility shift assay (EMSA), and super shift assay were used to characterize further the functional evidence for these sites in this study. Five oligonucleotide probes for putative NF- κ B-binding sequences and two probes for E2F-1 were designed and synthesized by the City of Hope National Medical Center DNA/RNA/peptide synthesis facility. As shown in Figure 4A, probe 1 contained the first and second NF- κ B-binding sites. The first site was mutated in probe 2, whereas the second site was mutated in probe 3. Both sites were mutated in probe 4, and probe 5 included only the second NF- κ B site. Sequences of the NF- κ B probes were as follows: probe 1: 5'-TCCGGGACTCTCCGCGGATCGGGAGGGGATTCCAGG-3'; probe 2: 5'-TCCTATTTCTCTCCGCGGATCGGGAGGGGATTCCAGG-3'; probe 3: 5'-TCCGGGACTCTCCGCGGATCGGGAAATCCATTCCAGG-3'; probe 4: 5'-TCCTATTTCTCTCCGCGGATCGGGAAATCCATTCCAGG-3'; probe 5: 5'-TCGCGCGCTGGAAATCCCGCG-3'. The two probes used for E2F-1 (wild type and mutant) were: probe 6: 5'-CTTTTC-TGGCATTCGCGGTACCTACCCG-3' (wild type); probe 7: 5'-CTTTTCTGGCATTTCGATTTCACCTACCCG-3' (mutant). Mutated bases are underlined. DNA fragments were annealed by incubating sense and anti-sense DNA strands at 72°C for 10 minutes followed by slow cooling to room temperature and then labeled with ³²P- γ -ATP using T4 polynucleotide kinase. Unincorporated nucleotides were removed using Micro Bio-spin P30 chromatography

columns (Bio-Rad). DNA-protein binding reactions were performed in solution with 4% glycerol, 1 mmol/L MgCl_2 , 0.5 mmol/L ethylenediamine tetraacetic acid, 0.5 mmol/L dithiothreitol, 50 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.5, and 0.05 mg/ml poly(dI-dC). Nuclear lysates were preincubated for 10 minutes, and then the labeled probes were added and incubated for an additional 20 minutes at room temperature. Competition analyses were performed in the presence of a 50-fold excess of unlabeled oligonucleotides. For the super shift assay, 2 μg of E2F-1 antibody (Santa Cruz Biotechnology) was included in the preincubation mixture. DNA-protein complexes were separated on a 6% DNA retardation gel (Invitrogen, Carlsbad, CA) in 0.5 $\times$ Tris borate-ethylenediamine tetraacetic acid at 250 V for 1 hour. The gel was dried and exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) or to a PhosphorImager screen (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 4 hours and then scanned by phosphorimager. Controls included nuclear proteins from SAME-treated KB cells, PC3 cells, and HeLa cells.

ELISA Assay for NF- κ B Active Form and DNA-Binding Activity

The specific DNA-binding activity of NF- κ B was measured by ELISA using the TransAM NF- κ B p65 transcription factor assay kit (Active Motif North America, Carlsbad, CA). Nuclear proteins were incubated in 96-well plates with immobilized oligonucleotide containing a consensus binding site for the p65 subunit of NF- κ B (5'-GGGACTTTC-3'), which specifically binds the active form of NF- κ B. A p65 antibody specific for the activated form was incubated with nuclear extract, and anti-IgG horseradish peroxidase-conjugated secondary antibody was used for visualization. The binding activity to NF- κ B was detected and quantified by spectrophotometry at 450 nm with a reference wavelength of 655 nm. HeLa cells nuclear extract and lysis buffer without proteins were used as positive control and blank control, respectively.

Western Blot Analyses

After treatment with SAME for 30 minutes, cells were washed once with PBS (pH 7.4) and incubated with 1.2 ml of RIPA buffer (1 $\times$ PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) with protease inhibitors (aprotinin, 30 $\mu\text{g}/\text{ml}$; leupeptin, 4 $\mu\text{g}/\text{ml}$; pepstatin, 2 $\mu\text{g}/\text{ml}$; and phenylmethyl sulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$). The lysates were transferred to 1.7-ml Eppendorf tubes, and the plates were washed with 1.2 ml of RIPA buffer to ensure complete retrieval. Lysates were incubated for 30 minutes on ice and then centrifuged at 10,000 $\times g$ for 10 minutes at 4°C. After centrifugation, the protease inhibitor cocktail was immediately added to the supernatant, and protein concentration was determined by Bradford assay. Total proteins (70 μg) were mixed

with electrophoresis sample buffer, boiled for 5 minutes, and separated on 14% Tris-glycine gels (Invitrogen). After electrophoresis, proteins were transferred to a PVDF membrane (American Pharmacia Biotech, Piscataway, NJ). Blots were probed with rabbit anti-human inhibitor κB - α (I κ B α) and I κ B β polyclonal antibodies (Santa Cruz Biotechnology). α -Tubulin was used as an internal control. Goat anti-rabbit alkaline phosphatase-conjugated IgG was used as the secondary antibodies. Blots were incubated with Tropix CSPD chemiluminescent substrate and detected by the Tropix Western-Light and Western Star detection system (Bedford, MA).

Transient Transfection of p53 and Promoter Assay of Hep3B

From the above study, GADD45 β expression in Hep3B could not be induced by SAME apparently as HepG2. Moreover, NF- κ B-binding ability and activity failed to respond to SAME administration. Based on the distinct difference of p53 status between HepG2 (p53 wild type) and Hep3B (p53-null), Hep3B cells were transiently transfected with 0.1 μg of pp53-EGFP (wild-type p53 fused to enhanced green fluorescent protein, GFP) (Clontech, Palo Alto, CA) by electroporation at parameter 80 $\mu\text{s}/650$ V. Mock transfection was included at the same time. Transfection efficiency was determined by counting the number of GFP-expressing cells per randomly chosen field of 100 cells 12 hours after infection. Then, promoter activity changes were investigated after SAME treatment by the luciferase reporter assay. Transcriptional activity modifications were further explored by EMSA analyses, ELISA, and Western blot as mentioned above.

Results

Influence on GADD45 β Expression in HCC Cells by SAME

Expression of GADD45 β , as shown by Northern blot, was low in HepG2 cells and could be significantly induced by SAME in a dose-dependent manner (Figure 1). There was approximately a fivefold increase in GADD45 β mRNA with 0.5 mmol/L SAME and an eightfold increase with 1.0 mmol/L SAME. Although a lack of GADD45 β expression was also observed in Hep3B as well as HepG2, induction by SAME was barely observed in Hep3B by SAME. Only a slight increase of GADD45 β occurred at 0.5 mmol/L SAME administration, and further escalation in SAME dose led to little increase in the induction. Quantitative real-time PCR was used to further confirm the results from Northern blot. The standard curve formulas $Y = 40.722 - 3.885X$ ($r^2 = 0.984$) for GADD45 β and $Y = 43.128 - 4.248X$ ($r^2 = 0.993$) for GAPDH were derived from the lines of the calibration curves. The mean ratio of GADD45 β to GAPDH mRNA in untreated HepG2 cells was 0.0052. The mean ratios significantly increased to 0.0282 and 0.0525 for cells treated with 0.5 and 1.0 mmol/L SAME, respectively ($P < 0.05$). Consistent with

Figure 1. Induction of GADD45 β expression by SAME in HepG2 and Hep3B. Northern blot validation of GADD45 β expression in HepG2 and Hep3B after SAME administration. The blot was probed with a 222-bp PCR product containing GADD45 β exon 3. GAPDH was used as an internal control for RNA loading.

the results from Northern blot, Hep3B did not demonstrate apparent GADD45 β induction. The mean ratio of GADD45 β to GAPDH was 0.0097, and the mean ratios were kept stable in the range of 0.0104 to 0.0113 ($P > 0.05$).

Analyses of GADD45 β Proximal Promoter

In our previous study, we identified several active promoter regions of GADD45 β in HepG2 and CL-48.⁵ Most of the active regions were located in GADD45 β promoter fragment spanning -618 to -436 . The promoter activity peak appeared at -470 with the deletion of as few as 50 bp from the 5'-end of -520 . Therefore, a putative inhibitory region located between -520 and -470 was taken into consideration. By means of 5-Aza-dC treatment, methylation-specific PCR (MSP) and sequencing of sodium bisulfite-treated DNA, we confirmed the hypermethylation in the putative inhibitory region at $-520/-470$.

As shown in Figure 2, although the promoter activity peak was detected at bp -470 , deletion of as few as 34 bases from the 5'-end of this region led to a 24-fold decrease in promoter activity. One putative E2F-1-binding site (-452 to -444) was located in this area with high score in search of the TRANAFAC database. Meanwhile, the removal of 27 bases from -618 caused a significant decrease in promoter activity as well. One putative NF- κ B-binding site (-602 to -593) was located in this deletion fragment with high score in the TRANAFAC database. Although further truncation from -592 and -547 could not influence promoter activity apparently, another two putative NF- κ B-binding sites between -591 and -520 (-581 to -572 and -537 to -528) were also identified with a relatively low score in the database. Of interest, Hep3B shared the same pattern of proximal promoter activity as HepG2 despite the difference in p53

Figure 2. Identification of GADD45 β proximal promoter region. The diagram illustrates the location of putative binding sites for the putative transcription factors. The comparison of relative promoter activity between HepG2 and Hep3B is also shown. Experiments were performed in triplicates, and the results are presented as the mean $\pm$ SD.

status. The overall activity level of Hep3B was relatively lower than that of HepG2 (Figure 2). Altogether, several active promoter regions and putative transcriptional factor-binding sites were identified based on luciferase report assay and consensus sequence. However, further evidence at the functional level is required to confirm the role of these regions in GADD45 β regulation.

Induction of GADD45 β by SAME Occurs through Regulation of NF- κ B

From above, we confirmed the induction of GADD45 β by SAME in a dose-dependent manner, which represents a very useful model for regulation study. Therefore, to reveal functional evidence of putative promoter regions, the effects of SAME on transcription factor-binding sites were focused on three putative NF- κ B-binding sites (-618 to -470) and one putative E2F-1-binding site (-470 and -436), as determined using the luciferase reporter assay system. As shown in Figure 3A, luciferase activity increased approximately fourfold on the addition of SAME in HepG2 transfected with the full-length reporter construct, which contained all three NF- κ B-binding sites (-618 to $+6$). In the absence of SAME, deletion of the first NF- κ B-binding site (-591 to $+6$) led to a 70% decrease in promoter activity compared with the full-length construct. However, this construct retained the ability to be activated approximately twofold by SAME treatment. The construct containing the third NF- κ B-binding site (-547 to $+6$) could only be induced by SAME approximately onefold. Meanwhile, there was not any response to SAME for the E2F-1-binding site (-470 to -436) as well. In Figure 3B, the consistent failure to response to SAME was observed, although even Hep3B had the same promoter activity profile, whereas the stimulator did not influence luciferase activity significantly.

Figure 3. Effect of SAME on GADD45 β promoter fragments encompassing putative transcriptional factor-binding sites in HepG2 and Hep3B. The diagrams illustrate the location of putative binding sites for the transcription factors NF- κ B and E2F-1. Fragments deleting each binding site were cloned into the pGL3 luciferase reporter plasmid, respectively. After SAME administration, luciferase activity was measured in HepG2 (A) and Hep3B (B) as mentioned in Materials and Methods. Experiments were performed in triplicates and the results are presented as the mean $\pm$ SD.

The Increase of NF- κ B Binding Activity after SAME Treatment in EMSA

Although both NF- κ B- and E2F-1-binding sites were identified in the proximal promoter region of GADD45 β , only the NF- κ B sites functioned positively to regulate transcription of GADD45 β in response to SAME treatment. We further validated the specificity of these sites in response to SAME treatment by EMSA analysis using γ -³²P-labeled oligonucleotide containing wild-type or mutant NF- κ B and E2F-1 recognition motif and HepG2 nuclear lysates. Furthermore, anti-human E2F-1 antibody was used for the super shift assay to confirm binding specificity.

As shown in Figure 4A, binding to the probe containing the first two NF- κ B-recognition motifs (probe 1) in HepG2 nuclear lysates was substantially enhanced by SAME in a dose-dependent manner. The addition of a 50-fold excess of unlabeled NF- κ B oligonucleotide shifted the binding, indicating the specificity of the observed interaction. Binding to probe 2, in which the first NF- κ B site was mutated, could not be readily detected. When the second NF- κ B site was mutated (probe 3), no NF- κ B-specific

binding occurred, and the binding pattern was altered. No binding could be observed with mutation of both NF- κ B-binding sites (probe 4). A probe containing only the third NF- κ B recognition motif (probe 5) also yielded no binding activity. A probe containing the consensus NF- κ B-binding sequence generated the same results as that obtained with probe 1 (data not shown). These results suggest that both the first and second NF- κ B-binding sites are essential for NF- κ B function after SAME treatment. When other cancer cell lines were examined, KB and PC3 cells only showed slight binding to the consensus NF- κ B probe, and no induction by SAME could be observed, suggesting that the effect may be specific to HCC cells. In contrast, there was significantly less binding to a probe containing the E2F-1-binding site and no apparent binding response to SAME than was seen for NF- κ B (Figure 4B). This result was further confirmed by competition and super shift assays. Taken together, we have validated that treatment of HepG2 cells with SAME specifically induced binding of NF- κ B to the GADD45 β promoter and activated its expression. E2F-1 may not play an important role in SAME-mediated transcription activation. In addition, the induction of GADD45 β by SAME via NF- κ B may be specific to liver cells.

The Increase of NF- κ B Active Form and DNA Binding Activity after SAME Treatment in ELISA

There are several methods to measure NF- κ B activity, directly or indirectly. The EMSA can only demonstrate DNA-binding capacity, and super shift analysis is crucial to confirming the specificity of binding. However, there are five subunits in the NF- κ B family in mammals,¹¹ and the available NF- κ B antibodies poorly recognize the NF- κ B DNA-protein complex. Others have reported that in super shift assays the NF- κ B DNA-protein-antibody complex could only be partially shifted.¹² Because activation of NF- κ B is controlled by I κ B α modification rather than NF- κ B protein synthesis,¹³ Western blot is also not very reliable for NF- κ B activity analyses. Because ELISA could be used to detect NF- κ B bound to an immobilized oligonucleotide encoding its recognition motif, the change in concentration of the active form of NF- κ B was examined quantitatively using ELISA. As shown in Figure 5, in HepG2, treatment with 0.5 mmol/L SAME led to a threefold increase in active NF- κ B relative to the untreated control, and a fivefold increase was seen with 1 mmol/L SAME. These results confirm that treatment with SAME led to a dose-dependent increase in active form of NF- κ B available to bind to the GADD45 β promoter and activate its expression.

Degradation of I κ B α Induced by SAME

NF- κ B normally exists in an inactive form in the cytoplasm bound to I κ B. Treatment of cells with various inducers results in the phosphorylation, ubiquitination, and subsequent degradation of I κ B α . This modification leads to the release of NF- κ B dimers that subsequently translocate to

Figure 4. EMSA and super shift analyses of NF- κ B and E2F-1-binding ability after SAME treatment. Nuclear protein lysates were prepared from HepG2 cells treated with 0, 0.5, or 1.0 mmol/L SAME and used for EMSA analysis. Specificity of binding was demonstrated by competition with a 50-fold excess of unlabeled probe. The **arrow** indicates the position of specific binding complexes. **A:** Five oligonucleotide probes of the three putative NF- κ B-binding sites were used for EMSA. Probe 1 contained the intact first and second NF- κ B-binding sites. The first site was mutated in probe 2, whereas the second site mutated in probe 3. The first and second sites were both mutated in probe 4. Probe 5 only included the third NF- κ B-binding site. The gray box showed the mutated binding site. KB cells were treated and collected as non-HCC cell control (last panel). **B:** Oligonucleotide probes for the putative E2F-1-binding site and its mutant counterpart were used in EMSA. E2F-1 antibody was included in the reaction complex for the super shift assay.

the nucleus and activate target genes. Therefore, we used Western blot to examine the effect of SAME on I κ B α and I κ B β in HepG2 cells and to confirm the role of NF- κ B in SAME-mediated induction of GADD45 β expression. As shown in Figure 6, the amount of I κ B α decreased 2.5-fold in cells treated with 0.5 mmol/L SAME and fivefold with 1.0 mmol/L SAME in HepG2. As expected, no apparent changes in the amount of I κ B β were observed. These findings are consistent with the above studies and further confirm the important role of NF- κ B in regulation of GADD45 β transcription by SAME.

GADD45 β Promoter Assay in Hep3B with p53 Transient Transfection

As the downstream effector of p53, GADD45 β was confirmed to be specifically down-regulated in HCC, which

was associated with the extent of p53 mutation.⁴ In our study, different from HepG2 (p53 wild type), Hep3B (p53-null) could not be induced by SAME (Figure 1), and NF- κ B-binding ability and activity also failed to respond to SAME (Figure 3B). With pp53-EGFP transfection, the mean ratio of GADD45 β to GAPDH in Hep3B was 0.0192 and 0.0371 after 0.5 and 1.0 mmol/L SAME treatment, respectively, as determined by real-time PCR. Compared with 0.0104 and 0.0113 in Hep3B without p53 transfection, GADD45 β induction could be partially provoked by SAME. However, even after reconstituting p53, Hep3B still did not demonstrate the same level of induction as HepG2: only 60 to 70% of the level in HepG2.

Figure 5. ELISA analysis of NF- κ B binding capacity after SAME treatment. Nuclear protein lysates were prepared from HepG2, Hep3B, and pp53-transfected Hep3B. After treatment with 0, 0.5, or 1.0 mmol/L SAME, ELISA was used to quantitatively measure NF- κ B-binding capacity. HeLa cell nuclear extract and lysis buffer alone were used as positive and negative controls. Experiments were performed in triplicates and the results are presented as the mean $\pm$ SD.

Figure 6. The influence of SAME on I κ B α and I κ B β in HepG2. HepG2 cells were treated by SAME at the designated dosage, and the amount of I κ B α and I κ B β was estimated by Western blot relative to α -tubulin as an internal loading control. The data shown here are representative of three independent experiments. Densitometry was performed using ImageQuant version 5.0, and the results are presented as the mean $\pm$ SD.

Figure 7. The influence of p53 transfection on Hep3B promoter activity. After p53 transfection, promoter activity was measured in Hep3B using the luciferase reporter system. HepG2 and nature type Hep3B were included in the same statistic analyses. Experiments were performed in triplicates and the results are presented as the mean $\pm$ SD.

Despite the same pattern as HepG2, the overall proximal promoter activity level of Hep3B was relatively lower (Figure 2). Partial increase of promoter activity in Hep3B was noticed after pp53-EGFP transfection, although a 20 to 30% gap between HepG2 and Hep3B + p53 (Figure 7). Furthermore, Hep3B did not show any apparent change in EMSA (Figure 8) and ELISA (Figure 5) even with pp53-EGFP transfection, which indicated that induction of GADD45 β expression may not be regulated only via NF- κ B pathway in Hep3B.

Discussion

Three members of the GADD45 gene family, GADD45 α , GADD45 β , and GADD45 γ , have been identified based on the extensive region of conserved sequence.¹⁴ GADD45 β shares the common function of the family,

which has been associated with influencing cell growth control, apoptotic cell death, and cellular responses to DNA damage.¹⁵ As a positive apoptosis modulator, activation of GADD45 β prevents the propagation of damaged cells, causing cell growth arrest and apoptosis after exposure to genotoxins.¹⁶ In our previous study, we confirmed the significant and specific GADD45 β decrease in HCC. Moreover, down-regulation of GADD45 β was strongly correlated with HCC differentiation and advanced nuclear grade.⁴ Our results suggest that the lack of GADD45 β expression in HCC might lead to the failure to inhibit atypical cell growth or to trigger apoptosis.

Therefore, to understand the molecular basis of GADD45 β and to seek possible pathways for GADD45 β induction could potentially be powerful therapeutic approaches in chronic liver disease or HCC. The p53-directed hypermethylation in proximal promoter of GADD45 β was verified in a serial set of HCC cell lines and clinical cases, which accounted for part of reasons in down-regulation mechanism.⁵ Furthermore, several transcriptional regulatory regions were also identified at the same time, although functional evidence and transcriptional regulation mechanism need be further elucidated.

To understand transcriptional regulation, evidenced by detailed promoter analyses, several active promoter regions were pinpointed in our current study. Two putative transcriptional factors, NF- κ B and E2F-1-binding sites, were identified by means of consensus sequence alignments and high scores in TRANSFAC database. As the requirement for functional assay, the significant induction of GADD45 β by SAME was confirmed by Northern blot and quantitative real-time PCR in HepG2 (p53 wild type). The role of transcriptional regulators was detected by luciferase assay, EMSA, and ELISA. The results showed that activity, binding capacity, and active form of NF- κ B were increased after SAME treatment in a dosage-dependent manner. Because activation of NF- κ B is mediated by the degradation of I κ B α rather than quantitative change of NF- κ B itself,¹³ a dramatically decreased level of I κ B α was further confirmed with SAME treatment by Western blot. All those data suggested that transcriptional responses to SAME were mediated by the NF- κ B-binding sites.

SAME has been known to possess multiple biological functions and is an essential compound in numerous metabolic reactions as a methyl donor.^{17,18} It has long been realized that SAME synthesis is impaired in patients with alcohol-induced or viral cirrhosis.¹⁹ Administration of SAME can protect liver from hepatotoxins, such as alcohol, CCl₄, galactosamine, acetaminophen, and bile acids, improve survival of cirrhosis patients, and prevent experimental hepatocarcinogenesis.^{20–22} Facilitation of methylation reactions and the restoration of depleted hepatocellular glutathione levels have been proposed to be the possible hepatoprotection mechanism of SAME.²³ However, the detailed molecular mechanism is primarily unknown and deserves further investigation. In our study, we found that expression of the DNA repair-related gene GADD45 β is specifically diminished in HCC but could be significantly induced by SAME. Therefore, we hypothe-

Figure 8. EMSA analyses of Hep3B with and without p53 transfection. Nuclear protein lysates were prepared from Hep3B with and without p53 transfection. Analysis system was described as mentioned in Materials and Methods. Competent probe included the intact first and second NF- κ B-binding sites.

sized that up-regulation of GADD45 β by SAME could be a mechanism of SAME-mediated hepatoprotection.

Activation of NF- κ B occurs when various stimuli trigger signal transduction pathways that lead to activation of I κ B kinase. Phosphorylation of I κ B α and I κ B β by I κ B kinase initiates polyubiquitination and rapid degradation by the proteasome, allowing NF- κ B to enter the nucleus. Accumulation of NF- κ B in the nucleus and DNA binding are necessary for positive regulation of gene expression.²⁴ Therefore, degradation of I κ B α is very important for NF- κ B function. It has recently been reported that the hepatoprotective action of SAME may be mediated through the modulation of nitric oxide production. That report also showed that SAME could accelerate the re-synthesis of I κ B α and blunt the activation of NF- κ B, which is different from our results.²³ However, normal rat hepatocytes were used in their study and human HCC cell line HepG2 was examined in our study. The disparity between normal and cancer cells may explain the different effects observed for SAME. In future work, we will examine the hepatoprotective and chemopreventive effect of SAME in normal human liver cells. Meanwhile, we noticed that GADD45 α and GADD45 γ could not be induced by NF- κ B, contrary to the regulation of GADD45 β .²⁵ In our previous study, we showed that the expression of GADD45 α remained the same level in both HCC and nontumor liver tissues.⁴ These findings suggest that, in HCC, different mechanisms may be involved in regulation of GADD45 α and GADD45 β , and these differences may be important for HCC malignant transformation.

In addition to the NF- κ B-binding sites, we identified an E2F-1-binding site on the GADD45 β promoter that was shown to be important for promoter activity. Deletion of the hypermethylation region (–520/–470), which is adjacent to E2F-1-binding region, nearly peaked GADD45 β promoter activity. Those results suggested that the hypermethylation of this area might play an important role in regulation of GADD45 β expression. However, our results showed that the hypermethylation region and E2F-1 site showed no response to SAME treatment (Figure 3), suggesting that only NF- κ B plays a specific role in SAME-mediated up-regulation of GADD45 β . The key to the puzzle may be back at the CpG islands located in the methylation region, which cannot be modulated by methyl donor SAME in *in vitro* luciferase activity assay. These findings further suggested the complicated structure of GADD45 β promoter and multiple roles of SAME in gene regulation. We are currently examining the role of methylation in the GADD45 β regulation by SAME on this area. The possibility of the existence of a balance among NF- κ B, E2F-1, and the methylation region in GADD45 β regulation or the existence of a provocation to impede biological functions need further exploration.

The p53-associated DNA damage repair pathway constitutes a very complicated network and GADD45 β was a downstream effector for cell cycle arrest. Not only has the p53 mutation been reported to be associated with GADD45 β down-regulation in HCC⁴ but hypermethylation regulation of GADD45 β might also be closely directed by p53 status. The distinct difference of p53 status between HepG2 (p53 wild type) and Hep3B (p53-null)

makes a perfect set of research models in HCC study. In our study, with p53 transfection, Hep3B could partially restore GADD45 β expression and partially provoked by SAME up to 60 to 70% of HepG2 level. However, there were no remarkable changes in EMSA and ELISA. Therefore, our data suggested that other mechanisms may be involved in GADD45 β regulation in addition to the NF- κ B pathway in Hep3B.

In summary, we have identified several important transcription factor-binding sites in GADD45 β proximal promoter and shown that only NF- κ B functions as a positive regulator for GADD45 β induction by SAME. Given the role of GADD45 β in HCC, we hypothesize that induction of GADD45 β expression by SAME via NF- κ B is likely to be the new mechanism of SAME-mediated hepatoprotection. Special regulation mechanisms in Hep3B and the role of p53 will be further elucidated.

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