Available online at www.sciencedirect.com
ScienceDirect
Free Radical Biology & Medicine 42 (2007) 44–51
www.elsevier.com/locate/freeradbiomed

Original Contribution

Oxalomalate regulates ionizing radiation-induced apoptosis in mice

Jin Hyup Lee, Jeen-Woo Park *

School of Life Sciences and Biotechnology, College of Natural Sciences, Kyungpook National University, Taegu 702-701, Korea

Received 12 June 2006; revised 25 August 2006; accepted 15 September 2006
Available online 23 September 2006

Abstract

Ionizing radiation induces the production of reactive oxygen species, which play an important causative role in apoptotic cell death. Recently, we demonstrated that the control of mitochondrial redox balance and the cellular defense against oxidative damage are primary functions of mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (IDPm) by supplying NADPH for antioxidant systems. In this paper, we demonstrate that modulation of IDPm activity in the kidneys of mice regulates ionizing radiation-induced apoptosis. When oxalomalate, a competitive inhibitor of IDPm, was administered to mice, inhibition of IDPm and enhanced susceptibility of apoptosis reflected by DNA fragmentation, the changes in mitochondria function, and the modulation of apoptotic marker proteins were observed upon exposure to 2 Gy of γ-irradiation. We also observed a significant difference in the mitochondrial redox status between the kidneys of the control and the oxalomalate-administered mice. This study indicates that IDPm may play an important role in regulating the apoptosis induced by ionizing radiation, presumably, through acting as an antioxidant enzyme.

Keywords: Ionizing radiation; IDPm; Oxalomalate; Apoptosis; Redox status

Introduction

Radiation therapy has been commonly used for the treatment of tumors. Ionizing radiation has been shown to generate reactive oxygen species (ROS) in a variety of cells [1]. When water, the most abundant intracellular material, is exposed to ionizing radiation, decomposition reactions occur and a variety of ROS, including superoxide, hydroxyl radicals, singlet oxygen, and hydrogen peroxide, are generated [2]. The secondary radicals formed by the interaction of hydroxyl radicals with organic molecules may also be of importance [1,2]. These ROS have the potential to damage critical cellular components such as DNA, proteins, and lipids and eventually result in physical and chemical damage to tissues that may lead to cell death or neoplastic transformation [3]. In many cases, ionizing radiation-induced cell death has been identified as apoptosis [4].

Biological systems have evolved an effective and complicated network of defense mechanisms which enable cells to cope with lethal oxidative environments. These defense mechanisms involve antioxidant enzymes, such as superoxide dismutase (SOD), which catalyzes the dismutation of O_2^- to H_2O_2 and O_2 [5], catalase, and peroxidases which remove hydrogen peroxide and hydroperoxides [6]. Since ROS appear to be mediators of the apoptosis by ionizing radiation [7], factors including antioxidant enzymes that regulate the fate of such species may be of great importance in the protection of cells against ionizing radiation-induced cell death.

The isocitrate dehydrogenases (ICDHs, EC1.1.1.41 and EC1.1.1.42) catalyze oxidative decarboxylation of isocitrate to α-ketoglutarate and require either NAD^+ or $NADP^+$, producing NADH and NADPH, respectively [8]. NADPH is an essential reducing equivalent for the regeneration of reduced glutathione (GSH) by glutathione reductase and for the activity of NADPH-

Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase; ICDHs, isocitrate dehydrogenases; IDPm, mitochondrial ICDH; DNPH, 2,4-dinitrophenylhydrazine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TRITC, tetramethylrhodamine isothiocyanate; DHR, dihydrorhodamine; PARP, poly (ADP-ribose) polymerase; JC-1, 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide; 8-OH-dG, 8-hydroxy-2′-deoxyguanosine; MDA, malondialdehyde; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

* Corresponding author. Fax: +82 53 943 2762.
E-mail address: parkjw@knu.ac.kr (J.-W. Park).

0891-5849/$ - see front matter

dependent thioredoxin system [9,10], both are important in the protection of cells from oxidative damage. Therefore, ICDH may play an antioxidant role during oxidative stress. We recently reported that mitochondrial ICDH (IDPm) is involved in the supply of NADPH needed for GSH production against mitochondrial oxidative damage [11].

In the present report, we demonstrate that the modulation of IDPm activity by oxalomalate in the kidneys of mice regulates apoptosis induced by γ-irradiation. Oxalomalate, a tricarboxylic acid (α-hydroxy-β-oxalosuccinic acid) formed in vitro and in vivo by condensation of oxaloacetate and glyoxylate, has been known to be a potent inhibitor of IDPm [12]. The results suggest that IDPm may play an important role in regulating the apoptosis induced by ionizing radiation, presumably, through acting as an antioxidant enzyme.

Materials and methods

Materials

β-$NADP^+$, isocitrate, 2,4-dinitrophenylhydrazine (DNPH), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), xylenol orange, propidium iodide (PI), oxalomalate, avidin-conjugated tetramethylrhodamine isothiocyanate (TRITC), and anti-rabbit IgG-conjugated TRITC secondary antibody were obtained from Sigma Chemical Co. (St. Louis, MO). 5,5,6,6-Tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide (JC-1), and dihydrorhodamine (DHR) 123 were purchased from Molecular Probes (Eugene, OR). Electrophoreses reagents and the Bio-Rad protein assay kit were purchased from Bio-Rad (Hercules, CA). Antibodies against Bcl-2, Bax, cleaved caspase-3, cleaved poly(ADP-ribose) polymerase (PARP), cytochrome *c*, Noxa, PUMA, p21, and p53 were purchased from Santa Cruz (Santa Cruz, CA) or Cell Signaling (Beverly, MA).

Mice

Six- to 8-week-old female C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD) and kept in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, MD). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

Animal treatment and whole-body irradiation

Two groups of 15 C57BL/6 mice each received either oxalomalate or 0.9% NaCl. Solutions of oxalomalate were freshly prepared in 0.9% NaCl and administered before irradiation at a dose of 25 mg/kg in volumes equivalent to 1% of each animal's weight once daily for 10 days. Control mice were given 0.9% NaCl, and all injections were administered ip. After irradiation with a ^{137}Cs source at a dose rate of 1 Gy/min, the mice were returned to climate-controlled cages for further experiment. Mice were sacrificed by a cervical dislocation at

Tissue dissociation

Dissociation of kidney tissue was performed using a petri dish, tweezers, and a fresh scalpel blade. Dissociated tissue was prevented from drying out by covering it with a few drops of PBS. During dissociation, the kidney epithelial cell suspension was aspirated repeatedly through a 20-gauge needle into a syringe, and consequently injected through a cell strainer into a 50-ml tube to retain clumps. Suspensions were centrifuged and resuspended with a defined amount of PBS, and final cell number was counted from aliquots.

Measurement of IDPm activity

For the preparation of mitochondrial fraction from kidney tissue, the tissue portions were homogenized with a Dounce homogenizer in sucrose buffer (0.32 M sucrose, 10 mM Tris-Cl, pH 7.4). Tissue homogenate was centrifuged at 1000 g for 5 min and the supernatants were further centrifuged at 15,000 g for 30 min. The precipitates were washed twice with sucrose buffer to collect mitochondria pellet. The mitochondrial pellets were resuspended in 1× PBS containing 0.1% Triton X-100, disrupted by ultrasonication (4710 Series, Cole-Palmer, Chicago, IL) twice at 40% of maximum setting for 10 s, and centrifuged at 15,000 g for 30 min. The supernatants were used to measure the activity of IDPm. The protein levels were determined by the method of Bradford using reagents purchased from Bio-Rad.

Determination of apoptotic cell death in tissue

Following dissociation of tissue, unfixed, dissociated epithelial cells from kidney tissue were washed again in PBS and then slowly injected into cold methanol (4 °C, 100%) to a final concentration of 70% methanol, and kept at 4 °C until staining. Methanol-fixed cell suspensions were stained by an indirect immunofluorescence technique. Detection of apoptotic cells in tissue was performed using PE-conjugated rabbit anti-cleaved caspase-3 IgG (BD Bioscience, San Diego, CA) according to the vendor's protocol after gating a cell population. The percentage of apoptotic cells was analyzed by flow cytometry.

DNA fragmentation

DNA fragmentation was evaluated by TdT-mediated dUTP nick-end labeling (TUNEL) assay. After the TUNEL reaction, cells or histologic paraffin-embedded sections were analyzed by fluorescence microscopy. The green fluorescence of fluorescein isothiocyanate (FITC) was recorded with excitation at 488 nm through a 515-nm bandpass, together with the transmission image.

Cellular oxidative damage

Intracellular hydrogen peroxide concentrations were deter-

[13]. Thiobarbituric acid-reactive substances (TBARS) were determined as an independent measurement of lipid peroxidation. The tissue homogenates (500 μl) were mixed with 1 ml TBA solution (0.375% thiobarbituric acid in 0.25 N HCl containing 15% (w/w) trichloroacetic acid and heated at 100 °C for 15 min. Then the reaction was stopped on ice, and the absorbance was measured at 535 nm [14]. The protein carbonyl content was determined spectrophotometrically using the DNPH-labeling procedure as described [15]. 8-Hydroxy-2-deoxyguanosine (8-OH-dG) levels of kidney tissue were estimated by using a fluorescent binding assay as described by Struthers et al. [16]. DNA damage was visualized with avidin-conjugated TRITC (1:200 dilution) for fluorescent microscopy with 540 nm excitation and 588 nm emission.

Mitochondrial redox staus and damage

NADPH was measured using the enzymatic cycling method as described by Zerez et al. [17] and expressed as the ratio of NADPH to the total NADP pool. The concentration of total glutathione was determined by the rate of formation of 5-thio-2-nitrobenzoic acid at 412 nm ($\varepsilon = 1.36 \times 10^4$ M^{-1} cm^{-1}) as described by Akerboom and Sies [18], and oxidized glutathione (GSSG) was measured by the DTNB-GSSG reductase recycling assay after treating GSH with 2-vinylpyridine. The mitochondrial membrane potential ($\Delta\varphi_m$) was semiquantitatively determined using the mitochondrial-specific lipophilic fluorescent cationic probe JC-1 as described [19]. Following dissociation of tissue, unfixed, dissociated cells were washed and resuspended in PBS, supplemented with 10 μg/ml JC-1. Then cells were incubated for 15 min at room temperature in the dark, washed, and resuspended in PBS for immediate flow cytometry using a FACScan cytometer. Photomultiplier settings were adjusted to detect JC-1 monomers and J-aggregate fluorescence on the FL1 (530 nm) and FL2 (585 nm) detectors, respectively. The fluorescence ratio at those wavelengths was used to monitor changes in mitochondrial membrane potential. Fragmented cells and debris were excluded from measurements by gating the remaining intact cells in a forward and side scatter analysis. Data were analyzed using the CellQuest software (Becton Dickinson) to calculate the percentage of JC-1-positive cells. To evaluate the levels of mitochondrial ROS dissociated cells from tissue in PBS were incubated for 20 min at 37 °C with 5 μM DHR 123 and cells were washed and resuspended in complete growth media, and ionizing radiation was applied to the cells. The cells were then incubated for an additional 40 min. FACS was used for fluorescence intensity quantification. Intracellular ATP levels were determined by using luciferin-luciferase as described [20]. Light emission was quantitated in a Turner Designs TD 20/20 luminometer (Stratec Biomedical Systems, Germany).

Immunoblot analysis

Proteins were separated on 10–12.5% SDS-polyacryla-

subsequently subjected to immunoblot analysis using appropriate antibodies. Immunoreactive antigen was then recognized by using horseradish peroxidase-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

Statistical analysis

The difference between two mean values was analyzed by Student's *t* test and was considered to be statistically significant when $p < 0.05$.

Results

IDPm activity

To study the relationship between IDPm activity and ionizing radiation-induced apoptotic cell death, mice were administered 25 mg/kg oxalomalate, a competitive inhibitor of IDPm, once daily for 10 days. When mice were unexposed and exposed to 2 Gy of γ irradiation, the enzyme activity of IDPm in the kidneys of oxalomalate-treated mice was decreased about 20% compared with that of the control mice (Fig. 1). Because cellular antioxidants act in a concerted manner as a team, it is important to investigate whether the modulation of IDPm activity caused concomitant alterations in the activity of other major antioxidant enzymes. The reduced activity of IDPm in the kidneys of the oxalomalate-treated mice did not significantly alter the activities of other antioxidant enzymes such as SOD, catalase, glucose 6-phosphate dehydrogenase, glutathione peroxidase, and glutathione reductase (data not shown), suggesting that the reduction of IDPm activity did not affect the activities of other enzymes involved in antioxidation.

Apoptosis determination

The effects of ionizing radiation and IDPm activity on the cellular markers of apoptosis were studied to determine whether these would correlate with changes in the apoptotic pathways. As shown in Fig. 2A, the percentage of active

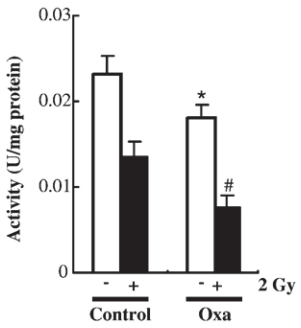

Fig. 1. Activity of IDPm in the kidneys of the control and the oxalomalate-administered mice unexposed and exposed to γ irradiation. Each value represents the mean ± SD of samples from five animals. *$p < 0.01$ vs unirradiated

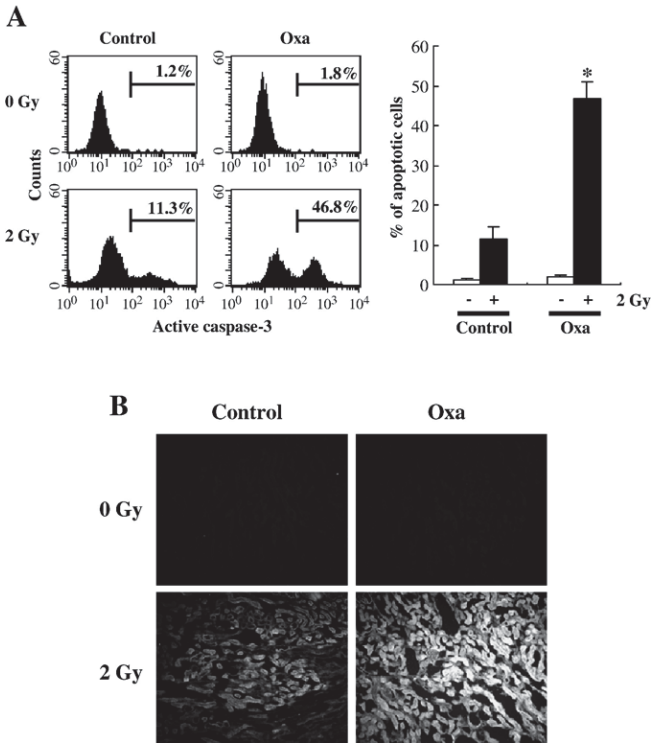

Fig. 2. Effects of oxalomalate on ionizing radiation-induced apoptosis in dissociated kidney epithelial cells from mice. (A) Activation of caspase-3 by 2 Gy of γ-irradiation can be followed by flow cytometry. Dissociated kidney cells from mice were stained with anti-cleaved caspase-3 IgG and analyzed by flow cytometry (left). Percentage of apoptotic cells calculated from data obtained by flow cytometry. Each value represents the mean±SD of samples from five animals. (Right) *$p<0.01$ vs irradiated control mice. (B) Characteristic DNA fragmentation in the kidney cells from mice was determined by the TUNEL assay and examined by fluorescence microscopy. TUNEL-positive cells were shown by bright FITC staining of nuclei.

caspase-3-positive cells had increased and was significantly higher in the kidneys of the oxalomalate-treated mice than in the control mice on exposure to a clinically relevant dose of 2 Gy. The DNA fragmentation evaluated by the TUNEL assay was significantly increased in the kidney cells of the oxalomalate-treated mice compared to the control mice when the mice were exposed to 2 Gy of γ-irradiation (Fig. 2B).

Cellular oxidative damage

To investigate whether the difference in apoptotic cell death of kidney cells from the oxalomalate-treated or the control mice on exposure to ionizing radiation is associated with ROS formation, the levels of intracellular hydrogen peroxide in the kidneys were evaluated. As shown in Fig. 3A, a significantly higher intracellular level of H_2O_2 was observed in the kidneys of the oxalomalate-administered mice compared to the control mice with the exposure of 2 Gy of γ-irradiation. These data strengthen the conclusion that IDPm provided protection from the ionizing radiation-induced apoptosis by decreasing the steady-state level of intracellular oxidants. As indicative markers of oxidative damage to cells, the occurrences of

tion were evaluated. It is well established that oxidative stress in various cells usually leads to accumulation of potent, cytotoxic lipid peroxides such as malondialdehyde (MDA) and 4-hydroxynonenal [21]. Exposure of 2 Gy of γ-irradiation increased the level of MDA in the kidney of the control mice; however, the increase in the MDA content of the kidneys of the oxalomalate-administered mice was significantly higher than that of the control mice (Fig. 3B). To determine whether reduced IDPm activity enhanced the sensitivity of protein damage, we performed carbonyl content measurements for protein oxidation after exposure to ionizing radiation. When 2 Gy of ionizing radiation was exposed, the kidneys of the oxalomalate-administered mice elicited an approximately 4.3-fold increase of carbonyl groups, compared to the unirradiated mice. Although the carbonyl content of the control mice also increased with irradiation, the increase was significantly lower than that of the oxalomalate-administered mice (Fig. 3C). The reaction of intracellular ROS with DNA resulted in numerous forms of base damage, and 8-OH-dG is one of the most abundant and most studied lesions generated. 8-OH-dG has been used as an indicator of oxidative DNA damage in vivo and in vitro [22]. Recently, it has been shown that 8-OH-dG level is specifically measured by a fluorescent binding assay using avidin-conjugated TRITC [16]. As shown in Fig. 3E, the fluorescent intensity which reflects the endogenous levels of 8-OH-dG in DNA was significantly increased in the kidneys of the oxalomalate-administered mice compared to control mice on exposure to ionizing radiation. These results indicate that IDPm appears to protect mice from oxidative damage caused by ionizing radiation.

Mitochondrial redox status and damage

One important parameter of GSH metabolism is the ratio of GSSG/total GSH (GSH_t) which may reflect the efficiency of GSH turnover. When the mice were exposed to 2 Gy of γ-irradiation, the ratio of mitochondrial [GSSG]/[GSH_t] was significantly higher in the kidneys of the oxalomalate-administered mice than the control mice (Fig. 4A). These data indicate that GSSG in the kidney mitochondria of the oxalomalate-administered mice was not reduced as efficiently as in that of the control mice. NADPH, required for GSH generation by glutathione reductase, is an essential factor for the cellular defense against oxidative damage. The ratio for mitochondrial [NADPH]/[$NADP^+$+NADPH] ($NADP_t$) was significantly decreased in mice treated with 2 Gy of γ-irradiation; however, the decrease in this ratio was much more pronounced in the kidneys of the oxalomalate-administered mice (Fig. 4B). Alterations in mitochondrial integrity and function may play an important role in the apoptotic cascade. MPT, associated with the opening of large pores in the mitochondrial membranes, is a very important event in apoptosis, and ROS is one of the major stimuli that change MPT [23]. The kidneys of the oxalomalate-treated mice showed a significant change in $\Delta\varphi_m$ determined with JC-1 (Fig. 4C). To determine if changes in MPT were accompanied

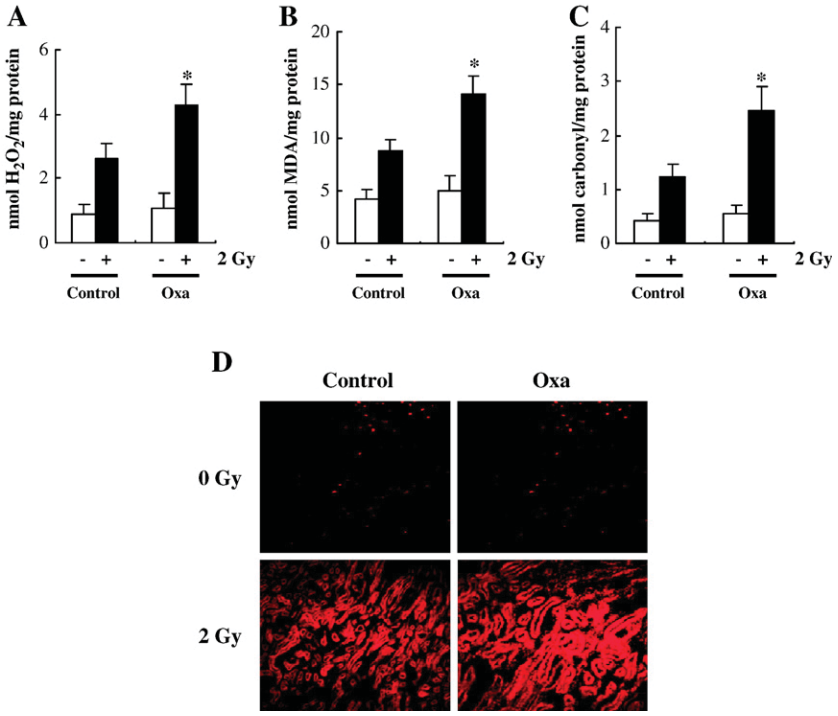

Fig. 3. Effects of oxalomalate on the cellular oxidative damage of the kidneys of mice exposed to ionizing radiation. (A) Production of hydrogen peroxide in the kidneys of mice exposed to ionizing radiation was determined by the method described under Experimental procedures. Each value represents the mean ± SD ($n = 5$). *$p < 0.01$ vs irradiated control mice. (B) Lipid peroxidation of the kidneys of mice after exposure to ionizing radiation. The level of MDA accumulated in the mice unexposed and exposed to γ-irradiation was determined by using a TBARS assay. Each value represents the mean ± SD ($n = 4$). *$p < 0.01$ vs irradiated control mice. (C) Protein carbonyl content of the kidneys of mice exposed to ionizing radiation. Protein carbonyls were measured in cell-free extracts with the use of DNPH. Each value represents the mean ± SD ($n = 5$). *$p < 0.01$ vs irradiated control mice. (D) 8-OH-dG levels in the kidneys of irradiated mice. 8-OH-dG levels reflected by the binding of avidin-TRITC were visualized fluorescence microscope.

peroxides in the mitochondria of mouse kidney were evaluated by confocal microscopy with the oxidant-sensitive probe DHR 123. As shown in Fig. 4D, the intensity of fluorescence was significantly higher in the kidneys of the oxalomalate-administered mice compared to that in the mitochondria of kidneys from the control mice when mice were exposed to 2 Gy of γ-irradiation. These results indicate that ionizing radiation most likely leads to increased mitochondrial injury while IDPm protects mitochondria from oxidative damage. Mitochondrial injury is often followed by the depletion of intracellular ATP level. As shown in Fig. 4E, when mice were exposed to 2 Gy of γ-irradiation the ATP level was significantly decreased in the kidneys of the oxalomalate-administered mice compared to the control mice, suggesting a protective role of IDPm against the loss of intracellular ATP levels.

Modulation of the apoptotic marker proteins

We evaluated changes in the apoptotic marker proteins as a result of ionizing radiation and the influence of IDPm expression on these proteins. The role of mitochondrial pathways of apoptosis in the ionizing radiation-induced cell death of mice kidneys was examined by immunoblot analysis of the abundance of Bax, Noxa, or PUMA, a proapoptotic

PUMA was increased after exposure to ionizing radiation, and it was significantly increased in the kidneys of the oxalomalate-administered mice compared to the control mice. The release of cytochrome c from mitochondria, a critical event provoking a cascade of caspases and eventually irreversible cell death [24], was increased after exposure to ionizing radiation. A marked increase of cytochrome c release was observed in the kidneys of the oxalomalate-administered mice compared to control mice. Caspase-3 activation in the mouse kidney was assessed by immunoblot analysis of kidney lysates from mice that had been exposed to 2 Gy of γ-irradiation. Ionizing radiation induced cleavage of caspase-3; however, the cleavage was significantly increased by the reduced activity of IDPm. Ionizing radiation also induced the formation of fragments which represents proteolytic cleavage of PARP, indicating an oncoming apoptotic process. The cleaved products of PARP increased markedly in the kidneys of the oxalomalate-administered mice compared to the control mice on exposure to ionizing radiation. Taken together, ionizing radiation-induced cleavage of procaspase-3 into the active form of caspase-3 and caspase-3 induces degradation of PARP. Ionizing radiation induces double-strand breaks in cells. One of the key components in damaged DNA recognition and signaling after γ-irradiation is the tumor suppressor protein p53 [25]. p53 protein levels determined by Western blotting

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.