Predicting Normal Tissue Complications In Radiotherapy

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Introduction
Patient-to-patient variations in the radiosensitivity of normal and neoplastic tissues are well known and recognized both in vivo(12) and in vitro studies. The typical example is the autosomal recessive disease ataxia-telangiectasia (AT). ATM patients are extremely sensitive to ionizing radiation. The patients develop severe to fatal radiation reactions when treated by standard radiotherapy regimens. Between those and normally sensitive patients, there is a wide range of radiosensitivity. These differences in the response to treatment raised the possibility of individualizing the dose prescription. Therefore, considerable interest in the development of predictive assays have emerged. The predictive test should have acceptable accuracy to enable screening between patients on the basis of their radiosensitivity in order to tailor the radiotherapy treatment to their expected clinical response.

Even though predicting the tumor response to radiotherapy has been attempted by many studies, this review will be limited to studies that are concerned with predicting the radiation effects on normal tissues. The tolerance of normal tissues is what actually constitutes the limiting factor for dose escalation in radiotherapy. Also, the concept of tumor resistance is relative since the tolerance of normal tissues prevents increasing the radiation dose to control the tumor. Therefore, a better knowledge of normal tissue tolerance would appear critical in the search for improving the efficacy of radiotherapy and to increase the therapeutic ratio. The objective is to tailor radiotherapy treatment to each individual patient's radiosensitivity. Radiation doses could be reduced for the small radiosensitive subset of patients (about 5%) and increased for the more resistant once. This dose modulation is expected to increase local control while reducing radiotoxicity or at least keeping it to an acceptable level. Although many factors such as the volume of normal tissue involved in the irradiated area, the total dose received, the fractionation regimen, age, medication, the presence of an associated disease (anemia, diabetes, hypertension, etc.), and chemotherapy could influence the severity of reactions to radiotherapy, large parts of inter patients variability remain unexplained. Studying breast cancer patients, Turesson et al. (9) estimated that such factors would only account for 30% of the total patient-to-patient variability. The remaining variability has been attributed to individual differences in cellular radiosensitivity, partly determined by genetic variations and partially by unknown epigenetic factors.

Radiosensitivity and predictive assays
The evaluation of normal tissue radiosensitivity of cancer patients before the start of treatment could improve radiotherapy results by prescribing the optimal dose for tumor cure without exceeding normal tissue tolerance. Although the relationship between acute and late normal tissue reactions is a matter of controversy, late effects are really the dose-limiting factor in radiotherapy because they are irreversible and usually impair quality of life. These include for example, fibrosis, telangiectasia, necrosis, fistula formation, non-healing ulceration, and damage to specific organs, such as spinal cord, lung, blindness, etc. The mechanisms of these phenomena are not fully understood, however cell depletion of the tissue renewal units seems to be an important factor. Cell to cell communication may also contribute to the spread of these reactions. On the other hand, acute effects of radiotherapy such as reactions of the skin or oral mucosa are now of minor importance as modern computer-aided treatment planning techniques and the use of linear accelerators have led to lowering radiation doses at
Many studies examined the beam entrance site. In addition, these reactions are usually noticed during the radiotherapy course and treatment plan is adjusted in order to minimize them. Acute radiation damage also tends to heal as soon as treatment ends.

Rare reports of patients for whom radiation doses were adapted to their normal tissue radiosensitivity can be found in the literature. Hali et al. (39) reported an 11-year-old boy with medulloblastoma and AT. The radiosensitivity of the patient's lymphoblastoid cells was measured in vitro and found to be 3 folds more sensitive than the normal. There fore, the patient was treated using one-third of the normal dose of standard radiotherapy regimen. Following treatment, the tumor regressed completely and acute and late normal tissue reactions were within the normal limits observed in non-AT patients. Another case was reported by Alsbeih et al. (38) who described a 3-year-old patient with astrocytoma associated with a family history of radiosensitivity and a chromosomal fragility syndrome not related to AT. The radiation oncologists were prompted to reduce the radiation dose to about 80% of what is usually prescribed in a standard protocol. The patient tolerated the treatment very well with no side effects. Studying skin fibroblasts in-vitro evidenced the presumed hypersensitivity of the patient since they showed 2 fold increase in sensitivity to radiation as compared to normal. These two reports show that severe reactions can be avoided in hypersensitive patients by reducing the dose with no obvious deleterious consequences on tumor control. In contrast, many reports described patients who developed fatal reactions following unadapted radiotherapy treatment. (32-34) This is in addition to the more frequent cases of patients developing serious late radiotoxicity. Furthermore, some hypersensitive patients could manifest severe acute reactions during the course of radiotherapy that necessitate interruption of treatment. Conversely, it has been estimated that a significant proportion of patients would be more radioreistant than the average. Those patients would tolerate higher doses that would translate to better tumor control. (11,12)

A predictive assay will be based on taking a biopsy of normal cells from cancer patients prior to the start of the radiation treatment and determining their radiosensitivity in vitro. The treatment can then be adjusted based on the degree of sensitivity of these normal cells. The choice of cell type to be used in an assay is important. Three cell types have been examined: skin fibroblasts, peripheral blood lymphocytes and keratinocytes. Fibroblasts and lymphocytes from hundreds of patients have been studied. Only fibroblasts gave reproducible results and a higher degree of correlation with the clinical outcome. In fact, fibroblasts are an important constituent of connective tissue, which is ubiquitous in the body and always involved in the irradiated areas.

Therefore, fibroblasts are good candidate cells to evaluate the general radiosensitivity of normal tissue and to compare between individuals. They are easy to establish and to culture in vitro. Their role in radio-fibrosis is uncontestable and illustrated by their scarcity in the irradiated field, which leads to slowing down the renewal of collagen molecules and their incomplete resorption. The accumulation of collagen molecules favors their crosslinking leading to loss of elasticity and the formation of fibrosis. (16)

Measuring Radiosensitivity by Clonogenic Assay

When tested in-vitro, fibroblasts from normal individuals and patients with a variety of genetic diseases show a wide range of intrinsic radiosensitivity. (15,28) Many studies examined a possible correlation between the in-vitro radiosensitivity, as measured by clonogenic assays, of skin fibroblasts and the clinical expression of normal tissue complications after radiotherapy. (11,29,32)

Although a general consensus is premature at this stage, (36) it could safely be concluded that late complications of radiotherapy are associated with increased fibroblast radiosensitivity in-vitro. Concerning acute reactions, there may be an association in the extreme cases of sensitivity. Rather unanimously, all the retrospective studies showed such a correlation or a trend toward a correlation. (92) However, prospective studies showed mixed results. (33,34) Even so, there is in the literature enough positive evidence to conclude that genetic differences in cellular radiosensitivity contribute to the extent of normal tissue reactions to radiotherapy. The other important conclusion is that the actual measurement of radiosensitivity using clonogenic assay has little chance of working in large-scale clinical screening. It is time consuming and very imprecise to be used with confidence as a predictive assay. Thus, a different approach is required if the goal of predicting normal tissue response is to be achieved.
DNA Double-strand Breaks Ds Bs
Determinant of Radiosensitivity

Since the early days of radiobiology, DNA damage has been recognized as the most important injury inflicted by ionizing radiation on living cells. Between the different types of DNA damage induced, double-strand breaks (dsbs) are those most associated with biological consequences. Unrepaired dsbs are likely to impair cell survival and if rejoined incorrectly can give rise to chromosome rearrangements and deletions. Therefore, they can potentially cause cell lethality, mutagenesis and carcinogenesis.

Early attempts to correlate the radiosensitivity of human cells to DNA dsbs have used the neutral filter elution technique (NFE). Normal and tumor cell lines were tested but mixed results were obtained, mainly because of non-reproducibility related to technical biases. DNA dsbs were then studied using the pulsed-field gel electrophoresis (PFGE) technique that quickly replaced NFE. More consistent results were obtained particularly with normal human cells where radiosensitivity was correlated with the DNA dsbs remaining unrepaired 24 hours after radiation. As an example, the radiosensitivities of AT homozygous and AT heterozygous (ATH) fibroblasts have been differentiated from each other based upon the number of residual lesions remaining after low dose rate irradiation that totaled hundreds of grays. These results also correlated with the survival data generated on these same cell lines. A number of studies evaluated the relationship between normal tissue reactions to radiotherapy and dsbs induced in genomic DNA. Although these studies have linked residual dsbs with cell survival and/or late radiotoxicity, the assay lacked the required sensitivity to distinguish with confidence between sensitive and normal cell lines and therefore it cannot be used as a predictive assay. In addition, the assay measures the total dsbs but does not give any idea about the quality of the rejoined (i.e., ligated) ends. Therefore, classical PFGE results were only regarded as indicative of DNA dsbs repair deficiencies in comparative studies. Similar conclusions were reached using the Comet 41 and the micronuclei assays.

DNA Repair Mechanisms and Cellular Radiosensitivity

The observation that some radiosensitive cell lines sustain a higher level of residual DNA dsbs after irradiation lends support to the hypothesis that genetic defects in DNA repair mechanisms could underline the causes of differences in radiosensitivity between cell lines and between patients. In fact, a number of mammalian cell lines have been shown to be sensitive due to defective repair of dsbs and, where radioresistance was restored by genetic means dsb rejoining function also returned. Recent advances in molecular biology allowed the identification of important genes that contribute to human radiosensitivity and predispose to cancer such as ATM, NBS, hMREII, LIG4, BRCA1 and BRCA2. The products of these genes are involved directly or indirectly in DNA repair. For instance, a subset of patients with the severe combined immunodeficiency (SCID) condition, attributed to a V(D)J [variable(division)joining] recombination defect, demonstrate also an increased radiosensitivity in vitro. These advances allow predictive assays based on DNA repair to be carried out at the mechanistic levels by trying to identify the protein(s) responsible for the defective signals or repair pathways that could lead to alteration in radiosensitivity. There are two major pathways for DNA dsb repair that have been identified in human cells: non-homologous end joining (NHEJ), and homologous recombination (HR). Non-homologous end joining (NHEJ) pathway is considered the major pathway of dsb repair in mammalian cells. Repair is achieved without the presence of extensive homology between the DNA ends to be joined. One major player in this repair pathway is the DNA-dependent protein kinase (DNA-PK). It is a primary DNA damage recognition system and it is expressed in constitutively high steady-state levels. Defects in the DNA-PK components confer radiosensitivity and dsb repair deficiency as was demonstrated in rodent mutant cell lines. Cells from radiosensitive, cancer-prone BALB/c mice showed a significant reduced expression level of the catalytic subunit of DNA-PK (DNA-PKcs) as well as a lowered DNA-PK activity level accompanied by inefficient end joining of dsbs as compared with cells from all of the other common y use strains. The other repair system is known as homologous recombination (HR) pathway. Cells are believed to perform mitotic recombination and preferentially repair dsbs by HR in late S and G2 phases of the cell cycle when an undamaged sister chromatid is available. In yeast, HR requires the Rad51, Rad52, Rad54, and Rad59 proteins in addition to Rad50, Mre 11 and Xrs2 which are also important. All these proteins have mammalian homologues.
Human RAD51 forms discrete foci in the nuclei of cells exposed to chemicals or ionizing radiation (but not UV). Over-expression of hRAD51 in human cells leads to a 2-3-fold increase in gene targeting (recombination between exogenous DNA and homologous chromosomal loci) and an enhanced resistance to ionizing radiation. Human cells are known to express two other RAD51-related proteins (XRCC2 and XRCC3) that interact with RAD51 and influence dsb repair by HR.50 Human RAD52 has a DNA double-strand break binding activity and it probably co-operates with hRAD51 in dsb repair.

Other proteins involved in DNA repair
Mutations in the ATM gene are responsible for the inheritance of the extremely radiation sensitive disease ataxia telangiectasia. AT is an autosomal recessive disorder characterized by a variety of clinical symptoms and cellular abnormalities. In normal cells, the ATM protein is recruited to the site of the DNA dsbs and acts as a sensor for DNA damage. The recruitment may be activated by an unknown mechanism and induce a signal to stabilize p53 which involves direct phosphorylation on serine 15 by the ATM, thereby regulating cell cycle, DNA replication, apoptosis, and DNA repair. The fact that AT homozygous patients can readily be identified by the Physicians and that their cancer treatment is usually tailored to their radiosensitivity makes AT heterozygosity more important in terms of predictive testing for radiosensitivity. It has been estimated that approximately 1% of the general population could be heterozygous for ATM gene mutations.

AT heterozygotes are clinically asymptomatic, though they display in-vitro different levels of radiosensitivity when compared with normal individuals, although not as pronounced as in AT patients. Accordingly, it may be expected that a substantial proportion of radiosensitive patients could be AT heterozygotes. Many groups of researchers studied this possibility particularly in breast cancer patients. Although these studies did not exclude a causative role for AT heterozygosity in the radiosensitivity of certain breast cancer patients and the risk of developing breast cancer, it would appear that the proportion of AT heterozygotes is less than the expected 4%.

However, the importance of the ATM protein in the radiosensitivity to ionizing radiation and its multi-interactions with other proteins put the ATM protein at the front of candidate causes leading to differences in radiosensitivity between radiotherapy patients. Cells from patients with the rare autosomal recessive disorder called Nijmegen breakage syndrome (NBS) have also been found to be hypersensitive to radiation. NBS 1 protein (also called Nibrin) is lost in NBS patients as judged by Western blotting. Stewart et al. described mutations in hMRE 11 gene located at 11q21, but not in ATM in certain individuals with a disorder virtually indistinguishable from AT. The cellular features resulting from these hMRE 11 mutations are similar to those seen in AT as well as NBS and include hypersensitivity to ionizing radiation, radioresistant DNA synthesis, genetic instability, and abrogation of ATM-dependent cell cycle checkpoints. The hMRE 11 hRAD50/ NBS1 complex has been proposed to act as a sensor of DNA damage.

The majority of hereditary forms of breast and ovarian cancers can be accounted for by germline mutations in two human breast cancer susceptibility genes, BRCA1 and BRCA2. These are involved in DNA repair in addition to other functions. Loss of functional BRCA1 results in moderate sensitization to radiation and DNA-damaging chemicals. BRCA2 cells are also sensitive to DNA-damaging agents, although more pronounced to ultraviolet (UV) than γ-radiation. Although BRCA1 and BRCA2 cells showed DNA repair deficiency and increased radiosensitivity in vitro the presence and the impact of BRCA1 and BRCA2 protein alterations (expression, activity) on patient sensitivity to radiotherapy in breast, ovarian and probably other malignancies remain to be explored.

The tumor suppressor protein p53 plays a central role in controlling the cellular DNA damage response following exposure to DNA-damaging agents such as ionizing radiation, UV radiation and DNA alkylating agents. It determines the fate of cells in which dsbs persist. Alterations in the p53 gene may occur as germline mutations in some cancer-prone families as part of Li-Fraumeni syndrome, and also as somatic mutations in about 50% of human tumors. p53 is a transcription factor that binds to sequence-specific sites in the promoter region of several genes, such as WAF1/CIP1, mdm2, WIP1, GADD45, bax and IGF-BP3. The transcriptional activation of these p53 target genes is associated with cell cycle arrest, DNA repair or apoptosis.
number of cellular genes with promoters lacking p53 binding sites, such as c-fos, c-jun, Rb and bcl-2. (68) Finally, several other gene products such as c-Abl, IRF-1 and ING1 (p33)—have recently been shown to be involved in the p53- mediated DNA damage response to ionizing radiation. Mutations in these genes can confer sensitivity to ionizing radiation or predisposition to cancer or both. Therefore, several of these genes may also be the underlying cause of sensitivity in at least a proportion of the radiosensitive patients and need to be investigated in the near future.

Conclusion
The idea of developing an assay for predicting normal tissue complications in radiotherapy is under intensive research in many laboratories around the world. An ideal assay is that which will avoid the drawbacks of the classical clonogenic survival method, including mainly the time consumption. Advances in molecular biology have introduced techniques that can potentially facilitate the development of an assay which can be used in routine radiotherapy. Hopefully, the mechanism(s) responsible for the increased radiosensitivity and risk to develop severe complications will be identified, in addition to the ability of screening patients according to radiosensitivity.

References:


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