

RADIATION-INDUCED ACTIVATION OF THE MITOGEN-ACTIVATED PROTEIN KINASE SIGNAL TRANSDUCTION PATHWAY IN IEC-6 CELLS

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ABSTRACT Effects of γ irradiation on mitogen-activated protein kinases (MAPKs) were examined in IEC-6 cells. In response to γ irradiation, Phosphorylation of intracellular signal-regulated protein kinase (ERK) was not significantly altered. The levels of phosphorylated forms of c-Jun NH₂-terminal kinase (JNK) and p³⁸ MAPK increased at 30 min following exposure to 6Gy γ irradiation, while the cell viability was lowered significantly at 12h. On the other hand, no clear changes were found in the total protein levels of ERK, JNK and p³⁸ MAPK. Chelation of intracellular Ca²⁺ suppressed γ irradiation induced JNK and p³⁸ MAPK phosphorylation almost completely, but removal of external Ca²⁺ did not. Activation of p³⁸MAPK, but not ERK, was correlated with γ irradiation induced apoptosis. The present results showed that γ irradiation is a potent activator of JNK and p³⁸ MAPK, and Ca²⁺ mobilized from intracellular stores plays an important role for the activation of MAPKs and the induction of apoptosis in IEC-6 cells.

KEYWORDS γ irradiation, ERK, JNK, p³⁸MAPK, Intracellular Ca²⁺, IEC-6 cells
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Exposure to γ irradiation may cause significant structural alterations to the intestinal wall including disruption of intestinal epithelial barrier by suppressing intestinal epithelial renewal. The molecular mechanisms of γ irradiation induced intestinal damage remain cumbersome^[1]. Physiologic intestinal epithelial cell growth and differentiation are closely regulated by a broad range of signals, for example, the signals from growth factor and hormone stimulation, however, the mechanisms involved in the signal transduction and interplay in the intestinal epithelial cells remain largely unclear^[2].

Mitogen-activated protein kinases (MAPKs) are a family of Ser/Thr protein kinase that transmits extracellular signals into the nucleus. MAPKs cascades are evolutionarily

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conserved in all eucaryotes and play a key role in the regulation of gene expression inasmuch as activated MAPKs can phosphorylate many transcriptional factors such as Elk-1, ATF-2 and c-Jun, and regulate the expression of c-fos and c-jun genes. In mammalian systems, three subfamilies of MAPKs have been identified, i.e., extracellular signal-regulated protein kinase (ERK), c-Jun NH₂-terminal kinase (JNK) also known as stress-activated protein kinase (SAPK), and p38 MAPK. These MAPKs require the phosphorylation on one Thr and one Tyr residue for their activation^[3]. While ERK cascades preferentially regulate cell growth and differentiation, JNK/SAPK and p38 MAPK mainly function in stress responses like inflammation and apoptosis. Recent studies reveal that MAPK pathways may play important roles in modulating physiologic and pathologic responses of intestinal epithelial cells to environmental stimuli. Some data obtained from in vitro studies using tumor cells indicate that activation of JNK/SAPK and p38 MAPK cascades may positively correlate with radiation induced apoptosis^[4,5]. However, we still do not know the signal transduction pathways for apoptosis induced by γ irradiation in intestinal epithelial cells^[6]. In the present study, to reveal the γ irradiation activated signaling pathway in intestinal epithelial cells, the phosphorylation status of ERK, JNK/SAPK and p38 MAPK was examined in IEC-6 cells following the exposure to γ irradiation. Since γ irradiation can cause a rapid increase in the intracellular Ca²⁺ concentration in various types of cells^[7], we also examined the effects of intracellular Ca²⁺ chelation on γ irradiation induced MAPKs phosphorylation in IEC-6 cells and their correlation with γ irradiation induced apoptosis in IEC-6 cells.

1 Materials and methods

1.1 Chemicals

1,2-Bis (O-aminophenoxy) theane-N, N, N, N-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA) AM) was purchased from Calbiochem (La Jolla, CA). Phospho-p44/42 MAPK (Thr 202/Tyr-204) antibody, phospho-JNK/SAPK (Thr 183/Tyr 185) antibody, phosphorylation state-independent JNK/SAPK antibody, phospho-p38 MAPK (Thr 180/Tyr 182) antibody, and phosphorylation state-independent p38. (Beverly, MA). Cell Count Reagent SF was purchased from Nacalai Tesque (Kyoto, Japan).

1.2 Cell culture and treatments

Rat intestinal epithelial cell line 6 (IEC-6 cells, Health Science Research Resources Bank, Osaka, Japan) was used in this study. IEC-6 cells were grown in RPMI 1640 (GIBCO BRL, Life Technologies, Inc., Rockville, MD) supplemented with 10% heat inactivated fetal bovine serum, 1000 units/mL penicillin and 100 μ g/mL streptomycin in a humidified atmosphere of 5% CO₂, 95% air at 37°C. Exponentially growing IEC-6 cells were diluted at 3×10^6 cells in 6-well culture plates or at 5×10^4 cells in 96-well culture plates, and subsequently used for the experiments.

An intracellular Ca²⁺ chelator, BAPTA/AM, was dissolved in DMSO. Cells were preincubated with serum-free RPMI 1640 containing either 0.03% DMSO or 10 μ mol/L BAPTA/AM for 30 min. After washing twice with Ca²⁺ and Mg²⁺ free phosphate-buffered saline (PBS), cells were exposed to γ irradiation.

When the effects of external Ca^{2+} -free condition on γ irradiation-induced MAPKs phosphorylation were examined, cells were washed twice with Ca^{2+} and Mg^{2+} -free PBS, and then exposed to 6Gy γ irradiation, Ca^{2+} free DMEM (GIBCO BRL) or Ca^{2+} free DMEM containing 2nmol/L EGTA for 20min. Untreated control cells were incubated with normal DMEM without γ irradiation for 20min.

1.3 Western immunoblotting

After exposure to γ irradiation, IEC-6 cells were washed twice with Ca^{2+} and Mg^{2+} -free PBS and lysed with SDS-polyacrylamide gel Laemmli sample buffer. Cell lysates were collected, sonicated and boiled for 5min. Aliquots equivalent to 4×10^5 cells were subjected to SDS/PAGE on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech, Buckinghamshire, England) as described previously^[3]. The membrane was blocked with 5% non-fat milk or 1% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 for 1h at room temperature. The membrane was incubated overnight at 4°C with the primary antibody diluted with 5% non-fat milk or 1% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20. Protein was detected with Phototope-HRP Western blot detection kit (New England Biolabs, Inc.). The blots were exposed to Hyperfilm (Amersham Pharmacia Biotech) for 1-5min.

1.4 In vitro kinase assay

The in vitro activities of ERK and p38 MAPK were measured using p44/42 MAPK assay kit and p38 MAPK assay kit (New England Biolabs, Inc.), respectively. Briefly, cell lysates equivalent to 4×10^6 cells were incubated with immobilized phospho-p44/42 MAPK (Thr202/Tyr204) monoclonal antibody and immobilized phospho-p38 MAPK (Thr180/Tyr182) monoclonal antibody, and the precipitated ERK and p38 MAPK were subjected to in vitro kinase assay using GST-Elk-1 (307-428) as substrate for ERK and GST-ATF-2 (19-96) for p38 MAPK. After SDS-PAGE of sample equivalent to 1×10^6 cells, phosphorylation of Elk-1 and ATF-2 was analyzed with immunoblotting using phospho-Elk-1 (Ser383) antibody and phospho-ATF-2 (Thr71) antibody, respectively. Densitometric analysis was performed.

1.5 Cell viability and assays for apoptosis

At 12h following exposure to γ irradiation, cell viability was determined with WST-8 assay, which is based on the conversion of tetrazolium salt WST-8 to highly water-soluble formazan by viable cells^[8]. Ten μL of the Cell Count Reagent SF which consists of 5mmol/L wst-8 (4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt), 0.2mmol/L 1-methoxy-5-methylphenazinium methosulfate and 150mmol/L NaCl was added to each well of 96-well culture plates. After the incubation for 1h at 37°C, the absorbance of each well was measured at 450nm with a reference wavelength at 655nm. Mitochondrial transmembrane potential ($\Delta\Psi_m$) was assessed using DiOC6 (3) staining and flow cytometry. Reduced mitochondrial potential is known to occur late in apoptotic process relative to the release of cytochrome c from mitochondria. Briefly, IEC-6 cells exposed to 6 Gy γ irradiation were detached at 12h

after irradiation, pooled with non-adherent cells, and washed with cold PBS. Cells were incubated with DiOC6(3) (45nmol/L in PBS) for 15 min at 37°C followed by analysis on flow cytometry (FACScan, Becton Dickinson, Sunnyvale, CA). Apoptosis was further verified by FITC-labeled annexin V and propidium iodide staining. FITC conjugated annexin V, which binds to phosphatidylserine, and propidium iodide were added to 10⁵ cells, then cells were incubated for 15min at room temperature in the dark according to the manufacturer's instructions (Apoptosis Detection Kit, R and D systems,USA), and analyzed by flow cytometry. Early apoptotic cells stained with annexin V alone, whereas necrotic cells and late apoptotic cell stained with both annexin V and propidium iodide.

1.6 Statistical analysis

Results were expressed as mean $\bar{x} \pm s$. The statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test. $p < 0.05$ was considered as statistically significant.

2 Results

2.1 Differential activation of MAPKs induced by γ irradiation in IEC-6 cells

In IEC-6 cells exposed to 6Gy γ irradiation, the levels of phosphorylated form of JNK/SAPK (p46 and p54, Fig.1b) and of p38 MAPK (Fig.1d) increased at 30 min after irradiation and reached peak levels at 120min after irradiation, while ERK (ERK2/p42 and ERK1/p44, Fig.1f) were not changed. Phosphorylation state-independent JNK/SAPK (Fig.1a), p38MAPK (Fig.1c) and ERK (Fig.1e) were not altered. Consistent with phosphorylation of JNK/SAPK and p38 MAPK by exposure to 6Gy γ irradiation, In vitro

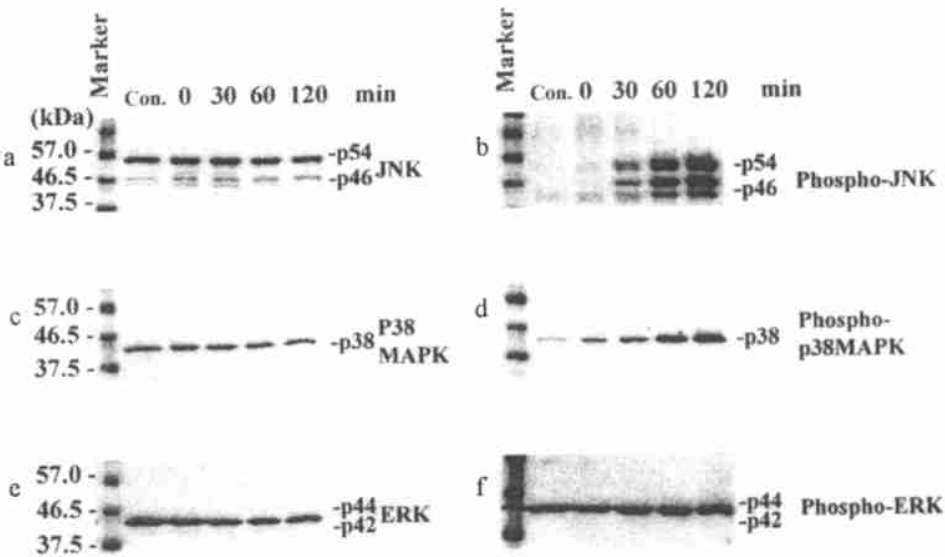


Fig.1 Differential activation of MAPKs by γ irradiation. IEC-6 cells were exposed to 6Gy γ irradiation. Accumulation of phosphorylated JNK/SAPK (b) and p38 MAPK (d), but not ERK (f), were immunoblotted. Phosphorylation state-independent JNK/SAPK (a) p38MAPK (c) and ERK (e) were not altered. Results shown are representatives of three independent experiments

activity of p38 MAPK determined using GST-ATF-2 as substrate was elevated in cells while ERK activity using GST-Elk-1 as substrate was unchanged (Fig.2).

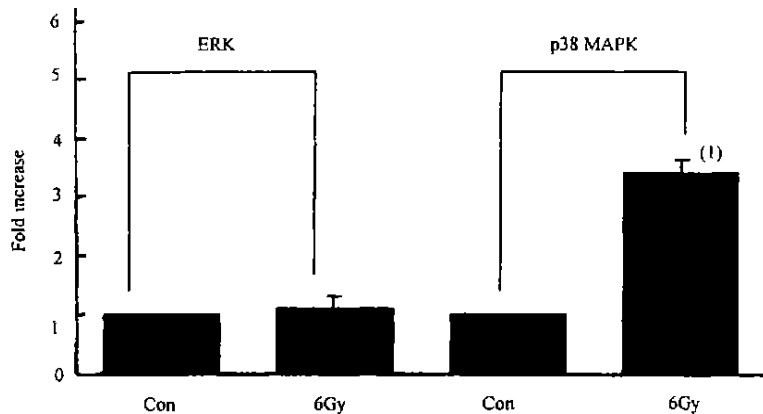


Fig.2 Effect of γ irradiation on the in vitro activities of ERK and p38 MAPK in IEC-6 cells. In vitro kinase activities were determined with GST-Elk-1 (307-428) as substrate for ERK or GST-ATF2 (19-96) as substrate for p38 MAPK. Each value (mean $\bar{x} \pm s$, $n=6$) was expressed as the fold increase with respect to control ⁽¹⁾ $p < 0.01$

2.2 Role of intracellular calcium in γ irradiation induced activation of MAPKs

Removal of external Ca^{2+} by suspending the cells in Ca^{2+} -free medium alone did not alter 6Gy γ irradiation induced phosphorylation of JNK/SAPK and p38 MAPK. In cells pretreated by BAPTA/AM, an intracellular Ca^{2+} chelator, 6Gy γ irradiation induced phosphorylation of JNK/SAPK and p38 MAPK was suppressed almost completely without changing total protein levels (Fig.3). This result suggested that calcium release from intracellular stores plays a major role in MAPKs activation.

2.3 Apoptosis and involvements of activated p38 MAPK and intracellular calcium in irradiated IEC-6 cells

The viability of IEC-6 cells was not changed at 1h after irradiation and lowered by 19% at 6h post exposure ($p < 0.05$, Fig.4). Cell viability decreased further to a lower level at 12h without recovery within the observation. In addition, the reduction of cell viability in IEC-6 cells exposure to 6Gy γ irradiation was inhibited significantly by pretreatment with BAPTA/AM ($p < 0.01$, Tab.1).

Apoptosis was induced in IEC-6 cells by 6Gy γ irradiation. The percentage of apoptotic cells was significantly elevated compared with control at 12h post irradiation (Tab.1). We further explored whether the differential activation of MAPKs was or not directly associated with irradiation induced apoptosis in IEC-6 cells by pretreating cells with specific inhibitors of MAP kinases for 4h prior to irradiation and then assessing apoptosis at 12h post irradiation. Pretreatment with 30 $\mu\text{mol/L}$ SB 203580, a specific p38 MAPK inhibitor, blocked p38 MAPK activation and suppressed irradiation induced apoptosis in IEC-6 cells while 30 $\mu\text{mol/L}$ PD98059, a specific MEK inhibitor offering a marked blockade on ERK activation and ERK activity, had caused a significant increase in apoptosis (Tab.1). These data clearly showed that activation of p38 MAPK, but

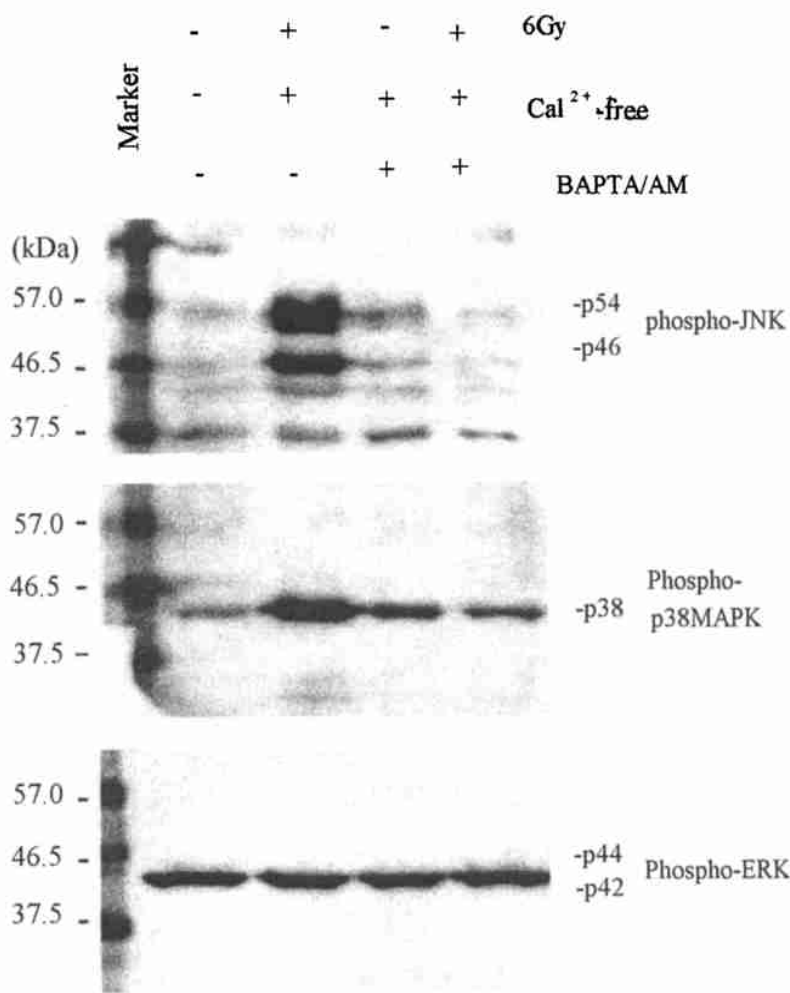


Fig.3 Effect of BAPTA/AM pretreatment on γ irradiation induced accumulation of phosphorylated JNK/SAPK, p38MAPK and ERK in IEC-6 cells. phosphorylation of JNK/SAPK and p38MAPK was blocked by BAPTA/AM pretreatment but not by calcium-free medium

not ERK, is an important event in γ irradiation induced apoptosis in IEC-6 cells. ERK seems to be a cell survival signal since inhibition of ERK pathway by PD 98059 obviously increase apoptosis following exposure to γ irradiation. Due to lack of specific inhibitor of JNK, we could not determine the direct link of JNK activation with apoptosis induced by 6Gy γ irradiation in this experiment. Furthermore, we tried to elucidate the role of intracellular calcium in γ irradiation induced apoptosis. Pretreatment with BAPTA/AM, but not calcium free medium, significantly blocked γ irradiation induced apoptosis in the cells. This revealed that increase of intracellular calcium is a pivotal event for γ irradiation induced apoptosis.

Tab.1 Apoptosis of IEC-6 cells in response to different treatments

Treatment	Apoptotic cells/%
Control	5.3± 0.6
6Gy irradiation alone	19.2±5.3 ⁽²⁾
PD98059 30μmol/L	16.6±7.2 ⁽²⁾
PD98059 + 6Gy	32.8±6.3 ^{(2),(3)}
SB203580 30μmol/L	6.7±0.4
SB203580 + 6Gy	11.4±2.1 ^{(2),(3)}
Ca ²⁺ -free	5.1±0.7
Ca ²⁺ -free + 6Gy	11.4±2.1 ^{(2),(3)}
BAPTA/AM 10μmol/L	4.9±0.7 ^{(2),(3)}
BAPTA/AM + 6Gy	7.2±1.3 ⁽³⁾

(n=6, mean $\bar{x} \pm s$)⁽¹⁾
(¹)Apoptosis determined at 12h after 6Gy γ irradiation
(²) $p < 0.05$, $p < 0.01$ vs control respectively
(³) $p < 0.05$, $p < 0.01$ vs 6Gy γ irradiation respectively

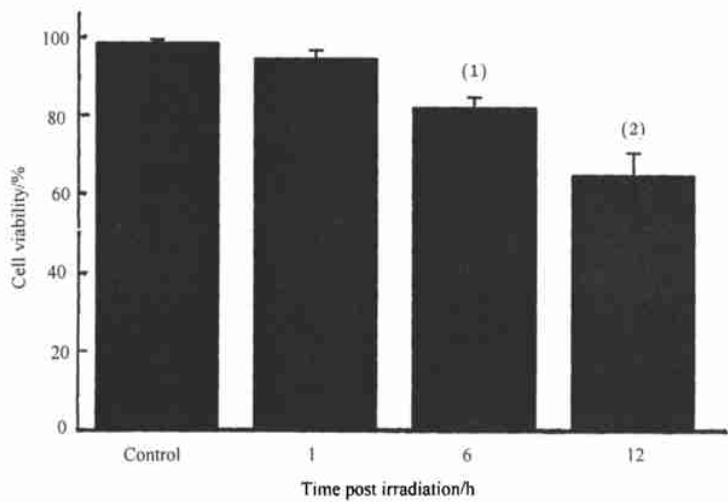


Fig.4 Time course of γ irradiation induced reduction of cell viability. Cell viability was determined with WST-8 assay. Each value (mean $\bar{x} \pm s$ n=6) represents the percentage of the control (¹) $p < 0.05$, (²) $p < 0.01$ compared to control

3 Discussion

The epithelial lining of the intestinal mucosa is maintained by continuous proliferation of cells in crypts, which are ultimately dependent on crypt stem cells. Proliferation is followed by cellular differentiation and migration up villi, where they replace the mature enterocytes that are shed from the tips. These intestinal epithelial cells are extremely radiosensitive and cytoablative dose of chemotherapy or radiotherapy compromises the absorptive and barrier function of mucosa by killing the intestinal epithelial cells and the crypt stem cells, thereby impairing normal regeneration. Therefore, protection of intestinal epithelium is a major approach for antagonizing γ irradiation induced gut damage.

Understanding the signal pathways of γ irradiation effects on intestinal epithelial cells at the molecular level is an essential requirement for developing the protective strategies.

In response to 6Gy γ irradiation, the marked phosphorylation of JNK/SAPK and p38 MAPK was found in IEC-6 cells, and their levels increased further in a dose-dependent manner. However, the phosphorylation of ERK was not observed. The cell viability was lowered significantly at 6h post-irradiation and apoptosis could be determined by fluorescent microscopic examination. On the other hand, no changes were found in the total protein (phosphorylation state-independent) levels of ERK, JNK/SAPK and p38 MAPK. We also found the elevation of phosphorylated JNK/SAPK and p38 MAPK levels in PC12 cells exposed to 6Gy γ irradiation without changing their total protein levels (data not shown). Therefore, γ irradiation activated MAPKs through their phosphorylation but not the elevation of protein levels in IEC-6 cells. In consistent with the differential activation of MAPKs, MAPK activities were also differentially altered.

Differential activation of MAPKs by γ irradiation, which was found at 30min following the exposure, does not seem to be a simple result of cellular damage because the cell viability was lowered much later (at 12h). However, activation of JNK/SAPK and p38 MAPK is a crucial step leading the cells to apoptosis. In the present study, we demonstrated that suppression of p38 MAPK activation could block apoptosis induced by 6Gy γ irradiation in IEC-6 cells by using specific p38 MAPK inhibitor. Furthermore, it was noticed that γ irradiation induced phosphorylation of JNK/SAPK and p38 MAPK was suppressed by pretreatment with intracellular Ca^{2+} chelator BAPTA/AM, but not by removing external Ca^{2+} . These results indicate that γ irradiation induced MAPKs phosphorylation depends on the mobilization of Ca^{2+} from intracellular stores rather than the influx of extracellular Ca^{2+} . Therefore, the elevation of intracellular Ca^{2+} concentration, particularly its mobilization from internal stores, seems to be prerequisite for γ irradiation induced MAPKs phosphorylation in IEC-6 cells. Pretreatment with BAPTA/AM suppressed apoptosis as well as reduction of cell viability in IEC-6 cells exposed to γ irradiation as has been reported previously in thymocytes. In addition, it has been reported that γ irradiation-induced activation of Caspase cascade in Jurkat T cells was blocked by Ca^{2+} chelation^[9]. Taken together, the pathways depending on intracellular Ca^{2+} elevation seem to play an important role in the apoptotic cell death induced by γ irradiation. Distinctions between necrosis and apoptosis have been highlighted as two distinct forms of radiation induced cell death. Perhaps surprisingly the characteristics of radiation induced cell death are not the same for all cells. Even among cells in which apoptosis is readily observed the pattern of timing is dramatically different between cell types. Recently Paris F and his colleagues reported endothelial apoptosis is the primary lesion initiating intestinal radiation damage^[1]. Our present study further support the view that in a moderate dose range of γ irradiation, intestinal epithelial cells mainly die in a manner of apoptosis following irradiation exposure.

In conclusion, this investigation first documented that γ irradiation could differentially activate MAPKs and the activation of JNK/SAPK and p38 MAPK is positively

correlated with the induction of apoptosis in IEC-6 cell. Elevation of intracellular calcium is an early event occurring before the differential activation of MAPKs and is a crucial step in γ irradiation induced apoptosis. These results could be of clinical relevance to tumor radiotherapy and to exploring the possible strategy for antagonizing γ irradiation induced intestinal damage.

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辐射诱导 IEC-6 细胞 MAPK 信号转导途径的激活

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摘要 研究检测了 γ 辐射对 IEC-6 肠上皮细胞丝裂原激活的蛋白激酶(MAPKs)的激活效应。 6Gy γ 辐射未引起 ERK 蛋白磷酸化的显著改变,而辐照后 30min JNK 与 p38 MAPK 蛋白磷酸化显著增强,ERK、JNK 和 p38MAPK 的总蛋白表达水平未见明显的变化。受照后 12h 细胞存活率显著降低。整合细胞内 Ca^{2+} 可几乎完全抑制 γ 辐照引起的 JNK 与 p38MAPK 的蛋白磷酸化,而清除细胞外 Ca^{2+} 却无此作用。p38MAPK 的激活与 γ 辐射诱导的细胞凋亡显著相关,而 ERK 则无明显的关联。实验结果表明, γ 辐射是一种强的 JNK 与 p38MAPK 激活因素,并且细胞内储存 Ca^{2+} 的释放在 γ 辐射诱导的 IEC-6 细胞 MAPKs 激活与细胞凋亡过程中起重要作用。

关键词 γ 辐射, ERK, JNK, p38MAPK, 细胞 Ca^{2+} , IEC-6 细胞

中图分类号 R811, R146