ERRATUM

GADD153 mediates celecoxib-induced apoptosis in cervical cancer cells

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Celecoxib, a selective cyclooxygenase 2 inhibitor, is known to have anti-inflammatory activity and to induce apoptosis in various types of cancer cells. Here, we examined the molecular mechanism of celecoxib-induced apoptosis in cervical cancer cell lines (HeLa, CaSki and C33A). Screening of a microarray cDNA-chip containing 225 different genes revealed that growth arrest and DNA damage inducible gene (GADD153), a transcription factor involved in apoptosis, showed the strongest differential expression following celecoxib treatment in all three cervical cancer cell lines. Notably, siRNA-induced silencing of GADD153 suppressed celecoxib-induced apoptosis in all the three cell lines, and exogenous expression of GADD153 triggered apoptosis in cervical cancer cells in the absence of other apoptotic stimuli. A luciferase reporter gene assay and mRNA stability tests revealed that expression of GADD153 was regulated at both the transcriptional and post-transcriptional levels following celecoxib treatment. The region between −649 and −249, containing an intact C/EBP-ATF binding site, was required for the basal activity and celecoxib-induced stimulation of GADD153 promoter activity. Also, mRNA stability test showed that celecoxib prolonged the half-life of GADD153 mRNA. In terms of signaling pathway, addition of the NF-κB inhibitor, N-tosyl-L-phenylalanyl-chloromethyl ketone (TPCK), had no effect on GADD153 expression levels. Celecoxib treatment induced Bak expression, whereas cell treated with siGADD153 or TPCK showed lower levels of celecoxib-induced Bak up-regulation. These novel findings collectively suggest that GADD153 may play a key role in celecoxib-induced apoptosis in cervical cancer cells by regulating the expression of proapoptotic proteins such as Bak.

Introduction

Celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor commonly used to treat chronic arthritic conditions, has recently been shown to induce apoptosis in various types of cancer cells, including colon, prostate, head and neck and cervical cancers (1–5). Several ongoing clinical trials have been undertaken to evaluate the use of celecoxib alone or in combination with other agents for the prevention or treatment in lung cancer (6). Thus, it is important that we understand the anticancer mechanism of celecoxib with the goal of enhancing its efficacy as valuable adjunct or single agent in anticancer therapy.

Celecoxib can induce apoptosis even in COX-2 negative cells, suggesting the presence of a COX-2 independent mechanism (5,7,8). A number of studies have indicated that COX-2 independent celecoxib-induced apoptosis may occur via inactivation of Akt signaling or modulation of the mitochondria-mediated death pathway (2,9). Celecoxib is also known to cause changes in the expression of genes in the target cells. We previously reported that celecoxib treatment activated NF-κB in cervical cancer cells, resulting in Fas death receptor expression and subsequent apoptosis, whereas inhibition of NF-κB partially blocked celecoxib-induced apoptosis in these cells (5). The identification of additional celecoxib-regulated genes will provide new insight into celecoxib-related signaling mechanisms, and may facilitate the development of new celecoxib-based anticancer strategies.

Here, we sought to identify genes that might be associated with celecoxib-induced apoptosis in cervical cancer cells. Analysis of a microarray cDNA-chip allowed us to identify several genes that were up-regulated in celecoxib-treated cervical cancer cell lines (HeLa, CaSki and C33A). In repeated experiments, of the up-regulated genes, the growth arrest and DNA damage inducible gene (GADD153) was most strikingly elevated, >1.0-fold consistently, in all the three cell lines.

GADD153, also known as CHOP (CEBP homology protein), has been demonstrated to be involved in growth arrest and apoptosis following DNA damage and a variety of stresses, such as nutrient deprivation and treatment with anticancer agents (10–12). Recently, Tsutsui et al. (13) showed that induction of apoptosis by NSAID is dependent on the function of GADD153 using cells from GADD153 knockout mice.

We further evaluated the role of GADD153 expression and regulation in this system, providing important new insight into the anticancer effect of celecoxib.

Materials and methods

Cell lines and reagents

HeLa, CaSki and C33A cell lines were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI1640 supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin (all from

Abbreviations GADD153, growth arrest and DNA damage inducible gene; TPCK, N-tosyl-L-phenylalanyl-chloromethyl ketone.

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In vitro, Carlsbad, CA). Celecoxib was a generous gift from Pharmacia, Korea.

The NF-κB inhibitor N-tosyl-l-phenylalanyl-chloromethyl ketone (TPCK) was purchased from Calbiochem (La Jolla, CA). Stock solutions were freshly prepared in dimethyl sulfoxide (DMSO) and added to the indicated final inhibitor concentrations. The final DMSO concentration was 0.001% and the same concentration was used as vehicle. DMSO alone (0.001%) was found to have no significant effect on cell function versus untreated cells. Propidium iodide and paclitaxel were obtained from Sigma Chemical (St Louis, MO).

Anti-GADD153 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bak was obtained from Cell signaling (Beverly, MA). The anti-α-tubulin antibody (used as a loading control) was obtained from Sigma and the LipofectAMINE 2000 transfection reagents were purchased from Invitrogen.

cDNA microarray analysis

Total RNA was isolated with the TRIZOL reagent (Life Technologies, Gaithersburg, MD). Total RNA (40 μg) was labeled and hybridized to Human Apoptosis CHIP Version 1.1 (Takara Shuzo, Japan) as described previously (14). In brief, fluorescence-labeled target cDNA was prepared with a labeling kit (Macrogen, Korea) in the presence of fluorescent labeled dNTPs (Cy3 dUTP or Cy5 dUTP). Labeled target cDNAs were hybridized to microarray cDNA-chip for 16 h in 3× SSC for 5 min. The Cy3 and Cy5 signals were obtained using a confocal laser scanner, and fluorescence intensity was analyzed with the iCycler IQ optical system software (version 3.0a, Bio-Rad Laboratories). Melting curve analysis was performed to confirm the peaks of interest in each samples. The results were normalized with regard to β-actin mRNA levels.

Construction and transfection of the GADD153 expression vector

For transfection of the plasmid expression vector encoding human GADD153 the DNA sequence containing the GADD153 open reading frame (ORF) flanked by BamHI–Xhol restriction sites was PCR amplified from HeLa cells. Primers were designed to introduce the Kozak (underline) sequence (16) to increase translation (5′-GGG GAT CCA CCA TGG CAG CTG CAT TGC CT and 5′-GGG GAT CCA CCA TGG CAG CTG CAT TGC CT). The resulting fragment was inserted into BamHI–Xhol precut pcDNA3 (Invitrogen) to generate pcDNA3-GADD153. The desired sequence was confirmed by direct DNA sequencing.

For transfection, cervical cancer cells were grown to 70% confluence and transfected in serum-free medium for 6 h with LipofectAMINE 2000 Transfection Reagent (Invitrogen), and pcDNA3-GADD153 or empty vector (control). After 48 h, cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), and processed for apoptosis analysis or western blot analysis.

Total RNA isolation and RT–PCR analysis

Total RNA was isolated and cDNAs were synthesized from 1 μg of total RNA using M-MLV Reverse Transcriptase (Invitrogen) with random hexamer primer. PCR was performed with specific primers (GADD153 sense 5′-GCT CTA GAG TGA TGC TCC CA-3′, antisense 5′-GGA ATT CGG GAC TGA TGC TCC AC-3′, β-actin sense 5′-ACA CCT CCA TCT ACG AGC-3′, antisense 5′-AGG GGC CGG ACT GTG CAT TGC CT and 5′-GGG GAT CCA CCA TGG CAG CTG CAT TGC CT). The resulting fragment was inserted into BamHI–Xhol precut pcDNA3 (Invitrogen) to generate pcDNA3-GADD153. The desired sequence was confirmed by direct DNA sequencing.

For transfection, cervical cancer cells were grown to 70% confluence and transfected in serum-free medium for 6 h with LipofectAMINE 2000 Transfection Reagent (Invitrogen), and pcDNA3-GADD153 or empty vector (control). After 48 h, cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), and processed for apoptosis analysis or western blot analysis.

Table 1. List of apoptotic genes up-regulated by celecoxib in three cervical cancer cell lines

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Log2 (activation fold)</th>
<th>Gene name</th>
<th>Log2 (activation fold)</th>
<th>Gene name</th>
<th>Log2 (activation fold)</th>
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<tbody>
<tr>
<td>AIF4</td>
<td>1.72</td>
<td>Cdc2-like1</td>
<td>2.11</td>
<td>APAF</td>
<td>1.18</td>
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<tr>
<td>Cdk2</td>
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<td>IGF-2</td>
<td>1.26</td>
<td>Cyclin C</td>
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</tr>
<tr>
<td>GADD153</td>
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<td>GADD153</td>
<td>2.92</td>
<td>GADD153</td>
<td>3.36</td>
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<tr>
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<td>Cdc10</td>
<td>2.01</td>
<td>IGF-2</td>
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</tr>
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<td>TP53</td>
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<td>Rb-like2</td>
<td>1.50</td>
<td>NTK</td>
<td>2.51</td>
</tr>
<tr>
<td>TNFRAF</td>
<td>1.23</td>
<td>NF-KI</td>
<td>3.16</td>
<td>Caspase-3</td>
<td>1.18</td>
</tr>
</tbody>
</table>

aAIF4, apoptosis inhibitor 4; cdc2, cell division cycle 2; GADD153, DNA-damage-inducible transcript; cdc10, cell division cycle 10; TP53, tumor protein p53; TNFRAF, tumor necrosis factor receptor 2 superfamily member 10; cdc2-like 1, cyclin-dependent kinase-like 1; IGF-2, insulin-like growth factor 2; Rb-like 2, retinoblastoma-like 2; NF-Kb, nuclear factor of kappa light polypeptide gene enhancer in B-0; APAF, apoptotic protease activating factor; NTK, neurotrophic tyrosine kinase type-2.

Fig 1. Induction of GADD153 expression by celecoxib in cervical cancer cells. (A) Cervical cancer cells were treated with celecoxib (50 μM) for the indicated times. Quantitative real-time RT–PCR was used to assess cellular GADD153 mRNA levels. The expression of mRNA of GADD153 was normalized to that of β-actin and the ratio of GADD153 to control mRNA was calculated. (B) Whole cell lysates (50 μg) were resolved by 12% PAGE, transferred to nitrocellulose membrane and probed with antibody against GADD153. Blots were stripped and reprobed with antibody to α-tubulin to verify equal loading. The blots were representative of three independent experiments, showing similar trends. The graph represents the mean ± SD of triplicate samples from three independent experiments (*P < 0.01 versus control).
Western blotting
Protein extraction was performed as described previously (5), and 50 μg of cell lysates were resolved by 12% SDS–PAGE, transferred onto nitrocellulose membrane, and immunoblotted with the indicated antibodies. Bound antibodies were detected using appropriate peroxidase-coupled secondary antibodies and visualized using an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ).

Construction of silencing RNA (siRNA)
The 21 nt siRNA duplexes (GADD153, GUGGCUACUGACUACCCUC; Bak, CAACCGACGCUAUGACUCA; GFP, AAGACCCGCGCCGAGGAGGAAG) were purchased from Dharmacon Research (Lafayette). The siRNA oligonucleotides were transfected into cultured cells using LipofectAMINE2000 according to the manufacturer’s recommendations. After 36 h post-transfection, the cells were treated with celecoxib (50 μM), incubated for an additional 6 h and then harvested for apoptosis analysis or western blotting.

Flow cytometry analysis of apoptotic cells
Treated cells were trypsinized and washed with cold PBS, fixed with 70% ethanol and stored at −20°C until use. The fixed cells were stained with 20 μg/ml of propodium iodide containing 10 μg/ml RNaseA and then incubated at room temperature for 30 min in the dark. The DNA content

![Diagram of protein expression and siRNA effects](image)

**Fig 2.** Inhibition of celecoxib-induced apoptosis by silencing of GADD153 expression with small interfering RNA (siRNA) Cervical cancer cells were cultured in 6-well plate and transfected with control (sictrl, 100 nM) or GADD153 small interfering RNA (siGADD153, 100 nM) for 36 h. After transfection, cells were treated with DMSO or 50 μM celecoxib for 6 h. (A) Whole cell lysates were prepared for western blotting analysis of GADD153 protein and α-tubulin. (B) Each of these groups was analyzed by flow cytometry after propidium iodide staining. The percentage of sub-G₁ phase cells was determined based on the DNA content histograms and represented as the mean ± SD of triplicate samples from three independent experiments.
mRNA stability. Cervical cancer cells were incubated with celecoxib for 6 h, the half-life of GADD153 mRNA was assayed as a measure of GADD153 mRNA stability. 

**Analysis of GADD153 promoter activities**

The GADD153 luciferase reporter gene construct was kindly provided by S.-H. Kim et al. (NorthRyde, Australia). The sub-G1 population was considered to represent apoptotic cells.

**Inhibition of celecoxib-induced apoptosis by silencing of GADD153 expression with small interfering RNA (siRNA)**

To determine the importance of the GADD153 in celecoxib-induced apoptosis, we used to siRNA methodology to silence the GADD153 gene. siRNA oligonucleotide specific for GADD153 or control oligonucleotide against GFP were transiently transfected into cervical cancer cells, which were then subjected to celecoxib treatment. Western blotting revealed that transfection of cervical cancer cells with 100 nM siGADD153 effectively suppressed celecoxib-induced increases in GADD153 expression (Figure 2A). Interestingly, transfection of siRNA to suppress GADD153 decreased the apoptotic sub-G1 fraction even in the presence of celecoxib (Figure 2B). To know that siRNA for GADD153 dose not inhibit induced by other stressor without GADD153 inducing activity, we examined the effect of siGADD153 on paclitaxel-induced apoptosis for the indicated time. Paclitaxel-induced apoptosis in cervical cancer cells. Interestingly, paclitaxel-induced GADD153 expression in C33A cells, but not in HeLa cells. Western blotting revealed that transfection of C33A cells with 100 nM GADD153 siRNA effectively suppressed paclitaxel-induced GADD153 expression in C33A cells. Furthermore, the transfection of GADD153 siRNA reduced the apoptotic sub-G1 fraction in the paclitaxel-treated C33A cells, but not in HeLa cells (data not shown).

**Effect of ectopic GADD153 overexpression on apoptosis in cervical cancer cells**

To further determine the effect of GADD153 on apoptosis, an expression plasmid containing the full-length cDNA of GADD153 was transiently transfected into cervical cancer cells. As shown in Figure 3, GADD153 overexpression increased the apoptotic sub-G1 fraction of the cells up to 24–30%, whereas transfection with the empty vector was associated with the apoptotic sub-G1 fraction <5%. These results indicate that expression of GADD153 is capable of inducing apoptosis in cervical cancer cells in the absence of other apoptotic signals.

**Celecoxib treatment increase the transcriptional activity and mRNA stability of GADD153**

To examine whether celecoxib increases GADD153 mRNA expression at the transcriptional or posttranscriptional level, we performed luciferase reporter gene assay and mRNA stability test. GADD153-luciferase reporter plasmids (954/+91, 649/+91, 249/+91, and 91/+91) were transiently transfected into cervical cancer cells, which were then treated with or without 50 μM celecoxib for 6 h.

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**Fig 3. Effect of ectopic GADD153 overexpression on apoptosis in cervical cancer cells**

Western blot analysis of GADD153 in cervical cancer cells transfected with the GADD153 expression plasmid (pcDNA3-GADD153) or vector control (pcDNA3) as described in Figure 1 (upper panel). The cells were also harvested for flow cytometric analysis of the percentages of cells with sub-G1 DNA content (lower panel). The blots are representative of three independent experiments showing similar trends. The percentage of sub-G1 phase cells was determined based on the DNA content histograms and represented as the mean ± SD of triplicate samples from three independent experiments (*P < 0.01 versus pcDNA3-transfected control).

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**Results**

**Celecoxib induces GADD153 expression in cervical cancer cells**

We previously reported that celecoxib effectively induced apoptosis in cervical cancer cells (5). Here we used a microarray cDNA-chip for detecting genes involved in apoptosis to examine differential gene expression in celecoxib-treated cervical cancer cells (HeLa, CaSki and C33A) treated with 50 μM celecoxib for 6 h. Out of a total of 225 different genes in the microarray, selected genes were consistently up-regulated ~1.1–10-fold in repeated experiments in celecoxib-treated cells (Table 1). GADD153, a transcription factor involved in apoptosis, showed the most striking differential expression in all three cervical cancer cell lines and was thus chosen for further study. To confirm the microarray results and examine celecoxib-induced regulation of GADD153 expression, we used real-time RT-PCR and western blotting analysis to determine celecoxib-induced regulation at the level of mRNA and protein over time in these cells. Consistent with the noted increase in the mRNA (Figure 1A), GADD153 protein level (Figure 1B) increased in a time-dependent manner in celecoxib-treated cervical cancer cells.

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**Analytic of GADD153 promoter activities**

The GADD153 luciferase reporter gene construct was kindly provided by S.-H. Kim et al. (NorthRyde, Australia). The sub-G1 population was considered to represent apoptotic cells.
As shown in Figure 4B, the wild-type −649/+91 construct showed a ~1.5–3-fold increase in luciferase activity in celecoxib-treated cells versus untreated controls. Deletion to −649 had no significant effect of the celecoxib-induced activation of GADD153 promoter activity, whereas deletion to −249 dramatically decreased the basal and celecoxib-induced stimulation of GADD153 promoter activity in all three cell lines. Further deletions of the promoter from −249 to −91 led to additional reduction in celecoxib responsiveness and a progressive reduction of basal activities.

These results suggest that the regulatory element(s) present between nucleotides −649 and −249 of the GADD153
Celecoxib-induced GADD153 expression is NF-κB-independent in cervical cancer cells. Cervical cancer cells were preincubated for 1 h with TPCK (0.1 μM), treated with celecoxib (50 μM) for 6 h, and then analyzed for GADD153 mRNA levels as described in Figure 1. The results are representative of three independent experiments, showing similar trends. The bar indicates the quantitative measurement of GADD153 band intensity adjusted versus that of β-actin (*P < 0.01 versus DMSO-treated control).

Celecoxib induces Bak expression in cervical cancer cells

Considerable evidence indicates that GADD153 mediates cellular response to oxidant injury, mainly by mediating ER stress (23–25). Furthermore some NSAIDs can induce ER stress response including GADD153 (13), and Bak has been found to be associated with the ER-stress pathway (26,27). We therefore examined the relationship between GADD153 and Bak in celecoxib-induced apoptosis.

Western blotting analysis revealed that Bak was induced in celecoxib-treated cervical cancer cells (Figure 6A), with a time course that resembled the GADD153 response. To check the apoptotic effect of Bak induced by celecoxib, we transiently transfected siRNA oligonucleotide specific for Bak (siBak) or control oligonucleotide against GFP into cervical cancer cells, which were then treated with celecoxib. Western blotting revealed that transfection of cervical cancer cells with 100 nM siBak partially suppressed celecoxib-induced expression of Bak (Figure 6B) and also decreased the apoptotic sub-G1 fraction in the presence of celecoxib (Figure 6C).

To test whether GADD153 is responsible for the induction of Bak, we examined the effect of siGADD153 on celecoxib-induced Bak expression. Bak expression was reduced by 50% in cells transfected with siGADD153 (Figure 6D). This suggests that GADD153 may directly regulate at least a portion of celecoxib-induced Bak expression. However, the observation that ~50% of celecoxib-induced Bak expression remained in siGADD153-treated cells suggests that celecoxib-induced Bak expression may be mediated by other GADD153-independent mechanisms.

In our previous report (5), we found that TPCK, NF-κB blocker, inhibited the apoptosis by celecoxib. Also, in this study, siGADD153 inhibited the apoptosis by celecoxib to the similar degree as shown in TPCK-treated cells. However, the induction of GADD153 was not affected by TPCK (Figure 5). These three data sets seem to be contradictory. To solve this problem, we tested the effect of TPCK on Bak expression. As shown in Figure 6E, the addition of TPCK reduced the expression of Bak in celecoxib-treated cells. Additionally, the simultaneous use of TPCK and siGADD153 reduced the expression of Bak, additively (Figure 6F).

Discussion

We herein showed that GADD153 is critical for celecoxib-induced apoptosis of cervical cancer cells. Two lines of evidence support this conclusion: first, silencing of the GADD153 gene by siRNA blocked celecoxib-induced apoptosis in cervical cancer cells, and second, ectopic expression of GADD153 was sufficient to induce apoptosis in cervical cancer cells in the absence of additional apoptotic stimuli.
The GADD153, a member of the CCAAT/enhance-binding protein family of transcription factors, is a stress-induced, low molecular weight nuclear protein, also known as C/EBP homologous protein 10 (CHOP) (28,29). GADD153 can negatively regulate C/EBP transcription factors that inhibit cell progression or can act as a positive regulator of target genes (30). GADD153 expression has been associated with apoptosis in response to a number of stress stimuli, including anticancer agents, retinoic acid and nutrient deprivation (10–12). In addition to its function as a transcription factor, GADD153 has been shown to mediate apoptosis through a non-transcriptional pathway (10). However, few studies have sought to establish a direct link between apoptosis and GADD153 expression.

Here, we showed that GADD153 has been shown to be up-regulated during the apoptotic pathway. Interestingly, silencing of GADD153 expression blocked apoptosis in celecoxib-treated cervical cancer cells (Figure 2), indicating that GADD153 up-regulation was not merely a consequence of apoptosis. We found that siRNA for GADD153 does not inhibit apoptosis induced by other stressor, such as paclitaxel that does not have GADD153-inducing activity (data not shown). This result suggests that GADD153 may have an important role in causing apoptosis of cancer cells by other various anticancer agents.

GADD153 expression may be regulated at both the transcriptional and post-transcriptional levels (10,31–33). GADD153 mRNA stability is reportedly increased by...
As GADD153 is induced by chemotherapeutics drugs in other tumor types (34,35,40) and plays a key role in drug-induced apoptosis, these findings provide important new insight into signaling involved in GADD153-induced apoptosis by celecoxib. This finding may facilitate the development of chemotherapeutic or chemopreventive strategies using celecoxib.

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Conflict of Interest Statement: None declared.

References


