Adenosine Promotes GATA-2-Regulated p53 Gene Transcription to Induce HepG2 Cell Apoptosis

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Key Words

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Abstract

Background/Aims: In our earlier study, adenosine induced apoptosis in HepG2 human hepatoma cells by tuning of apoptosis-mediator gene transcription. The present study aimed at understanding the regulatory mechanism underlying the apoptosis-mediator gene transcription under the control of adenosine. **Methods:** For HepG2 cells with and without knocking-down p53 or GATA-2, cell viability, mitochondrial membrane potentials, caspase activity, and transcriptional activity were monitored, and Western blotting, RT-PCR, electrophoretic mobility shift assay (EMSA), and chromatin immunoprecipitation (ChIP) assay were carried out. **Results:** Extracellular adenosine upregulated expression of the p53 mRNA and protein in HepG2 human hepatoma cells. Adenosine induced apoptosis, disrupted mitochondrial membrane potentials, and activated caspase-3, -8 and -9 in HepG2 cells, and those effects were inhibited by silencing the p53-targeted gene. In the assay of transcriptional activity using full-length p53 gene promoter and 5' deletion mutants combined

with the luciferase reporter vector, adenosine enhanced transcriptional activity for full-length p53 gene promoter, that was prevented by deleting from -240 to -146 bp on the promoter. In the EMSA using a ³²P-labeled DNA probe to detect binding to the putative GATA-2 binding site on the p53 gene promoter, adenosine produced ³²P-positive signals in nuclear extracts from HepG2 cells. In the Western blot analysis, adenosine increased presence of GATA-2 in nuclear extracts. In the ChIP assay, adenosine increased PCR products for the p53 gene promoter in chromosomal extracts from HepG2 cells, immunoprecipitated using an anti-GATA-2 antibody. Adenosine-induced upregulation of the p53 mRNA expression was suppressed by knocking-down GATA-2. **Conclusion:** The results of the present study show that p53 is a transcriptional target of GATA-2 and that adenosine upregulates GATA-2-regulated p53 expression, thereby activating caspase-3, -8, and -9 to induce HepG2 cell apoptosis.

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Introduction

Several avenues of evidence have shown that extracellular adenosine induces apoptosis in a variety of

cells via intrinsic pathways linked to adenosine uptake into cells and/or extrinsic pathways linked to adenosine receptors. Intracellularly transported adenosine induces apoptosis in GT3-TKB human lung cancer cells by activating AMP-activated protein kinase (AMPK) [1]. Adenosine induces apoptosis in Caco-2 human colonic cancer cells by activating caspase-3 and -9 via A_{2a} adenosine receptors linked to G_s protein [2]. Adenosine, alternatively, induces apoptosis in RCR-1 astrocytoma cells via both the intrinsic and extrinsic pathways each relevant to AMPK activation and A₁ adenosine receptors [3]. Additionally, adenosine is shown to induce apoptosis in hepatocellular carcinoma cells as mediated via A₃ adenosine receptors [4].

Amazingly, adenosine induces apoptosis in human hepatoma cell lines such as HuH-7 and HepG2 cells by tuning transcription of apoptosis-related genes. For HuH-7 human hepatoma cells, intracellularly transported adenosine activates caspase-8 and the ensuing caspase-3, by downregulating expression of Fas-associated death domain protein (FADD)-like interleukin-1 β -converting enzyme inhibitory protein (FLIP) [5]. Adenosine downregulates expression of Bcl-X_L and inhibitor of apoptosis protein (IAP), to directly inhibit caspase-3, -7, and -9, but otherwise adenosine upregulates expression of DIABLO, an inhibitor of IAPs [6]. Those actions account for adenosine-induced HuH-7 cell apoptosis.

For HepG2 human hepatoma cells, adenosine upregulates expression of mRNAs for tumor necrosis factor (TNF), TNF receptor 1-associated death domain protein (TRADD), TNF related apoptosis-inducing ligand receptor 2 (TRAIL-R2), TRADD/receptor-interacting protein kinase 1 (RIPK1), Fas-associated death domain (FADD), and caspase-9 [7]. TNFR1 is activated by TNF- α and forms a complex of TRADD/RIP1/FADD/procaspase-8, to activate caspase-8 [8, 9]. This pathway, accordingly, may account for adenosine-induced HepG2 cell apoptosis. How adenosine regulates apoptosis-related gene transcription in HepG2 cells, however, remains to be explored.

The tumor suppressor protein p53 is the major senescence- and apoptotic cell death-inducing transcription factor that targets hundreds of genes. p53 executes cancer cell apoptosis via transcription-dependent and -independent pathways. Bcl-2 homology 3 (BH3)-only Bcl-2 family members such as Puma and Noxa are transcriptional targets of p53 [10]. For p53-mediated transcription-dependent apoptotic pathway, Puma and Noxa release pro-apoptotic Bax subfamily members such as Bax and Bak from the association with anti-apoptotic

Bcl-2 subfamily members such as Bcl-2, Bcl-X_L, and Mcl-1, thereby disrupting mitochondrial membrane potentials, to allow cytochrome c efflux from the mitochondria into the cytosol, which triggers activation of caspase-9 and the effector caspase-3 to induce apoptosis [11, 12]. Puma and Noxa, alternatively, induces apoptosis by antagonizing Bcl-2 subfamily members [13]. For p53-mediated transcription-independent apoptotic pathway, p53 stimulates release of apoptogenic factors from the mitochondria, that include cytochrome c and Smac/DIABLO [14-17]. Cytochrome c forms the apoptosome that consists of the adapter protein Apaf-1 and caspase-9, to activate caspase-9 and the ensuing caspase-3 [14-17]. Smac/DIABLO activates caspase-3 through repression of IAPs [14-17].

GATA is a zinc-finger transcription factor, and the family consists of six proteins such as GATA-1, -2, -3, -4, -5, and -6, that engage in a development/cell type-specific manner. GATA-1, GATA-2, and GATA-3 play multiple vital roles in hematopoiesis in many cell lineages. GATA-1 and GATA-2 participate in lineage-specific transcription factor networks essential for proper differentiation of erythroid cells, that regulate erythroid gene expression, and GATA-1 and GATA-2 genes by themselves are also regulated by multiple transcription factors, including their own products GATA-1 and GATA-2 [18]. GATA-2 is required for genesis and/or function of hematopoietic stem cells, while GATA-1 drives differentiation of hematopoietic progenitors into a subset of the blood cell lineages [19]. GATA-1 directly represses GATA-2 gene transcription, thereby displacing GATA-2 from chromatin, a process termed a GATA switch [19]. GATA-3 serves as a master regulator of T-helper 2 cell differentiation in mature CD4⁺ T cells and is required at each stage of T-cell development [20]. Interestingly, GATA-3 still regulates normal cell development and tumor differentiation in non-hematopoietic cells including mammary cells [21].

An interaction between GATA family and p53 is not fully understood. For erythroid cells, GATA-1 inhibits activation of p53 gene promoter by binding to the GATA-1-binding site [22]. Multipotential hematopoietic progenitors lacking GATA-2 poorly proliferate and generate small colonies with extensive cell death, and the effect is restored by additionally deleting p53, suggesting that GATA-2 proliferates and protects early hematopoietic cells by inhibiting p53 function [23].

The present study focused upon GATA-2 and investigated GATA-2-regulated p53 gene transcription, responsible for adenosine-induced HepG2 cell apoptosis.

Materials and Methods

Cell culture

HepG2 cells, obtained from RIKEN cell bank (Ibaraki, Japan), were cultured in minimum essential medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (w/v) non-essential amino acids, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. ACHN human renal cell adenocarcinoma cells, kindly provided from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan), were cultured in Dulbecco's Modified Eagles Medium. Each medium was supplemented with 10% (v/v) heat-inactivated FBS, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

Assay of cell viability

Cell viability was assayed by the method using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as described previously [2]. MTT-reactive cells were quantified at an absorbance of 570 nm using a micro-plate reader (SPECTRAMax PLUS384, Molecular Devices, Sunnyvale, USA), and percentage of independent basal levels was calculated.

Assay of mitochondrial membrane potentials

Mitochondrial membrane potentials were measured using a DePispherTM kit. HuH-7 cells were untreated and treated with adenosine (3 mM) for 48 h. After washing with cold phosphate-buffered saline, cells were incubated in a DePispherTM solution at 37 °C for 20 min. Then, cells were washed with 1 ml of a reaction buffer containing a stabilizer solution. The fluorescent signals were observed with a laser scanning microscopes (LSM 510, Carl Zeiss Co., Ltd, Germany) equipped with an epifluorescence device using a fluorescein long-pass filter (fluorescein and rhodamine) at an absorbance of 590 nm for red aggregations and at an absorbance of 530 nm for green aggregations.

Assay of caspase activity

Activation of caspase-3, -8, and -9 was enzymatically measured using a caspase fluorometric assay kit (Ac-Asp-Glu-Val-Asp-MCA for a caspase-3 substrate peptide; Ac-Ile-Glu-Thr-Asp-MCA for a caspase-8 substrate peptide; and Ac-Leu-Glu-His-Asp-MCA for a caspase-9 substrate peptide) as previously described [2]. After 24-h treatment with adenosine (3 mM), HepG2 cells were harvested, pelleted, and frozen on dry ice. The pellet was resuspended in a cell lysis buffer and incubated on ice for 10 min, and the lysates were centrifuged at 15000 x g for 10 min at 4 °C. A total of 50 µg of cell lysates was incubated with 5 µl of 1 mM stock of the respective fluorescently labeled tetrapeptide at 37 °C for 2 h. The fluorescence was measured at an excitation of 400 nm and at an emission of 505 nm with a fluorometer (Fluorescence Spectrometer, F-4500, HITACHI, Tokyo, Japan).

Knocking-down p53 and GATA-2

The small interfering RNA (siRNA) silencing the p53-targeted gene (p53 siRNA) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). We constructed the siRNA silencing the GATA-2-targeted gene (GATA-2 siRNA): sense, 5'-GCG CAC AAC UAC AUG GAA CTT-3' and anti-sense, 5'-GUU CCA UGU AGU UGU GCG CTT-3'; sense, 5'-CGG AAG AUG UCC AAC AAG UTT-3' and anti-sense, 5'-ACU UGU UGG ACA UCU UCC GTT-3'; and sense, 5'-AGG AAA AAG GAU UAG GCA ATT-3' and anti-sense, 5'-UUG CCU AAU CCU UUU UCC UTT-3' (Cosmo Bio, Co. Ltd., Tokyo, Japan). The p53 siRNA, the GATA-2 siRNA, and the negative control siRNA (NC siRNA) were transfected using a Nucleofector II device (Amaxa biosystems, Cologne, Germany).

Extraction of nuclear and cytosolic components

HepG2 cells were suspended in a buffer A [10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 1% (v/v) protease inhibitor cocktail, pH 7.4] and lysed with 10% (v/v) NonidetP-40. After 15,000 x g centrifugation at 4 °C for 5 min, the pellet was resuspended in ice-cold buffer B [20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1% (v/v) protease cocktail inhibitor, pH 7.9] as a nuclear component and the supernatant was collected as a cytosolic component.

Western blotting

Lysates from total HepG2 cells or nuclear components were subjected to 10% (v/v) sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. Blotting membranes were blocked TBST [150 mM NaCl, 0.1% (v/v) Tween20, and 20 mM Tris] containing 5% (v/v) bovine serum albumin and reacted with antibodies against p53 (Calbiochem, CA, USA), and GATA-2 (Santacruz, CA, USA), β-actin (SIGMA-Aldrich, MO, USA), followed by an HRP-conjugated anti mouse or rabbit IgG antibody. Immunoreactivity was detected with an ECL kit (GE healthcare, NJ, USA) and visualized with an Image Gauge software (FUJIFILM, Tokyo, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were purified from cells by an acid/guanidine/thiocyanate/chloroform extraction method using a Sepasol-RNA I Super kit. After purification, total RNAs were treated with RNase free-DNase I (2 unit) at 37°C for 30 min to remove genomic DNAs, and 10 µg of RNAs were resuspended in water. Then, oligo dT primers, dNTPs, 5 x First Strand buffer, and SuperScript III RNase H-Reverse Transcriptase were added to the RNA solution and incubated at 65 °C for 5 min followed by 56 °C for 60 min, 58 °C for 60 min, 85 °C for 5 min to synthesize the first strand cDNA. Subsequently, 1 µl of the reaction solution was diluted with water and mixed with 10 x PCR reaction buffer, dNTPs, MgCl₂, oligonucleotide, dimethylsulfoxide (final concentration, 5% v/v) and 1 unit of Taq polymerase (Fermentas, St. Leon-Roth, Germany)(final volume, 20 µl). RT-PCR was carried out with a GeneAmp PCR system model 9600 DNA thermal cycler (Applied Biosystems, Indianapolis, USA) programmed as follows: the first one step, 94 °C for 4 min and

the ensuing 30 cycles, 94 °C for 1 s, 62 °C for 15 s, and 72 °C for 30 s. The primers used here were as follows: sense, 5'-GCC ATC TAC AAG CAG TCA CAG CAC AT-3' and anti-sense, 5'-GGC ACA AAC ACG CAC CTC AAA GC-3' for p53; sense, 5'-GAC GGA GAG CAT GAA GAT GGAAAG TGG C-3' and anti-sense, 5'-GAG GCC ACA GGC ATT GCA CAG GTA GT-3' for GAT A-2; and sense, 5'-GAC TTC AAC AGC GAC ACC CAC TCC-3' and anti-sense, 5'-AGG TCC ACC ACC CTG TTG CTG TAG -3' for GAPDH. PCR products were stained with ethidium bromide and visualized by 2% (v/v) agarose electrophoresis.

Assay of transcriptional activity

The full length of the human p53 gene promoter pp53-pl (-532 to +1)(forward, 5'-CTG CTA GCG GGA GAA AAC GTT AGG GTG TGG-3'; reverse, 5'-AGG CTA GCC CAA TCC AGG GAA GCG TGT C-3') and 5' deletion mutants such as pp53-pII (-240 to +1)(forward, 5'-CTG CTA GCG CGG ATT ACT TGC CCT TAC TTG-3'; reverse, 5'-AGG CTA GCC CAA TCC AGG GAA GCG T GTC-3') and pp53-pIII (-146 to +1)(forward, 5'-CTG CTA GCT GCT CAAGAC TGG CGC TAAAG-3'; reverse, 5'-AGG CTA GCC CAA TCC AGG GAA GCG TGT C-3') were combined with the luciferase reporter vector pGL4.12 (Promega, WI, USA). pp53-pl, pp53-pII, or pp53-pIII was transfected into HepG2 cells using a lipofectamine, and pGL4.73 vector (Promega) was co-transfected as an internal control. Cells were treated and untreated with adenosine 6 h after transfection and transcriptional activity was quantified by monitoring luciferase luminescent intensities with a luminometer (Berthold, Tokyo, Japan).

Electrophoretic mobility shift assay (EMSA)

To construct a ³²P-labeled DNA probe, the DNA element (GCG GAT TAC T) contained in the putative GATA-2 binding site on the p53 gene promoter (5'-GCA GGC GGA TTA CTT GCC CTT A-3') was incubated in a reaction medium (6.6 mM Tris-HCl, 50 mM NaCl, 6.6 mM MgCl₂, 0.5 mM dGTP, 0.5 mM dTTP, 1 mM dithiothreitol, pH7.4) containing 200 ng of DNA, 2 µl of [³²P]dCTP, and 1 unit of klenow fragment at 37 °C for 20 min, and the DNA probe was collected with a Sephadex G-50 column. Nuclear extracts from HepG2 cells were reacted with the ³²P-labeled DNA probe in a binding buffer [20 mM HEPES-NaOH, 80 mM NaCl, 0.3 mM EDTA, 0.2 mM EGTA, 0.2 mM phenylmethanesulfonyl fluoride, 10% (v/v) glycerol, 2 µg of poly(dI-dC), pH 7.9] at 25 °C for 15 min. DNA-protein complexes were separated by an electrophoresis on 4% (v/v) polyacrylamide gel.

Chromatin immunoprecipitation (ChIP) assay

Nuclear components extracted from HepG2 cells were immunoprecipitated using an anti-GATA-2 antibody (SANTA CRUZ, CA, USA). DNAs in the immunoprecipitants were extracted with phenol/chloroform and precipitated with ethanol after treatment with proteinase K at 45 °C for 1 h, followed by PCR using a primer to detect the p53 gene promoter (sense, 5'-GCG GAT TAC TTG CCC TTA CTT G-3' and anti-sense, 5'-CCA ATC CAG GGA AGC GTG TC-3'). PCR products were separated by an electrophoresis on 2% (v/v) agarose gel, stained with ethidium bromide, and analyzed and NIH Image.

Fig. 1. The p53 siRNA suppresses p53 expression in HepG2 cells. Western blotting using antibodies against p53 and β-actin was carried out in HepG2 cells transfected with the NC siRNA or the p53 siRNA. p53 immunoreactive intensities were normalized by respective β-actin immunoreactive intensities. In the graph, each column represents the mean (± SEM) normalized p53 expression (n=3 independent experiments).

Fig. 2. Adenosine induces HepG2 cell death in a p53-dependent manner. HepG2 cells transfected with the NC siRNA or the p53 siRNA were treated with adenosine (3 mM) for 24 h followed by MTT assay. In the graph, each column represents the mean (± SEM) percentage of basal levels (MTT intensities for cells untreated with adenosine) (n=12-14 independent experiments). P values, unpaired t-test.

Statistical analysis

Statistical analysis was carried out using unpaired t-test and analysis of variance (ANOVA).

Results

Extracellular adenosine induces HepG2 cell apoptosis in a p53-dependent manner

We initially examined whether p53 participates in adenosine-induced HepG2 cell apoptosis. Expression of

Fig. 3. Adenosine disrupts mitochondrial membrane potentials in a p53-dependent manner. HepG2 cells transfected with the NC siRNA or the p53 siRNA were untreated [Ado (-)] and treated with adenosine (3 mM) [Ado (+)] for 24 h, and then, mitochondrial membrane potentials were assayed using a DePsipher™ kit. Orange-red (A,C,E) and green fluorescent signals (B,D,F) were detected at an absorbance of 590 and 530 nm, respectively. Scale bars, 10 μ m. Note that a similar result was obtained with 4 independent experiments.

p53 protein in HepG2 cells was clearly suppressed by transfecting the p53 siRNA (Fig. 1), indicating p53 knock-down. For cells transfected with the NC siRNA, 24-h treatment with adenosine (3 mM) reduced HepG2 cell viability to approximately 70% of basal levels, and the effect was significantly inhibited by silencing the p53 gene (Fig. 2), suggesting the implication of p53 in adenosine-induced HepG2 cell apoptosis.

We next monitored mitochondrial membrane potentials using DePsipher™. DePsipher™, a mitochondrial activity marker, has the properties of aggregating upon membrane polarization forming an orange-red fluorescent compound. If the potential is disturbed, the dye has no access to the transmembrane space and remains in or reverts to its green monomeric form. For cells transfected with the NC siRNA before adenosine treatment, the mitochondria exhibited orange-red fluorescent signals at an absorbance of 590 nm (Fig. 3A) and no accumulation of green fluorescent signals at an absorbance of 530 nm (Fig. 3B). Treatment with adenosine (3 mM) for 24 h accumulated green fluorescent

Fig. 4. Adenosine activates caspase-3, -8, and -9. After 24-h treatment with adenosine (3 mM), activities of caspase-3 (A), -8 (B), and -9 (C) were assayed in HepG2 cells transfected with the NC siRNA or the p53 siRNA. In the graphs, each column represents the mean ($\pm$ SEM) ratio against basal caspase activities (before adenosine treatment) (n=4 independent experiments). *P* values, unpaired *t*-test.

signals (Fig. 3D) without orange-red fluorescent signals (Fig. 3C), but the effect was abolished by silencing the p53-targeted gene; accumulation of orange-red fluorescent signals (Fig. 3E) without green fluorescent signals (Fig. 3F). This accounts for adenosine-induced p53-dependent disruption of mitochondrial membrane potentials.

Fig. 5. Adenosine upregulates p53 expression in HepG2 cells. HepG2 cells were treated with adenosine (3 mM) for periods of time as indicated. (A) RT-PCR. PCR products for the p53 mRNA were normalized by those for the GAPDH mRNA. In the graph, each column represents the mean ($\pm$ SEM) relative expression of the p53 mRNA against the GAPDH mRNA ($n=3$ independent experiments). (B) Western blotting. p53 immunoreactive intensities were normalized by respective β -actin immunoreactive intensities. In the graph, each column represents the mean ($\pm$ SEM) ratio against basal normalized immunoreactive intensities at 0 h for cells untreated with adenosine ($n=6$ independent experiments).

Fig. 6. Adenosine enhances p53 gene transcription by activating the promoter region from -240 to -146 bp. HepG2 cells transfected with the reporter plasmids pp53-pI (-532 to +1), pp53-pII (-240 to +1), or pp53-pIII (-146 to +1) were untreated and treated with adenosine (Ado) (3 mM) for 24 h, and then, luciferase assay was carried out. In the graph, each column represents the mean ($\pm$ SEM) percentage of control levels (luciferase luminescent intensities for cells transfected with pp53-pI without adenosine treatment) ($n=6$ independent experiments). P values, unpaired t -test.

To see the role of p53 in adenosine-induced caspase activation, we enzymatically assayed activities of caspase-3, -8, and -9 in HepG2. Treatment with adenosine (3 mM) for 24 h significantly activated caspase-3, -8, and -9 for cells transfected with the NC siRNA, with huge activation of caspase-3 (Fig. 4A, B, C). The caspase activation was attenuated by silencing the p53-targeted gene, significantly for caspase-3 and -8 activation and not significantly for caspase-9 activation (Fig. 4A, B, C). This indicates that adenosine activates caspase-3 and -8, in part caspase-9, in a p53-dependent manner.

Taken together, these results suggest that adenosine activates caspase-8 and in part caspase-9 in association with mitochondrial damage and in turn, the effector caspase-3 in a p53-dependent manner, leading to HepG2 cell apoptosis.

Adenosine enhances p53 gene transcription through GATA-2 binding to the promoter region from -240 to -146

Our next attempt was to explore the effect of adenosine on p53 gene transcription in HepG2 cells. In the RT-PCR analysis, adenosine (3 mM) upregulates

expression of the p53 mRNA in a bell-shaped treatment time (0.5-6 h)-dependent manner, reaching a peak at 3 h (Fig. 5A). In the Western blot analysis, adenosine (3 mM) increased expression of p53 protein in a bell-shaped treatment time (12-48 h)-dependent manner, with a maximum (1.7 times of basal levels) at 24-h (Fig. 5B). Adenosine, thus, appears to govern p53 gene transcription, causing an increase in the expression of p53 protein, in HepG2 cells.

In the assay of p53 gene transcriptional activity in HepG2 cells, adenosine (3 mM) significantly enhanced the p53 gene transcriptional activity for cells transfected with the reporter plasmid containing the full-length p53 gene promoter (Fig. 6). The adenosine effect was inhibited by deleting from -532 to -240 on the p53 gene promoter, and more marked inhibition was obtained with deleting from -240 to -146 (Fig. 6). This implies that adenosine stimulates p53 gene transcription by activating the p53 promoter region from -240 to -146.

Then, we asked the question as to how adenosine regulates the p53 gene transcription. To address this question, we searched possible candidates that could bind to the p53 gene promoter region from -240 to -146 using

Fig. 7. Adenosine promotes GATA-2 translocation into the nucleus and binding to the putative GATA-2 binding site on the p53 gene promoter. Nuclear extracts were prepared from HepG2 cells untreated and treated with adenosine (3 mM) for periods of time as indicated. (A) EMSA was carried out in nuclear extracts using 32 P-DNA probe to detect binding to the putative GATA-2 binding site on the p53 gene promoter in the absence and presence of non-labeled DNA probe (1 μ g)(NL-DNA). Note that similar results were obtained with 4 independent experiments. (B) Western blotting was carried out in nuclear extracts using an anti-GATA-2 antibody. In the graph, each column represents the mean ($\pm$ SEM) ratio against basal immunoreactive intensities at 0 h for cells untreated with adenosine (n=3 independent experiments).

a TFSEARCH, and the most plausible hit was GATA-2. We, therefore, constructed a 32 P-labeled DNA probe, that can detect GATA-2 binding to the p53 gene promoter, and carried out EMSA. Adenosine (3 mM) produced signals reactive to the 32 P-labeled DNA probe in nuclear extracts from HepG2 cells, the signal intensity reaching a maximum at 3-h treatment, and the signals were neutra-

Fig. 8. Adenosine stimulates GATA-2 binding to the p53 gene promoter. Nuclear components were extracted from HepG2 cells untreated and treated with adenosine (3 mM) for 24 h and subjected to non-immunoprecipitation and immunoprecipitation (IP) using an anti-GATA-2 antibody (Ab). For another preparation, cells untreated and treated with adenosine (3 mM) for 24 h were lysed. PCR for the p53 gene promoter was carried out in the nuclear extracts and cell lysates (Input). In the graph, each column represents the mean ($\pm$ SEM) ratio against basal PCR products for nuclear extracts from cells untreated with adenosine without immunoprecipitation using an anti-GATA-2 antibody (n=3 independent experiments). *P* value, unpaired *t*-test.

lized by adding non-labeled DNA probe (Fig. 7). This suggests that adenosine promotes GATA-2 binding to the p53 gene promoter.

For nuclear extracts from HepG2 cells, adenosine (3 mM) increased immunoreactive signals against an anti-GATA-2 antibody in a treatment time (1-6 h)-dependent manner (Fig. 7). This indicates that adenosine accumulates GATA-2 in the nucleus, possibly due to GATA-2 binding to the p53 gene promoter.

To obtain further evidence for GATA-2 binding to the p53 gene promoter, we carried out ChIP assay. Nuclear extracts from HepG2 cells were immunoprecipitated using an anti-GATA-2 antibody followed by PCR using a primer to detect the p53 gene promoter. For immunoprecipitated samples, adenosine (3 mM) significantly increased PCR products for the p53 gene promoter (Fig. 8). Notably, such adenosine effect was not obtained with non-immunoprecipitated samples (Fig. 8). This confirms that adenosine promotes GATA-2 binding to the p53 gene promoter.

We finally examined whether adenosine upregulates p53 mRNA expression by stimulating GATA-2 binding to

Fig. 9. Adenosine promotes p53 gene transcription in a GATA-2-dependent manner. (A) HepG2 cells were transfected with the NC siRNA or the GATA-2 siRNA, and 24 h later RT-PCR was carried out. PCR products for the GATA-2 mRNA were normalized by those for the GAPDH mRNA. In the graph, each column represents the mean ($\pm$ SEM) relative expression of the GATA-2 mRNA against the GAPDH mRNA ($n=4$ independent experiments). P value, unpaired t -test. (B) HepG2 cells transfected with the NC siRNA or the GATA-2 siRNA were treated with adenosine (3 mM) for periods of time as indicated, followed by RT-PCR. In the RT-PCR analysis, PCR products for the p53 mRNA were normalized by those for the GAPDH mRNA. In the graphs, each column represents the mean ($\pm$ SEM) relative expression of the p53 mRNA against the GAPDH mRNA ($n=4$ independent experiments). (C) ACHN cells were transfected with the NC siRNA or the GATA-2 siRNA, and 24 h later RT-PCR was carried out. PCR products for the GATA-2 mRNA were normalized by those for the GAPDH mRNA. In the graph, each column represents the mean ($\pm$ SEM) relative expression of the GATA-2 mRNA against the GAPDH mRNA ($n=5$ independent experiments). P value, unpaired t -test. (D) ACHN cells transfected with the NC siRNA or the GATA-2 siRNA were treated with adenosine (3 mM) for periods of time as indicated, followed by RT-PCR. In the RT-PCR analysis, PCR products for the p53 mRNA were normalized by those for the GAPDH mRNA. In the graphs, each column represents the mean ($\pm$ SEM) relative expression of the p53 mRNA against the GAPDH mRNA ($n=5$ independent experiments).

the p53 gene promoter. Expression of the GATA-2 mRNA in HepG2 cells were clearly suppressed for cells transfected with the GATA-2 siRNA (Fig. 9A). For HepG2 cells transfected with the NC siRNA, adenosine (3 mM) increased expression of the p53 mRNA in a treatment time (0.5-6 h)-dependent manner (Fig. 9B). The adenosine effect was significantly prevented by silencing the GATA-2-targeted gene ($P=0.0012$ as compared with expression of the p53 mRNA for cells transfected with the NC siRNA, ANOVA) (Fig. 9B). The question raised is whether GATA-2-mediated regulation of the p53 gene transcription is restricted in HepG2 cells alone. To address this question, we examined expression of the p53 mRNA in ACHN human renal cell adenocarcinoma cells. In the preliminary study, we have obtained the data that adenosine (3 mM) induces ACHN cell apoptosis. Transfection with the GATA-2 siRNA into ACHN cells significantly reduced expression of the GATA-2 mRNA (Fig. 9C). Like for HepG2 cells, adenosine (3 mM) increased expression of the p53 mRNA in ACHN cells in a treatment time (0.5-6 h)-dependent

manner, and the effect was still inhibited by knocking-down GATA-2 (Fig. 9D) ($P<0.0001$ as compared with expression of the p53 mRNA for cells transfected with the NC siRNA, ANOVA). Overall, the results lead to a conclusion that adenosine stimulates p53 gene transcription through GATA-2 binding to the p53 promoter region from -240 to -146 to increase expression of p53 protein, responsible for adenosine-induced activation of caspase-3, -8, and -9, to induced HepG2 cell apoptosis

Discussion

The results of the present study clearly demonstrate that extracellular adenosine induced HepG2 cell apoptosis in a p53-dependent manner. Of particular interest is that p53 is a transcriptional target of GATA-2 and that adenosine upregulates expression of the p53 mRNA and protein in HepG2 cells. Intriguingly, adenosine-induced upregulation of the p53 mRNA in a GATA-2-dependent manner was also found with ACHN cells. This implies

that GATA-2-regulated p53 gene transcription is not restricted in HepG2 cells alone but common for other cell types. In the present study, HepG2 cells were treated with adenosine at 3 mM for 24 h to understand the mechanism underlying adenosine-induced HepG2 cell apoptosis. We have earlier found that adenosine induces HepG2 cell death in a concentration (0.1-20 mM)- and treatment time (24-72 h)-dependent manner [7]. This suggests that lower concentrations of adenosine could still induce HepG2 cell apoptosis by the mechanism similar to here. Adenosine receptors include A₁, A_{2a}, A_{2b}, and A₃ receptors. It remains unknown through what receptor adenosine promotes GATA-2-regulated p53 gene transcription to induce HepG2 cell apoptosis. Adenosine-induced HepG2 cell death was significantly inhibited by the A₁ adenosine receptor inhibitor 8-CPT, the A_{2a} adenosine receptor inhibitor DMPX, the A_{2b} adenosine receptor inhibitor MRS1706, and the A₃ adenosine receptor inhibitor MRS1523 (unpublished data). This interprets that a specific adenosine receptor might not mediate adenosine-induced HepG2 cell apoptosis.

GATA-2 is a member of the GATA transcription factor family. GATA-2 plays a critical role in proper differentiation and function of erythroid cells [18, 19]. Lines of evidence have pointed to GATA-2 interaction with pituitary-specific positive transcription factor 1, histone deacetylase 3, zinc finger and BTB domain-containing protein 16, LIM domain only 2, promyelocytic leukemia protein, and Spi/PU.1 transcription factor [24-29]. No direct evidence for GATA-2 interaction with p53, however, has not been provided, although there is a study suggesting the implication of p53 in GATA-2-dependent proliferation/survival of early hematopoietic cells and mast cell formation [23]. The present study is the first to provide direct evidence for GATA-2 binding to the p53 gene promoter to regulate p53 gene transcription.

p53 regulates cell cycling, DNA repair, and apoptosis through its transcriptional activity. Under the normal conditions, p53 forms a complex with Mdm2 or Mdmx, an E3 ubiquitin ligase [30, 31]. KAP-1 and 14-3-3-protein serve as a corepressor for Mdm2 and Mdmx, respectively. p53 is activated by DNA damage induced by a variety of factors that include UV light, ionizing radiation, chemical agents such as hydrogen peroxide, oxidative stress, osmotic shock, ribonucleotide depletion, and deregulated oncogene expression. DNA damage activates ataxia telangiectasia mutated (ATM) or AT- and Rad3-related (ATR), a protein kinase, which phosphorylates and activates checkpoint kinase 2 (Chk2) or Chk1, respectively. Activated ATM, on the other hand, phosphorylates p53 at Ser15,

dissociating p53 from a complex of p53/Mdm2 or Mdmx, and dissociated p53 is further phosphorylated at Ser20 by Chk2 or Chk1, to activate p53. Activated p53 promotes transcription of the p21/Waf1 gene and the p53R2 gene, and each protein arrests at the G₁ phase of cell cycling by inhibiting cyclin-dependent kinase and repairs DNA damage, respectively [32, 33]. In case of severe DNA damage, phosphorylated p53 at Ser15 and Ser20 is further phosphorylated at Ser46 by Ser46 kinase, which triggers transcription of the p53-regulated apoptosis-inducing protein 1 (p53AIP1) gene followed the Puma or Noxa genes [33]. The BH3-only Bcl-2 family Puma or Noxa dissociates Bax/Bak from a complex with Bcl-2, Bcl-X_L, or Mcl-1, to induce apoptosis through mitochondrial damage [11, 12]. For another pathway, Puma or Noxa induces apoptosis by neutralizing function of Bcl-2, Bcl-X_L, or Mcl-1 through its direct binding [13].

p53 can also induce apoptosis in a transcription-independent manner [14-17]. p53 directly binds to proapoptotic Bcl-2-family proteins, leading to efflux of cytochrome c and Smac/DIABLO from the mitochondria. Cytochrome c forms a complex with Apaf-1 and caspase-9, thereby activating caspase-9 and the effector caspase-3 to induce apoptosis. Smac/DIABLO indirectly activates caspase-3 by inhibiting IAPs, to induce apoptosis.

In the present study, adenosine activated caspase-3, -8, and -9 in HepG2 cells, and the effect was prevented by knocking-down p53. This accounts for the implication of p53 in adenosine-induced HepG2 cell apoptosis. The question addressing is whether activation of those caspases is p53 transcription-dependent or -independent. We have obtained the data that adenosine downregulates expression of the Bcl-X_L mRNA and protein but otherwise upregulates expression of the Bid mRNA and protein in HepG2 cells through A_{2a} adenosine receptors, without affecting mRNA and protein expression of Bcl-2, Bad, and Bax (unpublished data). This may interpret the p53 transcription-dependent effect in adenosine-induced HepG2 cell apoptosis. In further support of this note, adenosine upregulated expression of mRNAs for TNF, TRADD, TRAIL-R2, RIPK1, FADD, and caspase-9 in HepG2 cells [7]. No direct evidence for p53-mediated transcription of those apoptosis-related genes has not been provided as of yet. In addition, the regulatory pathway underlying p53 gene transcription through GATA-2 is presently far from understanding. To address these questions, we are currently carrying out further experiments.

In conclusion, the results of the present study show that adenosine promotes GATA-2-regulated p53 gene

transcription and induces HepG2 cell apoptosis by activating caspase-3, -8, and -9 in a p53-dependent manner. This may represent fresh insight into the molecular pathway underlying adenosine-induced HepG2 cell apoptosis.

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