Expression of phospho-Elk-1 in rat gut after the whole body γ-irradiation

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Radiation-induced entero-colitis; Phospho-Elk-1 expression; Biodosimetric marker.

Summary

Introduction: Gastrointestinal form is the second stage of Acute Radiation Syndrome (ARS) with a threshold dose of 8 Gy in man. It represents an absolutely lethal clinical-pathological unit, enteritis and procto-colitis necro-haemorrhagica with unknown causal therapy. Elk-1 is a protein acting as a transcription factor activating specified genes of DNA.

Objectives: The purpose of our study has been to examine the phospho-Elk-1 expression in irradiated jejunum and colon transversum via standard procedure of radiation-induced entero-colitis in rats and assess the contribution of this transcriptional factor as a biodosimetric marker of radiation-induced enteropathy.

Materials & Methods: The laboratory rats were randomly divided into 21 groups, 10 animals per group, and irradiated with whole body γ-irradiation of 1, 5, 10, 15, and 20 Gy. Samples of jejunum and colon transversum were taken 24, 48, 72, and 96 hours later, immunohistochemically stained, and the phospho-Elk-1 was examined with computer image analysis. A group of 10 sham-irradiated animals was used as control.

Results: Significantly increased expression of phospho-Elk-1 in rat jejunum has been found in all time intervals after irradiation by sublethal doses of 1 and 5 Gy. After irradiation by lethal doses, the expression of phospho-Elk-1 in rat jejunum significantly both increased and decreased, or did not indicate significant changes. Significantly increased expression of phospho-Elk-1 in rat colon transversum has been found in the first days after irradiation by sublethal doses of 1 and 5 Gy. After irradiation by lethal doses, the expression of phospho-Elk-1 in rat colon transversum significantly both increased and decreased, or did not indicate significant changes.

Conclusions: The detection of phospho-Elk-1 might be considered as a suitable and very sensitive biodosimetric marker of radiation-induced injury of small and large intestine. According to our knowledge, we firstly detected and quantified the phospho-Elk-1 expression in irradiated jejunum and colon transversum in the animal trial.

Introduction

Radiation-induced entero-colitis is a form of acute radiation disease developing after the absorption of dose of 8 Gy and higher. It concerns an absolutely lethal clinical-pathological unit, enteritis and colitis necro-haemorrhagica, of which its causal therapy is unknown (Fajardo 1982, Nguyen *et al.* 2002, Österreicher *et al.* 2003). The large morphological changes in small intestine mucosa develop rapidly and progressively during the first 4 days after irradiation with supralethal doses. Molecular biology explains the effect of irradiation on cells via the alteration of signalling pathways (Stulík *et al.* 2000, Suzuki *et al.* 2001, Torii *et al.* 2004). Elk-1 is a protein acting as a transcription factor inducing c-fos transcription and activating specified genes of DNA. Elk-1 is a component of the ternary complex that binds the serum response element (SRE) in response to serum and growth factor. Afterwards, Elk-1 C-terminal region at multiple sites is phosphorylated by mitogen-activated protein kinases (MAPK, extracellular signal-related kinases ERK1 and ERK 2), particularly phosphorylation on site of Ser383 is critical for transcriptional activation. Elk-1 appears to be a direct target of activated MAPK pathways, besides, the locus of Ser383 is also a target of the stress-activated kinases (SAPK/JNK1, JNK2 and p38) (Cavigelli *et al.* 1995, Kasza *et al.* 2005, Marais *et al.* 1993, Murai *et al.* 2002, Narang and Krishna 2004, Salinas *et al.* 2004, Whitmarsh *et al.* 1995). Phosphorylation of Elk-1 by ERK2 is a major link in the Raf-1 kinase-dependent signal transduction pathway that activates c-fos expression (Kortenjann *et al.* 1994). The Elk-1 transcription factor integrates MAPK signalling pathways in vivo to co-ordinate biological response to different extracellular stimuli (Whitmarsh *et al.* 1995).

In this study, we examined the expression of Elk-1 phosphorylated on Ser383 in rat jejunum and colon transversum and its dependence on dose and time after the whole body γ-irradiation.

Materials and methods

Randomly divided into 21 groups (10 animals per group), male Whistar rats (Konárovice, Czech Republic) aged 12-16 weeks and weighing 250-300 g were given the whole body γ-irradiation using a ^{60}Co unit (Chisotron Chirana, Czech Republic) at a dose rate of 1.0 Gy/min at a distance of 100 cm from the skin and at doses of 1, 5, 10, 15, and 20 Gy. The animals were lightly anaesthetised before irradiation or sham-irradiation using a mixture of one volume of Rometar (Spofa Company, Prague, Czech Republic), three volumes of Narkamon (Léčiva Company, Prague, Czech Republic) and 12 volumes of physiological saline. This solution was injected intramuscularly at 1.0 ml/kg. After 24, 48, 72, and 96 hours, the animals were sacrified by a cervical dislocation. The rats of a sham-irradiated group were killed after 72 hours. Only one animal of each group survived 96 hours after irradiation with doses of 15 and 20 Gy. These groups were expelled from the statistical analysis. All the procedures involving animals were approved by the Ethics Committee of Faculty of Military Health Sciences in Hradec Králové.

Histological examination

Samples were taken of the cranial part of jejunum (approximately 2 cm aborally from duodeno-jejunal flexure) and of the middle part of colon transversum. One transversal section of both jejunum and colon transversum per animal was examined. All the samples were incubated in 10 % formalin-buffered solution and fixated up to

24 hours. The immunohistochemical detection of phospho-Elk-1 was performed on epithelial cells with a standard immunoperoxidase technique. After blocking the endogenous peroxidase activity for 20 minutes, the tissue sections were incubated for 1 hour with the biotinylated mouse monoclonal antibody specifically recognising Elk-1 phosphorylated on Ser383 (Cell Signalling, USA) diluted 1:200 in phosphated buffer saline (PBS, pH 7,2). Afterwards, the sections were washed three times in PBS. All slides were then incubated for 20 minutes with a secondary antimouse antibody under the same condition. Excess antibodies were washed off with PBS. All slides were incubated with an enzymatic complex streptavidin-peroxidase (Universal large volume DAKO LSAB + Kit, Peroxidase - DAKO Corporation, Carpinteria, USA) and washed with PBS.

Finally, 0.05 % 3,3-diaminobenzidinetetrahydrochloride-chromogen solution (Sigma, St. Louis, MO, USA) in PBS containing 0.02 % hydrogen peroxide was added for 20 minutes to visualise the antigen-antibody complex in situ.

Measurement of phospho-Elk-1 expression

Stained samples were evaluated using a BX 51 microscope (Olympus Company, Prague, Czech Republic) and a computer image analysis ImagePro 4.11 (Media Cybernetics, USA). Immunoreactive structures in epithelium were detected in the ranges red 100-255, green 161-255, and blue 171-255. Six viewing microscopic fields at a 400-fold original magnification were randomly selected of each jejunum and colon sample so that 60 measurements were performed per group. To increase the validity of results, we have used 3 parameters of the computer image analysis for quantification of the phospho-Elk-1 expression: sum of density mean, density per area (percentual positivity per microscopic field) and integral optic density (IOD).

Data processing

The Mann-Whitney rank sum test was used for the statistical analysis. Significance level was set at $p < 0.05$. Data are given as arithmetic mean ± 1x standard error of mean (S.E.M.), that express 95 % confidence interval.

Results

1. Sum of density mean of phospho-Elk-1 positive cells of rat jejunum

The results of sum of density mean of phospho-Elk-1 positive cells in rat jejunum are shown in Figure 1. The value of sum of density mean is significantly higher in rats after irradiation by doses of 1 and 5 Gy in all time intervals with the maximum 24 hours after irradiation by dose of 1 Gy. Significantly higher values of sum of density mean were found in rats 96 hours after irradiation by dose of 10 Gy, 24 and 72 hours after irradiation by dose of 15 Gy, and 24 hours after irradiation by dose of 20 Gy. Significantly lower value was found in rats 48 hours after irradiation by dose of 15 Gy.

2. Per area density of phospho-Elk-1 positive cells of rat jejunum

The results of per area density of phospho-Elk-1 positive cells in rat jejunum are shown in Figure 2. The value of per area density is significantly higher in rats after irradiation by doses of 1 and 5 Gy in all time intervals with the maximum 72 hours after irradiation by dose of 1 Gy. Significantly higher values of per area density were found in rats 96 hours after irradiation by dose of 10 Gy. Significantly lower value was found in rats 24-72 hours after irradiation by dose of 10 Gy, and 48 hours after irradiation by dose of 15 Gy.

3. Integral optic density of phospho-Elk-1 positive cells of rat jejunum

The results of integral optic density of phospho-Elk-1 positive cells in rat jejunum are shown in Figure 3. The value of integral optic density is significantly higher in rats after irradiation by doses of 1 and 5 Gy in all time intervals with the maximum 72 hours after irradiation by dose of 1 Gy. Significantly higher values of integral optic density were found in rats 96 hours after irradiation by dose of 10 Gy. Significantly lower value was found in rats 24-72 hours after irradiation by dose of 10 Gy, and 48 hours after irradiation by dose of 15 Gy.

4. Sum of density mean of phospho-Elk-1 positive cells of rat colon transversum

The results of sum of density mean of phospho-Elk-1 positive cells in rat colon transversum are shown in Figure 4. The value of sum of density mean is significantly higher in rats 24-72 hours after irradiation by dose of 1 Gy with the maximum 24 hours after irradiation. Significantly higher values of sum of density mean were found in rats irradiated by dose of 5 Gy in all time intervals, 96 hours after irradiation by dose of 10 Gy, 24-48 hours after irradiation by dose of 15 Gy, and 24 hours after irradiation by dose of 20 Gy. Significantly lower value was found in rats 24-72 hours after irradiation by dose of 10 Gy, and 72 hours after irradiation by dose of 20 Gy.

5. Per area density of phospho-Elk-1 positive cells of rat colon transversum

The results of per area density of phospho-Elk-1 positive cells in rat colon transversum are shown in Figure 5. The value of per area density is significantly higher in rats 24-72 hours after irradiation by dose of 1 Gy with the maximum 48 hours after irradiation. Significantly higher values of per area density were found in rats 48 hours after irradiation by dose of 5 Gy, and 96 hours after irradiation by dose

of 10 Gy. Significantly lower values were found in rats 96 hours after irradiation by dose of 1 Gy, 48-72 hours after irradiation by dose of 10 Gy, 48-72 hours after irradiation by dose of 15 Gy, and 72 hours after irradiation by dose of 20 Gy.

6. Integral optic density of phospho-Elk-1 positive cells of rat colon transversum

The results of integral optic density of phospho-Elk-1 positive cells in rat colon transversum are shown in Figure 6. The value of integral optic density is significantly higher in rats 24-72 hours after irradiation by dose of 1 Gy with the maximum 48 hours after irradiation. Significantly higher values of integral optic density were found in rats 48 hours after irradiation by dose of 5 Gy, and 96 hours after irradiation by dose of 10 Gy. Significantly lower values were found in rats 96 hours after irradiation by dose of 1 Gy, 48-72 hours after irradiation by dose of 10 Gy, and 72 hours after irradiation by dose of 20 Gy.

Discussion

Ionizing radiation activates the typically mitogen-activated pathway (MAPK/ERK cascade) along with the activation of stress-responsing pathways, NF-κB, and many immediate early genes as c-jun, c-fos, and egr-1. According to PubMed, the original papers concerning signalling pathways alteration in enterocytes are particularly uncommon. Description of gene expression pattern in colonic crypts of adenomas with low grade dysplasia has shown that the activation of the MAPK pathway seems to be an early sign of carcinogenesis (Lechner et al., 2003). The works studying molecular changes in gastrointestinal tract after exposure to ionizing radiation are even more rare. Group of Österreicher, Driák at al. looks for new and sensitive biodosimetric markers of radiation-induced entero-colitis (Österreicher et al., 2007).

The most recent paper of Narang and Krishna describes the MAPK signalling pathway changes in rat liver (Narang and Krishna, 2004).

Ionizing radiation activates both the cytoprotective mitogen-activated protein kinases (MAPK, extracellular signal-related kinases ERK1, ERK2) and the cytotoxic stress-activated kinases (SAPK/JNK1, JNK2, p38). Each of them sends divergent signals to the nucleus. There is a chronological order of activation of the kinases and a dose-dependent activation. The cytoprotective ERK2 shows a progressive increase in expression with dose and a prolonged response to stimuli, whilst the cytotoxic SAPK is activated by very low doses of ionizing radiation (0.1 Gy) at early period. The duration of activation of kinases seems to be the deciding factor of whether the cell goes into proliferation and differentiation or cell-cycle arrest. A transient activation of ERK leads to proliferation, whereas persistent activation leads to cell-cycle G2/M arrest, and apoptosis is possible. Low doses of ionizing radiation (1 Gy) cause a prolonged activation of MAPK and SAPK. Higher doses of radiation (6 Gy) cause a much weaker activation of the MAPK cascade, but a similar degree of SAPK activation (Narang and Krishna 2004).

Transcription factor Elk-1 is a common target of activated MAPK pathways and the SAPK pathways, the two signalling pathways converge by means of Elk-1, as it can be seen on Figure 7. The expression of Elk with response to dose correlates very well with the activation of ERK2 at higher doses (1-5 Gy) in rat liver (Narang and Krishna 2004). The expression of the precursor to SAPK/JNK was induced at a lower dose with maximum at the dose of 0.5 Gy and declined at the dose of 1 Gy. A complete inhibition of induction of expression occured at the doses of 2-5 Gy. The expression of phospho-Elk did not increase at the doses of 0.1-0.5 Gy. According to the study on irradiated rat liver, ionizing radiation induces both the ERK and the JNK

pathway to phosphorylation of Elk-1, however, in a different chronological order and in a dose-dependence (Narang and Krishna 2004).

According to our knowledge, we firstly detected and quantified the phospho-Elk-1 expression in irradiated rat gut. In our study, we observed a significantly increased expression of phospho-Elk-1 in rat jejunum in all time intervals after irradiation by sublethal doses of 1 and 5 Gy. After irradiation by lethal doses, the expression of phospho-Elk-1 in rat jejunum was significantly both increased and decreased, or did not indicate significant changes.

We also observed a significantly increased expression of phospho-Elk-1 in rat colon transversum in the first days after irradiation by sublethal doses of 1 and 5 Gy. After irradiation by lethal doses, the expression of phospho-Elk-1 in rat colon transversum was significantly both increased and decreased, or did not indicate significant changes. Although epithelial denudement after lethal irradiation is usually described, our time-dose intervals were focussed to clinically prodromal and latent phases when lower number of enterocytes is compensated by their flattening. Therefore, we have had opportunity to measure the phospho-Elk-1 expression in enough number of enterocytes. Only one animal of each group survived 96 hours after irradiation by doses of 15 and 20 Gy, where large devastating effects and remarkable loss of enterocyte were observed. Due to the low amount of epithelial cells, these groups were expelled from the statistical analysis.

The variance of values after irradiation by lethal doses can be explained with a different velocity of signalling pathway changes after irradiation by various doses. The other mode of explanation is an exhaustion of produced signalling molecules in injured cells. The increased values 96 hours after irradiation by dose of 10 Gy may be due to the resynthesis of signalling molecules. After irradiation by doses higher

than 10 Gy, the resynthesis is not sufficient. Our hypotheses have to be verified by further trials investigating the phospho-Elk-1 expression in the first hours after irradiation (1, 2, and 4 hours). In the study on irradiated rat liver, the expression of the signalling factors was looked at 4 hours after irradiation (Narang and Krishna 2004).

In comparison with radiation-induced enteritis, increased values of phospho-Elk-1 expression in colon transversum have not been observed 96 hours after irradiation with dose of 1 Gy and in several time intervals after irradiation with dose of 5 Gy. This might be explained by a rather different (more slow) cytokinetics and repopulation of large intestine mucosa and earlier exhaustion of signalling molecules. After irradiation by supralethal doses the dynamics of changes in both jejunum and colon transversum are very similar.

Based on the results of our study, we suppose that phospho-Elk-1 is a very sensitive parameter of radiation-induced gut injury, significantly elevated after irradiation by sublethal doses. The detection of phospho-Elk-1 might be considered as a suitable biodosimetric marker useful under clinical conditions, and possibly in military field conditions. However, this suggestion has to be verified by further experiments detecting the phospho-Elk-1 expression in other tissues and after other nuclear, biological and chemical agents than ionizing radiation.

Besides radiation, the phosphorylation of Elk-1 is stimulated by a wide variety of extracellular stimuli, e.g. visible light, UV light (Price *et al*. 1996, Coogan and Piggins 2003, Kaminska *et al.* 1999). Besides the oxydative stress, other stressors could induce the phosphorylation of Elk-1. Receptor-bound growth and neurotrophic factors induce Elk-1 phosphorylation and c-fos transcription through ERK activation, UV irradiation stimulates Elk-1 activity through JNK activation (Cavigelli *et al.* 1995). In such case, it might be a universal response to the cellular stress caused by an

extracellular signal. However, there will be probably a different order of time- and dose-dependence of activation.

Conclusion

Significantly increased expression of phospho-Elk-1 in rat jejunum in all time intervals (i.e. 24, 48, 72, and 96 hours) after irradiation by sublethal doses of 1 and 5 Gy has been observed. We can suppose that, at this time, phospho-Elk-1 is the most sensitive biodosimetric marker of radiation-induced enteropathy. After irradiation by lethal doses, the expression of phospho-Elk-1 in rat jejunum was significantly both increased and decreased, or did not indicate significant changes. Significantly increased expression of phospho-Elk-1 in rat colon transversum in the first days after irradiation by sublethal doses of 1 and 5 Gy have been observed. After irradiation by lethal doses, the expression of phospho-Elk-1 in rat colon transversum was significantly both increased and decreased, or did not indicate significant changes. The increase of phospho-Elk-1 expression was higher in irradiated jejunum than colon transversum, that confirms the suggestion that the small intestine is more radiosensitive than the large one.

The detection of phospho-Elk-1 might be considered as a suitable and sensitive biodosimetric marker of radiation-induced injury of small and large intestine. Further experiments verifying the expression of phospho-Elk-1 in other tissues are needed. We propose further testing of other agents, in addition to the ionizing radiation. If our results will be confirmed, phospho-Elk-1 might be supposed as a useful parameter under clinical conditions, and possibly in military field conditions.

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Figure 1. Sum of density mean of p-Elk-1 positive cells in irradiated rat jejunum in arithmetic mean ± 1x S.E.M.

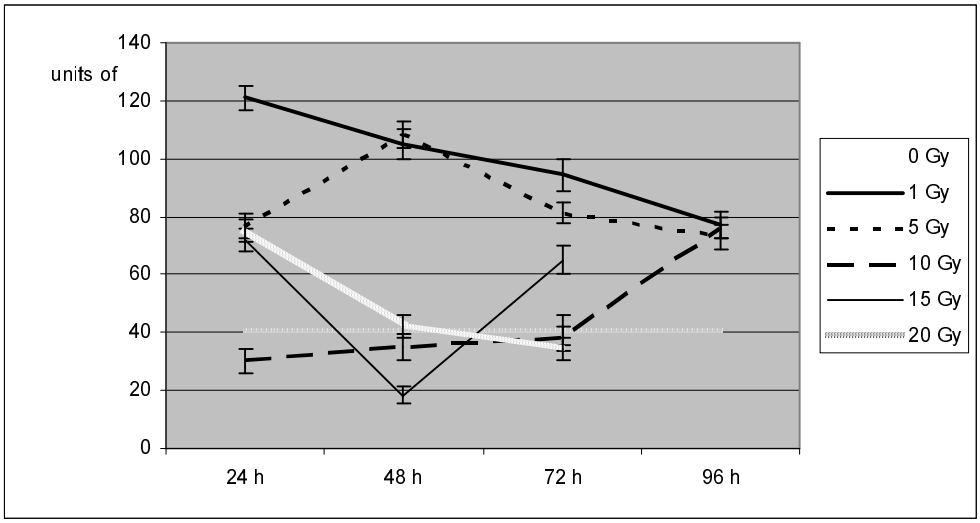

Figure 2. Per area density of p-Elk-1 positive cells in irradiated rat jejunum in arithmetic mean ± 1x S.E.M.

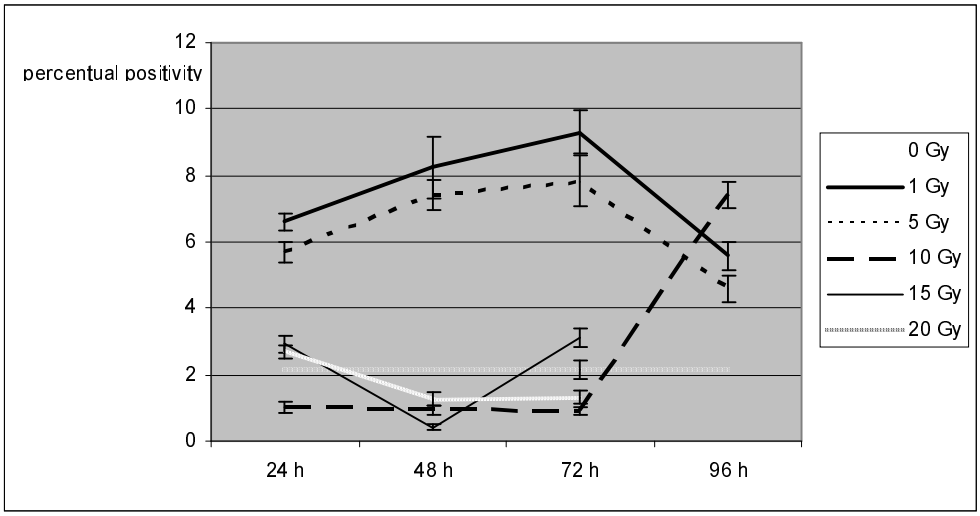

Figure 3. Integral optic density of p-Elk-1 positive cells in irradiated rat jejunum in arithmetic mean ± 1x S.E.M.

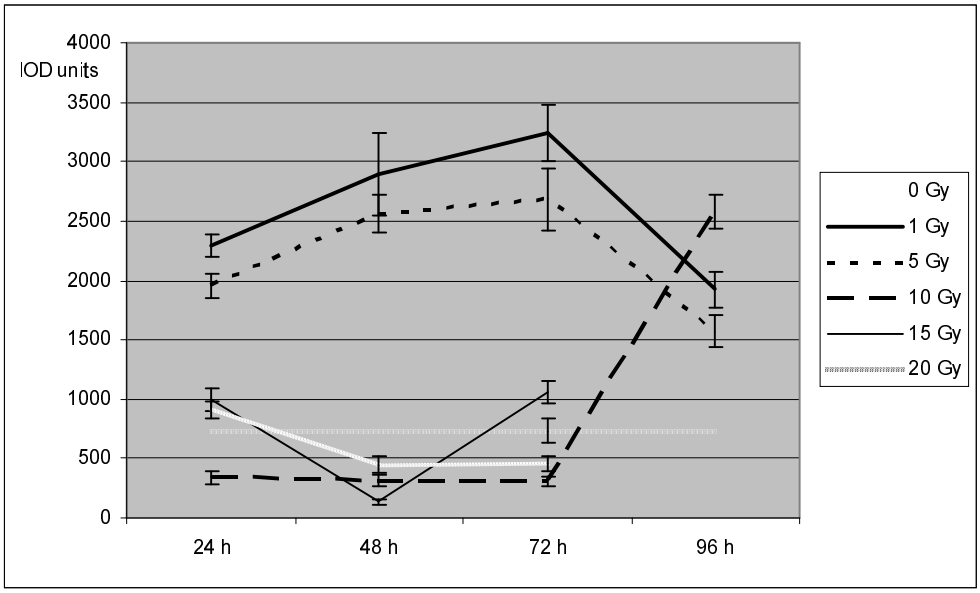

Figure 4. Sum of density mean of p-Elk-1 positive cells in irradiated rat colon in arithmetic mean ± 1x S.E.M.

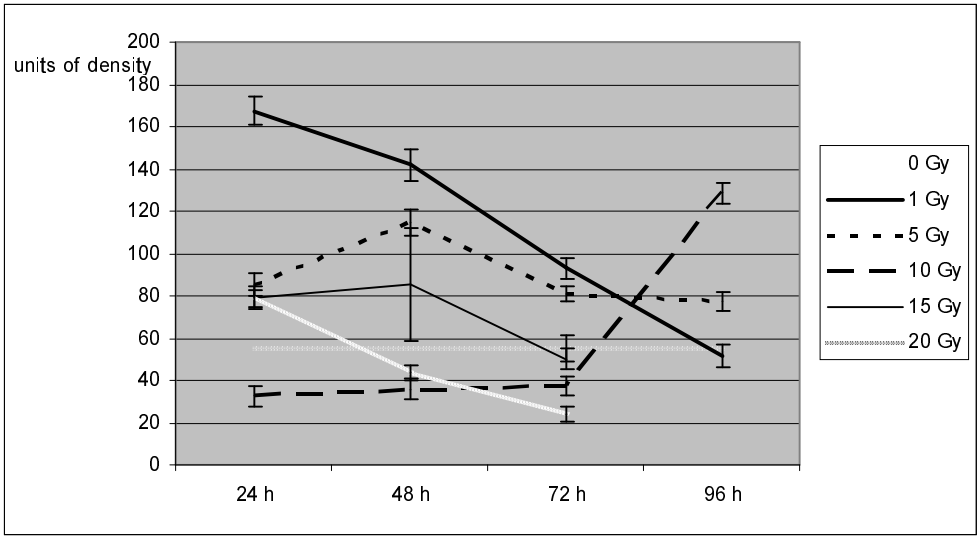

Figure 5. Per area density of p-Elk-1 positive cells in irradiated rat colon in arithmetic mean ± 1x S.E.M.

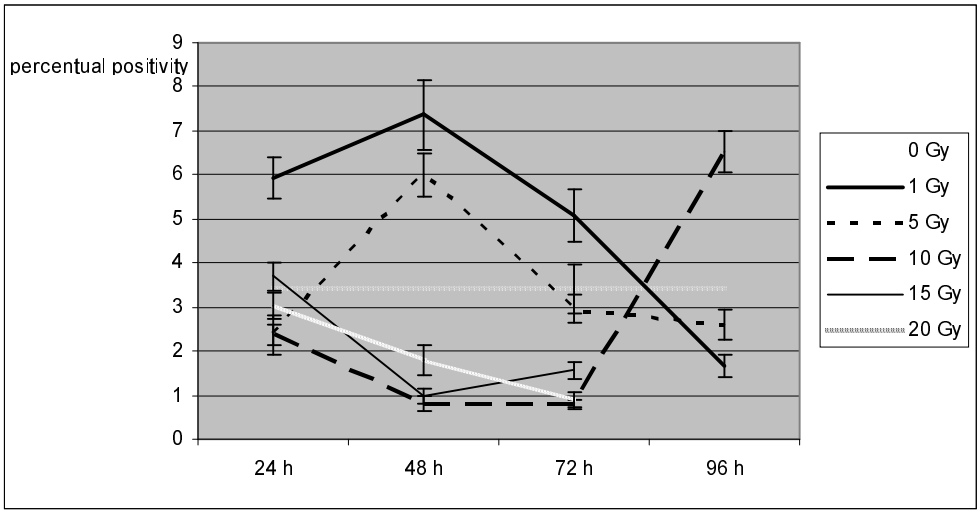

Figure 6. Integral optic density of p-Elk-1 positive cells in irradiated rat colon in arithmetic mean ± 1x S.E.M.

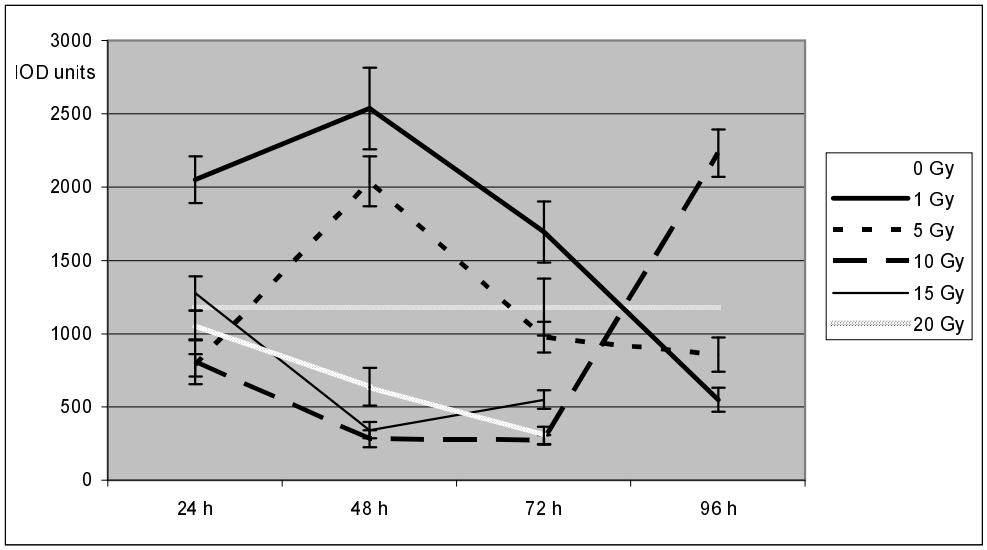

Figure 7. Signalling map of 3 kinases pathways