Studies of DNA repair strategies

in response to complex DNA damages

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Abstract

The main aim of this thesis was to study the role of the indirect actions of γ-rays and α-particles on the complexity of primary DNA damages and the repair fidelity of major DNA repair pathways: non-homologous end joining (NHEJ), homologous recombination repair (HRR) and base excision repair (BER). The complexity of radiation-induced damages increases and the proximity between damages decreases with increasing LET due to formation of ionization clusters along the particle track. The complexity of damages formed can be modified by the free radical scavenger dimethyl sulfoxide (DMSO). In addition, the effects of low doses of low dose rate γ-radiation on cellular response in terms of differentiation were investigated.

Paper I investigates the role of the indirect effect of radiation on repair fidelity of HRR, NHEJ and BER when damages of different complexity were induced by radiation or by potassium bromate. We found that potassium bromate induces complex DNA damages through processing of base modifications and that the indirect effect of radiation has a high impact on the NHEJ pathway. Results in **paper II** confirmed our conclusions in paper I that the indirect effect from both γ-rays and α-particles has an impact on all three repair pathways studied and NHEJ benefits the most when the indirect effect of radiation is removed.

In **paper III** we investigated the effects of low dose/dose rate γ-radiation on the developmental process of neural cells by using cell models for neurons and astrocytes. Our results suggest that low dose/dose rate γ-radiation attenuates differentiation and down-regulates proteins involved in the differentiation process of neural cells by an epigenetic rather than cytotoxic mechanism.

List of original publications

This thesis is based on the following publications:

I. **Bajinskis, A.**, G. Olsson and M. Harms-Ringdahl (2012). "The indirect effect of radiation reduces the repair fidelity of NHEJ as verified in repair deficient CHO cell lines exposed to different radiation qualities and potassium bromate." Mutat Res **731**(1-2): 125-132.

II. **Bajinskis, A.**, Erixon, K., Natarajan, A.T., Harms-Ringdahl, M. (2012). "The response of HRR-deficient Chinese hamster ovary cell line reveals significant contribution of the indirect effect from both γ-rays and α-particles on NHEJ pathway." Manuscript.

III. **Bajinskis, A.**, H. Lindegren, L. Johansson, M. Harms-Ringdahl and A. Forsby (2011). "Low-dose/dose-rate gamma radiation depresses neural differentiation and alters protein expression profiles in neuroblastoma SH-SY5Y cells and C17.2 neural stem cells." Radiat Res **175**(2): 185-192.

Additional publications, not included in this thesis:

IV. Brehwens, K., **A. Bajinskis**, E. Staaf, S. Haghdoost, B. Cederwall and A. Wojcik (2012). "A new device to expose cells to changing dose rates of ionising radiation." Radiat Prot Dosimetry **148**(3): 366-371.

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Abbreviations

8-oxodG	8-oxo-7,8dihydro-2'-deoxyguanosine
AP	Apurinic/apyrimidinic site
APE1	AP endonuclease 1
ATM	protein kinase ataxia telangiectasia mutated
BER	base excision repair
CHO	Chinese hamster ovary
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DNA-PK	DNA-dependent protein kinase
$DNA\text{-}PK_{cs}$	DNA-dependent protein kinase catalytic subunit
DRAG	detection of repairable adducts by growth inhibition
dRP	deoxyribose phosphate
DSB	double strand break
DTPA	diethylene-triamine-pentaacetic acid
e^-_{aq}	hydrated electron
eV	electron volt
FEN1	flap endonucleases 1
Gy	Gray
GFAP	Glial fibrillary acidic protein
γH2AX	phosphorylated histone H2AX
H·	hydrogen atom
H_2O_2	hydrogen peroxide
HRR	homologous recombination repair
IR	ionizing radiation
$KBrO_3$	potassium bromate
LET	linear energy transfer
Lig I	DNA ligase 1
Lig III	DNA ligase 3
LigIV	DNA ligase 4
LMDS	locally multiple damaged sites
LNT	linear non-threshold hypothesis

MALDI-ToF	matrix-assisted laser desorption ionization - time-of-flight mass spectrometer
MN	micronuclei
MRE11	meiotic recombination protein 11
NBS1	Nijmegen breakage syndrome 1 protein
NHEJ	non-homologous end joining
·OH	hydroxyl radical
OCDL	oxidative clustered DNA lesion
PAR	poly ADP-ribose
PARP-1	poly (ADP-ribose) polymerase-1
PNK	polynucleotide kinase
Pol-β	DNA polymerase β (beta)
Pol-δ/ε	DNA polymerase δ (delta)/ ε (epsilon)
RBE	relative biological effectiveness
RMF	response modifying factor
RPA	replication protein A
SSB	single strand break
XRCC1	X-ray repair cross-complementing group 1 protein
XRCC3	X-ray repair cross-complementing group 3 protein
XRCC4	X-ray repair cross-complementing group 4 protein

Ionizing radiation

Exposures

Each living organism in our environment is continuously exposed to ionizing radiation (IR). Cosmic radiation is one of the sources of natural radiation and it amounts to about 13 % of the background radiation. It comes from the sun and outer space and consists of positively charged particles and γ-radiation. Another natural source of radiation comes from radioactive substances in the earth surface, for ex., radon gas is the decay process of radium that is found in the soil. Exposure to radon and its decay products is one of the greatest risks of IR from natural radiation sources, especially in countries like Sweden and Great Britain. In lungs the decay products of radon attach to the surface of the respiratory tract thus damaging cells in the outer layer. Radon progenies polonium-218 (^{218}Po) and polonium-214 (^{214}Po) decay and emit high energy α-particles that increase the lung cancer risk [1, 2]. In addition to the cosmic radiation and radioactive substances in our environment, all people have low levels of radioactive isotopes such as ^{40}K, ^{14}C, ^{210}Pb and other isotopes inside their bodies. Radioactive materials in the air, soil and water can be ingested through the food chain.

Besides natural radiation sources, there are also artificial (man-made) sources of radiation like medical equipment (x-ray devices) to which most people will be exposed. The proportion of annual radiation dose to the general population from medical sources of radiation is about 20 % [3] and it has tendency to increase due to advanced examination methods that requires higher radiation doses for better image resolution. Other radiation sources are consumer products like smoke detectors, tobacco products [4] as well as radioactive fallouts from nuclear weapon testing in the atmosphere and emissions of radioactive materials from nuclear facilities.

Radiation protection

To limit the cancer risk of the general population from background radiation and from diagnostic radiology as well as the workers in nuclear industry, dose limits have been established for the public as well for occupational exposures. Currently, the linear non-threshold (LNT) hypothesis is applied in radiation protection, where cancer risk increases linearly with increasing radiation dose. This hypothesis is primarily based on the epidemiological data from atomic bomb survivors where dose response relations for cancer have been established for doses over 100 mGy [5]. One should be aware that A-bomb

survivors received acute high dose rate radiation exposures while in radiation protection dose rates are very low, equivalent to 20 mGy per year for radiation workers and 1 mGy per year for the general public. There is a lack of solid data for radiation doses below 100 mGy and that rises discussions about the linearity of the cancer risk at low radiation doses [6]. Besides the LNT model, other models have been proposed as alternative dose response relations in the low dose range for radiation-induced cancer risk [7], see Figure 1.

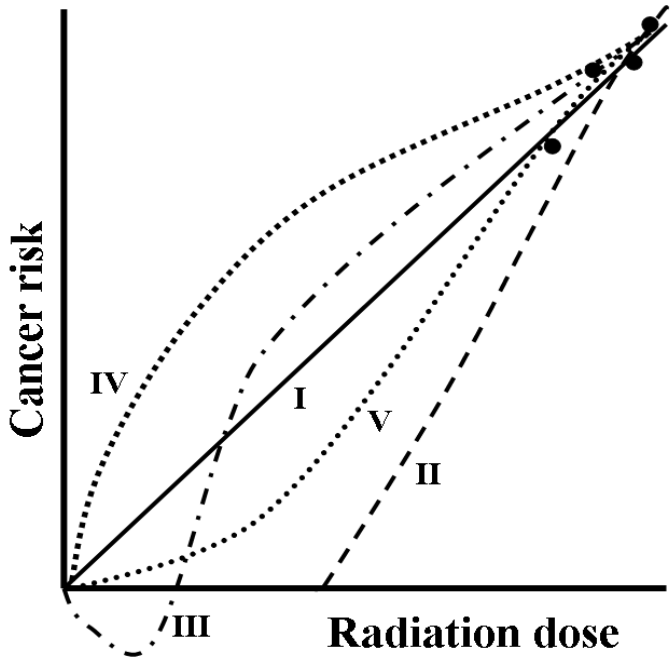

Figure 1. Five suggested models for radiation-induced cancer risk, (I) the LNT concept, (II) the threshold dose model, (III) the protective effect or hormesis model, the risk (IV) underestimation and (V) overestimation models.

There is a general concern whether epidemiological studies could verify any of these models [8] and that even very large population studies will not necessarily solve this problem due to uncertainties in the radiation doses and variations raised by confounding factors and by individual sensitivity [9, 10]. Moreover, there are recent evidences of radiation-induced non-cancer effects, such as cardiovascular diseases and eye cataract, and possibly diseases of respiratory and digestive systems after both high and low dose exposures [11-13]. To bridge the gap in the low dose range, where knowledge is missing, a mechanistic understanding of cellular responses to low doses may provide the missing evidence regarding the shape of the dose response relation.

The mechanistic models should take into consideration that the genome of the human cells is constantly attacked and damaged by endogenous and exogenous reactive molecules. One of

the exogenous sources is IR while the major source of endogenous damages is reactive oxygen species (ROS). Highly reactive ROS are generated from oxygen during the normal cellular metabolic activity and cause oxidative stress and damages in DNA, proteins and lipids in each cell every hour [14]. Cells have evolved efficient defence systems against induction of damages and mechanisms for repairing damages [15-18]. Accumulation of ROS-induced oxidative damages or changes in balance between levels of ROS and antioxidants may lead to mutations, carcinogenesis and ageing [19-22].

As will be further discussed below, IR induces both direct damage to DNA as well as damage induced by indirect effects. The indirect effect is caused by radiolysis of water in the cellular compartments and will lead to formation of ROS. The average number of DNA modifications from endogenous ROS is around 10^6 modifications per cell per day while the number of DNA alterations from radiation-induced ROS is on average 2×10^2 per cell per 20 mGy single dose [14, 23]. Thus the endogenous ROS production may add or interact with the IR-induced damage, and the relative impact on the IR-induced DNA damage will be largest in the low dose region.

It has recently been shown that low doses of IR will also trigger a stress response that lead to a transient increase in endogenous ROS which are several magnitudes higher compared to the ROS produced by the indirect effect of IR [24-26] and that will contribute to the genotoxic action of the IR exposure. These results may be indicative of that low doses of radiation might be more genotoxic than what should be expected from a linear extrapolation from high doses. A central part of this thesis is to explore how the indirect effect of radiation, mainly ROS, contributes to the quantity and quality of the DNA damages produced by ionizing radiation and how the fidelity of the repair systems of the cell is influenced. Considering that ROS will also be produced through endogenous stress response mechanisms as a consequence to low doses of IR, research on the interaction of ROS, indirect effects of radiation and the mechanisms behind the genotoxicity of low doses of IR are of basic interest.

Radiation qualities

Ionizing radiation is a high-energy type of radiation that is capable to produce ionizations in target substances through which it passes. There are two main types of ionizing radiation: electromagnetic radiation and particle radiation. X-rays and γ-rays are electromagnetic radiations with short wavelengths and high energies. X-rays are produced by energetic electrons hitting a specific target. Gamma-rays are very similar to x-rays in their effects to

living organisms and are generated when unstable atoms, such as ^{137}Cs and ^{60}Co, decay. Examples of particle radiation are protons, neutrons, electrons, α-particles and heavy ions. They are emitted during the decay of radionuclides or produced in specific devices – cyclotrons, betatrons and linear accelerators.

The deposition of energy and the production of ionization tracks depend on the quality of the radiation. Thus, IR can be divided into sparsely ionizing radiations (X-rays, γ-rays) and densely ionizing radiations (particle radiations). The characteristics for these types of radiation are described by their linear energy transfer (LET) – the amount of energy transferred to matter per unit length when ionizing particles pass through it. Sparsely ionizing radiations are low-LET radiations with LET values less than 1 keV/µm while densely ionizing radiations are high-LET radiations with LET values around 10-100 keV/µm for neutrons, 100-200 keV/µm for α-particles and more than 1000 keV/µm for heavy ions.

The effectiveness of different qualities of IR for various endpoints can be evaluated by the so called relative biological effectiveness (RBE). This parameter is obtained by comparing the dose needed to cause a specific effect from one type of radiation quality, which usually is low-LET radiation, with the dose needed to obtain the same effect from another type of radiation quality. The RBE values for low-LET radiations are close to 1 and increases with higher LET, reaching a maximum at 100 to 200 keV/µm, and then decreases with higher LET, for review see [27]. RBE values for one type of radiation may differ for various endpoints and different cell types.

Direct and indirect action of IR

The interaction between a target molecule in the cell and the initial radiation can be categorized into direct and indirect action. The direct action is the event when the energy of the initial radiation is deposited directly in the target molecule (DNA). In the event of an indirect action the initial radiation interacts with molecules (H_2O) surrounding the target and the reactive radiolysis products formed will cause damage on the target molecule (DNA). Around 70% of damages formed by sparsely ionizing radiation are caused by the indirect action. For DNA, the direct effect is the major mode of action of high-LET radiations (α-particles, neutrons). These high-LET radiations produce a dense track of ionization and excitation events along the particle path (Figure 2). A single α-particle track produces around 10’000 ionizations when traversing a cell nucleus [28]. In contrast to the high-LET radiation, a γ-ray track produces only around 30 ionizations within the cell nucleus. Thus the single α-

particle track produces much more DNA damages in the cell nucleus than a single γ-ray track explaining the higher RBE of high-LET radiation.

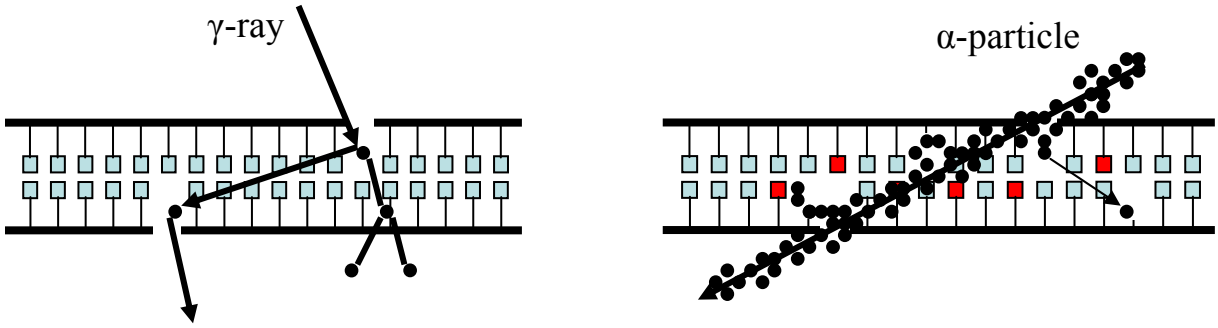

Figure 2. Track structures of γ-rays and α-particles at DNA level. Each dot represents event of ionisation or excitation. Gray squares represent DNA bases, red squares are damaged bases, and broken lines are DNA strand breaks.

Free radical species and scavengers

The energy from ionizing radiation is transferred into water that surrounds the DNA molecules and there are free radicals produced: hydroxyl radicals (·OH), hydrogen peroxide (H_2O_2), hydrated electrons (e^-_{aq}), hydrogen atoms (H·) and hydrogen molecules (H_2). These are highly reactive radicals that react with surrounding molecules. The yield of radicals per unit dose is LET-dependent thus with higher LET the yield of e^-_{aq}, H· and ·OH decreases and the yield of H_2O_2 and H_2 increases due to combination reactions of ·OH or H· radicals within the particle track, for review see [29]. As cells have a natural scavenging system, consisting of proteins and soluble scavengers, free radicals, produced within a diffusion range of 4 – 6 nm, may interact with DNA (size of DNA helix is 2 nm) and produce DNA damages [30]. The yield of free radicals from the indirect action of IR is reduced by the scavenging of these reactive species before they reach a specific target thus decreasing the indirect effect of radiation [31, 32].

One commonly used radical scavenger that efficiently protects against the indirect action of radiation is dimethyl sulfoxide (DMSO) [33, 34]. It scavenges hydroxyl radicals and to a lesser extent hydrated electrons [35]. Early studies on the protective effect of DMSO in Chinese hamster V79 cells showed that 2M DMSO is efficient in increasing the cell survival in G1-synchronized cells both after X-rays and heavy ions [33]. The degree of protection by 2M DMSO for heavy ions was lower than for X-rays and was around 50 %. Theoretically, removal of all the indirect actions of sparsely ionizing radiations would be possible at high concentrations of DMSO (3-4M), however these concentrations are toxic to cells [36]. It was

also suggested that the protective effect of DMSO has a maximum at a LET of around 100 keV/μM and that the level of protection diminishes with radiation qualities of higher LET. Other mechanisms than free radical scavenging have been proposed at very low concentrations of DMSO. Irradiation of DNA repair-deficient cells in the presence of 64 mM DMSO showed no radioprotective effect on cell survival but the level of remaining DNA damages was lower in the cells treated in the presence of DMSO [37]. Although the interaction of DMSO with ·OH radicals may lead to the formation of reactive methylperoxyl radicals (CH_3·) it has been shown that these radicals do not increase the yield of DNA SSBs [35].

DNA damages and repair

Ionizing radiation produces a variety of DNA lesions, such as single-strand breaks (SSBs), double-strand breaks (DSBs), base modifications and DNA-protein cross-links. DSBs are the most lethal lesions and can be repaired by two major DSB-repair pathways: non-homologous end joining (NHEJ) and homologous recombination repair (HRR). NHEJ is a fast repair pathway that is active during the whole cell cycle [38]. As NHEJ rejoins DNA double-strand breaks in juxtaposition and even incompatible ends, this DSB-repair pathway is more error prone and may lead to mutations or cell death [39]. Current models of NHEJ repair (Figure 3) suggest that heterodimer Ku70/Ku80 binds to the broken DNA ends and forms a repair complex by recruiting the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), for review see [40]. DNA-PKcs recruits and activates additional repair proteins, including the Artemis nuclease for end processing. The XRCC4-DNA ligase IV complex is then recruited by DNA-PK and this complex is responsible for the ligation of the DNA ends in the presence of DNA-PK complex [41, 42].

HRR is active during the S- and G2-phase of the cell cycle. This DSB-repair pathway is regarded as error free, since HRR uses an undamaged homologous sister chromatid as a template. DSB-repair has a fast and slow component [43, 44] and HRR is involved in the slow component of DSB-repair [45]. Initial processing of DNA ends (Figure 3) is performed by the MRN protein complex (MRE11, RAD50 and NBS1) and this complex recruits the phosphatidylinositol 3-kinase ATM [46]. ATM phosphorylates histone H2AX in the vicinity of DSB and triggers activation of the cell cycle signalling proteins [47]. DNA ends are resected to form a single-stranded region of DNA and the single-stranded DNA-binding protein RPA covers this DNA region.

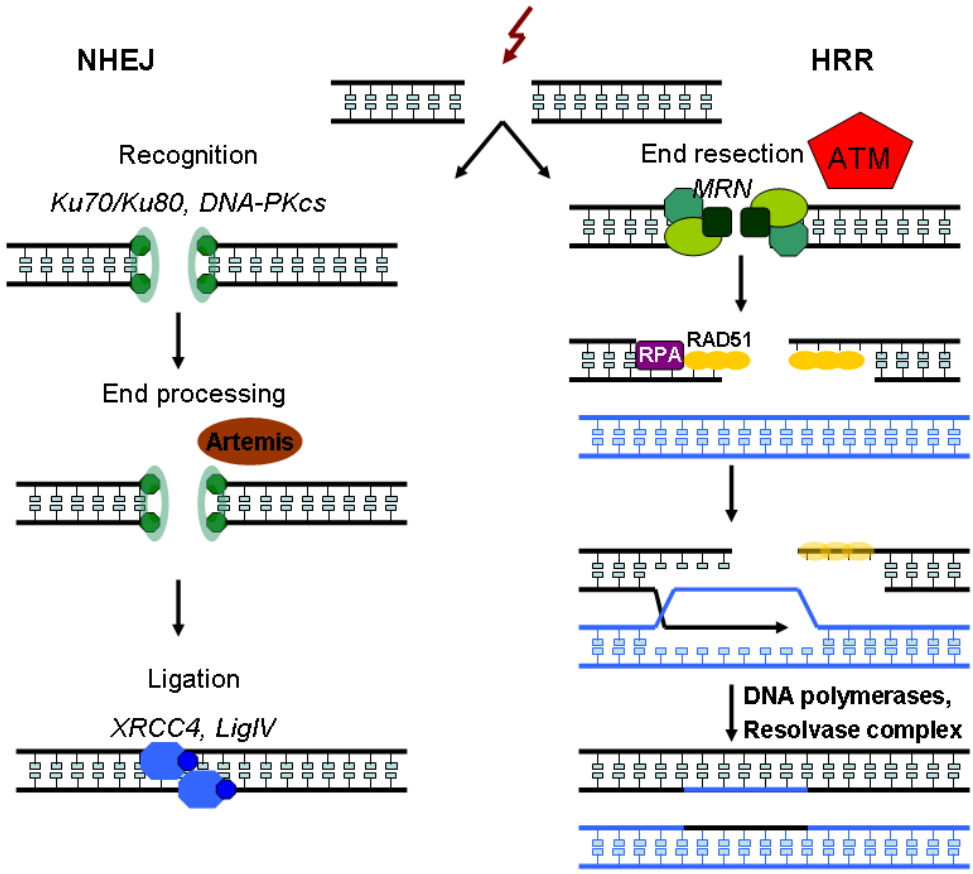

Figure 3. Simplified models of DSB-repair by NHEJ or HRR.

In the following steps RPA is replaced by Rad51, which searches for the homologous double-stranded DNA and invades this template strand by the creation of a Holliday junction. DNA polymerases fill in the gap in the strand and a resolvase complex cleave the junction for accurate repair of DNA [48]. The XRCC3 protein and RAD51 paralogue RAD51C make a complex CX3 which functions during late steps of HRR by binding and resolving of the formation of four DNA strands or Holliday junctions [49, 50].

SSBs, oxidized bases and abasic (AP) sites, induced by free radicals formed during radiolysis of water as well as from endogenous production of ROS, are repaired with high fidelity by BER therefore BER is a major player in the defence machinery [51].

In the enzymatic processing of modified DNA bases by BER, an early step is the recognition and excision of the altered base by a DNA glycosylase (Figure 4). The next step is binding of the endonuclease APE1 to the AP site that generates a single strand break with 5'-deoxyribose phosphate (dRP). Pol-β removes dRP and adds a nucleotide into the gap. XRCC1 and Ligase 3 (Lig III) complex then ligates the DNA ends. If the 5'-terminus of the break is a dRP what Pol-β cannot remove, the Pol-β and Pol-δ/ε conduct the filling of a gap

with 2-12 nucleotides in long-patch repair and flap endonucleases 1 (FEN1) then removes the flap structure, and finally, the ligase 1 (Lig I) ligates the long-patch, for review see [52].

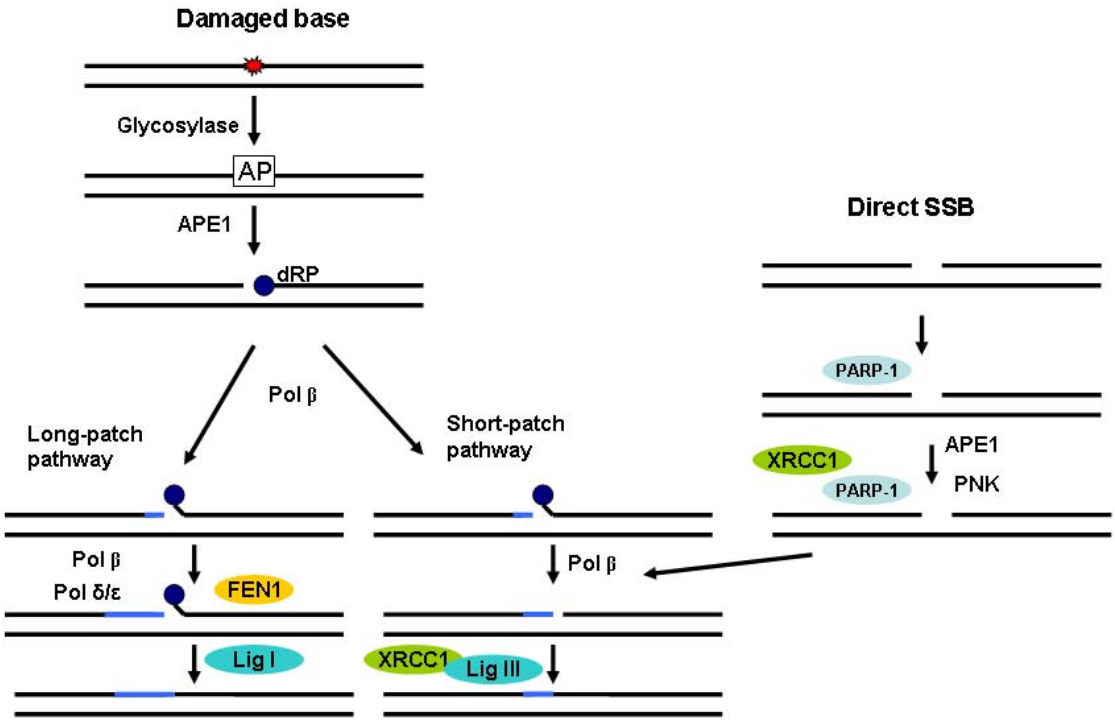

Figure 4. Model for BER of damages bases or direct SSBs, adapted from [20].

An early step in the repair of SSB (Figure 4) is the rapid binding of poly (ADP-ribose) polymerase-1 (PARP-1) to the strand break site where PARP-1 is activated and starts synthesizing chains of poly ADP-ribose (PAR) [53]. PARP-1 has a role in coupling and accumulating the XRCC1 and ligase 3 (Lig III), then it dissociates from the SSB [54]. During the processing of the DNA ends, enzymes are recruited including AP endonuclease 1 (APE1), polynucleotide kinase (PNK) and DNA polymerase β (Pol-β), for review see [52]. In the gap filling step Pol-β binds the DNA end and XRCC1, and insertion of a nucleotide occurs, then Lig II ligates the DNA ends.

Multiple damaged sites

A single radiation track can produce both isolated DNA lesions and locally multiple damaged sites (LMDS) that are two or more lesions within approximately one helical turn of DNA [55]. Densely ionizing radiations, such as α-particles and heavy ions, induce more complex damage within DNA due to the high concentration of ionizing events along the

particle track and the complexity of DNA damage thus depends on the LET of radiation [56]. Up to 70% of all DSBs induced by high-LET radiation can be complex DSBs [57, 58]. Besides DSBs, multiple SSBs and base lesions of different complexity may form oxidative clustered DNA lesions (OCDLs) within one helical turn of DNA [59, 60] that can be transformed into DSBs by cellular processes of repair [61]. These complex DSBs might be more difficult to repair by the cell [62] and thus be more toxic. An important factor besides complexity is proximity of clustered lesions, especially after exposure to densely ionizing radiation, like α-particles, because this can lead to formation of short double-stranded DNA fragments [63]. It has been suggested that HRR is the major pathway in repair of complex DSBs as the ability of NHEJ to repair short fragments is reduced [64].

Chromatin structure

Recently it has been suggested that many DSB-repair proteins are involved in both NHEJ and HRR, i.e., MRN complex, histone H2AX, DNA-PK and ATM, for details see reviews by [65, 66]. Compactness of chromatin affects the choice of DSB-repair pathway. HRR takes place at condensed or heterochromatin and compactness of chromatin restricts access for other DSB-response proteins to the break [67]. Migration of DSBs to the periphery of heterochromatin has been observed and suggests that by this process DSBs become more accessible to DSB-repair proteins [68, 69]. Less condensed or euchromatin is more accessible to DNA-repair proteins and thus has been associated with the NHEJ pathway for DSB-repair and with base-excision repair for SSBs and base damages [70, 71]. The role of the compactness of chromatin has been suggested already from observation of fast and slow components of DSB-repair by DNA fragmentation analysis in different cell types after irradiation to low- and high-LET radiations [72-75]. Observed fast component (5 to 30 min) of biphasic DSB-rejoining has been associated with NHEJ and slow component - with HRR [76, 77].

Differentiation process of neural cells

The nervous system consists of two main cell types, neurons and glia, and they are generated from neural stem and progenitor cells during the development of the nervous system in the ventricular zones of brain during the embryonic period. Proliferating neural cells migrate from the ventricular zone to the periphery of the developing brain where they

differentiate into neurons or two main sub-types of glial cells, astrocytes or oligodendrocytes [78]. The neuronal differentiation process involves formation of neurites that during further extension develop to axons and dendrites [79]. Each neuronal cell contains one axon, responsible for transmission of impulses, and several dendrites that are responsible for receiving and carrying of impulses to a neuron. Glial cells support the neurons and there is recent evidence that astrocytes play an important role in formation of neuronal synapses and reduction of reactive oxygen species by release of antioxidants [80].

Sensitivity of the nervous system is related to the stage of development of the organism and, in general, the nervous system is more sensitive in the early stages of development when cell proliferation occurs. Endogenous sources of damage in the nervous system are related to high oxygen level in the brain and production of ROS, which can lead to DNA damages. If DNA damages in proliferating cells are not repaired, they can lead to cell death. Both HRR and NHEJ pathways are active in the proliferating neural cells. HRR is the major pathway during early stage of active cell proliferation while NHEJ is the prevalent pathway during differentiation [81]. However, in proliferating cells DSBs often lead to apoptosis, instead of repair, as cells can be replaced by new cells from neural progenitor cells [82].

The present investigation

The indirect effect of radiation reduces the repair fidelity of NHEJ as verified in repair deficient CHO cell lines exposed to different radiation qualities and potassium bromate (Paper I)

The response of HRR-deficient Chinese hamster ovary cell line reveals significant contribution of the indirect effect from both γ-rays and α-particles on NHEJ pathway (Paper II)

Aim

The aim of these studies was to analyze the involvement of DNA repair pathways in response to damages of different complexity induced by low-LET γ-rays, high-LET α-particles, or potassium bromate ($KBrO_3$) by using cell lines deficient in repair pathways: HRR, NHEJ, and BER. In particular the indirect effects of radiation on the repair fidelity were of interest. Complexity of radiation-induced damages was modified by using free radical scavenger DMSO, thus reducing the indirect action of radiation.

Cell lines

There are two ways of discussing the response of DNA repair-deficient cell lines, either by the effects due to the pathway, which is missing or the role of the pathway which is still operating in the cell line instead of the deficient one. In our studies we discussed the effects of the NHEJ pathway in the HRR-deficient cell line, of the HRR pathway in the NHEJ-deficient cell line and the role of BER pathway in the BER-deficient cell line.

AA8

The Chinese hamster ovary cell lines used in this study were derived from CHO-K1 cells that have a mutation in the p53 gene [83, 84]. The CHO AA8 fibroblast cell line lacks the G1/S checkpoint due to a mutation in p53, since p53 is required for this checkpoint [85]. Since this cell line has no detected DNA repair deficiencies it is used as a wild type cell line in many studies [86, 87].

irs1SF

The irs1SF cell line is a derivative of the AA8 cell line and it has a defective XRCC3 gene [88]. The lack of the normal function of XRCC3 in the irs1SF cells raises their sensitivity to different DNA damaging agents, including IR [89]. XRCC3 has protein-protein interaction

with the RAD51 paralogue (RAD51C) during the homologous recombination repair thus the deficiency in XRCC3 makes the irs1SF cells HRR-negative [90, 91].

V3-3

The V3-3 cell line is a clone of the AA8 cell line with increased sensitivity to DSB-inducing agents, including IR [92]. It has a defect in non-homologous end joining due to absent DNA-PKcs [93], which is needed to activate additional proteins in NHEJ pathway [94, 95].

EM9

The XRCC1 protein is not detectable in this cell line therefore it is defective in BER [96, 97]. The cell line is sensitive to IR, alkylating agents and reactive oxygen species [98]. It has been suggested that the deficiency in XRCC1 has an impact on the ligation step while the base incision is not influenced [99]. If the incision rate in the EM9 cells is not influenced but only the ligation rate is decreased, the level of intermediate SSBs during BER will be enhanced [100]. SSBs present during the S-phase in the cell cycle will result in DSBs at replication forks that may need HRR for repair [101].

Radiation sources

Irradiation of cells to γ-rays was performed with a ^{137}Cs source "Scanditronix" at a dose rate of 0.43 Gy/min. Flasks with cells were kept on ice before and during irradiation in order to inhibit any repair. DMSO was added directly before the irradiation and removed after irradiation by washing 3 times with Hanks Balanced Salt Solution (Gibco).

For irradiation of cells with high-LET radiation a medium containing α-particles from 214-Polonium (^{214}Po) which is one of the 226-Radium (^{226}Ra) decay products (Figure 5) was used. 96 well plates with cells in were kept on ice in a cold room during irradiation to α-particles.

Radon (^{222}Rn) is a gas produced as a natural decay product of ^{226}Ra. Radon decay emits α-particles and has a half-life of 3.8 days. The energy of α-particles from polonium ^{214}Po decay is 7.69 MeV (mean LET 108 keV/μm) and the range in water is 69.9 μm [102]. The secular equilibrium process between the half-life of parent radionuclide (^{222}Rn half-life is 3.82 days) and short lived decay products with relatively negligible half-lives (^{218}Po half-life is 3.05 min) has been applied in order to obtain ^{214}Po α-particles.

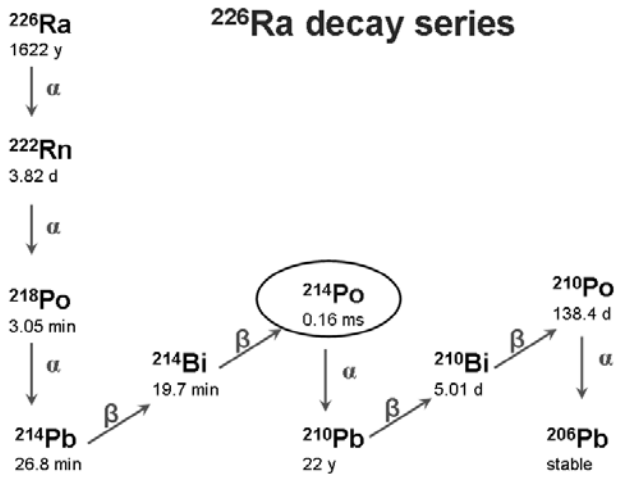

Figure 5. Decay of ^{226}Ra with decay products, their half-lives and type of emitted radiation.

The secular equilibrium is a process when short lived decay products over the time reach the activity of the parental nuclide and this process should be around 10 half-lives. Radon gas was produced in a shielded container containing ^{226}Ra source and for irradiation 1.2 ml gas were aspirated into a glass syringe with 1.2 ml medium. The syringe was then placed on agitator for 3 hours to reach the secular equilibrium between ^{222}Rn and its decay products. After that medium was transferred into a polypropylene tube and bubbled with sterile air to remove radon gas from the medium and left for 30 min, allowing total decay of ^{218}Po (half-life 3.05 min). This ensured that only ^{214}Pb, ^{214}Bi and ^{214}Po were left in the medium. The activity of ^{214}Po was measured by liquid scintillation counter in all samples and the contribution of β- and γ-rays from ^{214}Pb and ^{214}Bi was negligible. The α-particle dose to the cells was calculated from the accumulated activity in medium, taking into account the range of ^{214}Po α-particles. Cells were attached to the well surface and irradiated from the medium above the cells, therefore factor of 0.5 for radioactivity was used. The dose (D) (in Gy) to the cells from ^{214}Po in the medium was calculated by using the following equation:

$$D = \left[\left(\frac{0.5 \times 7.69 MeV / Bq \times 1.6 \times 10^{-19} J / MeV \times Bq / ml}{\text{volume in ml} \times 10^{-3} \text{ kg/ml}} \right) \right],$$

where 7.69 MeV – energy of ^{214}Po α-particles,

Bq/ml is activity of ^{214}Po per ml.

To ensure homogenous distribution of radon progeny and to study the possible binding of radon progeny to the surface of cells, exposures were also done in the presence of 100 mM Diethylene-triamine-pentaacetic acid - calcium trisodium salt hydrate (DTPA, CAS Nr 17034-67-2, Sigma). DTPA is a chelator that binds ions and therefore prevents attachment of radon progeny to cells and plastic surfaces thus ensures diffusion controlled irradiation of cells. Cells were treated in the absence or presence of DTPA and 10 µl samples of medium or total volume of scraped cells from individual wells were taken 30 minutes and 2 hours after starting of treatment. The fraction of activity associated with cells was recalculated and assimilated to the total activity present in the 69.9 µm range of ^{214}Po α-particles in water. The fraction of activity adsorbed to the cells in the absence of chelator DTPA was after 30 minutes 1.05 % ± 0.02 of total number of counts per minute (CPM) and after 2 hours the activity was 1.39 % ± 0.08. In the presence of DTPA activity associated with cells was 0.04 % ± 0.005 and 0.14 % ± 0.01, respectively. However, the calculated cellular dose from the attached fraction of progeny was negligible in comparison to the dose coming from progeny in the medium; therefore further experiments were done without DTPA.

Potassium bromate

Potassium bromate ($KBrO_3$) has been used widely as a food additive, especially in the bread making process. During the baking process it is usually decomposed, however there might be traces of potassium bromate in bread. The International Agency for Research on Cancer has classified potassium bromate as a possible human carcinogen [103], however it is still used for bleaching of flour. Potassium bromate is a well known DNA oxidizing agent that induces specifically the DNA base modification 8-oxodG [104]. Recently, several DSB-specific endpoints like chromosomal aberrations, micronuclei and γH2AX foci have been observed in response to potassium bromate treatment in various cell types [105-108]. These results suggest that complex DSBs might be formed during processing of 8-oxodG lesions [109, 110].

Growth inhibition assay

For detection of inhibition in cell growth we used the DRAG assay that has been adapted to 96 well plates for detection of genotoxic and cytotoxic effects of different chemicals [111]. We modified this assay by increasing the incubation time to 120 h after treatment since we did not observe any significant effect on cell proliferation upto 72 – 96 hours. Cells were

seeded into 96 well plates, incubated for 24 h in humidified 95% air and 5% CO_2 atmosphere. Then cells were exposed to radiation or potassium bromate, incubated for 120 h in humidified 95% air and 5% CO_2 atmosphere, fixed and stained with neutral red that binds to cellular proteins.

Clonogenic cell survival assay

Due to limited volume of media for irradiation with α-particles (1.2 ml), the clonogenic survival assay was modified, cells were seeded into dishes directly after irradiation. Equal conditions for all irradiated cells were achieved by performing the same procedure also after γ-irradiation. Cells were stained with methylene blue in methanol after 8 to 14 days and colonies containing at least 50 cells were counted.

Micronucleus assay

Misrepaired or unrepaired DSBs may lead to formation of acentric chromosome or chromatid fragments and result in micronuclei (MN) [112]. The cytokinesis-block micronucleus test is based on inhibition of cytokinesis by cytochalasin B thus cells have gone through mitosis and contain two nuclei within one cell cytoplasm. MN are scored in binucleated cells and scoring of binucleated cells provide information of the number of cells that has undergone one cell division after irradiation. Radiation-induced MN can also be formed from directly induced DSBs, from processing of oxidative clustered DNA lesions (OCDLs) or from SSBs that may form one-ended DSBs at replication forks [113].

Single-cell gel electrophoresis or comet assay

The comet assay is a sensitive method for measuring DNA strand breaks, including SSBs, DSBs and oxidized bases in individual cells [114, 115]. Agarose-embedded cells on a microscope slide are subjected to lysis with high salt and detergent that results in nucleoids with supercoiled loops of DNA. Only loops containing strand breaks are then relaxed in an alkaline or neutral buffer and subjected to electrophoresis. During electrophoresis those relaxed, negatively charged DNA strands are pulled out into the agarose, forming comet-like structure with a head and a tail. Analysis of the tail intensity relative to the comet head after staining with fluorescent dye reflects the amount of DNA damage. In general, alkaline comet assay (pH >13) measures SSBs while neutral comet assay detects both DSBs and SSBs. Modification of neutral comet assay with cell lysis at 50 °C allows to detect only DSBs [116],

however high temperature may induce heat-labile sites and these sites in close vicinity may form additional DSBs [117, 118].

Results and discussion

The results on cell proliferation by both growth inhibition and clonogenic survival assays revealed increased sensitivity of both DSB-repair deficient cell lines irs1SF and V3-3 after irradiation with γ-rays or α-particles. There was a good correlation between 37% of cell survival values (D_{37}) and 50% inhibition in cell growth values (IC50) for γ-rays with r^2 values of 0.974 and 0.999 in the absence and presence of DMSO, respectively. The correlation was good also for α-particles –DMSO with r^2 equal to 0.946 while there was no correlation for α-particles +DMSO due to response of the HRR-deficient cell line observed both for survival and growth inhibition.

The results from these studies reveal that the indirect effect of γ-rays or α-particles contributes significantly to the fidelity of repair pathways, especially to the NHEJ pathway, which gained most from the scavenging effect of DMSO. Base damages and SSBs are produced in close vicinity of DSBs in the absence of radical scavenger and may inhibit the NHEJ pathway [119]. In the presence of DMSO damages induced by the indirect effect are diminished and mainly DSBs induced by the direct effect are left. It was reported that the NHEJ pathway repairs directly induced, simple DSBs that are rejoined by fast DSB-repair [71].

The intermediate sensitivity and the significant protection of the BER-deficient cell line by DMSO suggest that BER is important for avoiding the formation of DSBs. Since the incision rate in the EM9 cells is not influenced but only ligation rate is decreased, the enhanced level of SSBs during the S-phase of the cell cycle will result in one-ended DSBs at replication forks [101]. In addition, if OCDLs formed from the indirect effect are not properly ligated in the BER-deficient cells, they are prone to be processed into complex DSBs and repaired by HRR [109, 110].

We also observed higher sensitivity of DSB-repair deficient cell lines after $KBrO_3$ treatment and our results together with earlier studies suggest that $KBrO_3$ induces complex DSBs through the formation of OCDLs.

From high to low radiation doses

In the last decade studies have shown that unique gene and protein expressions are induced in response to low doses (in the range of 10 – 100 mGy) compared to high doses (1 to 4 Gy) of γ-rays or iron ions are compared [120-124]. Studies showed that different numbers of genes or proteins are altered in response to radiation and only a small number of genes or proteins was the same for low and high doses.

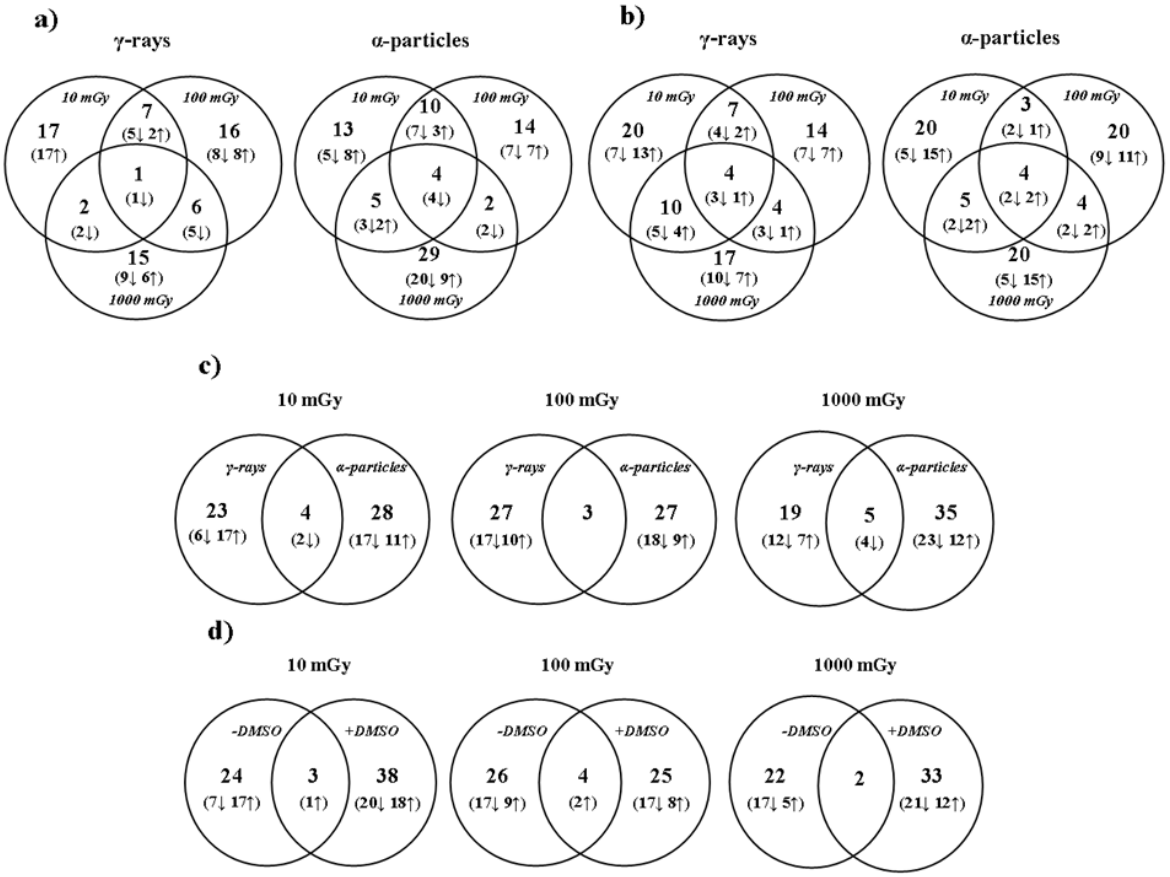

Figure 6. Number of altered protein spots in response to 10, 100 or 1000 mGy doses of (a) γ-rays or α-particles –DMSO, (b) γ-rays or α-particles +DMSO, (c) for both types of radiation – DMSO at different doses, (d) for DMSO effect for γ-rays at different doses.

We aimed to investigate this in more details by irradiating cells with two different doses in the low dose region and also study if the radical scavenger DMSO could modify the protein response. CHO cells were exposed to acute doses of 10, 100 or 1000 mGy γ-rays (dose rate 0.43 Gy/min) or α-particles in the absence or presence of DMSO. The proteins were extracted from cells 3 hours after irradiation and used for protein expression analysis by two-dimensional polyacrylamide gel electrophoresis.

About 1200 to 1700 spots per gel were detected and results revealed that 3 hours after irradiation each radiation dose generated very specific response of protein spots. To handle results of the differentially expressed protein spots after two radiation qualities in the absence or presence of free radical scavenger and three radiation doses, multivariate data analysis by SIMCA (Umetrics) was applied. Each circle represents one dose and one radiation quality and the figures in the circle show the number of differentially expressed protein spots. Numbers in overlapping areas show the number of spots that were identical for the two exposure conditions.

The protein expression induced by 10, 100 or 1000 mGy of γ-rays differed markedly between the doses (Figure 6a) suggesting that unique DNA damage signaling pathways were induced for each dose with little overlap. Only 1 altered protein spot was the same for all three doses of γ-rays -DMSO while the total number of altered protein spots was 27, 30 and 24 for 10, 100 and 1000 mGy respectively (Figure 6a). A similar type of response was observed after α-particle irradiation -DMSO in terms of number of the altered protein spots, where only 4 altered protein spots were observed the same for all three doses. Thus for both low- and high-LET radiation unique protein expression patterns for the three doses were observed.

In Figure 6c the protein expression pattern for γ-rays and α-particles were compared for the same doses. Also here is evident that unique differences in protein expression were induced for the two radiation qualities, supporting the view that the DNA damage response pathways triggered depend on the dose as well as the radiation quality.

When the indirect effect of radiation was decreased by 2M DMSO (Fig 6 b and d), new patterns of protein expression were observed in response to the changes in the quantity and quality of the primary DNA produced.

The results presented demonstrate that unique patterns of up or down regulated protein spots are produced in response to different radiation qualities as well as doses. Work is now in progress to identify the proteins involved and future work will aim to provide a better understanding of the cellular processes induced by low doses of radiation from high or low LET radiation.

Low-dose/dose rate γ radiation depresses neural differentiation and alters protein expression profiles in neuroblastoma SH-SY5Y cells and C17.2 neural stem cells (Paper III)

Aim

The aim of this study was to evaluate new endpoints for cellular responses to low doses and low dose rates of γ-radiation on neuronal and glial cells.

Cell lines

SH-SY5Y

The neuroblastoma cell line SH-SY5Y is a clone of SK-N-SH cells which is of human origin and has been widely used in experimental studies as in vitro cell model for neurons [125-127]. The SH-SY5Y cells have capacity to proliferate in culture for long times and the differentiation process can be initiated by various agents, including retinoic acid (RA) [128]. The undifferentiated cells have a high rate of proliferation and addition of RA decreases proliferation and stimulates neurite outgrowth [128].

C17.2

C17.2 is a multipotent stem cell line derived from mouse cerebellum that has been immortalized by avian myc (v-myc) oncogene [129]. Due to multipotency this stem cell line has ability to differentiate into mature neurons or glia depending on the culturing conditions [130].

Irradiation

Irradiations with acute high dose rate of 0.43 Gy/min (25800 mGy/h) were carried out with ^{137}Cs γ-rays (Scanditronix) at room temperature in the dark thus keeping RA stable.

A specially designed cell incubator with ^{137}Cs γ-radiation source placed beneath was used for low dose rate exposures of 5 or 15 mGy/h. Cells were irradiated at 37 °C in humidified 95% air and 5% CO_2 atmosphere.

Neurite formation

The number of neurites per cell in the SH-SY5Y cells was scored 6 days after the start of cell exposure to RA in order to evaluate the effects of radiation on neurite outgrowth. The number of neurites with a length exceeding the diameter of the cell body in irradiated cells compared with control cells was scored [131].

Immunochemistry of astrocyte differentiation by GFAP

Astrocyte differentiation in the C17.2 cells was evaluated by glial marker – glial fibrillary acidic protein (GFAP) [132]. This marker is specific for glial cells and it is not expressed in neural type cells [133]. The intensity of GFAP staining relative to DAPI staining was compared for irradiated cells and control cells.

2D-PAGE and mass spectrometry

Alterations in protein expression profiles for both neural and glial type cells after irradiation with low dose/ dose rate γ-rays were evaluated by two-dimensional polyacrylamide gel electrophoresis with subsequent identification of proteins from the peptide mass fingerprints by MALDI-ToF. Peptide mass fingerprints were analyzed by search database PROFOUND and protein functions were obtained from UniprotKB/Swiss-Prot database. The protein functions were compared for both cell types.

Results and discussion

Our results revealed that there was no linear dose response relation for the neurite outgrowth of both immature and semi-differentiated SH-SY5Y cells. Surprisingly, low dose rate γ-rays significantly reduced the neurite outgrowth at low doses (10, 30, 100 mGy) but at the same time did not affect cell proliferation, suggesting that the response to oxidative stress might be due to low dose hypersensitivity. Low dose rate γ-rays showed significantly reduced neurite development together with general cytotoxicity at high doses of 1020 and 2100 mGy. Interestingly, SH-SY5Y cells have been observed as radiation resistant to high dose rate γ-rays (1 Gy/min) therefore cytotoxicity might be associated with apoptosis in immature neuronal cells which undergo apoptosis rather than trying to repair DNA damages.

Astrocyte development was decreased at low dose rate irradiation for the doses 10 and 30 mGy, therefore proteome analysis was performed on both glial and neuronal cells irradiated for 30 mGy γ-rays. Functional classification of altered proteins provided similar resonses for both cell types, with 1/3 of the proteins involved in development and differentiation. The next major group identified was cell cycle and proliferation associated proteins and, since they remained altered after several cell divisions, we suggest that radiation exposure induced epigenetic responses that controlled the differentiation of the neural cells.

Additional research is needed to explore if epigenetic changes are induced in response to low dose and low dose rate radiation with possible impact for cancer as well as non-cancer diseases.

Future perspectives

The studies in papers I and II have revealed the important role of the indirect effect on complexity of damages and the choice of repair pathway. The role of chromatin compactness on repair pathways in the repair of DNA breaks might be studied by pulsed-field gel electrophoresis by analyzing repair-deficient cells after irradiation ±DMSO. If DNA-fragment size distribution changes in the presence of DMSO then DSBs should be induced in euchromatin and the HRR-deficient cell line may be influenced the most. However, the number of short DNA fragments after α-particle irradiation +DMSO should stay the same if they are induced in heterochromatin since DMSO may have limited access to condensed chromatin. In addition, cell cycle distribution in those cells should be analyzed which would provide an additional information on repair pathways involved.

The role of OCDLs on the complexity of lesions could be studied by applying modified PFGE together with modified comet assay using lesion-specific endonucleases and if DMSO affects particularly formed base lesions.

Another strategy to gain knowledge about the mechanisms of repair is to study the protein expression changes after low- and high-LET radiation ±DMSO, this is an ongoing project. I have showed that both types of radiation have very specific protein expression profiles and that protein alterations are dose-dependent and influenced by DMSO. Analysis of peptides may provide information on the molecular mechanisms of DNA damage repair, especially at low doses of radiation. Since radiation doses were selected from high and low dose regions (10, 100, 1000 mGy) there is a possibility to find proteins with same or similar functions to the ones observed in the neural cells, i.e., oxidative stress response proteins.

Oxidative stress responses observed in neural cells after low doses/ low dose rate radiation is another interesting research subject. The persistence of epigenetic changes could be studied by different methods of DNA methylation analysis. Irradiation of neural stem cells at the beginning of differentiation to very low doses and dose rates of radiation could provide more knowledge on the role of oxidative stress on induction of neurodegenerative diseases.

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