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Introduction

Rational judgements in radiation protection require a detailed knowledge of the basic mechanisms by which radiation induces cancer and genetic disorders. The identification of most of the genes involved in DNA damage responses to ionising radiation and the generation of knockout mice defective in these processes provide the basis for unravelling the mechanisms of the different DNA damage response pathways and their interactions. Based on these results, genes in three pathways for the repair of DNA double-strand breaks (the most critical lesion after ionising radiation, IR) have been identified, namely homologous recombination, DNA end-joining and replication-associated repair.

In this project we exploited new technologies (e.g. scanning force microscopy, DNA chips, proteomics, fluorescent redistribution after photo bleaching) and utilised a number of different organisms (human, mouse, *Drosophila, Xenopus, C. elegans*, budding and fission yeast) to further dissect the mechanistic pathways. To carry out the research plan, the work was divided into five different work packages. Six laboratories were involved to carry out the various aspects.

We investigated the precise functions of the proteins involved in each of these pathways and the interactions within and between pathways using genetic and biochemical approaches (WP1). The research in WP2 was focussed on cell-cycle regulation after ionising radiation. In addition to repair of DNA double-strand breaks, cells arrest the cell cycle in response to DNA damage, and the genes involved in these cell-cycle checkpoints have been successfully characterised. The biochemistry of the checkpoint proteins will be explored with the goal of reconstituting the checkpoint signal transduction pathway. Furthermore, it is evident that the response to DNA damage is not carried out simply by a collection of proteins. It takes place within the context of chromatin in highly ordered nuclear structures. In WP3 we investigated the heterogeneity of repair within chromatin, chromatin assembly and the involvement of nuclear foci as repair factories. In WP4 we assessed the radiation-induced mutations and other genetic changes that result from a lack of fidelity of damage responses.

Ultimately our goal is to understand the consequences of deficiencies in radiation damage responses in man. To this end, we analysed and identified the defects in damage response mechanisms in rare radiosensitive individuals. In addition, we exploited *Drosophila melanogaster* mutants and the knockout mouse models, which we have generated (in which one or more repair genes have been deleted) to assess the effects of ionising radiation in whole organisms.

Objectives

A detailed knowledge of the basic mechanisms by which radiation induces cancer and genetic disorders is essential for informed judgements to be made in radiation protection. The achievements of the objectives of our previous collaborative programme on the molecular basis of radiosensitivity has led to the identification of most of the genes involved in the DNA damage responses to ionising radiation, and to the generation of a battery of knockout mice defective in these processes. The aim of the current programme was to take this work forward to obtain a detailed understanding of the mechanisms of the different DNA damage response pathways and their interactions. DNA double-strand breaks (DSB) are the critical lesions induced by radiation. These lesions are the central focus of the project. DSB also arise as

intermediates during cellular processes such as meiotic recombination, transposition and V(D)J recombination. Furthermore, repair of certain DNA lesions or replication past DNA breaks or gaps may also result in the formation of DSB. A double-strand break, which is either not repaired or misrepaired, will result in cell death, deletions and chromosomal rearrangements. It is generally believed that these genetic alterations ultimately give rise to cancer and other diseases. To counteract the deleterious effects of DSB, several repair pathways have evolved. Non-homologous end-joining (NHEJ) and homologous recombination repair (HR) are the major pathways for repairing DSB. As well as repairing DNA lesions such as DSB, cells arrest the cell cycle in response to DNA damage, and the genes involved in these cell-cycle checkpoints have also been successfully characterised in the course of the last contract.

The relative importance of each pathway depends on the phase of the cell cycle, on the cell type, and the stage of development. In addition, the contribution of each pathway to repair may be influenced by the complexity of the DSB. All these damage response mechanisms are highly conserved in evolution. In order to achieve the goals of the current project, we will exploit new technologies (e.g. scanning force microscopy, DNA chips, proteomics, and fluorescent redistribution after photo bleaching). We will also utilise a number of different organisms (human, mouse, *Drosophila, Xenopus, C. elegans,* budding and fission yeast), which each have their own acknowledged advantages in helping to dissect the mechanistic pathways that are highly conserved throughout eukaryotes. Using genetics and biochemistry we will investigate the precise functions of the proteins involved in each of these repair and cell-cycle checkpoint pathways and the interactions within and between pathways both *in vitro* and in cells.

Ultimately our goal is to understand the consequences of deficiencies in radiation damage responses in man. To this end, radiation-induced mutations and other genetic changes will be assessed and we will analyse and identify the defects in damage response mechanisms in rare radiosensitive individuals (in which we have had notable success in the last contract) and experimental animals. Regarding the latter, we will also exploit the knockout *Drosophila* and mouse models, which we have generated, in which one or more repair genes have been deleted. Our work will provide important links with other consortia within the radiation protection programme, e.g. our knockout mice will be available for study by the radiation cytogenetics and radiation carcinogenesis groups. The tools and concepts that we will develop will assist in the understanding of genetic changes and radiation carcinogenesis in man and will ultimately help to extrapolate to the effects of low doses and low dose rates.

Results

The major lethal lesions induced by ionising radiation are the DNA double-strand breaks (DSB). For its repair two major evolutionary conserved repair pathways have been identified: homologous recombination repair (HR) and non-homologous end-joining (NHEJ). However, protection against IR-induced DNA damage not only needs repair pathways but also the coordinated action of the cell-cycle checkpoint pathways.

Homologous recombination. Error-free repair of DSB requires recombination between homologous DNA molecules. Genes belonging to the yeast RAD52 group (RAD50, 51, 52, 54, 54B, 55, 57, 59, MRE11 and XRS2) are the key players in this process. The Rad51 protein has a critical role in HR, as this protein is the leading factor in the search for

homology between DNA molecules. The large number of repair proteins involved in HR is concentrated in nuclear foci and also includes the Brca proteins implicated in breast cancer predisposition. In this project we further unravelled the role of the various genes in recombination repair. Most notably, we showed that Rad54 has a motor activity on double-stranded DNA. This activity can function to facilitate homologous DNA pairing by the hRad51 protein directly. In addition, the induction of super coiling by hRad54 could stimulate recombination indirectly by displacing histones and/or other proteins packaging the DNA into chromatin. Recent investigations on interactions of repair proteins have yielded interesting results on the interplay between HR and other pathways. The mammalian Rad-51-like protein Xrcc2 plays a key role in HR as reported previously; interestingly, new data indicate an association with the RNA polymerase II transcription regulatory factor SRB7. This interaction provides evidence that XRCC2 (and potentially the homologous recombination repair system) is 'talking to' the transcriptional machinery, possibly in a similar way to other repair pathways i.e. excision repair.

Another novel finding during this contract period was the discovery by our consortium and others that the gene defective in some Fanconi anaemia (FANCD1) patients is in fact BRCA2, explaining the severe sensitivity of these cells to DNA damage and the chromosomal instability phenotype.

The NHEJ pathway. A number of genes involved in non-homologous end-joining, has been identified encoding Ku70/80 subunits, Artemis, a DNA-dependent protein kinase (DNA-PKcs) and Xrcc4/DNA ligase IV. In vitro and in vivo systems with defined DSB have been successfully employed to study the functions of some of these proteins. In this contract period we have reconstituted NHEJ in vitro and further investigated the function of proteins involved (Ku proteins, DNA PKcs, Xrcc4/ligase IV as well as BRCA and Fanconi genes). A novel finding in yeast was that the isolation of the Ku70/80 complex not only revealed expected partner proteins such as ligase IV, but also of RNA polymerase III subunits suggesting a possible coupling of NHEJ to transcription. The challenging outcome of these studies is that both HR and NHEJ may interplay with transcription. It is unknown whether this interplay is mechanistically related to the well-known repair pathway transcription coupled repair (TCR). More insights in the recognition of DSB by the Ku and Xrcc4/ligase IV have been gained from in vitro studies: our findings suggest that Xrcc4/ligase IV binds to the DNA ends and causes inward translocation of Ku required for ligation activity. Moreover, the ligase IV protein appears to protect ends of DNA in addition to ligation function and also independent of ligase function.

In this contract period we gained more insights in the role of BRCA1 and FANC genes in NHEJ. Mutations in BRCA1 or FANC genes did not affect significantly the NHEJ efficiency, but strongly reduce the NHEJ fidelity suggesting that BRCA1 and FANC genes are not components of the NHEJ machinery by itself but rather may function either in the sensing of specific DNA damage or in signalling the presence of specific lesions (DNA cross-links, DSB) to the repair and cell-cycle checkpoint machinery.

Nbs1/Mre11/Rad50 complex. The complex of the human Mre11 (hMre11) protein with hRad50 and Nbs1 plays a pivotal role in DSB repair. It has generally been assumed that this complex plays an essential role in both homologous recombination and end-joining repair pathways, even though they are mechanistically distinct. In this study this model has been challenged by demonstrating that the Nbs1/Mre11/Rad50 complex is not a core component of NHEJ neither in mammalian cells (i.e. NBS-deficient cells do perform NHEJ) nor in the yeast

Schizosaccharomyces pombe. Scanning force microscopy has revealed a human Rad50/Mre11 complex and this study suggested that this complex provides a flexible, possibly dynamic, link between DNA ends. We showed that a common function of the Mre11 complex in two mechanistically distinct DNA double-strand break repair, end-joining and homologous recombination, could be to tether broken DNA molecules. This finding is more in agreement with a role of Nbs1/Mre11/Rad50 complex in the recombination and end-joining repair. Although a common mechanism may link recombination repair and end-joining, our analysis of DNA binding by the Rad52 and Ku proteins makes it unlikely that Rad52 and Ku compete as "gatekeepers" of different DSB break repair pathways. Rather they interact with different DNA substrates produced early in DNA double-strand break repair.

Cell-cycle response. After DSB induction, the cell cycle is arrested to allow repair. The complexity of the interactions of the checkpoint pathway with the cell-cycle machinery is well illustrated in yeast envisaging DNA damage sensing, signal transduction and interaction with down stream effectors as critical steps. Recent work in yeast demonstrates that via components of the signalosome, nucleotide metabolism plays an important role in cell-cycle regulation after DNA damage in G2 phase under control of checkpoint function. Attempts are now made to identify genes involved in signalosome in other organisms than yeast (ultimately mammals), starting with *C. elegans*. Homologues to seven subunits have been identified in this organism and by RNA interference methodology we have established that signalosome depletion increases the duration of the DNA damage response.

Mutations/polymorphisms in DNA damage response genes and cancer. Mutations in a number of HR and NHEJ genes have been found to be related to human diseases, mostly associated with cancer predisposition, radiosensitivity and chromosomal instability: mutations in MRE11 give rise to the AT-like syndrome, in NBS1 to the Nijmegen breakage syndrome, both associated with cancer predisposition, radiosensitivity and chromosomal instability. In this project period a new syndrome called the LIG4 syndrome, which is characterised by immunodeficiency, developmental delay and microcephaly, has been identified. These patients display mutations in the DNA ligase IV gene and cells of these patients are radiosensitive and defective in DSB repair. Another syndrome (the Seckel syndrome) has been identified with mutation in the ATR during the course of this project. However it is clear that fully inactivation of the damage response genes in mammals is frequently lethal as demonstrated by the inability to generate transgenic knockout mouse models for certain repair genes; thus only hypomorphic mutations are viable. An essential and strong point in this contract is the use of various organisms to study DNA damage responses and this has allowed studying mutations in genes in lower organisms such as *Drosophila* that tolerate the inactivation of some of these genes and that allow to make double mutants as well. The results obtained with LIGASE IV and RAD54 *Drosophila* mutants demonstrate that the two major DNA double-strand break repair pathways in mammals have overlapping as well as specialised roles, and that the relative contribution of these pathways towards repair of ionising radiation-induced DNA damage changes during development of the animal. Similar conclusions have been reached with mice deficient in the RAD54 and DNA-PKcs genes.

In this project period we have started for the first time to apply the knowledge of genes involved in IR response (gained in this and previous projects) to assess individual risk for cancer. Our focus was on polymorphisms in the XRCC2 gene. This gene plays a key role in homologous recombination. The data are suggestive of an association of XRCC2 genotype and breast cancer, but it is important to note here that confirmation requires a larger data-set.

The results obtained in this period of the contract have been reported in 176 publications in peer-reviewed journals.

Implications and future perspectives

The picture that emerges is that the response to ionising radiation-induced DNA damage is carried out by multi protein complexes assembled in dynamic foci rather than by single proteins. These complexes are capable somehow to interact with different pathways including novel pathways such as chromatin remodelling and transcription. Deficiencies in these cellular response mechanisms (either at the level of repair or cell-cycle control) generally lead to mutations and chromosomal aberrations as shown for many mutants, but the frequency of chromosome exchanges induced by radiation is dependent on the operation of DSB repair pathways. Homologous recombination-deficient cells show an elevated frequency, suggesting that this pathway protects against exchange formation, while cells involved in end-joining of double-strand breaks have a reduced frequency, especially following alpha irradiation. Mutations in DNA damage response pathways do not necessarily predispose to cancer. For example, in humans DNA ligase IV deficiency (an obvious repair defect) is generally not associated with predisposition to cancer, whereas NBS deficiency (a cell-cycle checkpoint defect rather than a repair defect) leads to cancer predisposition.

The biological consequences of mutations in the major DNA damage response genes can be assessed in two ways. The development of mouse models with defects in repair or cell-cycle control genes provides the way to look at tissue and whole-body consequences of IR exposure, especially to understand the role played by these genes in mitigating the development of cancer. In addition, screenings for polymorphisms in these and other genes involved in radiation responses might reveal enhanced cancer risk and ultimately identify individual susceptibility to IR.

The lethality of several knockout mouse models for damage responsive genes demonstrates that more subtle mutations are required to generate viable genotypes and these mutations are the more relevant mutations for human health. For example, whereas RAD17 knockout mice are embryonic lethal, we found a hypomorphic mutation in RAD17 that allows viability of ES cells and revealed a role for RAD17 in DNA damage repair.

For the near future we expect that novel genomic techniques will allow large screens for mutations ands polymorphisms in these and other genes involved in radiation responses to identify individual susceptibility to ionising radiation. One way to do this is to analyse expression profiles. Using microarrays, we have identified many fission yeast genes whose transcript levels were altered in response to ionising radiation. Some of these genes were part of a core environmental stress response set, but 117 genes defined a radiation-specific gene-expression response signature in yeast. The roles of specific genes were explored to show that there is a complex network of regulatory pathways coordinating gene expression responses to radiation in eukaryotes. It is conceivable that mutations in core network genes will hallmark the transcription profiles.

Some other exciting possibilities were already fulfilled in this project. We have identified radiosensitive (RS) SCID patients over the past several years. From several of these patients we identified the genetic defect: they were all mutated in the recently discovered Artemis gene. One step further ahead is to investigate possible effects of polymorphism in these genes. The identification of a DNA polymorphism in the human XRCC2 gene allowed the testing of

its role in breast cancer. Tests on more than 500 cases suggested a small level of increased breast cancer risk. There is no doubt that this is only the beginning of the identification of polymorphic changes in ionising radiation response genes and the assessment of spontaneous and radiation-induced health effects.