Anhang/Appendix S

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3 Radiosensitivity of Mammalian Cells

3.5 Cellular and Molecular Aspects

3.5.1 Sources of DNA damage

In reviewing differences in DNA repair capacities, it is important to define the kinetic parameters involved and the specific lesions to which they apply. Ionizing radiations produce a gamut of lesions in DNA, none of which is unique to ionizing radiations; *i.e.*, the same lesions are produced by naturally occurring biochemical entities in the foods we eat, in the liquids we drink, and by the background radiation to which we are exposed. This background radiation comes from: (a) cosmic radiations which reach the earth from space ($\approx$ 8% of average effective dose to the US population [1]), (b) radioactivity in the earth's crust and the building materials we make from it (also $\approx$ 8% [1]), (c) ingested naturally occurring radionuclides (primarily ^{40}K; $\approx$ 11% [1]), and (d) inhaled naturally occurring radon ($\approx$ 55% [1]). To this 82% we add: (e) man-made exposures ($\approx$ 18% [1]) - which includes Medical (and Dental) X-rays ($\approx$ 11% [1]), Nuclear Medicine ($\approx$ 4% [1]), consumer products (including television sets)($\approx$ 3% [1]), and Other ($<$ 1% [1]), which includes occupational exposure (0.3%), Fallout ($<$0.3%), the Nuclear Energy fuel cycle ($\approx$ 0.1%). A brief review of the relative importance of these contributions can be found in [2].

3.5.2 DNA lesions

The major forms of damage to DNA consist of: altered bases, missing bases, incorrect bases, bulges in the DNA backbone due to the deletion or insertion of a nucleotide, linked pyrimidines, strand breaks, cross-linked strands, DNA-protein cross links, and 3'-deoxyribose fragments [3]. Each of these lesions can be repaired by cellular DNA repair systems. Excellent reviews of the causes of these lesions and the repair systems by which

cells repair these lesions can be found in [4-9]. Although all of these lesions can be repaired, what determines the fate of a cell is the fidelity with which they are repaired, the probability that not all of them are repaired, and the locus of the remaining error, if any.

The consequences of repair infidelity are mutation and/or cell death (usually loss of reproductive integrity). That efficient repair of the vast majority of these lesions occurs widely in living systems can be seen from the fact that even bacteriophage T4, a large genome virus of the bacterium *E. coli*, can repair all of these lesions (using enzymes coded by its monomolecular DNA genome), except DNA double-strand breaks (DSB), under the conditions of a standard plaque-forming assay. Indeed, some 80 % of loss of plaque-forming ability (reproductive integrity) can be ascribed to one DSB per genome (86% [10], 76% [11]). Smaller viruses, such as PM2, do not code for the enzymes needed to repair certain types of base damage and single-strand breaks, so that these lesions are also lethal for them.

For acute doses below the several tens of Gy (kilorad) range, loss of reproductive integrity is the major (if not sole) determinant of radiation-induced organismal lethality and of specific organ-system failures (*e.g.*, gut and bone marrow mortality syndromes in irradiated animals [12-18] and severe growth inhibition in plants [19-23]. This relationship is well understood for hierarchically organized tissues (*i.e.*, those with a precursor or stem cell population which proliferates to maintain its size and which provides replacements for the functional differentiated cells of the tissue) [24] and for the flexibly organized tissues (*i.e.*, those with functional cells which can upon demand be called upon to repopulate the tissue) [25,26].

For carcinogenesis, the underlying problem is misrepair and consequent mutagenesis, followed by additional mutations which, together, release the cell from normal growth control, allow the cell to bypass programed cell death (apoptosis), and become

invasive/metastatic. This process is less well understood, although many of the genes which can be involved in carcinogenesis (through lack of, or error in, their gene product) are now known [7-9,27,28]. First, we shall consider the causes of survival/loss of reproductive integrity (which we shall argue can be explained in terms of DSB repair kinetics), and then carcinogenesis (in which all of the DNA lesions can be important). Survival of reproductive integrity is a critical component of carcinogenesis because a cell which has acquired carcinogenic mutations needs to be able to reproduce itself *ad inifinitum* in order to produce a cancer. The bell-shaped radiation dose dependence of cancer induction owes its decreased cancer yield at high dose to the fact that more cancer cells are killed (sterilized) than are produced in that dose range. This can be seen clearly when one plots the incidence of cellular transformation *vs* dose and the cell surviving fraction *vs* dose, the terminal slopes of both curves are exponential and have a common slope (*e.g.*, D_0 = 1.5 Gy for 10T1/2 cells [29]).

3.5.3 Cellular reproductive integrity as the basis for most radiation-induced organismal mortality syndromes.

The reason why T4 cannot repair DSB in the standard plaque assay conditions is that in order to be able to count plaques, one mixes the virus with *E. coli* cells in the ratio $1:10^5$, plates the cells, and subsequently counts plaques. Under such conditions, the probability that two virus particles infect the same cell is negligibly small, and when the virus has been irradiated to a dose which produces an average of 1 DSB/genome, then two copies of the genome are needed to permit genetic recombination from which an intact genome can be made. A plaque-forming assay can be done under conditions which allow such recombination by using an infectious center assay, *i.e.*, the cells are infected at viral multiplicities of 1 or

more, the viral particles which have not infected the cells are removed by centrifugation and/or by use of antibody against the viral protein coat, and the infected cells (infectious centers) are then mixed in the ratio $1:10^5$ with unexposed *E. coli*, plated and subsequently, plaques counted. Under such conditions, DSB are repaired, and survival is considerably higher [30-33] than would be predicted by Classical Target Theory [33,34], which assumes the absence of repair processes [34]. However, it is clear that bacteriophage T4 [30-33], bacteria (*E. coli*)[35,36], and yeast (*S. cerevisiae*)[37-43] can repair DSB under the appropriate conditions. For the latter, haploid cells prior to DNA synthesis (G_1) cannot repair their DSBs [39] while post-DNA-synthesis G_2 and diploid cells can [38-43]. The importance of DSB repair in determining the shape of the survival curve for reproductive integrity was further demonstrated by the use of a diploid temperature-sensitive mutant of the gene *RAD*54-3 in *S. cerevisiae* [37, 40-43]. At the permissive temperature, DSBs are repaired and the cells are quite radioresistant, having a shouldered survival curve with shoulder size and reciprocal slope increasing with postirradiation (liquid holding) incubation time. At the nonpermissive temperature, DSB are not repaired and the survival curve is a very steep simple exponential. Mammalian cells have also been shown to repair DSBs [44-47].

3.5.4 DNA DSB repair kinetics as the basis for survival of cellular reproductive integrity.

Knowing the above, it was reasonable to construct a model which assumes that the only cause of cellular loss of reproductive integrity is the failure of a cell to repair all of its DSB within the time allotted for this repair (by the cell's metabolism, *etc.*). The quantitative statement of this assumption is the DSB model [48-54]. A critical test of this, and other alternative models which are not so specific in their definition of the critical lesion which

leads to reproductive death, lead to a demonstration that only the DSB model correctly predicted subsequently observed data [54-55]. There were several predictions: (1) qualitatively: in a delayed plating experiment, where cells are irradiated in monolayer culture and then incubated in either fresh medium or conditioned (nutritionally depleted) medium before trypsinizing and plating the cells for colony formation, that after cell survival had reached the plateau of maximal survival for cells in fresh medium, that survival would increase to the higher survival maximum plateau typical of that for delayed plating with incubation in conditioned medium from the start [54-55]; (2) quantitatively: that the rate of DSB repair (τ_{DSBR}) could be predicted quantitatively from split-dose recovery kinetics data and that it would be equal to that measured directly from DNA repair measurements. This too was correct [54]; (3) quantitatively: the time available for DSB repair (t_{rep}) could be predicted from the initial slope of the cell survival curve and the rate of DSB repair (τ_{DSBR}) determined either as above, or directly from DSB repair measurements in pulsed-field gel electrophoresis experiments, and that this would equal the time at which changing the delayed plating incubation medium from fresh to conditioned would be too late to provide the increased survival characteristic of delayed plating in conditioned medium. This also was correctly predicted [54]. Furthermore, measurements of DSB rejoining showed that there were two rates, fast and slow, which corresponded to two values of t_{rep}, and which agreed with the delayed plating survival data [54]. Molecular genetics studies also demonstrated two pathways for DSB rejoining in mammalian cells: the end rejoining pathway (Ku70/86 DNA-PK) which is also responsible for V(D)J and immunoglobin class-switch recombination, and the recombination pathway (RAD 54)[56]. Thus, it seems clear that the kinetics of DSB induction and repair (including both rejoining rates and durations, (*i.e.*, times available for repair)), when applied to the DSB model, can correctly predict cell survival of reproductive

integrity (further tests of this model are in progress). It is also noteworthy that (as mentioned above) from a knowledge of the cell survival curve parameters for the underlying precursor cells in hierarchically organized (stem cell based) tissues, one can predict the organ failure probability, and if that organ is a vital one, the mortality probability of a multicellular animal [12-18,24-26,57,58].

3.5.5 Cancer as a Genetic Disease

All cells in a tumor arise from a single cell in which the normal mechanisms which control proliferation have been disrupted. Cancer cells also differ in the normal cell-cell interactions, a difference which allows them to invade and metastasize. Multiple events (at least two) are needed to induce malignancy, and in humans it may take decades for the second event to occur. Normal cell regulation represents a balance between the expression of genes which control growth promotion and those which control growth inhibition. The former are called "oncogenes" and the latter "tumor suppressor genes". Thus, mechanisms of cancer predisposition can be classified as: (1) germline tumor suppressor gene inactivation, (2) germline oncogene activation, (3) DNA repair defects, and (4) ecogenetic traits (i.e., hereditary disorders involving hypersensitivity to common carcinogens)(see [59]). The first two classes can lead to hereditary or familial cancers.

Hereditary retinoblastoma (eye tumors which occur in young children, usually before the age of 4) provides an example of the first class, and a demonstration of the two-hit model for carcinogenesis. Knudson [60] proposed that all children with hereditary retinoblastoma have inherited one genetic lesion (hit) which is in every cell in their body and that tumors arise when a second lesion has occurred. The Rb gene has been mapped (chromosome 13q14, [61]) and shown to be mutated in, or deleted from, one copy of chromosome 13 in

affected families. The second lesion (loss of the second, functioning, homologous gene) can occur due to mistakes in replication or an exogenously caused mutation, and explains the need for "promotion" after an initial mutation in a growth regulatory gene. Since the first hit already exists in every cell in individuals who have inherited this mutation, and there are large numbers of retinoblasts in each eye, the acquisition of the second hit need not take a long time and multiple cells can be expected to have acquired both hits. This is consistent with the finding of multiple retinoblastomas in 30% of hereditary retinoblastoma patients before the age of 2, but their being quite rare in nonhereditary sporadic retinoblastoma patients (in which both hits must occur in the same somatic cell) with the peak incidence occurring later in sporadic cases, between ages 2 and 4. However, for other tumor suppressor genes, such as CDKN2 [62], germ line homozygous mutation of both homologues does not produce a more severe form of the disease (melanoma) and additional mutations in other genes are required for tumorigenesis.

The second mechanism, activation of a germline oncogene, although once thought to be an important mechanism, appears to have very few examples: namely, multiple endocrine neoplasia types IIA and IIB [63-66]. Protooncogene expression is highly regulated in normal cells and hence germline activation, unless limited to some selected cell lineages, may well be lethal for the offspring. However, constitutional (not germline) activation of an oncogene can be seen in the example of the endocrine tumors often found in McCune-Albright syndrome patients [67].

The third mechanism, DNA repair mutations which predispose to cancer induction, has been extensively studied and remains a very productive and rapidly growing field. It is now clear that there are a large number of genes (and their gene products) involved in the normal housekeeping of DNA (see [3-9,27-28,56,68]). Defects in, or the absence of, the

gene products of many of these genes can lead to carcinogenesis by creating new mutations due to lowered fidelity of DNA replication, by allowing endogenously or exogenously induced DNA lesions to persist (due to absence of a repair pathway or by shortening the time available to a cell for repair of such lesions), by inducing new errors by misrepair, or by failure to prevent cells with such genetic damage from undergoing uncontrolled growth or avoiding apoptosis (programed cell death). Many DNA repair-deficiency related genetic diseases have been studied: *e.g.*, Xeroderma pigmentosum (XP), cells of which are hypersensitive to UV light; Cockayne's Syndrome (CS) and Ataxia telangiectasia (AT), cells of which are hypersensitive to x-rays; Fanconi's Anemia (FA), cells of which are hypersensitive to DNA cross-linking agents; Bloom's Syndrome (BS) and Fragile X Syndrome, cells of which are subject to a high incidence of spontaneous chromosome breaks; *etc.* All of these syndromes are rare autosomal recessive disorders associated with an increased risk of cancer. XP has been classified into complementation groups representing defects in at least eight different genes active in the same pathway for the repair of DNA lesions induced by ultraviolet light. Several of these genes have been cloned and their activities include binding of photoproducts, helicase and endonuclease activities [68]. The AT gene has been cloned and its mutant shown to be defective in a signal transduction pathway which mediates DNA damage responses such as cell cycle check point control, activation of DNA repair enzymes, and control of apoptosis [69,70]. Interestingly, the rate of DNA double-strand breaks in these cells appears to be same as in normal cells but repair terminates earlier in AT cells. Mutations in mismatch repair genes (MSH2 on chromosome 2, a human homolog of *E. coli* MUTS; MSH1 on chromosome 3, a human homolog of *E. coli* MUTL; PMS1 on chromosome 2, homologous to yeast PMS1 and *E. coli* MUTL; PMS2 on chromosome 7, homologous to yeast PMS1) are involved in hereditary nonpolyposis colon

cancer (HNPCC)[71,72].

The fourth mechanism, ecogenetic traits, can be considered to include the DNA repair disorders as extreme examples. However, a more typical example is epidermodysplasia verruciformis. In this disease, UV radiation and papilloma virus act as cocarcinogens to produce squamous cell carcinoma in genetically susceptible individuals [73]. Genetic factors influencing individual responses to environmental carcinogens can also be found in pathways involving detoxification of those carcinogens. One example is the polymorphisms in the cytochrome P450 gene: CYP1A1, CYP2D6 and CYP2E1 and their role in lung cancer susceptibility [74], although the risk attributable to hereditary factors is small compared to that for cigarette smoking. Another example may be the role of genetic variation in epoxide hydrolase for aflatoxin B1 induced hepatic carcinogenesis [75].

3.5.6 Variations in DNA repair capacities in human cells among individuals

Although the frequency of homozygous recessives resulting in any of the DNA-repair deficiency diseases is rare, the frequency of heterozygous carriers can be significant; *e.g.*, AT heterozygous individuals have been estimated to occur in about 0.5 to 5% of the population and they have a twofold higher lifetime risk of cancer induction [76]. Given the number of proteins involved in DNA surveillance and repair, one might expect that a significant fraction of the general population may carry a mutation (one or more of several possible ones) predisposing to higher cancer risk. This should therefore be a significant concern in the setting of radiation, and other genotoxicity, protection standards.

However, a contrary example is provided by the BRCA1 gene. The proportion of breast cancers attributable to a mutated BRCA1 gene is 1.7% in women under age 70 and 7.5% for women under age 30, while the frequency of this mutation is 6×10^{-4} in the

population of females under age 70. For Ashkenazi Jewish women under age 30, the BRCA1 mutation frequency is 4.7×10^{-3} and 38% of breast cancers in this group are attributable to mutations of this gene [77]. Sankaranarayanan and coworkers [78-81] have developed a model which estimates the risk of radiation-induced cancer in relatives of those known to carry a particular gene predisposing them to cancer, relative to the general population. Applying this model to test the effect of genetic heterogeneity (in BRCA1 mutation frequency), they showed that unless a cancer-predisposing mutation is common (higher than that for young Ashkenazi women) and the radiosensitivity differential and predisposition strength are conjointly large, the risks of radiation-induced cancer even in first-degree relatives should not be substantially different from those for unrelated individuals.

Another example of heterogeneity in radiation sensitivity comes from the age and gender dependence of induction and rejoining of DNA DSBs [82-87]. Using neutral filter elution to estimate DSBs in the unstimulated peripheral blood lymphocytes of humans between 23 and 78 years of age, Mayer *et al.* [82-84] found that: (1) DNA in older donors sustained fewer DSBs after X-irradiation than in younger donors, (2) older donors rejoined only half the fraction of DSBs that younger donors did, (3) there was a gender difference in that the decrease in DSB rejoining was more pronounced in older women than in older men (and this more pronounced decrease may begin after age 65 in women), and (4) at comparable levels of DSB induction, cells from men rejoin a higher fraction of DSBs than those from women. Findings (1) and (2) were the first to show an age difference in human DSB rejoining and (3) & (4) were the first report of a gender difference in DSB rejoining (and in DNA repair). Grossman [85-87,28] also reported age and gender differentials in DNA repair, and showed that this was related to increased mutability [86-87].

From the perspective of cancer therapy, the population heterogeneities discussed

above pose another problem. Since the maximum treatment dose is usually determined to be that which produces severe complications (life-threatening) in no more than 5% of patients, if 5% or more of cancer patients possess a repair deficiency, then the rest of the cancer-bearing population is being under-treated. If radio- or chemosensitive individuals could reliably be detected prior to cancer treatment, then they could be given treatments suitable for their higher sensitivity while the repair proficient patients could be given higher dose treatments to improve their probability of cure [88].

Thus, although we have ample reason to expect that the human population has many genetic reasons for a range of levels of increased risk of cancer induction by environmental carcinogens and ionizing radiations, and that this should lead to individual variations in sensitivity to genotoxic agents, there is a paucity of human data showing what fraction of the population is at what level of increased risk (or what the shape of the distribution curve is), who needs extra protection, and from what. We have much research left to do.

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