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P1

TP53 foci formation at sites of radiation-induced DNA damage.

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The tumour suppressor protein TP53 plays an essential role in the cellular radiation response and in the coordination of cell cycle arrest and DNA repair.

We examined the subnuclear localisation of TP53 after ionising radiation by means of immunocytochemical staining and confocal microscopy. After exposure to X-rays and charged particles, even at low doses, a dose or fluence dependent accumulation of TP53 foci was observed in nuclei of human fibroblasts. For ion irradiation, a direct correlation was proven between TP53 foci and sites of particle traversals. In addition, the TP53 foci were found to co-localise with proteins interacting with radiation-induced double-strand breaks (DSBs) such as hMre11 and γ -H2AX, the correlation with γ -H2AX signals being strongest at longer times post-irradiation (24 h). The kinetics of TP53 foci formation was found to depend on the radiation quality, foci appearing earlier after high-LET than after X-irradiation. Furthermore, an increase in ionisation density led to a longer persistence of foci at the sites of DNA damage. The number of persisting foci was also increased in DSB repair-deficient cells. The formation of TP53 foci appears to be dependent on early phosphorylation by ATM, as observed in experiments with PI-3 kinase inhibitors and in AT cells. Furthermore, immunostaining for Ser15-phospho-TP53, the phosphorylated protein was also detected within radiation-induced foci.

We conclude that TP53 accumulation is connected to slowly repaired or irreparable DNA DSBs. We postulate that the extent and density of lesions could influence the outcome of the cellular damage response at the level of the TP53 protein.

P2

DSB signalling and repair after micro-irradiation with energetic ions

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In recent years, our understanding of the biochemical mechanisms of double-strand break (DSB) repair has increased substantially. A thorough understanding of the chain-of-events taking place during repair in the context of the three-dimensional functional architecture of the nucleus requires, however, that topological factors also be considered. These factors include the position of the damage with respect to functional chromatin organisation and to other damaged sites, potential movement of damaged chromatin within the nucleus, and the dynamics of DSB signalling and repair proteins. The analysis of these factors can greatly be facilitated by targeted induction of DSB, using a micro-irradiation facility, where the site to be damaged can be pre-determined by the experimenter, and where any signals observed after irradiation can be related to the initial damage site.

We adapted the ion microprobe SNAKE at the Munich 14 MV tandem accelerator for micro-irradiation of cells. In the present set-up, we achieve an accuracy of irradiation of about 500 nm, i.e. small enough to target specific substructures of the nucleus. Patterns of histone H2AX phosphorylation and accumulation of a variety of signalling and repair proteins (53BP1, Mdc1, Rad51) agree reasonably well with irradiation patterns, so that we can exclude large-scale movement of damaged chromatin within the nucleus. Foci fine-structure does, however, suggest that small-scale movements occur readily. Currently we investigate how the kinetics of foci appearance and disappearance depends on damage density (which can be varied by variation of the ion and ion velocity) and on cell cycle state.

P3

Spatial dynamics of gamma-H2AX foci after precise ion micro-irradiation

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Gamma-H2AX, the phosphorylated form of the histone H2AX is a well established marker of double-strand breaks (dsb). The temporal dynamics of phosphorylation and de-phosphorylation of histone H2AX after dsb-induction has been studied in a large number of cell-lines. In contrast to this, the spatial dynamics of gamma-H2AX, which could represent movement of broken chromosome ends, is not well studied yet.

SNAKE, the ion microprobe beam at the Munich tandem accelerator is now adapted for precise micro-irradiation of cells. Following micro-irradiation with single O16 or C12-ions, we analyze the dynamics of gamma-H2AX phosphorylation along the particle track. First experimental results, using non-confocal 2D- and 3D-microscopy, show a good agreement between irradiation pattern and gamma-H2AX foci. For quantitative analysis the centers of mass of the gamma-H2AX foci within a nucleus are determined and used to calculate the distance of the foci. First analysis of a small 2D-data subset shows an increase in the variation of the calculated distances with increased incubation time post irradiation, possibly representing a small scale movement of gamma-H2AX foci. The small scale movement of the foci could imply a small scale movement of damaged chromosomes.

This study will be extended to a larger number of samples and to 3D follow-ups of single ion tracks.

P4

Artemis and the ATM-dependent NHEJ pathway: contribution in different phases of the cell cycle

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Two functionally different pathways, non-homologous end-joining (NHEJ) and homologous recombination (HR), are active in the repair of DSBs. In mammalian cells, NHEJ is the major repair pathway and active in all phases of the cell cycle, whereas HR contributes to double strand break repair in S and G2 phase where sister chromatids facilitate homology directed repair mechanisms.

Five core components of NHEJ have been identified (Ku70/Ku80, DNA-PKcs, DNA ligase IV-XRCC4) and cells lacking any of these components show radiosensitivity and impaired ability to rejoin DSBs. Recently, Artemis was identified as a sixth NHEJ component. Although Artemis-deficient cells are IR sensitive, they do not display the pronounced DSB repair defect characteristic of cells lacking any of the five core NHEJ proteins. We have now identified Artemis as a component of a subpathway of NHEJ. This pathway is ATM-dependent and also includes other known factors of DSB repair. Using PFGE and γ -H2AX foci analysis of wild type and mutant fibroblasts we demonstrate that ~ 90% of IR-induced DSBs are repaired by core NHEJ, whereas about 10 % of the breaks require the ATM-dependent repair pathway. The contribution of core and ATM-dependent NHEJ to DSB repair was assessed in the G1 and G2 phase of the cell cycle using the γ -H2AX foci approach. Whereas confluent cells were utilized for G1 analysis, repair in G2 was assessed in exponentially growing cells, labelled with G2-specific marker proteins. The role of Artemis in DSB repair throughout the cell cycle was further substantiated by analysis of chromosomal aberrations shortly after irradiation. Artemis-deficient cells revealed a higher frequency of aberrations when compared to wildtype cells. In conclusion, our findings link NHEJ with ATM-dependent signalling and demonstrate a role for core NHEJ and ATM-dependent repair in all phases of the cell cycle.

P5

UV(A)-radiation induces replication-independent DNA double-strand breaks (gamma H2AX foci)

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UV-radiation is a complete carcinogen. It plays important roles in the steps of initiation, promotion and progression of skin cancer. Different wavelength regions (UVB: 280 – 315 nm, UVA, 315-400 nm) induce different kinds of primary DNA lesions which can lead to mutations causing skin cancer.

In this contribution we report that UVA is able to induce (aside from other DNA-lesions) DNA double-strand breaks (DNA-dsb) in a replication-independent way in primary human skin fibroblasts. UVA-induced DNA-dsb were detected via immunofluorescent microscopic visualization of phosphorylated histone H2AX (gamma-H2AX-foci). Split-dose experiments and the use of radical scavengers indicate that UVA-induced DNA-dsb are formed by reactive oxygen species (ROS) and/or radicals which are produced through the interaction of cellular photosensitizers (e.g favins, porphyrins, etc.) with UVA-radiation. Because DNA-dsb are known to be precursor-lesions for the formation of chromosome aberrations, which play an important role in the etiology of skin cancer, our results shine new light on the mechanisms of UVA-induced carcinogenesis of the skin. This will have important consequences for radiation protection in the field of UV-radiation.

P6

Deficient XRCC4 but not Ku80 reduces frequency and fidelity of non-homologous end-joining of restriction enzyme-induced DNA double-strand breaks

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Non-homologous end-joining (NHEJ) of DNA double-strand breaks (DSBs) can be an error-free or error-prone repair process depending the structure of DNA ends created. Complementary ends can be rejoined by precise ligation (error-free or high-fidelity repair). All non-compatible ends require DNA end modification and hence sequence alteration prior to ligation (error-prone). This error-prone repair may promote chromosomal rearrangements and genomic instability. The DNA-Pk dependent repair pathway is known to largely control NHEJ. In this study, we investigate the role of the mayor players, namely Ku80, DNA-PKcs and XRCC4. We applied a novel plasmid-based assay to monitor the repair of chromosomal DSBs created by the I-SceI endonuclease in isogenic mouse fibroblast lines. In XRCC4^{-/-} cells the frequency of errorprone NHEJ was 10-fold reduced as compared to the wildtype. The cells showed significantly longer deletion formation, less rejoining of distant DSB and made frequently use of terminal microhomologies. In contrast, neither inhibition of DNA-PKcs by Wortmannin nor the Ku80^{-/-} influenced the rate and fidelity of NHEJ. However, all three deficiencies slowed down the velocity of NHEJ. This NHEJ phenotype largely contrasts to the extremely enhanced radiosensitivity and DSB repair deficiency after ionizing radiation. Our results suggest important differences between radiation and restriction enzyme-induced DSBs with possible implication for carcinogenesis and repair after irradiation.

P7

Gamma irradiation-induced frameshift mutations in cell cycle-arrested yeast cells largely depend on DNA ligase IV

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Mutations occurring while cells remain in a quiescent state are assumed to considerably contribute to the overall incidence of mutations. During previous studies on the occurrence of spontaneous reversions of an auxotrophy-causing frameshift allele in the yeast *Saccharomyces cerevisiae*, we noticed that about 50% of the adaptive reversions depended on a functional non-homologous end joining (NHEJ) pathway of DNA double-strand break (DSB) repair. In order to evaluate the effect of an excess of DSBs on mutagenesis we exposed cell cycle-arrested cells to gamma irradiation. This treatment dramatically increased the frequency of frameshift mutations of repair-proficient strains. By the use of NHEJ-deficient DNA ligase IV-deleted strains we detected that the majority of the gamma-induced mutations was required a functional NHEJ pathway. These results suggest that the same mutagenic NHEJ mechanism acts on ionizing radiation-induced as well as on spontaneously arising DSBs. Inaccurate DSB repair by NHEJ may represent a source of mutations in cell cycle-arrested cells not only during tumor development (due to spontaneous DSBs) but also following radiation therapy (due to ionizing radiation-induced DSBs).

P8

DNA double-strand break repair and the formation of chromosomal aberrations

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DNA double-strand breaks (DSB) – occurring either spontaneously during the cell cycle or after exposure to ionising radiation – are considered the critical primary lesions in the formation of chromosomal aberrations (CA) which can lead to cell death or oncogenic cell transformation. To eliminate this dangerous damage, different evolutionarily well conserved repair pathways related to homologous recombination (HR) and non-homologous DNA end joining (NHEJ) have evolved.

To investigate the potential contributions of NHEJ and single-strand annealing (SSA), an error-prone sub-pathway of HR, to the formation of CA, we constructed artificial DSB-repair templates containing two closely spaced cleavage sites for the rare-cutting I-SceI endonuclease which permit the induction of single, well defined DSB in living cells. Furthermore, the design of the DNA constructs facilitates the discrimination of the different NHEJ- and SSA-repair products by differential expression of a GFP-fusion protein formed by successful SSA.

Firstly, these NHEJ/SSA constructs were stably transfected in CHO-K1 cells. As a preliminary measure for the induction of CA after electroporation of I-SceI nuclease into these cells, we performed micronucleus assays and analysed metaphase spreads for structural changes. Both methods revealed a 2-fold increase in the frequency of CA indicating, that a single DSB is sufficient to induce a chromosomal aberration.

Secondly, the I-SceI-linearized NHEJ/SSA constructs were employed in cell-free DSB-repair assays using extracts derived from different CHO cell lines proficient for (CHO-K1) or deficient in (xrs6: Ku80^{-/-}; XR-C1: DNA-PKCS^{-/-}; XR-1: XRCC4^{-/-}) NHEJ. In wild-type extracts, all constructs were repaired exclusively by NHEJ. By contrast, extracts derived from mutant cells also used SSA in addition to NHEJ indicating that NHEJ is the major pathway of DSB-repair and replaced by homology-driven mechanisms in the absence of proteins crucial for NHEJ.

P9

The role of homologous recombination, nonhomologous end-joining and single-strand annealing in the cell cycle-dependent repair of DNA double-strand breaks induced by sparsely or densely ionizing radiation in mammalian cells

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We have studied the relative contributions of error-free homologous recombination (HR), potentially error-prone nonhomologous end joining (NHEJ) and error-prone single-strand annealing (SSA) in the rejoining of dsbs induced by sparsely ionizing 200 keV X-rays or densely ionizing ²⁴¹-americium alpha-particles in Chinese hamster ovary cells in G1- and S-phase of the cell cycle. For this study we have used the parental cell line AA8 and its not genetically engineered derivatives V3 (NHEJ-deficient), irs1SF (HR-deficient) and UV41 (SSA-deficient). These four cell lines were synchronized in G1 or in S-phase and exposed to either X-rays or alpha particles to produce dsbs of different complexities. The survival response yielded the following results:

SSA plays no role at all for dsbs rejoining in G1 phase cells after exposure to X-rays or alpha particles. In contrast, in S-phase SSA is efficiently operating on simple and complex dsbs.

NHEJ is the most important mechanism in G1 cells for the rejoining of simple and complex dsbs. Even in S-phase cells is NHEJ more relevant than SSA in coping for simple dsbs, but SSA is more important than NHEJ for the rejoining of complex dsbs.

HR plays a surprising role in G1 cells although it is less important than NHEJ for acting on simple and complex dsbs. In S-phase cells, HR is the most important mechanism to rejoin both simple and complex dsbs. Additional studies with an irs1SF cell line whose defect was complemented with human XRCC3 cDNA showed that overexpression of XRCC3 confers a higher radioresistance to G1 cells than that observed for the parental cell line AA8. Furthermore, overexpression of XRCC3 in S-phase cells results in a phenotype specifically hyperresistant against alpha particles, suggesting an important role of HR for the repair of complex dsbs.

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P10

DNA Double Strand Break Repair defects in Breast Tumorigenesis

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Activity changes of DNA double strand break repair (DSB) factors have been suggested to play a fundamental role during the development of both familial and sporadic forms of mammary carcinoma. Thus, the involvement of the breast cancer susceptibility genes, BRCA1, BRCA2, and p53, in the homologous recombination pathway for DSB repair suggests that pathogenesis of breast cancer may be driven by DSB-initiated chromosome instability and the mechanisms involved in DSB repair are of vital etiological importance during breast tumorigenesis.

The aim of the present study was to determine whether recombinative DNA rearrangements could serve as potential indicators for breast cancer predisposition. Five breast cancer cell lines have been established from familial and sporadic Indian breast tumors. These cell lines were used to study the role of defects in distinct recombination repair pathways in this malignancy. Alterations in BRCA1, BRCA2, and p53 expression levels were determined in three breast cancer cell lines and correlated with genomic instability as measured by the fluorescence based *in vitro* assay for the quantitative and qualitative determination of recombination activities.

Variations in recombination frequencies were observed in these established sporadic and familial breast cancer cell lines. The role of conservative homologous recombination (cHR) and non-homologous end joining in sporadic and hereditary breast cancers will be discussed. The results provide evidence for an association between specific functional defects and loss of BRCA1 protein signal leading to breast cancer particularly in subjects at high risk for developing breast cancer. Therefore, determination of recombination activities may be useful in identifying genetic alterations in *BRCA1* and carries a high diagnostic potential for breast cancer risk assessment

P11

Does inhibition of PARP change the frequency of homologous recombination?

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The aim of this project is to examine the role of PARP-1 (poly(ADP-ribose) polymerase-1) in repair of DNA double strand breaks. In mammalian cells DSB are repaired by nonhomologous end-joining (NHEJ) and by homologous recombination (HR). CHO-K1 wild type and xrs-6 mutant cell line were transfected with pLrec plasmid carrying two nonfunctional copies of the beta-galactosidase (lacZ) gene in a tandem array. In result of recombination they give rise to a functional copy of beta-galactosidase. Isolated transfected clones were used to examine the effect of ADP-ribosylation inhibition on the frequencies of spontaneous and X-ray (2 Gy) induced recombination. The cells were incubated with the PARP-1 inhibitor (AB) and recombination frequency was determined with histochemical or flow cytometry methods. Cells were cultured in the medium containing G418 or left to grow without G418 (5-6 days before and 2 days after treatment). This treatment allows to distinguish between reciprocal gene exchange/HR (loss of neo gene) and gene conversion (neo gene is still present and cells are resistant to G418). The level of beta-galactosidase activity (reflecting the frequency of spontaneous recombination) in transfected CHO-K1 cells was 2-3 times lower than in xrs-6 cells, whereas frequency of recombination per generation measured by flow cytometry was one order of magnitude lower. However, significant differences between individual clones have been observed. Irradiation of the cells with 2 Gy of X-rays insignificantly elevated the level of the enzyme in both cell lines. As expected, in non-irradiated cells, release of the selective pressure (7 days without G418) significantly elevated the level of beta-galactosidase activity as compared with that in cells cultured with G418. These results suggest that the defect in NHEJ-mediated DSB repair pathway results in elevated frequency of HR. No effect of inhibition of poly(ADP)rybosylation was observed in this experimental model.

P12

Function of Rad5p and Rad18p in DNA Double Strand Break Repair

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A DNA Double strand break (DSB) is a general phenomenon which may happen endogenously or exogenously. There are several pathways to repair DSBs, such as homologous recombination (HR), single-strand annealing (SSA), and non-homologous end joining (NHEJ). NHEJ can be further divided into error-prone and error-free pathways. Regions of ssDNA are involved in the repair of DSB, where 3'-ssDNA tails are generated for initiation of recombination at the site of the break.

Rad5p and Rad18p are two E3s involved in the ubiquitination of PCNA in the context of postreplication DNA repair. Both proteins were shown to preferentially bind to ssDNA in vitro. Rad5p also has potential ATPase function. A function of Rad5p in DNA DSB repair has reported. Little is known about Rad18p on DSB repair.

By means of chromatin immunoprecipitation assay (ChIP) and immunofluorescence we have observed that Rad5p and Rad18p bind to ssDNA tails of an HO-induced DSB in different patterns. Furthermore, Rad5p inhibits Rad18p's ssDNA binding through its ATPase activity. In consistent with their binding of DSB termini, Rad5p and Rad18p play important roles in DSB repair and in particular influence the repair accuracy in plasmid repair assays.

P13

Homologous recombination in human B cells

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The diversification of the antibody repertoire in germinal center B cells involves two genetic processes that abundantly introduce targeted DNA double strand breaks (DSB): class switch recombination and somatic hypermutation. While DSBs during class switch recombination have been shown to be repaired by non-homologous end joining, the mechanism of DSB repair during hypermutation has been speculated to involve homologous recombination. B cells need to properly regulate these repair activities, as aberrant repair of these DSBs has been shown to lead to lymphoma development.

To study the regulation of DNA double strand break repair in human B cells, we have analysed the expression of DNA repair factors. Normal as well as transformed human B cells show interesting differences in the expression of DSB repair factors at both the transcript and protein levels, in particular for factors involved in homologous recombination. A functional assay for homologous recombination activity in these cell lines, based on a tandem GFP recombination reporter cassette, detects hyperrecombination in germinal center like Burkitt's lymphoma cell lines. Surprisingly, most recombination products in these cells are due to non-conservative recombination, which may contribute to genetic instability. We are now analysing the molecular basis of hyperrecombination in human B cells, the causes for preferential non-conservative recombination, and the relationship between hyperrecombination and somatic hypermutation. We expect insight into the normal regulation of DSB repair in B cells, as well as into pathways of its deregulation that may lead to cancer development.

P14

BUB1b haploinsufficiency leads to an impaired spindle checkpoint in vitro and to an accelerated lymphomagenesis in Bub1b+/-/ Mlh1-/- double transgenic mice

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Chromosomal instability is a hallmark of malignant transformation. Recent work indicates that the complete loss of the highly conserved mitotic spindle assembly checkpoint leads to chromosomal instability and/or cell death. In addition it was shown that defects in the spindle checkpoint system occur frequently in human tumor cell lines. On the other hand, mutations in spindle checkpoint genes can be detected rarely in cancer cells. From these observations it was suggested that alterations in the epigenetic control of spindle checkpoint components might play a role in chromosomal instability and tumorigenesis. To prove this hypothesis we analyzed the effect of haploinsufficiency in the important spindle checkpoint gene *Bub1b* for spindle checkpoint activity. Therefore we isolated mouse embryonic fibroblasts (MEFs) from *Bub1b* heterozygous mice, which we previously had generated by gene targeting. We could show that incubation of *Bub1b*+/- MEFs with the spindle depolymerising agent nocodazole leads to an increased number of cells, characterized by premature sister chromatid separation and aneuploidy. These results indicate that the spindle checkpoint is impaired in *Bub1b*+/- MEFs. To further analyze the effects of *BUB1b* haploinsufficiency in vivo, *Bub1b* heterozygous mice were monitored for tumor events. As already observed in other spindle checkpoint defective mouse models, *Bub1b*+/- mice also develop lung adenomas with an increased frequency at very late latencies. To additionally characterize the impact of *Bub1b* heterozygosity for tumor development we generated a *Bub1b*+/-/ *Mlh1*-/- double transgenic mouse line. In this mouse model we could show that *BUB1b* haploinsufficiency leads to accelerated lymphomagenesis compared to the lymphomagenesis observed in *MLH1* deficient mice. In future we are interested to study the mechanism of *BUB1b* mediated tumorigenesis using tumors derived from *Bub1b*+/-/*Mlh1*-/- mice. In summary, our results demonstrate that haploinsufficiency for *BUB1b* leads to spindle checkpoint impairment and chromosomal instability in vitro and to an accelerated tumor development in vivo.

P15

The rate of extrachromosomal homologous recombination within a novel reporter plasmid is elevated in cells lacking functional ATM protein

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Homologous recombination between identical stretches of DNA depends on the coordinated action of many tightly regulated proteins. Cellular defects in homologous recombination are strongly associated with increased genomic instability and tumorigenesis. In cells of the cancer-prone syndrome ataxia telangiectasia (A-T), increased intrachromosomal recombination has been demonstrated, while extrachromosomal recombination has been discussed controversially. We constructed a novel, episomally replicating pGrec recombination vector containing two mutated alleles of the enhanced green fluorescent protein (eGFP) gene. Homologous recombination can reconstitute functional wildtype eGFP, thus allowing detection of recombination events based on cellular eGFP fluorescence. Using an isogenic cell pair of A-T fibroblasts and derivatives complemented by an ATM expression vector, we were able to demonstrate in A-T cells high extrachromosomal recombination rates, which are suppressed upon ectopic ATM expression. We thus found that ATM deficiency increases spontaneous recombination not only in intrachromosomal but also in extrachromosomal substrates, suggesting that lack of ATM increases homologous recombination independent of the chromatin structure.

P16

New cellular roles of Nbs1: Functional analysis in patient cell lines and after knockdown by RNAi in epithelial cells

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The rare genetic disorder Nijmegen Breakage Syndrom is characterized by various cellular phenotypes including radiation sensitivity, cell cycle checkpoint deficiencies, DNA repair defects and genomic instability. The NBS1 cDNA encodes a ubiquitously expressed protein of 754 aa. The N-terminus includes a breast cancer carboxy-terminal domain (BRCT) and a fork-head-associated domain (FHA), while the Mre11 binding domain is located in the C-terminal part. Nbs1 is a member of the DNA double strand break (DSB) repair complex Nbs1/Mre11/Rad50 and binds directly to γ -H2AX. However, the efficiency of DSB rejoining is not affected in NBS^{-/-} cells.

To further elucidate the obviously complex functions of Nbs1, we analyze cellular functions of NBS^{-/-} patient cell lines and their NBS^{+/-} controls. Only the heterozygous cell lines express the full length Nbs1 protein with a molecular mass of 95 kDa. A 70 kDa fragment, produced by internal translation initiation, can be detected in the nucleus and cytoplasm of both cell lines and retains association to Mre11 and Rad50. We could show increased induction of apoptosis after γ -irradiation in NBS^{-/-} cells, suggesting a role for Nbs1 in mediating or initiation of apoptosis. Analysis of gene expression on the transcriptional and translational level showed differential amounts of some important proteins involved in apoptosis and ubiquitin metabolism between the homozygous and heterozygous cells. Additionally new interaction partners of Nbs1 could be identified by immunoprecipitation.

To exclude partial activity of truncated Nbs1 variants and to analyze Nbs1 functions in a well characterized genomically stable cellular background we knockdown NBS1 by RNAi.

We have identified suitable RNA sequences for a Nbs1 knockdown of 80% by vector-based siRNA expression for 96h. Now work is in progress to establish a stable transfected NBS1 knockdown for long term studies. Additionally we investigate the applicability of chemically synthesized siRNA in our short term studies.

P17

Rad50 deficiency causes a variant form of Nijmegen Breakage Syndrome

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The MRN (Mre11/Rad50/p95(nibrin)) complex assumes a central function in DNA double-strand break repair. Mutations of Mre11 and p95/nibrin are associated with the radiation sensitivity syndromes ataxia-telangiectasia-like disorder (ATLD) and Nijmegen breakage syndrome (NBS), respectively. Here we report that Rad50 deficiency also occurs as an inherited condition in man due to hypomorphic RAD50 germline mutations. This was found in one 18-year-old German who has a variant form of the NBS without immunodeficiency. She is a compound heterozygote for a nonsense and a stop codon mutation in the RAD50 gene. Rad50 protein expression is reduced to less than one tenth in her fibroblasts and lymphoblastoid cells. The Rad50 deficiency is associated with a high frequency of spontaneous chromatid exchanges and with the failure to form MRN nuclear foci in response to irradiation. ATM autophosphorylation, phosphorylation of p53 at serine 15 and the transcriptional induction of p21/WAF1 mRNA are decreased in Rad50 deficient cells, and Ser343 phosphorylation of p95/nibrin is undetectable following irradiation. These defects could be complemented by transient transfection of wildtype Rad50 cDNA into the Rad50 deficient lymphoblastoid cells. Our findings expand the spectrum of DNA double strand break repair disorders and provide evidence that Rad50 modulates some functions of the ATM kinase.

P18

Differences in the Association of p53 Phosphorylated on Serine 15 and Key Enzymes of Homologous Recombination

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Phosphorylation of p53 on serine 15 by ATM or ATR is a frequent modification and initiates a cascade of post-translational modifications. To identify possible mechanisms that modulate p53 functions in recombination surveillance, we compared the nuclear localization of p53 phosphorylated on serine 15 (p53pSer15) and the key enzymes of homologous recombination (HR) after replication fork stalling. We demonstrate an almost mutually exclusive subcompartmentalization with Rad52, while p53pSer15 was colocalizing with 40-60% of the Rad51 and Mre11 foci. Therefore, possible sites of p53pSer15-dependent regulation seem to be sites of Rad51- rather than Rad52-dependent HR processes. Remarkably, the association of p53pSer15 with repair complexes containing Rad51 or Mre11 was transient, because less than 20% of the Rad51 and Mre11 foci overlapped with p53pSer15 after 6 h. When we examined colocalization of p53pSer15 and the RecQ helicase BLM with recombination surveillance and pro-apoptotic functions, we observed colocalization within approximately 70% of the BLM foci both immediately and 6 h after replication arrest. Our data suggest that p53pSer15 plays a dual role in the functional interactions with early complexes of Rad51-dependent recombination and with BLM-associated surveillance and signalling complexes within distinct nuclear subcompartments.

P19

Radiation-induced EGFR nuclear translocation is linked with DNA repair

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Ionizing radiation triggers EGFR translocation from membrane into nucleus. During this process, the proteins Ku70/80 and the phosphatase PP1 are also translocated to the nucleus. As a consequence, an increase in the nuclear kinase activity of DNA-PK and increased formation of the DNA end binding protein complexes containing DNA-PK – essential for repair of DNA-strand breaks - occurred. Blockade of EGFR translocation using the anti-EGFR monoclonal antibody C225 abolished radiation-induced activation of DNA-PK. In contrast inhibition of EGFR kinase activity by the specific inhibitor AG1517 failed to block radiation-induced activation of DNA-PK. Our data implicate a novel function of the EGFR during DNA-repair processes.

P20

Role of XRCC2 in the response of cells to alkylating agents

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Xrcc2 is a member of the Rad51 paralog family involved in homologous recombination repair. Mice with disrupted Xrcc2 gene are not viable. Xrcc2^{-/-} embryonic fibroblasts show increased sensitivity to X-rays and DNA cross-linking agents, such as mitomycin C. They also show elevated level of spontaneous and X-ray induced chromosomal aberrations. Recently we showed that proteins involved in DSB repair such as ATM can also play a role in the response of cells to simple alkylating agents which is presumably due to impaired repair of secondary derived DSB. Here we report that XRCC2 is also involved in such response. We show that Xrcc2^{-/-} embryonic fibroblasts are hypersensitive to the methylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) as shown by metabolic WST-1 assay. Sensitivity of Xrcc2 knockout cells to MNNG is due to increased apoptosis as measured by subG1 quantification and annexin V staining. The frequency of necrosis was only marginally enhanced. Transfection of Xrcc2^{-/-} cells with Xrcc2 cDNA restores resistance to MNNG to almost wild-type level. Xrcc2 deficient fibroblasts display a lower level of MNNG induced sister chromatid exchanges and a higher level of chromosomal aberrations compared to Xrcc2 complemented cell line. Screening with laboratory and clinically used chemical agents reveals that Xrcc2 knockout cells are cross-sensitive to alkylating agents, such as methyl metanesulfonate (MMS) and temozolomide, and to various bifunctional alkylating drugs, such as fotemustine and mafosfamide which produce crosslinks in DNA. Altogether the data suggest that XRCC2 plays a role in the response of cells to alkylating agents most likely by its involvement in the repair of secondary DNA lesions that were produced by DNA replication-dependent processing of O6-methylguanine.

P21

Hypersensitivity of DNA double-strand break repair deficient cells to alkylating agents

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Ataxia telangiectasia mutated protein (ATM) is a serine/threonine nuclear kinase, which is of particular interest in radiation biology since ATM mutated cells exhibit different defects in response to γ -irradiation. Upon irradiation, ATM binds to DNA double-strand breaks (DSB's) and initiates signalling for DSB repair and cell cycle arrest. Here we provide evidence that ATM plays a role in case of DNA damage caused by chemical agents such as MNNG and MMS. We show that mouse fibroblasts deficient for ATM are more sensitive than corresponding wild-type cells to methylating DNA damaging agents, which was shown in clonogenic survival and apoptosis experiments. Inactivation of the DNA repair enzyme MGMT by a specific inhibitor induced increased apoptosis after low dose treatment with MNNG and MMS and decreased survival, whereas transfection with a MGMT expression plasmid provoked an opposite effect. From this we conclude that O6-methylguanine (O6MeG) is a critical DNA lesion being responsible for death of ATM knockout cells in case of MNNG and partially in case of MMS. Apoptosis was a late effect starting 48 h after treatment with both agents, which was shown by subG1 quantification and caspase activation. Finally we show that resistance of wild type cells is due to ATM kinase activity as treatment with caffeine (ATM inhibitor) increase the level of apoptosis of ATM proficient but not mutated cells. In summary, we conclude that ATM is not only involved in the cellular response to ionising radiation but also in the processing of alkylating agent-induced DNA damage.

Debiak M., Nicolova T., Kaina B. 2004: Loss of ATM sensitizes against O6-methylguanine triggered apoptosis, SCEs and chromosomal aberrations. *DNA Repair* 1;3(4):359-68.

P22

Pleiotropic effects of statins on ionizing radiation-inducible cellular stress responses and apoptosis

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HMG-CoA-reductase inhibitors (i.e. statins) are known to interfere with a variety of cellular stress responses provoked by either inflammatory cytokines or by DNA damaging agents. This effect of statins is believed to be due to attenuation of the activity of small GTPases (i.e. Ras and Rho). In the present study, we investigated the effect of lovastatin on ionizing radiation (IR)-induced activation of gene expression and apoptosis in primary human endothelial cells (HUVEC). We present evidence that lovastatin is able to attenuate IR-triggered activation of NF- κ B as well as p53 induction. Since NF- κ B is essential for the expression of a variety of cell adhesion molecules, we analyzed the effect of lovastatin on the expression of E-selectin, which is hypothesized to be related to tumor progression. We found that therapeutically relevant concentration of lovastatin abrogates E-selectin expression on the level of the protein, mRNA and promoter. Furthermore, low dose pretreatment with lovastatin largely protected HUVEC from IR-induced cytotoxicity as measured by a cell viability assay. Interestingly, viability of human fibroblasts was only marginally enhanced by lovastatin and resistance of HeLa cells was even reduced by statin treatment. Obviously, the effect of lovastatin on cellular susceptibility to radiation damage is cell type specific. Protection of HUVEC by low concentration of lovastatin is due to reduction of radiation-induced apoptosis. At higher concentration, lovastatin promoted radiation-induced apoptotic death. The molecular mechanisms involved in the protective effect of lovastatin against radiation-induced apoptosis will be discussed. In summary, our data shows that statins exert pleiotropic effects on radiation-stimulated stress responses in human endothelial cells, affecting both cell adhesion and apoptosis. Thus, statins might be suitable drugs to modulate the therapeutic efficiency of radiation-based tumor therapy.

P23

The topoisomerase I damage response

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The human topoisomerase I (htopoI) is known to be important for the relaxation of torsional stress that is generated throughout several processes of DNA metabolism. In the course of this reaction, a transient covalent enzyme-DNA complex is formed. Previous studies have shown that long-lived covalent htopoI-DNA complexes accumulate in response to various kinds of DNA damage *in vivo*.

This complex formation can be investigated using the ICT-assay (in vivo complex of topoisomerase I). After the separation of free protein from DNA on a CsCl gradient the presence of covalent htopoI complexes on DNA can be analyzed.

Exposure to genotoxic stress provoked an elevated and highly stable htopoI-DNA complex level that was detectable primarily in cells destined to apoptosis. The finding that these complexes were associated with apoptotically degraded DNA further supported the idea that stable htopoI-DNA complex formation may be part of an apoptotic pathway. Cells that were not exposed to any external genotoxic stress, but underwent apoptosis under normal cell culture conditions, also showed an elevated htopoI-DNA complex level. On the other hand, non-apoptotic cells after treatment with genotoxins contained only few covalent complexes that could be repaired. The pathway of this newly discovered damage response is yet unknown, but our data imply an involvement of the tumor suppressor protein p53. Preliminary results further indicate that htopoI-DNA complex formation may not be absolutely dependent on DNA damage, but rather be part of a more general stress response.

P24

Physical and functional association of the DNA topoisomerase II binding protein 1 (TopBP1) with Poly (ADP-ribose) polymerase (PARP-1)

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TopBP1, a protein containing eight BRCT motifs, appears to be involved in DNA replication and in DNA damage checkpoints. A region within the 6th BRCT domain of TopBP1 is similar to auto-(ADP-ribosyl)ation sites of PARP. We purified this 6th BRCT domain of TopBP1 and a bigger C-terminal fragment of TopBP1 including the 6th, 7th and 8th BRCT domains as GST-fusion proteins and performed GST-pull down experiments in two ways. First, a N-terminal DNA-fragment of PARP including the DNA-binding and the automodification domains was used as a template in an in vitro transcription/translation experiment. The ³⁵S-labelled protein fragment specifically interacted with the GST-TopBP1-fragments but not with GST alone as a control. In a second experiment, the 6th BRCT domain-GST-fusion protein bound to glutathione-sepharose was incubated with lysates from HeLa-cells, the associated proteins were pelleted by centrifugation, washed and analyzed for PARP by Western blotting. Only with the 6th BRCT domain but not with a neighbouring fragment as control PARP could be detected in the Western blots. More significantly, the in vivo interaction of endogenous TopBP1 and PARP-1 proteins could be shown in synchronised HeLa S3 cells by coimmunoprecipitation. Furthermore, the auto-(ADP-ribosyl)ation of PARP is negatively regulated by association with TopBP1. The results show that TopBP1 may be involved in the regulation of PARP-1 activity during DNA repair and replication.

P25

Impact of p53 status on sensitivity / resistance of non-transformed mouse embryonic fibroblasts (MEFs) to the anticancer drug topotecan

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The clinically used anticancer drug topotecan (TPT) blocks the religation reaction of topoisomerase I (Topo I), which leads to stabilization and accumulation of Topo I-DNA covalent complexes. Consequently, lethality induced by Topo I inhibitors occurs only upon their irreversible conversion to DNA strand breaks (Topo I poisoning). To dissect pathways leading to cellular sensitivity / resistance to TPT, and to attribute a possible role of p53, non-transformed mouse embryonic fibroblasts (MEFs) proficient or deficient for p53 were used. In addition, we made use of MEFs proficient for p53 but deficient for the Apaf-1 protein, a key player of the mitochondrial apoptotic pathway. In comparison to wild-type and apaf-1 null MEFs, viability of TPT-treated p53 deficient MEFs was extremely reduced (WST-1 metabolic assay), and apoptosis was significantly induced (annexin V flow cytometry). Most of the caspase-9/-3 activity induced by TPT was found in p53^{-/-} cells, less in wild-type, and none in apaf-1 null cells. Despite significant Fas receptor activation (western-blot) and enhanced transcription of Fas ligand mRNA in wild-type and apaf-1^{-/-} cells (not in p53^{-/-}), caspase-8 was not activated, neither in wild-type nor in p53^{-/-} cells. Cleavage of PARP in p53 deficient MEFs occurred earlier than in wild-type cells, and no PARP cleavage was found in apaf-1 null cells. Exposure to TPT led in all MEFs to activation of Chk1, Cdc25A degradation, S- and G2/M arrest. In comparison to wild-type and apaf-1 knock-out cells, p53 null fibroblasts underwent significant apoptosis directly from the S- or G2/M block. A hallmark of cellular sensitivity / resistance was degradation of Topo I in the cleavable complex, observed only in p53 proficient (wild-type and apaf-1^{-/-}) but not in p53 deficient MEFs. TPT-induced down-regulation of Topo I could be abrogated by co-incubation of cells with a 26S proteasome inhibitor MG132. This indicates that hypersensitivity of p53^{-/-} cells to TPT could result, at least partially, from a defect in Topo I degradation in these cells.

P26

Similar UV-A induced apoptosis in p53 proficient and deficient human keratinocytes

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The tumor suppressor p53 plays an important role as transcription factor in DNA-repair, apoptosis and cell-cycle. UV-A irradiation induces DNA damage, which in turn activates p53 as regulator for apoptosis and DNA repair.

In this study we analyzed the time course of apoptosis in p53 proficient and deficient HaCaT keratinocytes.

After UV-A irradiation p53 proficient and deficient keratinocytes show the same time course of apoptosis with two distinct peaks at approx. 12h and 48h post UV-A exposition. To analyse these apoptosis peaks we monitored the pro and anti-apoptotic proteins bax and bcl by ELISA and correlated them to distinct cell cycle positions as determined by flow cytometry. We found an S-phase arrest in both cell types from 24h to 48h post exposure, which is accompanied by an increase in annexin V positive cells as well as an increased bax level in both cultures. In contrast anti-apoptotic bcl-2 is different in p53 $-/-$ and p53 $+/+$ cells. While p53 negative HaCaT cells showed only a weak peak after 8 h but not after 72h post UV-A, the p53 proficient HaCaT cells showed a strong increase in bcl-2 after 72 h post UV-A exposition. These results show that there is a significant dependency between p53 and bcl-2 expression, but not on the occurrence of apoptosis.

P27

Damage of DNA is not sufficient to (re)activate p53 protein HeLa cervical carcinoma cells

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It is widely known that exposure of cells to a variety of DNA damaging agents leads to upregulation and activation of wild-type p53 protein. We investigated the (re)-activation of p53 protein in human cervix carcinoma HeLa cells and in human breast cancer MCF-7 cells by different genotoxic and cytotoxic stimuli. Treatment of human cells with an alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or exposure to ionising radiation (IR) resulted in a strong DNA damage as evidenced by Comet assay and subsequently induction of p53 response. However, in HeLa cells the expression of p53 protein was not elevated after MNNG treatment despite strong DNA damage.

In HeLa cells p53 protein is targeted E6 oncoprotein, which is encoded by high risk HPV for accelerated ubiquitination and degradation. Therefore, the activation of p53 protein in HeLa cells seems to depend on its decreased targeting by E6 oncoprotein. We determined the expression of E6 protein in HeLa cells exposed to a variety of genotoxic agents. We observed that the upregulation of p53 protein coincides with the repression of E6 protein. It implicates that the capability of different agents to activate p53 in HeLa cells depends strongly on their inhibitory effect on E6 oncoprotein.

P28

RNA interference: a method to investigate the influence of carcinogenic metal compounds on DNA repair processes and cell cycle control in cultured human cells

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RNA interference (RNAi) is a method for inactivation of genes first described by Fire *et al.* in 1998. In mammalian cells, RNAi acts by introducing short double-stranded RNA fragments (siRNA) into the cells (McManus and Sharp 2002). The siRNA bind to RISC, a protein complex, and they are unwound to single strands which then guide the RISC-complex to the complementary mRNA. The mRNA is cleaved by RISC, degraded by cellular exonucleases and can no longer be used for protein synthesis. By the use of plasmid vectors coding for siRNA it is even possible to create cell lines with stable inactivated genes. In our experiments we transfected three different human cell lines (HeLa S3, MCF7 and A549) with a plasmid construct directed against the tumor suppressor gene p53 and succeeded in isolating stable transfected colonies of each cell line carrying a resistance marker gene. The isolated colonies were examined by western blot for p53 protein. On RNA level, we used real-time RT-PCR to quantitate the knock down efficiency. From every cell line there were colonies with a reduction of p53 mRNA from 72% up to 79% compared with non transfected cells. Further experiments in combination with carcinogenic metal compounds are planned to investigate their influence on DNA repair processes and cell cycle control.

Fire *et al.* (1998): Nature 391(6669), 806-11.

McManus and Sharp (2002): Nat Rev Genet 3(10), 737-47.

P29

Virtual radiation biophysics: implication of nuclear structure

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The spatial organization of the genome in the interphase nucleus has far reaching consequences for radiation biophysics. For example, the existence of chromosome territories and their spatial distribution in the human cell nucleus is expected to pose serious constraints on the induction of specific chromosome aberrations by ionizing radiation. Experimentally, using Fluorescence in situ hybridization (FISH) chromosome painting, confocal laser scanning microscopy, and digital image analysis, a non-random, non-statistical positioning of chromosome territories (CTs) in human lymphocyte cell nuclei has been observed for certain CTs. This distribution has been maintained throughout primate evolution, suggesting a functional significance. Such a clearly non random organization of nuclear genome SmacroT structure has raised the question whether systematic chromosome-chromosome associations exist which have significant influence on interchange rates. In such a case the spatial proximity of certain CTs or even of clusters of CTs have to increase the respective exchange yields significantly in comparison to a random association of CTs. Computer simulations of nuclear genome structure based on experimentally known general features allow to predict chromosome aberration yields induced by ionizing radiation in interphase nuclei on the single cell level. As an example, in this report computer simulated arrangements of CTs in human cell nuclei models (S1-Mbp Spherical Chromatin Domain ModelT) were assumed to calculate interchange frequencies between all heterologous CT pairs. For the positioning of CTs in the virtual nuclear volume, both a statistical and a gene density correlated arrangement was assumed. The predicted exchange rates may be directly compared with the results of observations on the single cell level.

P30

Abstract: The modelling of the radiation induced chromosomal exchange aberrations

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One of the most striking and direct consequences of the DNA misrepair is chromosomal aberration. In an effort to assess the influences of higher order chromosome geometry of interphase nucleus on inter and intra chromosomal misrepair based exchanges, we mechanistically quantify the radiation induced aberrations by implementing the established SCD model (Spherical 1 Mbp Chromatin Domain; G. Kreth *et al*, 2002), which is explicitly based on non-random positioning of chromosome territories (C. Cremer *et al*, 2001). The model based on simulated human lymphocyte cell nuclei comprising the interphase chromosome territories according to the gene density was established for the measurement of the aberrations such as translocations, dicentrics and centrics. Apparently, an emerging conclusion is that the higher order spatial genome distribution pattern is a potential contributing factor to the acquisition of chromosomal aberrations. By using a theoretical model, radiation induced aberrations were measured for calculated low LET doses ranging from 0 to 5 Gy and compared to the experimental aberration spectra. We observed that the dose response measurements found to be in better accordance with the experimental data. As seen in previous studies, the dependency of one chromosome yield and DNA content found to adopt the pattern:

yield \sim (DNA content)^{2/3}.

P31

Wortmannin treatment reveals a repair deficiency in the radioresistant tumor cell line WiDr

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Purpose: To investigate the role of DNA DSB repair in the process of radiation-induced chromosome aberration formation with special emphasis on the impact of NHEJ and the PI3-kinases DNA-PK and ATM.

Methodology: Two tumor cell lines (WiDr, M059K), which are radioresistant with respect to colony formation and radiation-induced chromosome aberrations received a combined treatment consisting of 20 mM wortmannin and irradiation with 200 kV X-rays. Cells were irradiated in G0/G1 and chromosome-type and chromatid-type aberrations were scored in first-division metaphase cells stained by either Giemsa or FISH. DNA-PK activity was measured in the absence and in the presence of wortmannin.

Results: Both cell lines showed DNA-PK activity, which was completely inhibited by 20 mM wortmannin. ATM can also be affected by this wortmannin concentration. As expected, irradiation in G0/G1 led to the sole formation of chromosome-type aberrations, including simple and complex exchanges and excess acentrics in both cell lines. In M059K cells, wortmannin caused a marked increase in the yield of simple stable and unstable and of complex aberrations. In WiDr cells, after addition of 20 mM wortmannin, the formation of chromosome-type exchange aberrations was completely suppressed. The G0/G1-irradiated cells displayed exclusively chromatid-type aberrations including simple and complex chromatid exchanges and chromatid/isochromatid breaks.

Conclusion: Treatment with repair inhibitors is a useful tool to detect cell lines with hidden repair defects, which may be an appropriate model for further studies. While M059K cells can recruit a DNA-PK independent NHEJ, WiDr cells resort to recombination repair processes to cope with the DNA damage.

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Comparing the radioprotective effect of vitamins A, E and Cotoneaster nummularia on mouse bone marrow cells against Gamma irradiation

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Study of the different aspects of protection against spontaneous exposure to ionizing radiation, always, has been an active area of research. High cost and toxicity of radioprotective drugs has limited their use. So, search for new drugs with high protection value and lower cost and toxicity seems a necessity.

In this study radioprotective effect of vitamins A, E, and Cotoneaster nummularia, regarding their high accessibility and low side effects on human as well as animals, against Gamma irradiation, was analyzed using micronucleus assay on bone marrow cells of male mouse (Balb/c).

Vitamins E, A, water soluble extract as well as alcoholic extract of Cotoneaster nummularia have been administered (IP) with doses of 50, 100, 200 and 0.6, 1.2, 2.4 and 250, 500, 1000 and 3750, 7500, 15000 mg/kgBW respectively for five days. One hour after last administration, mice were exposed to 2 gray of Gamma radiation. Micronucleus assay performed on bone marrow cells 24 hours post-exposure. One thousands nucleated cells were scored per slide and the frequency of micronucleus calculated.

High frequency of micronucleus was observed in non treated gamma-exposed mice, which represented the clastogenic effect of irradiation. Vitamin E, A, and Cotoneaster nummularia treated mice represented the lower frequency of micronucleus ($P < 0.01$).

The results showed a 5.56, 3.93, 3.32 and 2.1 times decrease in the gamma induced micronucleus frequency in vitamin E, A, water soluble and alcoholic extract of Cotoneaster nummularia respectively.

Key words: Gamma irradiation, vitamin A, vitamin E, Cotoneaster nummularia, micronucleus