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Introduction

The discovery of radiation-induced genomic instability has raised concern for its implication in the radiation protection of the public, especially quantification of human risk at low doses and high LET exposure. The main source of information on radiation-induced human cancer risk comes from epidemiological data of exposed populations. However, direct information is available only at relatively large doses, and mostly for low-LET X- and gamma-rays. A linear extrapolation from these data is applied at lower doses, which are more relevant in terms of exposure of general population and radiation workers. Additional extrapolation is applied to other radiation types. The shape of the cancer dose-response curve at low doses is a matter of constant debate; arguments are ranging from threshold (or even hormesis) to non-threshold supralinear responses. Biological modelling of radiation carcinogenesis may offer a tool to study the risk in the low-dose region. Non-targeted radiation effects, such as genomic instability (novel mutations and cell death in the progeny of irradiated cells) and bystander effects (effects observed in the neighbouring cells not directly impacted by radiation), may be important early steps in the development of radiation-induced cancer. Understanding of underlying mechanisms may have profound consequences on cancer risk assessment.

Objectives

The RADINSTAB project brought together all major European laboratories involved in the discovery, characterisation and mechanistic investigation of radiation-induced genomic instability. The role of radiation-induced genomic instability in carcinogenesis was studied with animal models and mechanistic investigations of induction and perpetuation. Genes and genome regions involved were studied and the basis of individual susceptibility was investigated. The relationship of genomic instability end-points to radiation dose, dose rate, and quality was studied.

Results

Genomic instability end-points

To determine the role of radiation-induced genomic instability in carcinogenesis, a comprehensive study of responses and the mechanisms underpinning them is required in a range of models. The RADINSTAB project has produced extensive new data for a range of genomic instability end-points. These end-points have included extending existing data for both transmissible and non-transmissible damage and new end-points previously not studied. In particular, this has included new data on gene expression and hypomethylation changes and a careful examination of the relationship between apoptosis and chromosomal instability in animal models. For many of the end-points studied, data was obtained in both cellular and animal models. For transmissible damage, the end-points studied have included micronuclei and chromatid aberrations, gene expressions changes, hypomethylation changes, calcium changes, reactive oxygen species (ROS) and nitric oxide, mitochondrial and other membrane changes, inflammatory responses, macrophage activation and neutrophil infiltration, differentiation, transformation and tumourogenesis. For non-transmissible (lethal) damage, loss of clonogenicity and apoptosis has been studied.

Dose dependency and effect of radiation quality

A key aspect of the non-targeted responses investigated in the RADINSTAB project is that they have been found to have fundamentally different types of response to radiation from those well established for the classical targeted responses. In general, targeted responses increase progressively with radiation dose. They also show a marked dependence on radiation quality, with maximum RBE values generally between 2 and 20, depending on the system, and peaking at LETs in the 100-200 keV/micron region. Much of the dose-effect behaviour and LET dependence of targeted responses can be explained in terms of DNA damage as the initial lesion. Effects of radiation quality can be explained by its known influence on the complexity of damage induced by the radiation tracks directly in the DNA. In contrast, the initiating event with non-targeted effects appears to be energy deposition in targets other than nuclear DNA. Also, the damage is expressed distantly in space and time and the responses are clearly governed by cell-signalling pathways rather than by the stochastic induction of DNA damage followed by its repair or misrepair.

In general, the dose-effect relationship for genomic instability shows a plateau but is a function of time at which the effects are scored. High LET is more effective than low LET in inducing genomic instability, but LET also influences temporal pattern of expression. For the bystander effect, the dose-effect relationship invariably shows a plateau (< 1 Gy) whether measured by a medium-transfer method or by low-fluence direct irradiation. Moreover, the effect appears mainly to be determined by the *dose per hit cell*, rather than by *number of cells hit*, and high- and low-LET radiations appear to be almost equally effective. The plateau in the dose-effect behaviour needs further investigation. As yet, there is no explanation of what determines which cells respond and which do not. The plateau does not appear to be explainable in terms of sensitive and insensitive subpopulations.

Mechanisms of instability induction and perpetuation

The key problem relating to mechanisms was to accept or reject an epigenetic mechanism of induction and or transmission of the unstable state. Many characteristics of genomic instability cannot be accommodated within the framework of conventional genetics. The idea that radiation damage can persist and that death or mutation of cells could occur in healthy colonies defined as “survivors” seriously challenges the central role of the DNA strand break. All the progeny of the cell should be affected by the same mutation if a mis-repaired break caused a mutation. Since this does not happen, the delayed effect cannot be due to the initial DNA break. The ability to link dose with effect in any simple way is challenged by these data since the level of the reduced fitness of the progeny of irradiated cells is not dose dependent and is not always reduced or eliminated during expansion of the irradiated progeny. The delayed effect appears to be maintained or perpetuated in some way so that the initial irradiation triggered some process, which led to an increased probability of mutation in future progeny. The idea of a “mutator phenotype” or a repair defect was considered by the team in an attempt to fit the new phenomena within a framework allowing the DNA strand break to remain the key lesion, but the facts do not fit. Delayed genomic instability or death is a high-frequency event which affects many more cells in the field than could have been hit. The effect is induced by extremely low doses (a gamma-ray dose as low as 2 mGy can turn on the process and lead to detection of high yields of whatever end-point is being measured). The effect does not either disappear or dominate with time or passage of the progeny. The team tested progeny of irradiated cells out to 12 passages or approximately 100 cell population doublings without seeing the cells return to normal. The incidence of non-clonal mutations in clonal progeny is in or-

ders of magnitude greater than mutation frequencies. All these facts suggest an epigenetic driver for radiation-induced instability and cannot be accommodated by a mutation in a mutator gene. However this does not exclude the possibility that a DNA-damaging event somewhere in the population of cells or in a tissue may be necessary to start up genomic instability.

A very popular hypothesis is that the driver is the "bystander mechanism", i.e. that the signalling of damage from hit cells to unhit cells itself induces and perpetuates genomic instability in the population. The bystander mechanism could be induced by this initial DNA damage in part of the cell population and allow the whole population to become unstable. The dose response for the bystander component of the total radiation response is remarkably similar to the dose response for delayed effects. It is now thought likely that much of the delayed death and delayed non-clonal chromosomal instability induced by radiation may be due to bystander signal mechanisms and not a direct result of energy deposition in DNA. Bystander effects are now known to occur in many cell types, many species and following high or low LET radiation exposures. The signals produced are capable of inducing death, genomic instability, mutations or transformation in cells not themselves directly hit by radiation. The bystander effect once induced is perpetuated in progeny of cells which received either direct irradiation or signals from directly irradiated cells. Two members of the team have shown plateau or saturated type dose responses for low-LET and high-LET bystander effects. In both cases extremely low doses (one particle or 2 mGy) can induce the full bystander effect.

There are two main ways that bystander effects can be detected and studied. Either a field of cells is exposed in such a way that certain cells in the population are targeted specifically and every cell does not receive a hit by an ionising track or every cell receives a hit but the effect is detected in a totally separate population which only receive signals expressed into a fluid medium by the hit cells. In the first case, effects are sought in cells which are known not to have been targeted by the radiation. In the latter case, effects are measured in cells not exposed to radiation which received conditioned medium from hit cells. In both cases controls are of the utmost importance. The first detectable effect in untargeted cells receiving media-borne signals from hit cells is a rapid (1-2 min) calcium pulse which is followed 30 min to 6 h later by changes in mitochondrial membrane permeability and induction of reactive oxygen species. This has now been shown by team members in both microbeam and medium transfer model systems. Persistent elevation of reactive oxygen species in cells subject to bystander signals would be a plausible epigenetic driver for genomic instability which fits the facts. There appears to be individual patient and mouse genotypic variation in the ability of bladder tissue biopsies to produce the factor. The critical role of mitochondrial metabolism is also suggested by the lack of signal production by cells which do not have a functional G6PD enzyme.

While the team are of the opinion that genomic instability is driven by bystander mechanism, we considered two other DNA-based epigenetic mechanisms – DNA methylation and genomic imprinting. The latter was found not to be involved, but there is evidence that both hypermethylation and hypomethylation can be induced under different circumstances, in a stable fashion by irradiation. There is a high frequency of induction and the effects are stably transmitted from generation to generation. It is possible that this mechanism may be a secondary consequence of the induced changes in oxidative stress and may help to facilitate the formation of mutations in the long term.

In conclusion, DNA damage in an individual cell is not required for that cell to become unstable. This does not exclude the possibility that DNA damage somewhere in the system is

sensed by the population, which then become unstable. The most likely drivers for genomic instability are bystander effects. DNA methylation effects have been detected and these are probably secondary to the bystander-induced cellular oxidative stress.

Identification of genes and genome regions involved

Gene expression changes seen at early time points were different from those at delayed points. The process of a cell culturing itself had an effect on gene expression patterns, regardless of irradiation status. No individual gene could be consistently related to induction of chromosomal instability across all the experiments. Our methods showed high heterogeneity in gene expression patterns. It seems plausible that a cell may end up into processes leading to instability in a variety of ways and, thus, causing different types of changes in the expression of genes, gene families, or metabolic routes. The net effect on measured gene expression levels, especially in bulk cultures, can therefore be subtle. In cultures of clones derived from a single cell we observed a tendency towards a more heterogeneous general gene expression pattern among irradiated cell clones than among controls. This could either be a consequence of the genomic instability or possibly a manifestation of an epigenetically deregulated state of the cell with a globally relaxed control of normal regulatory constraints. A set of candidate irradiation-related genes has been defined.

Susceptibility to radiation-induced genomic instability and predisposition to cancer

The ability to maintain genome integrity in the face of DNA damage is critical for healthy survival. The means by which organisms achieve this are complex, involving intricate homeostatic mechanisms that have evolved to allow cellular adaptation to physiological and pathological stresses. Such responses must be carefully coordinated, and there has been considerable progress in identifying the mechanisms by which eukaryotes respond to harmful exogenous insults by initiating processes that either enhance cell survival or lead to the regulated loss of damaged or unwanted cells. Human genetic diseases in which the homeostatic processes have broken down are recognised, resulting in complex and often multi-system effects. A high level of spontaneous chromosomal aberrations and gene mutations characterises these so-called chromosomal instability syndromes. They all exhibit a significant predisposition to malignancy. In addition to the highly penetrant mutated genes that confer such significant predisposition, there is growing interest in genes with lower penetrance that may also be regarded as predisposition genes, for example those responsible for the correlation between chromosomal radiosensitivity and cancer predisposition detected by the G2 assay.

Challenging the conventional model for radiation-induced genetic lesions initiating the malignant process, there is now considerable evidence that unirradiated cells that are the progeny of cells irradiated many cell divisions previously may express a high frequency of gene mutations, a variety of chromosomal aberrations, and may also exhibit an increased frequency of cell death. These effects are collectively known as radiation-induced genomic instability. The genomic instability induced by ionising radiation is superficially similar to the cellular phenotypes in the chromosomal instability syndromes but cannot be explained by conventional mutagenic effects of direct DNA damage. An additional aspect of non-targeted effects of radiation is that chromosome aberrations, mutations, and cell death can also be demonstrated in unirradiated cells that receive signals from irradiated cells via mechanisms collectively regarded as radiation-induced bystander effects. RADINSTAB investigations indicate that these indirect effects may be responsible for many aspects of the instability phenotype. Non-targeted effects can readily be accommodated into a view of malignancy that rather than ask-

ing how normal cells become cancer cells (the simplistic model of malignant change) asks how normal multi-cellular tissues are subverted into malignant tissues (the approach now favoured by many cancer biologists).

The expression of both radiogenic tumours and radiation-induced genomic instability is influenced by genetic factors, but at present there is no reason to assume that there is a simple relationship between these genetic predispositions. However, there is accumulating data consistency with the expression of delayed death and delayed cytogenetic aberrations being inversely related and reflecting genotypic differences in signalling pathways associated with damage recognition and apoptotic response, i.e. a sectoring of cells into growth arrest/repair (with the possibility of misrepair) or apoptosis as downstream responses to damage recognition mechanisms. In some circumstances a permanent growth arrest or terminal differentiation may be a consequence of instability, but these effects are functionally equivalent to apoptosis in that affected cells do not proliferate. These conclusions reflect data obtained by RADINSTAB investigations for both haemopoietic cells and urothelial cells and are indicative of those genotypes that tend to have a more effective apoptotic response being less predisposed to the development of malignancy. The investigations also indicate the importance of tissue-specific gene expression in these processes. Responses in individuals with such genotypes would deviate from a linear-no-threshold model in a protective direction. Conversely, those genotypes that have less effective responses are those in which chromosomal instability is readily expressed. This may increase risk at low doses.

At the present time, it is difficult to see how general principles can be extracted to comment on risk. It could be argued that inter-cellular, inter-tissue and inter-individual differences in response to radiation exposure reflect the biological realities, particularly at low doses, and whilst contributing to the genetically-determined differences in responses to radiation may not help in refining epidemiologically-based risk estimates. Elucidation of the genetically influenced processes involved in radiation-induced genomic instability would help our understanding of the mechanisms underlying the stochastic effects of radiation exposure. And according to one view, mechanistic insight will contribute to risk assessment, particularly at low doses; the dose region where epidemiology is a very ‘blunt tool’.

Relevance for cancer risk assessment and radiation protection

A basic paradigm in radiobiology is that after exposure to ionising radiation, the deposition of energy in the cell nucleus and the resulting damage to DNA, the primary target, are responsible for the harmful biological effects of radiation. The radiation-induced changes are thought to be fixed already in the first cell division following the radiation exposure and health effects are considered to result as a consequence of clonal proliferation of cells carrying mutations in specific genes.

These basic assumptions have now been challenged by the new findings on radiation-induced genomic instability and bystander effects showing that deleterious effects can be observed also in cells that were not irradiated. Genomic instability and bystander effects are observed already after very low doses. In fact, some dose-response data indicate that the relative contribution of these indirect effects as compared to damage caused by direct hits may well be more pronounced in the low-dose region, thus giving some support to a potential supralinear response in the low-dose region. These effects also provide a potential mechanistic explanation for the development of non-cancer diseases.

The cancer risk of low doses of ionising radiation will probably never be fully elucidated by epidemiological studies, as this would require very large populations and accurate dosimetry. The dosimetry of protracted exposures is even more demanding than dosimetry for single exposures. Uncertainties in dosimetry of epidemiological studies makes it more difficult to observe a dose response, which in turn tends to lead to lower risk estimates (especially for the protracted exposures). Biological modelling of radiation carcinogenesis may offer a tool to study the risk in the low-dose region. The input data should contain not only the conventional direct radiation effects, but also the non-targeted effects which may be important modifiers of radiation response in the low-dose region.

The genomic instability and bystander end-points are both transmissible (mutational) and non-transmissible (lethal). The balance of these in the different cellular systems may lead either to an increased or decreased risk. Some scientists indeed argue that these non-targeted radiation effects are in fact part of the adaptive response to ionising radiation. More research is needed on the delayed damage response systems, such as adaptive response and premature differentiation.

Individual sensitivity seems to play a role both in genomic instability and bystander effects. Genotypes that have a more effective apoptotic response seem to be less predisposed to the development of malignancy. The genetic basis for this variability requires further research. Future research should preferably be carried out in *in vivo* systems.