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Original

The Role of GADD45A in Resistance to Oxidative Stress-Mediated Cell Death in Human Colon Tumor Cell Lines

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Abstract

Objective: GADD45A is a growth arrest-associated gene, directly involved in the maintenance of genomic stability. In fact, in the absence of this protein cells became sensitized to death by ultraviolet irradiation or cisplatin. However, this role with respect to cell damage triggered by Reactive Oxygen Species (ROS) is not well understood. Thus, in an attempt to evaluate its role in oxidative stress-induced apoptosis, we analyzed its expression after induction of ROS in human colon cell lines. **Material and methods:** Cell lines derived from human colon tumors (RKO-AS45-1 and HuTu-80) were treated with Menadione (MEN) and Hydrogen Peroxide (HP). Next, the expression of GADD45A was evaluated by semi-quantitative RT-PCR analysis. **Results:** The results indicated that expression of this gene was associated with resistance to apoptosis. Additionally, cells with high expression of GADD45A were resistant to treatment with oxidative stress-inducing compounds. **Conclusion:** In conclusion, we propose that the expression of the GADD45A gene can be used as a functional tool to predict cellular responses to antitumor treatments.

Keywords: Apoptosis. Colorectal cancer. GADD45A. Oxidative stress.

Introduction

GADD45A is considered to be an effective indicator of poor prognosis or malignant potential of human tumors such as breast, lung and hepatocellular carcinomas.[1-3] It is thus worthwhile to study its expression profile in cell lines derived from human cancers such as colorectal tumor, which constitutes an excellent system for studying both carcinogenesis and the molecular events involved in the development of human cancer. Colorectal cancer is one the most prevalent tumors and is characterized by the accumulation of multiple mutations in tumor suppressor genes and oncogenes that affect the balance between cell proliferation and apoptosis.[4-6] This etiology highlights the importance of tumor suppressor genes such as *GADD45A,* which is well-known to play a key role in the maintenance of cellular homeostasis. *GADD45A* is a growth arrest-associated gene, its expression is known to be regulated by p53 in response to the genomic instability triggered by ultraviolet irradiation, hydroxiurea, and ionizing radiation, and also by the NFκB/IkB signaling pathway.[7-8]

While the role that the GADD45A protein plays in apoptosis remains unclear, its role in the G_2/M checkpoint in response to DNA damage is well-established. GADD45A activates p53-dependent G_2/M arrest and inhibits cdc2 kinase, which can be blocked by overexpression of cyclin B1, providing a link between the p53-dependent cell cycle checkpoint and DNA repair.[9-11] Overexpression of *GADD45A* in normal human fibroblasts causes G2/M arrest but not apoptosis. Nonetheless, genotoxic stress or

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BRCA1-induced apoptosis seem to involve GADD45A-mediated activation of the stress-responsive c-jun NH2-terminal kinase and/or p38 mitogen-activated protein kinase.[12-13]

GADD45A is directly involved in the maintenance of genomic stability. In fact, in the absence of this protein cells became sensitized to death by ultraviolet irradiation or cisplatin, a cancer chemotherapy drug which produces DNA cross-links.[14] However, this role with respect to cell damage triggered by ROS (reactive Oxygen Species) is not well understood. Thus, in an attempt to evaluate its role in oxidative stress-induced apoptosis, we treated cell lines derived from human colon tumors (RKO-AS45-1 and HuTu-80) with Menadione (MEN) and Hydrogen Peroxide (HP), both of which are known to induce oxidative stress and affect cell proliferation and survival, leading to apoptosis. The RKO-AS45-1 cell line was generated from the human colon carcinoma RKO cell line by transfection of these cells with the open reading frame portions of human antisense cDNAs for *GADD45A* cloned into the expression vector pCMV.[14]

The expression of *GADD45A* after induction of oxidative stress was analyzed, and the results indicated that expression of this gene was associated with resistance to apoptosis. Additionally, cells with high expression of *GADD45A* were resistant to treatment with oxidative stress-inducing compounds. In conclusion, we propose that the expression of the *GADD45A* gene can be used as a functional tool to predict cellular responses to antitumor treatments.

Material and Methods

Cell Lines and Cell Culture

The human colon cancer cell lines RKO-AS45-1 (CRL-2579™) and HuTu-80 (HTB-40™), both p53 wild types, were obtained from the American Type Culture Collection. Cells were cultured in modified minimum essential medium eagle - EMEM (Sigma) supplemented with 10% v/v fetal bovine serum (FBS) at 37°C in 5% CO2.

Drugs

To promote oxidative stress-induced cell death in both human colon cancer cell lines, treatments with Menadione (MEN) and Hydrogen Peroxide (HP) obtained from commercial sources (Sigma) were administered. Drugs were prepared freshly for each experiment.

MTT Assay

The effect of MEN and HP on cell viability was evaluated by a colorimetric assay that is based on the activity of mitochondrial dehydrogenase, which cleaves the yellow dye MTT (3-[4, 5-dimethylthiaolyl]-2, 5-diphenyl-tetrazolium bromide) to form purple formazan crystals in living cells.[15] Twenty-four hours before treatment, exponentially growing human colon cancer cell lines were seeded in 96-well microtiter culture dish (Corning®) at a density of 10^5 cells/well, in a final volume of 100µL of EMEM medium supplemented with 10% w/v FBS. After washing once with PBS 1X, cells were treated with MEN or HP. Untreated cells (EMEM 1.0% v/v FBS) served as controls. Replica-plated well for each treatment condition were incubated for 3 h at 37°C with a solution of MTT (Sigma) at a final concentration of 0.45 µg/µL. The cells were lysed at room temperature in 50 µL of DMSO (Merck). For each well, the absorbance of the reduced intracellular formazan product was read at 492 nm in a microtiter plate reader (Labsystems Multiskan MS**)**.

Survival Rate

To determine the IC_{50} for MEN and HP, cell lines RKO-AS45-1 and HuTu-80 were submitted to a dose-dependent treatment for 30 minutes at 37°C. The IC_{50} for both drugs was defined as a 50% reduction of absorbance in the MTT assay.

Cell Viability Analysis

The LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes®), which provides a simple fluorescence-based method for determining viability of adherent or nonadherent cells, was used to analyze oxidative stress-induced cell death mediated by MEN and HP. This system comprises two probes: calcein AM and ethidium homodimer-1. Calcein AM is a fluorogenic esterase substrate that is hydrolyzed to a green-fluorescent product (calcein) only in living cells. Ethidium homodimer-1 is a high-affinity, red-fluorescent nucleic acid stain that is only able to pass through the compromised membranes of dead cells. Human colon cancer cell lines were plated in 48-well microtiter dishes at a density of 1.2 x 10^5 cells/well in a final volume of 200µL of EMEM medium

supplemented with 10% w/v FBS. Twenty-four hours after the initial seeding, cells were washed once with PBS 1X, and treated with 25mM of MEN or 15mM of HP. Untreated cells were used as controls. After washing once with PBS 1X, the live/dead assay was performed according to manufacturer's instructions.

RNA Extraction, cDNA Synthesis, and Semi-Quantitative RT-PCR

Exponentially growing human colon cancer cell lines were seeded in 6-well microtiter culture dishes (Corning®) at a density of 1.5 x 10^6 cells and treated with MEN or HP, as described above. Total RNA of drug-treated and untreated-cells was extracted using TRizol® (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized using IMPROM™ Reverse Transcriptase (Promega) and used for semi-quantitative PCR employing intron-spanning primers (Table 1). We normalized *GADD45A* data with β-actin expression (*ACTB*) as an internal control. Gel images were digitalized and analyzed using Kodak 1D Image Analysis Software (Kodak®).

Statistical Analyses

Statistical analyses were performed using t-student test. For each test, p-values less than 0.05 were considered as statistically significant.

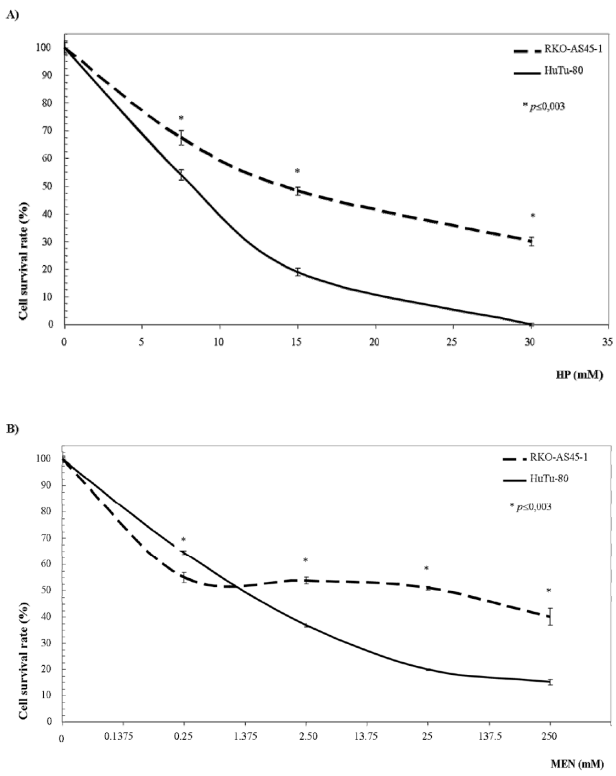
Table 1- Intron-spanning primers utilized on RT-PCR semi-quantitative analysis

Primers	TM
GADD45A	
FW: 5'- AGA TCC ACT TCA CCC TG -3'	52° C
RV: 5'- ACC GTT CAG GGA GAT TA -3'	
ACTB	
FW: 5'- CTG GAC TTC GAG CAA GA -3'	56° C
RV: 5'- TTC TGC TTG CTG ATC CA -3'	
ATM	
FW: 5'- TTA CGG GTG TTG AAG GTG TCT TC -3'	56° C
RV: 5'- GGA TTC ATG GTC CAG TCA AAG AG -3'	
p53	
FW: 5'- GGA GGA GCC GCA GTC AGA -3'	56° C
RV: 5'- CAA GAA GCC CAG ACG GAA AC -3'	
p27	
FW: 5'- ACC TGC AAC CGA CGA TTC TT -3'	56° C
RV: 5'- CCC TTC CCC AAA ATT GCT TC -3'	

Results

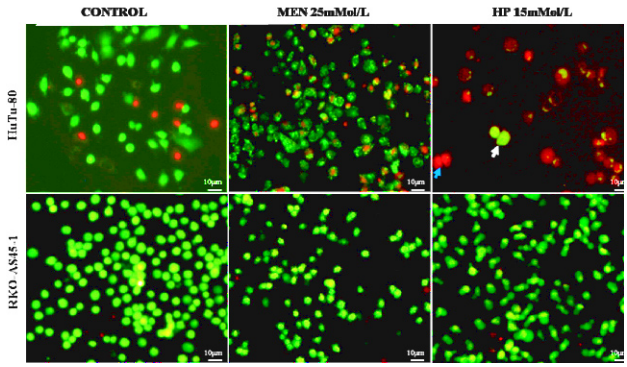
Statistically Significant Differences in Responses of the Cell Lines After Induction of Oxidative Stress

According to the MTT assays, MEN and HP treatment induced a profound concentration-dependent reduction in the proliferation of both human colon cancer cell lines. Nevertheless, the RKO-AS45-1 cell line was more resistant to both treatments when compared to HuTu-80 (Figure 1). The same difference in responses to



Legend: Dose-dependent effects of Hydrogen Peroxide (A) and Menadione (B) on survival rates of RKO-AS45-1 and HuTu-80 cell lines, after 30 minutes at 37°C, 5%CO2. Cell growth was performed using the MTT colorimetric assay. The results are the average of at least four replicates and two-independent experiments, and bars represent standard deviation values. Statistical differences between MEN and HP on survival rates of RKO-AS45-1 and HuTu-80 cells was calculated by a parametric test (t-Student). Statistically significant differences on survival rates for all comparisons were observed with p-values<0.003.

Figure 1 - Survival curve for RKO-AS45-1 and HuTu-80 after MEN and HP treatment



Legend: Cell viability of treated and untreated RKO-AS45-1 and HuTu-80 cells as assessed using two probes: calcein AM and ethidium homodimer-1. Calcein AM is a fluorogenic esterase substrate that is hydrolyzed to a green-fluorescent product (calcein) only in living cells (white arrow). Likewise Ethidium homodimer-1 is a high-affinity, red-fluorescent nucleic acid stain that is only able to pass through the compromised membranes of dead cells (blue arrow). In this assay we used doses estimated to be the IC50 of RKO-AS45-1 cells according to the MTT cell viability assay.

Figure 2 - Cell viability after induction of oxidative stress by MEN and HP.

oxidative stress was observed after analyzing cell viability using fluorescent probes specific to live and dead cells. Again, the results demonstrated that HuTu-80 was less resistant to the oxidative stress-induced cell death induced by MEN and HP (Figure 2).

Expression Profile Analysis of Genes Related to the Maintenance of Cellular Homeostasis

In an attempt to explain the statistically significant differences in the rates of cell death observed between the RKO-AS45-1 and HuTu-80 lines, semi-quantitative RT-PCR analysis of genes known to be responsible for the maintenance of cellular homeostasis after oxidative stress, *GADD45A*, *ATM*, *p27* and *p53,* was performed. *GADD45A* was found to be differentially expressed between treated RKO-AS45-1 and HuTu-80 cells (Figure 3C). Additionally, *GADD45A* was down-regulated in MEN-treated RKO-AS45-1 cells in a dose-dependent manner (Figure 3E), in a pattern similar to the survival curve (Figure 1B). Surprisingly, after oxidative stress was induced for 3 hours with HP, *GADD45A* was up-regulated by 1.4-fold in RKO-AS45-1 cells (Figure 3C). *p53* was down-regulated in the RKO-AS45-1 cell line treated with MEN or HP by 1.7- and 1.4-fold, respectively, and by 1.5-fold in MEN-treated HuTu-80 cells. After oxidative stress induced by HP, *p27* was down-regulated by 3.2-fold in RKO-AS45-1 cells. In contrast, *ATM* was not differentially expressed between the cell

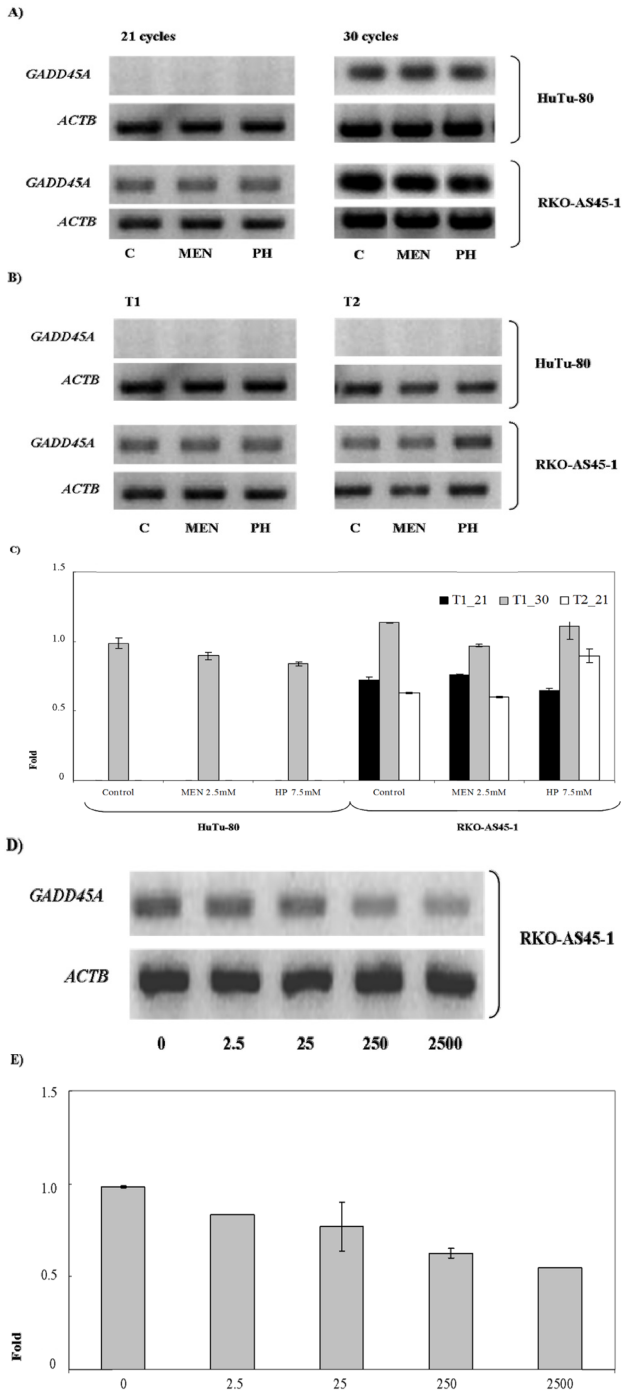


Figure 3 (A-E) - RT-PCR semi-quantitative analysis for GADD45A gene. (A) GADD45A expression in RKO-AS45-1 and HuTu-80 cells after treatment with MEN (2.5mM) and HP (7.5mM) for 30 minutes (T1) at 37°C, by RT-PCR using 21 or 30 cycles. (B) GADD45A expression in RKO-AS45-1 and HuTu-80 cells after treatment with MEN (2.5mM) and HP (7.5mM) for 30 minutes (T1) or 3 hours (T2) at 37°C, by RT-PCR using 21 cycles. (C) The graph shows normalized GADD45A data obtained from (A) and (B) with ACTB as an internal control. (D) GADD45A expression in RKO-AS45-1 cells after a dose-dependent treatment (2.5, 25, 250 and 2500mM) with MEN for 30 minutes (T1) at 37°C, by RT-PCR using 21 cycles. (E) The graph shows normalized GADD45A data obtained from (D) with ACTB as an internal control. The results are the average of at least two-independent experiments, and bars represent standard deviation values.

Figure 3 - Semi-quantitative RT-PCR analysis of the GADD45A gene.

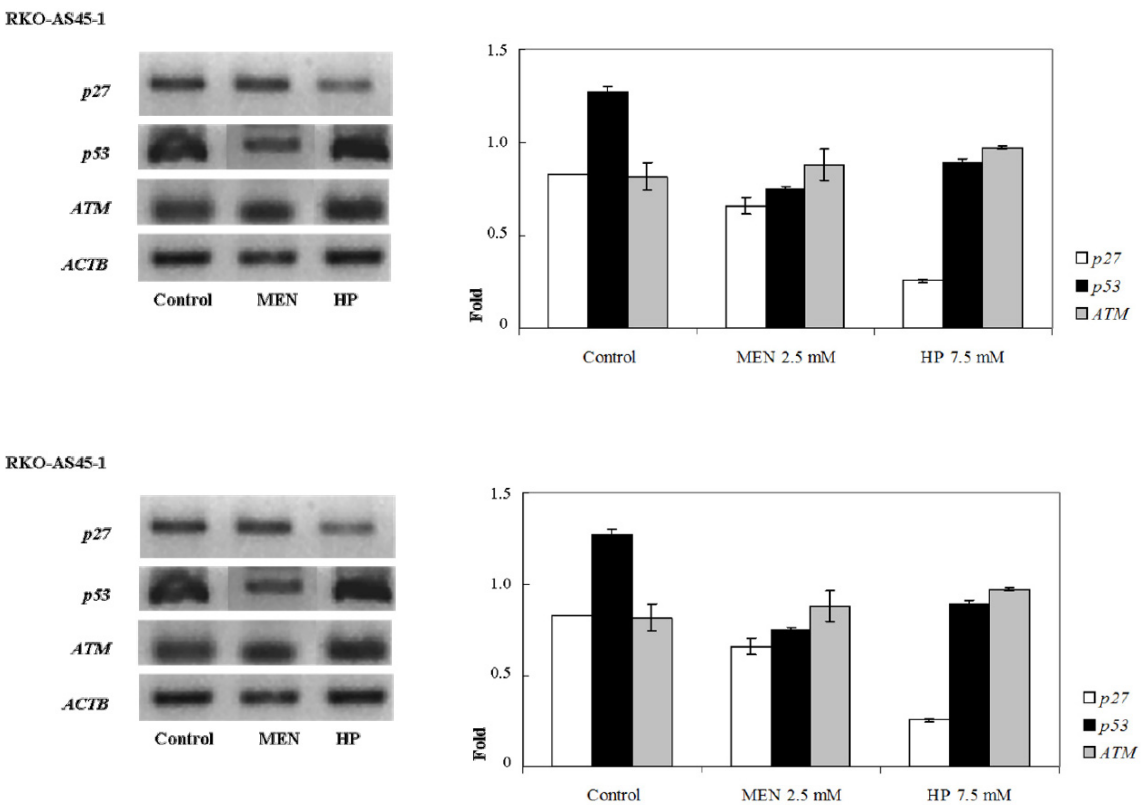
lines either before or after the induction of oxidative stress (Figure 4).

Discussion

Over the past decade, significant progress has been made in the treatment of localized colorectal cancer due to advances in surgery, radiotherapy, and chemotherapy. However, the greatest problem associated with effective cancer treatment is drug resistance. In recent years, a large number of studies have attempted to define molecular and biochemical markers that may be useful predictors of tumor response to treatment.

As *GADD45A* has been considered to be an effective indicator of poor prognosis or the malignant potential of some human tumors,[1-3] we decided to study its expression by semi-quantitative RT-PCR analysis after the induction of oxidative stress. To this end, we treated tumor-derived cell lines with the compounds MEN and HP, which are both known to be cytotoxic and to induce oxidative stress in many human cancers.[16-18] This study examined the oxidative stress responses of the RKO-AS45-1 cell line, which expresses antisense *GADD45A* and is well-known to be sensitivity to ultraviolet-irradiation or cisplatin.[14,19] Intriguingly, the results of these experiments indicated that the RKO-AS45-1 cell line showed resistance to cell death induced by both drugs, as seen in MTT and live cell staining experiments. This resistance was probably due to higher basal expression of *GADD45A,* which was both detected previously in the parental cell line RKO[14] and demonstrated here by semi-quantitative RT-PCR analysis.

Suppression of *GADD45A* expression might alter cell survival after induction of ROS by MEN and HP. Accordingly, HuTu-80 cells had lower expression of *GADD45A* than RKO-AS45-1, even after 30 cycles and for both times tested. However, it is worthwhile to consider possible biases in the expression analysis of *GADD45A* by RT-PCR after 30 cycles. In particular,



Legend: Semi-quantitative RT-PCR analysis (21 cycles) of p27, p53, ATM and ACTB in RKO-AS45-1 and HuTu-80 cells after treatment with MEN (2.5mM) and HP (7.5mM) for 3 hours (T2) at 37°C. The graphs show GADD45A data normalized with ACTB as an internal control. The results are the average of at least two independent experiments, and bars represent standard deviation values.

Figure 4 - Semi-quantitative RT-PCR analyses for p27, p53, ATM and ACTB genes

GADD45A expression in RKO-AS45-1 treated-cells could not be reliably measured since the analyzed bands were saturated (Figure 3).

The role of GADD45A in cell cycle arrest and DNA repair is well-established, but its role in ROS-mediated apoptosis remains unclear. Our results suggest that it may play an important role in this process, as its expression profile was associated with augmented resistance of RKO-AS45-1 to ROS-mediated cell death.

The higher basal level of *GADD45A* expression in RKO-AS45-1 cells compared to other human cells has been previously observed,[14] as has the increase in its expression in response to oxidative stress induction.[1] In addition, *GADD45A* suppression has been shown to result in decreased in DNA repair and sensitize cells to ultraviolet-irradiation or cisplatin.[14, 19]

Conclusion

The analysis of *GADD45A* expression conducted in this study suggests that this gene is involved in apoptosis resistance. Additionally, cells with high expression of *GADD45A* were resistant to treatment with oxidative stress-inducing compounds, including an antitumor drug. In conclusion, we propose that the *GADD45A* gene could be used as a functional tool to predict cellular responses to antitumor treatments.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

MPM was involved in MTT and live cell staining experiments, cell treatments, and gene expression analysis. LMS was involved in live cell staining experiments and supplied both cell lines used in this study. WKM participated in the design of the study and manuscript preparation. All authors have read and approved the final version of the manuscript.

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