Contract FIGH-CT-1999-0012

Title: INDUCTION, REPAIR AND BIOLOGICAL CONSEQUENCES OF DNA DAMAGES CAUSED BY RADIATIONS OF VARIOUS QUALITIES ("RADNA")

Introduction

The RADNA project focuses on the role of radiation-induced DNA damage, particularly double-strand breaks (DSB), in the production of chromosome aberrations and mutations as critical events in the induction of cancer. Underlying these studies are investigations into the role of DNA repair processes, particularly those involving DSB, in subsequent biological effects. The project utilizes conventional irradiation methods, and innovative microbeam technologies coupled with single-cell assays of response. The objective is to map out responses at the level of individual cells. The overall aim of the project is to provide data that will contribute to mechanistic modelling of radiation risk, particularly in respect of radiation quality factors at low doses.

The project is based on the concept that radiation-induced cancer is initiated by discrete damage to the DNA of an individual cell, leading to genetic changes which transform it to the malignant state. At the dose levels that are relevant to radiation protection, the process starts at the level of a single track of radiation passing sufficiently close to the DNA to cause dsb and other lesions, most of which are repaired by the cell. A small fraction of lesions are not repaired correctly and some of these lead to gross changes in the organisation of the genome (chromosomal aberrations) and mutations. A fraction of these damages can lead to the genetic changes that initiate the carcinogenic process.

The work programme is divided into work packages that reflect these stages of effect. WP 1 provides novel techniques developed to enable the processes that result from a single radiation track passing through a cell to be studied precisely. The technology that it delivers to the experimental WPs, 2 to 5, enables them to follow the respective stages of radiation effect outlined above in relation to the early stages of the carcinogenic process. These use both conventional and microbeam approaches. The structure is outlined below:

- WP 1: Microbeam techniques to measure responses of individual cells to single tracks of radiation.
- WP 2: Induction of DNA damage.
- WP 3: Cellular processing of DNA damage.
- WP 4: Formation of chromosomal aberrations.
- WP 5: Mutation induction.

In general, the flow of information follows the sequence of the work packages. WP 2 provides data for WP 3 on the spectrum of damages induced in DNA as a function of radiation quality. WP 3 provides information about how cells process damage which relates to studies of the mechanisms of chromosomal aberration formation (WP 4) as also to mutation induction (WP 5). WP 5 also requires inputs from WPs 2 and 3 in relation to interpretation of the molecular spectra of radiation-induced mutations.

The project brought together 11 European Laboratories from 7 different countries with unique expertise to collaborate in this multidisciplinary consortium for 42 months (2000-2003).

Objectives

The main objective of the RADNA project was to make experimental measurements of the actions of low doses of radiation on cellular systems. It is based on the concept that the low dose risks of cancer induction and genetic injury are the result of damage that takes place at the cellular level. There was emphasis on the role of DNA damage and repair in the production of chromosomal aberrations and mutations. The aim was to improve the quantification of radiation risk at low doses. The experimental work employed radiation types that cover an important range of LETs in respect of radiation quality factors, or radiation weighting factors that are used in radiation protection. Studies using counted-particle irradiations were designed to provide new information about dose-effect relationships, down to the ultimate low-dose of a single track traversal per cell. These measurements related to the development of mechanistic models that will improve the low dose extrapolation of high-dose epidemiological risk data, which come predominantly from the follow-up of A-bomb survivors.

Results

Workpackage 1: Development of microbeam probes of radiation effectiveness

A major output was the increase of European competence in the area of the development of microbeam approaches for studying the lowest possible dose of a single particle traversal. During the programme technology transfer occurred between the partners leading to the completion and start of experiments with charged particle microbeams developed at PTB Braunschweig (GAG) and at INFN Legnaro (INFN-LNL). The existing charged particle microbeam at GLCRT was complemented with a new soft X-ray microprobe. This gives a significant capability at the European level with both the ranges of ions and X-ray energies which can be used for experiments. Initial studies with these systems have started to measure cell killing at low doses and develop models for studying bystander and adaptive responses. Significant new data has already been obtained showing bystander killing after only a single cell within a population has been targeted and that it is energy deposited within a targeted cell and not the number of cells exposed which governs the effectiveness of the bystander response. These studies are showing the importance of cellular signalling at individual cell level, and will allow us to identify source and receptor of the signals. Microbeams also allow subcellular precision (e.g., cytoplasmic versus nuclear targeting). However, with the larger range of energies of ions and X-rays coming on line with the new facilites developed under FP5, microbeams may also give us a window on how organised tissues respond to targeted irradiation. For this project however the key goal has been to understand effects of relevance to the low dose risk associated with single tracks and individual electrons. These approaches will have significant benefit for studies of DNA damage and repair (See WP2 and WP3).

Workpackage 2: DNA dsb induction and distributions

DNA lesions, especially DNA double-strand breaks (DSB), are considered to be closely related to health effects. Therefore, their yields, distribution in the genome and complexity were studied in relationship to the roles of LET and chromosome

ultrastructures. Understanding the yields and distributions of dsb after exposure to radiations of differing LETs has been a key goal of this project and the information obtained feed directly into the other workpackages. A key output has been the development of clear criteria for the analysis of conventional pulsed-field electrophoresis data. This has led to more quantifiable yields of dsb from different radiation qualitites. Specifically, mathematical tools were developed to calculate the yields of DSB per Gbp per Gy using experimental DNA fragment size distributions and extrapolation to the entire genome based on random breakage. DNA fragment size distributions were taken into account and corrected for background-dependent random breakage. DNA fragment size distributions as obtained in different types of mammalian cells after exposure to radiations of various qualitites were used to calculate the yields of DSB per Gbp per Gy applying mathematical tools mentioned above. An important development was the development of a new lysis and PFGE protocol to avoid the significant (30%) contribution of DSB originating from heat labile sites to the total DSB yield. The main outcome from these studies was that evidence was found for the organisation of DNA in chromatin fibre and higher-order structures being responsible for the majority of non-randomly distributed DSB by high-LET radiations. A method has been developed to quantify DNA fragments in the range 80 bp to several kbp using DNA ladders as internal standards.

As well as these pulsed field techniques, pilot studies using the combination of microbeam technologies and γH2AX foci formation showed its high potential to investigate important molecular and cellular mechanisms occurring in the low dose range. Individual tracks could be observed by detecting individual γH2AX foci for both microbeam particle and conventional γ-ray exposures.

To relate distributions of radon deposition in the lung with dose and dose-distributions at the cellular level, a model was developed to simulate distributions in central airway geometries. Based on a numerical method to compute three-dimensional physiologically realistic central airway bifurcation geometries it was found that in "deposition hot spots" the α-particle hit probabilities are about two orders of magnitude higher than the average values. Correlation was found between sites of "deposition hot spots" and sites of enhanced tumour incidences within bronchial branching sites. These observations have important implications for assessing the likely distributions of radiation tracks within individual cells at the tissue level.

Work Package 3: Rejoining and repair fidelity of DNA

The observation that the amount of initial damage to cellular DNA, such as double strand breaks (DSB's), does not generally correlate well with the cellular effects that are manifested later, indicates that cellular processing of DNA damage is the link between initial damage and stable or non-stable rearrangements of the genome.
Central to the key role of dsb after radiation exposure is a cells ability to repair these lesions. In WP3, patterns of dsb induction and distributions induced by radiations of differring qualities were considered after repair. Several approaches were used to monitor repair of dsb and to simulate this using both numerical and analytical models.
These specifically assessed the role of non-random distribution of fragments induced by different radiations and how these were rejoined. After high LET radiation exposure, the half-life of the fast rejoining of fragments was related to fragment size, with larger fragments having a longer half-life. Using a numerical model based on Monte Carlo analysis and a Generalized Broken Stick Model it was shown that repair

of dsb fragments could be simulated using a random removal of breaks independent of fragment size. These experimental measurements and simulations suggested that lesion complexity due to the localised clustering of damage may be a more critical determinant of rejoining kinetics than the spatial correlation of breaks over larger distances.

These studies did not consider misrejoining. For this an assay developed under FP4 has been used to quantify correct rejoining of DNA double-strand breaks and compare this with total (correct *and* incorrect) rejoining. The misrejoining frequency has a strong dose dependency after X-rays and α-particles with the misrejoining frequencies being significantly higher for high LET and dose-rate dependent. The NHEJ repair pathway is the predominant pathway for repairing DSB induced by low LET and the complex breaks induced by high LET. Significant differences in rejoining are also observed in different regions of the genome, with increased correct rejoining in centromeric regions which may be related to the condensation state of the DNA. However differentiation status did not influence correct rejoining kinetics. The role of different repair pathways for radiation-induced studies has been studied using both PFG approaches and foci formation of γH2AX. A new protocol for PFG was developed which reduced the measurement of heat labile sites and showed that these were related to the fast component of repair which is measured. With this protocol, no fast component was observed in cells deficient in NHEJ. Further studies on misrejoining showed that NHEJ and not HR is involved. Cell cycle studies showed that, as expected, the NHEJ pathway was important in G1, however, it also played a role in G2 along with HR. Further complimentary studies using γH2AX foci showed that HR was important for the repair of newly replicated chromatin.

Workpackage 4: Mechanisms of chromosomal aberration formation

Chromosome aberrations are an important consequence of radiation exposure and are highly relevant to radiation protection as these can be scored in individuals after exposure and used as a biological dosimeter. The cellular processing of DNA damage that is involved in the production of chromosomal aberrations has been studied using premature chromosome condensation (PCC) and fluorescence *in situ* hybridisation (FISH) techniques including COBRA MFISH. The fast and slow temporal components of the formation of chromosomal aberrations have been successfully quantified. Radiation-sensitive cell mutants and DNA repair inhibitors have been used to correlate defined DNA damage processing pathways with the formation of chromosomal aberrations. Effects of the cell cycle status upon the formation of chromosomal aberrations by different radiations have been studied using wild-type cells. Treatments that modify chromatin structure have also been considered. The formation kinetics and persistence of complex exchange aberrations have been quantified, visualising all human chromosomes using FISH. The proportion of complex aberrations increased with LET and could be used a signature for high-LET exposure. FISH has also been employed to estimate low- and high-LET induced complete and incomplete chromosome exchanges.

Important supporting evidence for a lesion-non-lesion model of chromosome aberration formation has been found, and involvement of rapid chromatin decondensation. For exchange aberration formation the α coefficient is LET dependent and has been explained on the basis of pairs of local energy deposition events or around 100eV leading to deletions in the 30 nm chromatin fibre. Using

carbon-K soft X-rays, evidence for a single hit mechanism for the induction of chromatid aberrations was observed. Studies with inhibitors of repair provided evidence for latent formation of telomeres due to amplification of interstitial telomeric repeat sequences possibly playing a role in genomic instability.

Workpackage 5: Mutation induction

Radiation induces mutations in DNA and these play an important role in the initiation of cancer. In WP5, analysis of mutations at the molecular level has been made using the polymerase chain reaction (PCR). Measurements have been made of the cross sections for the induction of mutations in the HPRT gene in Chinese hamster cells for a range of radiation qualities. Also the spectra of damage have been observed in terms of mutational type. Deletion patterns were determined in V79 Chinese and several human cell lines with a number of ions and photons of different energy. It was found that there is a clear tendency for increased proportions of deletions with increasing LET. It has to be noted, however, that even with very high LET there is still a sizeable fraction of mutants with no detectable exon deletions. Although differences in radiation response were noted in dependence of p53 status the determination of deletion patterns proved to be not possible because of very poor growth of the cell lines so that mutants at the HPRT-locus could not be recovered.

Mutation induction at the HPRT-locus was studied in the presence and absence of the repair inhibitor Wortmannin in human cells. Mutant frequencies were significantly higher under conditions of inhibited repair. The molecular analysis revealed a small increase of deletions with inhibited repair. The yield of translocation was determined in mutants of human cells exposed to photons of different energy. The fraction of mutants showing translocations of the q-arm of the X-chromosome (site of the HPRT-gene) was found to be comparatively small and did not depend significantly on LET. A comparison of the cross sections for mutation DSB and cell killing as functions of radiation quality showed differences consistent with the functional importance of cellular processing of DSB. Measurements were also made of preferential deletion within the HPRT gene. In particular the significance of non-contiguous deletions.

Both, adaptive response and bystander effect, were demonstrated in alpha-exposed cells but their interrelationship remains unclear at present. Genomic instability was demonstrated in the progeny of irradiated cells using the micronucleus assay. Mutation and transformation studies are in progress.

Conclusions

The results of the RADNA project provide new datasets and insights into the effects of radiation exposure in cellular systems. Significant mechanistic information in the radiation quality dependent processes leading from energy deposition through to DNA damage, repair, chromosome aberrations and mutations has been obtained. Also some new data on adaptive, bystander and instability phenotypes have been obtained to add to the continuing debate regarding non-targeted responses. Overall this information will input into biophysical models of radiation effectiveness which will ultimately help develop more accurate predictors of low dose risks for populations exposed to ionising radiations. Current risk estimates are based on epidemiological data from exposed populations which covers only a limited range of exposure conditions and is

highly limited in its applicability at low doses. Thus mechanistic studies of the biological effects of radiation in model systems have an important role to play as an important predictor of radiation response at low doses.

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