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Impact of Phospho-ELK-1 Expression in Enterocytes in Biodosimetry after Low-dose Irradiation

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ABSTRACT

Previously the higher phospho-ELK-1 (*p*-ELK-1) expression in enterocytes 24-96 h after sub-lethal irradiation was measured. Therefore, purpose of this study is to examine *in vivo* expression of this common ERK1/2 and JNK/SAPK target during the first hours after low-dose irradiation to assess ability of this transcriptional factor as a biodosimetric marker after the single whole-body irradiation. The laboratory rats were randomly divided into 12 groups and irradiated with whole-body gamma radiation dose of 0.25 Gy, 0.5 Gy, and 1.0 Gy. Samples of jejunum were taken 1 h, 2 h, 4 h, and 24 h later, the *p*-ELK-1 was immunohistochemically detected and then its expression was measured by computer image analysis. A significantly increased expression of *p*-ELK-1 in enterocytes was measured 1 h, 2 h, and 4 h after 0.5 Gy and 1.0 Gy irradiation, 24 h after all radiation doses, respectively. Peak of *p*-ELK-1 expression was observed at 0.5 Gy at all time points after irradiation. The detection of *p*-ELK-1 might be considered as a perspective qualitative *in vivo* biodosimetric marker for military purposes expressed by proliferative cells 24 h after irradiation with doses from 0.25 Gy to 1.0 Gy.

Keywords: Radiation, biodosimetry, phospho-ELK-1, image analysis, immunohistochemistry, biomarkers

1. INTRODUCTION

Development of biomarkers for biodosimetry was given high priority in the consensual agreement among countries as part of readiness for countermeasures against the possibility of a terroristic attacks using radiological and nuclear devices¹. One perspective and non-invasive approach is to find dose-dependent reliable and sensitive markers or altered protein expression in epithelial cells such as enterocytes.

At cell level, pathological changes in a gastrointestinal tract epithelium have been described even after irradiation by sub-threshold doses. Those changes such as flattening of the width of enterocytes,

depletion of enterocytes, etc. are compensable^{2,3}. Recent *in vitro* studies presented that alterations of signal transduction pathways would have a biodosimetric impact and they would be more sensitive than classic histopathologic patterns⁴.

Radiation-induced DNA damage leads to G2/M cell cycle arrest of proliferative cells⁵. G2/M arrest is attributed to ERK 1/2 pathway⁶. Common direct member of ERK 1/2 and JNK/SAPK mitogen-activated protein kinases (MAPK) pathways, the ELK-1 protein⁷, is activated by phosphorylation⁸ on site of its serine 383 and subsequently binds DNA repair protein genes such as XRCC1⁹.

Narang and Krishna¹⁰ observed phospho-ELK-1 (*p*-ELK-1) expression in irradiated liver. They found higher *p*-ELK-1 expression during the first 24 h by the dose of 3.0 Gy and a dose-dependent expression in the range 0.5 Gy-5.0 Gy after 4 h after single-dose irradiation. Their results also showed gradually increased phospho SAPK expression during the first day, which peaked after 0.5 Gy irradiation, while the second ELK-1 activating pathway member, ERK 2, was overexpressed during 0.5 h - 4 h without dose-dependency trends.

In addition, *p*-ELK-1 is overexpressed in irradiated cells with proliferative activity. As published previously³, this protein was detected in irradiated enterocytes *in vivo*, unlike in fibroblasts, fibrocytes and blood cells. Therefore, *p*-ELK-1 production is linked to proliferative cells.

Previously, a significantly higher expression of the phosphorylated form of ELK-1 was found in irradiated rat jejunum and colon transversum lasting up to 96 h after the whole-body single dose of 1.0 Gy - 5.0 Gy gamma irradiation.

Above-mentioned facts led to investigate phosphorylated ELK-1 expression in irradiated enterocytes *in vivo* during the first day after low-dose irradiation and its potential impact in field biodosimetry.

2. MATERIAL AND METHODS

Male Wistar rats (Konarovice, Czech Republic) aged 12-16 weeks weighing 250-300 g were given a whole-body irradiation performed in a jig using ⁶⁰Co unit (Chisotron, Chirana Company, Czech Republic) at a dose rate of 0.95 Gy.min⁻¹ with a target distance 1 m. The animals were slightly anaesthetised before irradiation using a mixture of one volume of Rometar (Spofa Comapany, Prague, Czech Republic), three volumes of Narkamon (Zentiva Company, Prague, Czech Republic) in 12 volumes of physiologic saline. This solution was injected intramuscularly at 10 mg.kg⁻¹.

Four groups of rats were used irradiated by the single dose of 0.25 Gy, 0.5 Gy, 1.0 Gy, and sham-irradiated control group. Irradiated rats were

then examined 1 h, 2 h, 4 h, and 24 h after irradiation in subgroups consisting of 10 animals.

2.1 Histological Examination

Rats were killed by cervical dislocation and central parts of colon transversum were carefully fixed with a 10 per cent neutral buffered formalin. Samples were subsequently embedded into paraffin and 1 µm thick tissue sections were cut and Gram's staining for bacteria and also immunohistochemic detections for phosphorylated ELK-1 were provided.

Immunohistochemical detection of phosphorylated ELK-1 was performed with a standard peroxidase technique. After blocking the endogenous peroxidase activity for 20 min, the tissue sections were incubated for 1 h with a monoclonal antibody recognising *p*-ELK-1 (Cell signaling, Prague, Czech Republic) diluted 1:200 in phosphate buffered saline (PBS, pH 7.2) and then washed three times in PBS. All slides were then incubated for 45 min with biotinylated anti-mouse antibody (Biogenex, Prague, Czech Republic) diluted 1:200 in PBS. Subsequently, all slides were incubated with streptavidin horseradish peroxidase (Biogenex, Prague, Czech Republic) under the same conditions as the secondary antibody. Excess antibodies were then washed off with PBS. Finally, 0.05 per cent 3,3'-diaminobenzidinetetrahydrochloride-chromogen solution (Sigma, Prague, Czech Republic) in PBS containing 0.02 per cent hydrogen peroxide was added for 10 min to visualise the antigen-antibody complex *in situ*.

2.2 Image Analysis

Stained samples were evaluated using the BX-51 microscope (Olympus, Prague, Czech Republic) and computer image analysis ImagePro 4.11 (Media Cybernetics, MD, USA). Six microscopic fields at a 400 fold original magnification were randomly selected from each rat sample.

The *p*-ELK-1 immuno-reactive structures were detected in the inverted RGB range: red 100-255, green 171-255, and blue 161-255, where 0 is white and 256 is black colour. Subsequently, integral optical density, density mean (of positive objects), and percentage of positive stained areas of viewing fields were measured.

2.3 Data Processing

The Mann-Whitney test was used for the statistical analysis giving mean $\pm 2 \times$ SEM (Standard error of mean).

3. RESULTS

No animal died prior to the time of examination and no infectious agents were found. In the central parts of colon transversum, positively stained cytoplasm of enterocytes and goblet cells were seen.

In comparison to values of non-irradiated animals, a significantly higher density means of phospho-ELK-1 were measured in colon transversum of all rats irradiated by the doses of 0.5 Gy and 1.0 Gy except the group examined 4 h after 1.0 Gy irradiation. During 24 h after irradiation, a dose-dependency of *p*-ELK-1 expression measured as density means of immuno-reactive objects was not observed (Table 1).

Significantly higher values of the *p*-ELK-1 expression in per cent were measured in animals examined 24 h after 0.25 Gy irradiation as well as in all animals irradiated with 0.5 Gy and 1.0 Gy

than in non-irradiated rats except the group examined 4 h after 1.0 Gy irradiation. A dose dependency of *p*-ELK-1 expression after irradiation in the range of 0.25 to 0.5 Gy was observed 24 h after irradiation (Table 2).

During the measurement of the integral optical density, it was found that behaviour of irradiated enterocytes is similar to that of the density means of *p*-ELK-1 (Table 3).

4. DISCUSSION

ELK-1 is a transcription factor which activates by several MAPK, it is involved in ERK 1/2 and JNK/SAPK pathways conducting the immediate early responses of the c-FOS promoter to ionising radiation. ELK-1 is a component of the ternary complex that binds the serum response element (SRE) in response to serum¹¹. ELK-1 is inducible by pro-inflammatory cytokine cascade member Interleukine-1 beta¹² and growth factors such as vascular endothelial growth factor¹³ and epidermal growth factor¹⁴, which are up-regulated after irradiation *in vivo*. This protein might be also activated by

Table 1. Average values of *p*-ELK-1 positive structures density means per microscopic field \pm SEM

	Radiation dose 0 Gy	Radiation dose 0.25 Gy	Radiation dose 0.5 Gy	Radiation dose 1.0 Gy
1 h post-irradiation		45.3 \pm 4.5	128.2 \pm 7.6***,+++	132.7 \pm 5.4***
2 h post-irradiation		26.0 \pm 2.7	130.9 \pm 6.8***,+++	137.1 \pm 6.5***
4 h post-irradiation		40.3 \pm 4.7	157.5 \pm 7.6***,+++	63.3 \pm 4.4+++
24 h post-irradiation	59.5 \pm 6.2	111.6 \pm 6.9***	162.6 \pm 7.4***,+++	151.4 \pm 7.1***

Significant differences among animals sham-irradiated (0 Gy) and animals irradiated by the dose of 0.25 Gy-1.0 Gy: p < 0.05 - *; p < 0.01 - **; p < 0.001 - ***.

Significant differences between values of irradiated animals to animals irradiated by the neighbouring lower dose: p < 0.05 - +; p < 0.01 - ++; p < 0.001 - +++.

Table 2. Average value of *p*-ELK-1 positive structures in per cents per microscopic field (field area is 100 %) \pm SEM

	Radiation dose 0 Gy	Radiation dose 0.25 Gy	Radiation dose 0.5 Gy	Radiation dose 1.0 Gy
1 h post-irradiation		1.28 \pm 0.17	10.15 \pm 1.00***,+++	8.36 \pm 0.54***
2 h post-irradiation		0.60 \pm 0.10	9.28 \pm 0.90***,+++	8.66 \pm 0.58***
4 h post-irradiation		1.44 \pm 0.24	14.9 \pm 1.22***,+++	1.95 \pm 0.24+++
24 h post-irradiation	3.57 \pm 0.56	4.15 \pm 0.34***	25.15 \pm 1.54***,+++	10.01 \pm 0.94***,+++

Significant differences among animals sham-irradiated (0 Gy) and animals irradiated by the dose of 0.25 Gy-1.00 Gy: p < 0.05 - *; p < 0.01 - **; p < 0.001 - ***

Significant differences between values of irradiated animals to animals irradiated by the neighbouring lower dose: p < 0.05 - +; p < 0.01 - ++; p < 0.001 - +++

Table 3. Average value of integral optical density of p-ELK-1 positive structures per microscopic field ± SEM

	Radiation dose 0 Gy	Radiation dose 0.25 Gy	Radiation dose 0.5 Gy	Radiation dose 1.0 Gy
1 h post-irradiation		407±55	3433±351***,+++	2788±186***
2 h post-irradiation		207±39	3106±307***,+++	2858±198***
4 h post-irradiation		457±77	5069±425***,+++	630±78+++
24 h post-irradiation	1141±163	1385±113	8486±526***,+++	3249±304***,+++

Significant differences among animals sham-irradiated (0 Gy) and animals irradiated by the dose of 0.25 Gy-1.0 Gy: p < 0.05 - *; p < 0.01 - **; p < 0.001 - ***

Significant differences between values of irradiated animals to animals irradiated by the neighbouring lower dose: p < 0.05 - +; p < 0.01 - ++; p < 0.001 - +++

other stress stimuli such as hyperosmolarity¹⁵. An early ELK-1 activation after radiation-induced DNA damage leads to higher cell proliferation and higher resistency to apoptosis probably via action of anti-apoptotic MCL-1¹¹, whereas persisting activation has link to G2/M cell cycle arrest and/or apoptosis. Suzuki⁴, *et al.* suggested that apoptosis seen in MAPK active condition after irradiation is due to p53 accumulation, which was not detected at doses up to 0.5 Gy, while between 1.0 Gy and 6.0 Gy, the level of p53 protein increased in a dose-dependent manner. This suggestion supports Lee,¹⁵ *et al.*, who observed acceleration of apoptotic pathway after inhibition of ERK1/2 activity.

In this study, expression of ELK-1 phosphorylated was measured by MAP kinase 1 predominantly at serine 383, although MAP kinase 1 can also phosphorylate Elk-1 at serine 324, 389, and 422 and at threonine 336¹⁶. It is not still clear whether ELK-1 phosphorylation at different site than serine 383 regulates different mechanisms.

From results it follows, that the p-ELK-1 saturation was observed during the first 4 h and during the 24 h after irradiation by a dose of 0.5 Gy and 0.25 Gy respectively. For better clarity of p-ELK-1 action it is crucial to answer why p-ELK-1 activation in enterocytes is lower after sub-lethal and lethal irradiation. One of possible reasons could be low fraction of enterocytal stem cells or activation of different pathways after serious DNA damage^{4, 17}. However, generally lower p-ELK-1 expression was observed in low-differentiated cells localised between mucous plicas than in mature and well-differentiated cells at the top of mucous plicas. Therefore,

p-ELK-1 expression might be directly dependent on cell differentiation and for practical biodosimetric evaluation it should be measured in the most differentiated part of mucosa. Probable explanation was published by Townsend¹⁸, *et al.*, who measured proliferative and differentiation effect of MCL-1, the ELK-1-mediated protein, to immature monocytes is that ELK-1-mediated up-regulation of MCL-1 through mechanisms similar to those utilised by c-FOS, EGR1, NUR77, and others might aid in maintaining viability as these cells move along the differentiation pathway. Therefore, consequences of ELK-1 up-regulation in irradiated cells might be strong differentiation as well as proliferation factor mobilising pool of surviving stem cells to compensate low number of enterocytes after irradiation.

The early response mechanism may also serve to restrict MCL-1 expression to specific windows of time (e.g., the initiation of a step forward in differentiation), preventing prolonged exposure of cells to the viability-promoting gene product and minimising the possibility of transformation¹⁸.

Biodosimetric abilities of p-ELK-1 enhances results published by Sautin¹⁹, *et al.*, when ELK-1 activation did not change significantly in *Clostridium difficile* toxin-treated murine hepatocyte cell line AML12. Otherwise, bacteria themselves such as *Staphylococcus aureus*, provide time- and dose-dependent ERK1/2 activation. Therefore, in irradiated patients, it is necessary to exclude ELK-1 activation due to any other microbial and physical stress stimulation. In addition, p-ELK-1 expression is more sustainable than for example p53 accumulation. Previously, p-ELK-1 positive enterocytes were detected even 96

h after 1.0 Gy and 5.0 Gy irradiation, otherwise p53 accumulation with subsequent phosphorylation at serine 15 was observed during the first day within 3 h after irradiation²⁰. A higher and prolonged expression of ELK-1 in irradiated enterocytes might be linked to enhanced stimulation of stem cells to compensate diminished number of mature enterocytes after irradiation until this cell population would reach a sufficient number that allows them to restore and keep all functions. Therefore, ELK-1 up-regulation can be measured even 96 h after sub-lethal irradiation.

On basis of *in vivo* results, it is suggested:

- (a) *p*-ELK-1 activation is strongly linked to radiation response of proliferating cells, and
- (b) Viable biodosimetric 'window' is 24 h after single 0.25 Gy - 5.00 Gy gamma irradiation due to sustainability of *p*-ELK-1 expression.

The detection of *p*-ELK-1 might be considered as a suitable qualitative biodosimetric marker of proliferative cells useful under clinical conditions. In military field conditions should be used more accessible proliferating cell line such as keratinocytes or epithelial cells from cavum oris. However, this suggestion has to be verified by further experiments detecting the *p*-ELK-1 expression in other tissues and after other agents than ionising radiation, which can be produced by weapons of mass destruction, namely nervous paralytic compounds and lethal infections.

5. CONCLUSIONS

Significantly increased expression of *p*-ELK-1 in rat colon transversum 24 h after sub-lethal irradiation has been observed and subsequent experiments including preparation of ELISA (the most suitable military field molecular biologic technique) calibration curves of human samples as rectal smears might be conducted.

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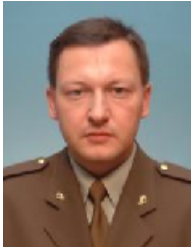
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