IAEA-TECDOC-1297

Predictive assays and their role in selection of radiation as the therapeutic modality

INTERNATIONAL ATOMIC ENERGY AGENCY IAEA

July 2002

The originating Section of this publication in the IAEA was:

Applied Radiation Biology and Radiotherapy Section
International Atomic Energy Agency
Wagramer Strasse 5
P.O. Box 100
A-1400 Vienna, Austria

PREDICTIVE ASSAYS AND THEIR ROLE IN SELECTION OF RADIATION AS THE THERAPEUTIC MODALITY

IAEA, VIENNA, 2002
IAEA-TECDOC-1297
ISSN 1011–4289

Printed by the IAEA in Austria
July 2002

FOREWORD

Radiation therapy is a modality to treat cancer patients using ionising radiation. According to recent studies by the World Health Organization, cancer incidence in the world is increasing rapidly in both developed and developing countries. About 9 million new cancer cases are recorded each year; about 5 million of these in the developing world. Generally, half of the cancer patients receive radiation treatment for either curative or palliative intent. Radiation therapy is used, often in combination with other modalities.

Ionising radiation kills cancer (or malignant tumour) cells and hopefully cures the patient. If some cancer cells survive the treatment, those cells would ultimately multiply again and eventually kill the patient. Although radiation is intended to focus on the cancer, irradiation of the normal tissues is unavoidable. The higher the radiation dose given to the cancer, the greater the chance of eradicating it. On the other hand, the greater the radiation dose, the greater the probability of severe morbidity or side effects. Thus, optimising the dose for the patients is crucial.

Human cancers have variable radiation sensitivities. Many factors influence the sensitivity. These factors include simple parameters such as tumour size, cellular sensitivity, such as repair capacity, and tumour environment, such as oxygen content. If we can predict the radiation sensitivity of the individual tumours prior to radiation therapy, or even during the radiation therapy, it would give us valuable information with regard to determining the optimal dose. Such an approach has led to the search for predictive assays. Recent advances in molecular technology and equipment have facilitated progress in this field. Some of the studies relied on tumour tissues taken by biopsy, while others focused on cell cycle parameters, which could suggest optimal fractionation schedules.

The IAEA's sub-programme on Applied Radiation Biology and Radiotherapy aims to assist Member States in establishing or upgrading radiotherapy facilities to contribute effectively to cancer treatment for palliative or curative purposes and to provide assistance towards the enhancement of radiation-induced therapeutic gain. The Co-ordinated Research Project (CRP) on Radiation Responsiveness Criteria for Human Tumours as Determinant for Therapeutic Modality Planning was initiated in 1992 to address this problem. This publication was assembled as a monograph based on the CRP results. It reviews the current status of this field. Some information about the cost of analyses and the stage of research is also provided.

Although less widely discussed as part of this CRP, normal cell radiosensitivity is an area of predictive testing which has considerable overlap with determinants of tumour response to radiotherapy. Many of the assays and endpoints used in studying tumour radiosensitivity can be used to measure normal cell radiosensitivity which is of continuing interest in the future.

The IAEA officer responsible for this technical publication was H. Tatsuzaki of the Division of Human Health. Dr. K. Skov, Canada, and Dr. W. Dobrowsky, United Kingdom, assisted with the compilation. It is hoped that the publication will both be helpful to the researchers in Member States and also contribute to health-welfare in Member States through the effective applications of radiation.

EDITORIAL NOTE

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CONTENTS

1. OVERVIEW OF PREDICTIVE ASSAYS FOR RADIATION RESPONSIVENESS

1.1. The need for predictive assays

The reason for the investigation of predictive assays in clinical oncology is the potential role they could have to select patients individually for tailored therapy thereby optimising treatment for a higher probability of cure.

The prognosis of each patient is determined by various factors. Firstly, the general condition of each patient characterised by factors such as performance status, age, gender and presence of other concomitant non-malignant diseases, is of great importance. The outcome of the patient is further determined by the status of the tumour. Tumour characteristics influencing the prognosis of the patient include the stage (TNM), histology, primary site of the tumour and response to therapy (radiation therapy, surgery, combined modality therapy) and the natural history of the disease. The outcome will ultimately be determined by the form of therapy or the combination of strategies and order of therapy, as well as by the skills of the personnel who administer the treatment.

The aim of radiation therapy is to contribute to local tumour control. This can be achieved either by treatment of primary tumours or by adjuvant treatment, where mostly surgery is the main form of therapy. Local therapies clearly determine the outcome in tumours which are locally invasive, without a high incidence of distant metastases. Tumours of the head and neck region, cervical uterine cancers and brain tumours are examples of such tumour sites. Even in cancers regarded as prone to distant metastases such as breast and rectal cancer, it has been shown that local radiation therapy reduces local recurrence which has a major influence on survival. This leads to the question of how the patient is best treated by means of local radiotherapy, and how to optimise this treatment.

A number of factors determine the local tumour control after radiation therapy. The question of the extent to which it is necessary to be able to identify various predictive factors for response to radiation therapy and how this affects the consequences arises. The importance of predictive assays can easily be demonstrated by the following example: If in a trial, a strong prognostic or predictive factor is ignored or unknown and unevenly distributed, significant effects of therapy might be overlooked or concealed. On the other hand, conclusions may be made with regard to the benefit of a certain therapy when this observed benefit is actually due to different distribution of a predictive factor in the treatment groups. It should be noted that gains in clinical trials are often smaller than the effect of some known predictive factors.

The ultimate goal of predictive assays must be the change of treatment policy in patients with less favourable features or changes that circumvent the negative parameters. If, for instance, a patient is found to have a low haemoglobin value and to have a tumour expected to respond favourably to radiation therapy, then anaemia might contribute to recurrence due to radioresistance of hypoxic cells in the tumour, since it is well known that tumour oxygenation is partly dependent on the haemoglobin status. In this example, a transfusion of erythrocytes, treatment with erythropoietin or combination of radiation therapy with hypoxic cell sensitizers or cytostatics, predominately toxic to hypoxic cells, could be of benefit.

For many years, different forms of atypical fractionation have been tested by various centers. The identification of which fractionation scheme would ultimately be of importance for each individual, not only for local tumour control but also with regard to early and late radiation effects in normal tissue, would be an advantage.

Patients who have been found to have a more radioresistant tumour may benefit from additional therapy (surgery, combined modality therapy) or other forms of radiation therapy such as brachytherapy, high linear energy transfer (LET) radiation modalities or dose intensification. In tumours which are usually treated by radical surgical operations, resulting in a degree of mutilation, those patients who have radiosensitive tumours could be identified, a different treatment policy and more organ preserving therapy could be applied.

Many biological factors which have value in predictive tests for radioresponsiveness have been identified. Whether all these factors are useful in clinical oncology is questionable. A panel of tests might be of more value than a single probe. Ultimately, for clinical use, the cost-effectiveness of the investigation will have to be determined.

Intensified research on the mechanisms of radioresistance is necessary to elucidate the potential of predictive assays in the optimisation of radiation therapy. The reduced radiosensitivity under hypoxic conditions is well known and accepted to be a major cause of failure in radiation therapy of malignant tumours. Studies on intrinsic and cellular radioresistance, tumour heterogeneity, and biochemical modulation of radioresponsiveness will further contribute to understanding in this field. These and others factors are addressed in the first part of this monograph.

Experimental and clinical oncologists must further interact in future research in this field which potentially can be of great benefit for patients. Biological parameters applicable to clinical radiation oncology are expected to provide more accurate information on the prediction of radiation sensitivity and provide methods for individual optimisation of treatment.

1.2. Biological factors determining tumour response to radiotherapy

There are three important radiobiological factors involved in determining tumour response to radiotherapy: cellular radiosensitivity, hypoxia and proliferation. The clinical relevance of these parameters is currently receiving considerable attention. Studies have been published suggesting the potential of all three as prognostic factors for radiotherapy. Interest in tumour radiosensitivity follows reports from the Institut Gustav-Roussy [1,2], confirmed by the group at the Institute for Cancer Research [3], that there is a correlation between the ability to control various classes of tumours and parameters that describe the initial portion of the *in vitro* cell survival curves. These parameters are the surviving fraction at 2 Gy (SF_2), the initial slope of the survival curve (α) and the mean inactivation dose (ö). Experimental studies have subsequently supported these observations and have shown that, in animal models, SF_2 measured *in vitro* can predict tumour response to *in vivo* irradiation [4,5].

There is general agreement that not only tumours, but apparently also normal individuals, differ in their intrinsic radiosensitivity. This was initially supported by the discovery in 1975 of the correlation between cellular radiosensitivity of skin fibroblasts and severe reaction to radiotherapy in an individual with the genetic disorder ataxia telangiectasia (A-T) [6], and later observations on a small number of patients suffering severe normal tissue damage following radiotherapy [reviewed in 7]. Significant differences have also been demonstrated in the *in vitro* radiosensitivity of cultured skin fibroblasts [8] and peripheral blood lymphocytes [9] of normal individuals. (see also section 2.6; cancer susceptibility genes) There have now been several independent studies that have shown a correlation between the in vitro radiosensitivity of skin fibroblasts and the severity of complications following radiotherapy in the patients from whom the cells were cultured [10,11].

It is also generally recognised that the rate of tumour cell proliferation can be a major factor in determining the success of fractionated radiotherapy [12,13]. For example, an extension of overall treatment time leads to a reduction in local control levels for T3 and T4 larynx cancers

[14,15]. The ability to measure the rate of tumour cell proliferation accurately and rapidly may be of predictive value. Tumour cell proliferation kinetics have been shown to be prognostic for several types of cancer treated by surgery [reviewed in 16], in particular breast carcinomas [e.g. 17]. The methods used have been either thymidine-labelling index (LI) or flow cytometry to determine the proportion of cells in S phase (SPF). The former is tedious and time-consuming, and the latter may overestimate proliferative capacity as there is evidence that cells may become arrested in phases other than G_1 [18]. Gratzner's [19] development of a monoclonal antibody to halogenated pyrimidines iododeoxyuridine (IUdR) and bromodeoxyuridine (BUdR) allowed the establishment of a rapid technique for the simultaneous measurement of DNA content in tumours, labelling index, the duration of S phase, and, thus, tumour potential doubling time (Tpot) [20,21]. A wide range of proliferation rates have been reported in human tumours, even within the same histopathological type [22,23]. Animal studies, however, have not shown a clear relationship between pre-treatment measurements of Tpot and either tumour cure [24] or effective tumour doubling time [25]. A recent meta-analysis suggested that the flow cytometric measurement of LI following IUdR/BUdR may be a better than Tpot in predicting the outcome of radiotherapy [26].

Following considerable experimental evidence that hypoxia causes radioresistance, there is evidence that hypoxia in human tumours influences radiation response. Clinical studies have shown that reduced blood haemoglobin levels are associated with reduced local control following radiotherapy [27]. In addition, improvements in local control have been achieved using hyperbaric oxygen [28] and radiosensitizers [29,30]. More recently direct measurements have been made of the levels of tumour hypoxia using oxygen electrodes and the Eppendorf histograph. Results from these studies have shown that pre-treatment hypoxia in tumours is significantly associated with a reduced probability of survival following radiotherapy and surprisingly, after surgery [31].

1.3. Resistance mechanisms (intrinsic radiosensitivity)

The inherent radiosensitivity of a cell has been defined by many different factors. *In vitro* studies suggest that three factors produce most of the variation seen in tumour cells: DNA repair, cell growth characteristics, and genome instability. It has been suggested that one or more of these may be related to increased radioresistance at low doses which follows hyper radiosensitivity.

1.3.1. DNA repair

There is a large body of evidence to suggest that a primary factor underlying both histology-based differences in sensitivity and inter-individual differences in sensitivity is the alteration in the repair of DNA double-strand breaks [32, 33]. While early studies reported correlation between the initial break frequency and radiation sensitivity [34, 35], these were likely to have been an artefact of the assays used to measure break frequency [35]. In 1988, the first observations were published that suggested a relationship between radiosensitivity and break rejoining rate [32, 33]. More radiosensitive cells rejoined breaks more slowly. Recently, there have been reports that radiation sensitivity also corresponds with the residual levels of DNA double-strand breaks [33, 36]. The more sensitive cell lines show higher levels of break frequencies when measured up to 4 h after irradiation. Thus both slower overall rates of break rejoining and higher levels of residual breaks are associated with more radiosensitive phenotypes. Alterations in DNA break rejoining influence chromosome aberration frequency and thereby overall survival [33]. The variations in DNA repair may reflect alterations in chromosome structure or DNA protein interactions [37].

1.3.2. Cell growth characteristics

The cell growth characteristics are another key factor in the response to radiation. Both the distribution of cells in different phases of the cell cycle and the rate of cell growth exert an influence on radiation sensitivity [38]. Cell growth characteristics are defined in part by cell cycle checkpoint control mechanisms [39]. Cell cycle checkpoint controls, the processes that controls cell progression through the cell cycle, are often deregulated in tumour cells. One of the key mediators of G_1 cell cycle checkpoint control is p53. Approximately half of all solid tumours have mutations in p53. Inactivation of p53 is associated with a loss in G_1 cell cycle checkpoint control [40] (see also section 2.7). Normally, cells arrest at the G_1/S border following exposure to radiation. In p53-deficient cells, cells continue to progress into S phase following radiation exposure. In general, loss of p53 is associated with a more radioresistant phenotype, but there appear to be many exceptions where loss of p53 either has no effect on radiation sensitivity or is associated with a more sensitive phenotype [40]. Some recent studies suggest that the effects of alterations in p53 are highly dependent on the growth conditions of the cell [41]. The G_2 cell cycle checkpoint control may also play an important role in response [39]. There are wide variations in G_2 cell cycle checkpoint integrity in tumour cells, and in some cases, there are correlation between G_2 checkpoint integrity and radiosensitivity [39]. Attenuated G_2 checkpoint control is associated with a more radiosensitive phenotype. Many additional approaches and references can be found in the annex.

1.3.3. Genome instability

One hallmark of tumour cells is that they show genome instability which is characterised by chromosome rearrangements, additions and deletions, and alterations in DNA ploidy [42]. The relationship between radiation sensitivity and DNA ploidy has been extensively studied [reviewed in 32]. Most investigators report that diploid tumours show better responses to radiation therapy than aneuploid cells [35], although, as with studies on cell cycle checkpoint responses, there appear to be many exceptions where no relationship between ploidy and response is found. Similarly, in vitro studies on the relationship between radiation sensitivity and chromosome content do not consistently show a relationship between chromosome content and radiosensitivity.

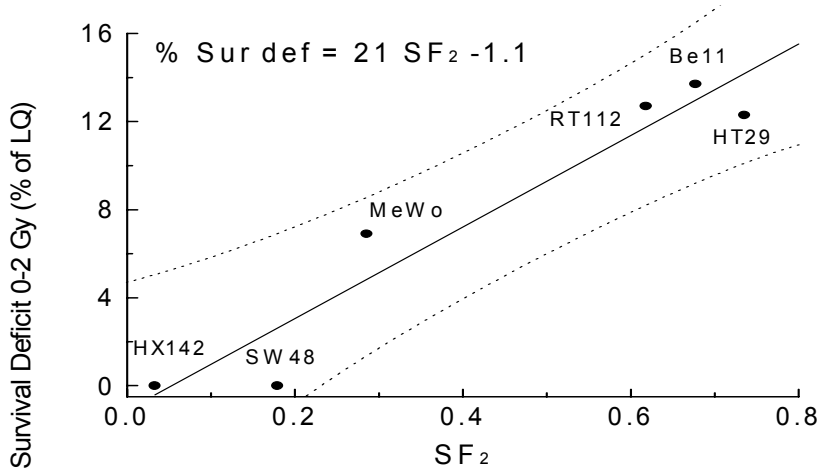

FIG. 1. The relationship between intrinsic radioresistance (SF_2) and extent of hypersensitivity or IRR, measured as the "deficit" from the linear quadratic fit, as presented by Lambin et.al. [46], with results from various human tumour lines.

1.3.4. Hyperradiosensitivity (HRS) at low doses

The nature of the effects of low doses of radiation on biological systems and the possibility of induced or increased radioresistance (IRR) has been the subject of several reviews. [e.g. 43]. It is important to note that there is now also clinical evidence for HRS in human skin response [44, 45]. More importantly in the context of predictive assays, there is support for the concept that the IRR is related to intrinsic radioresistance (SF_2) [46] (FIG. 1). SF_2 is the surviving fraction after a dose of 2 Gy, used to compare cell sensitivity, and being explored as a predictive assay [32] (see also section 2.5). The mechanism may involve DNA repair, DNA structure, or cell cycling.

1.4. Conclusions

Both *in vitro* and *in vivo* studies have identified four factors that define the inherent radiosensitivity of a cell: DNA repair, cell growth characteristics, genome instability, and hypersensitivity at low doses. In some cases, measurement of one or more of these parameters has been shown to correlate with tumour response to radiation therapy. However, exceptions to the general relationships have been shown for each parameter that has been studied. One complicating factor is that as radiation sensitivity is defined by more than one variable, the influence of one factor may be masked by differences in other factors. The relative importance of any one factor may be histology-specific.

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2. CURRENT EXPERIMENTAL APPROACHES

2.1. Hypoxia

It was suggested 60 years ago that hypoxic cells are radioresistant [1]; we now know that low levels of oxygen in certain tumours limit the success of cancer therapy. There is evidence of hypoxia in human tumours [2–5]. Furthermore, more recently, compelling evidence implies that hypoxia is related to the aggressiveness of tumours. Thus, modification of treatment may be desirable if the oxygenation status in a given tumour is known. Over the last 2 to3 decades, significant research effort has been put into both developing agents to circumvent the hypoxic problem and means to measure hypoxia/oxygen [6, 7]. Promising technologies have evolved from quite different approaches to the detection of hypoxia (physical, chemical, biochemical) [8, 9]. It is now possible to study the effects of both transient hypoxia as well as chronic hypoxia [10].

The OER, the hypoxic problem in resistance to treatment: possible solutions

Hypoxia has been studied for many years by radiobiologists and radiotherapists, because many cellular responses to ionising radiation show an oxygen effect. In mammalian cells, the

oxygen enhancement ratio (OER) for clonogenic survival has generally been assumed to be ~3[1] for low LET radiation such as X rays or γ rays. Early studies suggested that hypoxia posed problems in the treatment of cancer using radiotherapy — and also with chemotherapy [20–25]. This area, which is a subject of studies in many centres, (e.g. recent conferences on "Chemical Modifiers of Cancer Treatment", a series at which hypoxia has traditionally been the focus [6]) was summarised eloquently by Coleman [7]. Hypoxia is also present in metastases [26]. Over the last decades, approaches to the hypoxic problem have included radiosensitizers and hypoxic cytotoxins.

2.1.1. Radiosensitizers

While 2-nitroimidazoles were expected to be the best sensitizers, most of the drugs developed specifically for this purpose did not succeed in the clinic (misonidazole, metronidazole, pimonidazole and etanidazole). Other analogues, being examined in Japan, may be successful. It has been known for some time that the 5-nitroimidazole, nimorazole, is beneficial and these results are now available [27] (see also [1]). All hypoxic sensitizer studies, *including* those agents now known to be *in*effective were drawn upon, involving over 10,000 patients, showing a 14% improvement in outcome [28]. Thus, to reiterate: hypoxia *is* a problem, and there is a need to find better methods of detection, to select those patients who would benefit, and to use effective radiosensitizers. Alternate treatments include carbogen breathing (+/- nicotinamide), transfusion for anaemic patients, and mild temperature hyperthermia, which has recently been shown to improve tumour oxygenation [29–34].

2.1.2. Hypoxic Cytotoxins

"Hypoxia: the picture has changed in the 1990's" [35] refers to exploiting hypoxia for bioreduction rather fighting it, a result of "the search for specificity" [36]. Manipulation of tumour hypoxia [37] included increasing hypoxia with vasoactive drugs. Concurrently, more effective hypoxic cytotoxins were developed [38] to exploit hypoxia as a solution — a property of the tumour which could be targeted. Early hypoxia-selective agents include Mitomycin C; a newer agent is tirapazamine (SR 4233) [38] which has reached clinical trials [39]. For nitroimidazoles and related nitroaromatics ($ImNO_2$), their "electron affinity" is related to reduction potential which permits their activities as radiosensitizers, hypoxic cytotoxins and hypoxic markers (below):

$$ImNO_2 \xrightarrow{\bar{e}} ImNO_2^{\bullet} \xrightarrow{\bar{e}} \rightarrow \text{binding to cellular components, toxicity in some cases;}$$

$$O_2^{\bullet} \leftarrow O_2 \quad \text{first step reversible in air (selective toxicity/binding in its absence).}$$

$E_{½} \simeq -385$mV for first electron (for $ImNO_2$ = misonidazole, etanidazole)

2.1.3. Aggressiveness

More recent evidence strongly suggests that hypoxia may have further implications, being linked to aggressiveness of tumour cells. For example, in both cervical cancer [40] and sarcoma [41] patients whose tumours were treated with surgery, the outcome was significantly better in

[1] With technical improvements which permit studies at lower radiation doses (below 2 Gy), there is strong evidence for a lower OER [11–13] as had been suggested earlier [14–16] (see also section 5). Electron affinic radiosensitizers also show diminished enhancement ratios at low doses, as measured by three independent methods [12, 17]. Like cisplatin [18], nimorazole is effective at low doses, but misonidazole does not [19].

those whose tumours were relatively well oxygenated! These rather surprising results may be related to the many reports suggesting that hypoxia promotes genomic instability, increases metastatic potential, selects for mutant p53, increases levels of a factor required for angiogenesis, enhances mutation frequency and increases growth factors such as VEGF [42–56] (and others in [6]). This has led to a new chapter in the relevance of hypoxia to treatment of solid tumours — hypoxia is more than the absence of O_2, which has radiobiological consequences (high OER) even for transient hypoxia [10], but it is also a stress which turns on/off proteins and genes [57], and which leads to mutations which seem to favour uncontrolled growth. If it is true that tumour cells exposed to low oxygen levels may become more aggressive, then we need, more than ever, to be able to assess which patients have "aggressive" tumours containing hypoxic cells? — regardless of whether hypoxia is a cause or an effect of this aggressiveness. Finding treatments which will not only eradicate the hypoxic cells in the primary tumour, but also be directed towards the metastatic disease accompanying the aggressiveness caused by hypoxia is imperative. Increasing hypoxia, for example to exploit cytotoxins, may not be an appropriate option if hypoxia causes aggressiveness.

2.1.4. Detection of hypoxia — quantitation of oxygen

Chemical, physical and biochemical approaches have been developed, particularly with a goal of a predictive assay for radioresistance, such as:

— Nitroimidazole markers
— Microelectrodes
— Comet assay

The comet assay is based on the fact that DNA damage caused by radiation is ~3x greater in an oxic cell. This assay was developed and refined for clinical use following FNA (fine needle aspirates) by Olive [59, 60]. Advantages of the comet assay include direct assessment of the O_2 level in the *nucleus* of the cell, and analysis on a cell-by-cell basis. Unfortunately, larger doses than normally used (~2Gy) were needed to perform the assay initially, and many radiotherapy centres are unable to change clinical treatment protocols in response to the assay results. However, greater sensitivity has recently been achieved.

Of many additional methods (Reviews [8,9]), a few examples and references are: positron emission tomography (PET): one assessment of hypoxia prior to treatment using F-miso can provide selection of resistant tumours [61]; technecium agents (Squibb) [62]; MRI (magnetic resonance imaging)(needs high dose of drug) [58, 63], single positron emission tomography (SPECT) [64, 65]; phosphorescence using a Pd-porphyrin [66]; phosphorescence, detection of radioactivity [8, 9]; time resolved fluorescence using a fibre optic sensor (luminescence of ruthenium complex) [67]. Most of the above methods are somewhat invasive (either a drug is administered, a biopsy taken or a probe inserted) except ^{31}P NMR.

2.1.5. Nitroimidazole markers and "measuring hypoxia"

The methods listed here do not give information on tumour architecture. This may be offered by some of the many additional approaches which rely on the selective reduction in hypoxia of agents such as nitroimidazoles ($ImNO_2$) as shown in the schematic in cytotoxins section above. Chapman's group first exploited a tritiated misonidazole for hypoxia detection in tumours [68, 69] in Edmonton; members of this group have continued elsewhere in this field of markers [9, 70, 71]. An isotope directed to hypoxic areas via $ImNO_2$ is proposed for diagnostic purposes [62, 64, 72]. Halogenated $ImNO_2$ are used with PET detection as one non-invasive method [61, 73]; others include SPECT using DNA targeting via a sugar moiety [64, 74] and MRI e.g. using SR4554 [63] (a compound similar to EF5 below). Detection of the adducts to

biomolecules formed in the absence of air has exploited either incorporation of an isotope into the agent (tritium for autoradiography, ^{18}F for PET, ^{125}I, technetium, etc. SPECT) or recognition of reduction products by antibodies raised to certain adducts.

Spatial resolution is possible with the use of antibodies to detect bioreduced adducts [9, 69, 75, 76]. This was refined using a misonidazole derivative [70, 77] or pimonidazole [78]. Another agent (NITP) is progressing well [76], but may suffer from formulation problems. Most recently, a pentafluorinated analogue of etanidazole was approved by the National Cancer Institute (USA) for large scale synthesis, toxicology and Phase I clinical trials. Although the initial trials use immunocytochemical detection with a monoclonal antibody (MoAb) with a fluorescent tag (Cy3) which is invasive like many of the above methods (biopsy or fine needle aspirate), further development will lead to an ^{18}F agent for detection using PET. The agent is currently called EF5[2], and was developed by C. Koch [71, 79]. While EF5 has many advantages over some of the existing agents which have been developed using similar approaches (*e.g.* up to 100-fold higher fluorescence in hypoxic versus aerobic cells *in vitro*; low toxicity; good biodistribution), the agent pimonidazole (pimo, pimonidazole = Ro-03-8799) also has good properties, and has the advantage that it was previously used in patients (as a radiosensitizer), such that the toxicity and pharmacokinetics have been determined in Phase I trials. On the other hand, EF5 offers additional applications using MRI (Siemann, personal communication and presented 1998 [6]) or PET, using the same agent as that for the architectural studies.

The long term goals in this area are to find a non-invasive accurate means to detect and quantitate the extent of hypoxia in a tumour, such that the appropriate adjutant treatment can be given (or not) to treat the primary tumour. If results of studies continue to support the relationship between aggressiveness and hypoxia [40, 41], then such measurement of hypoxia in the primary tumour would be important for planning of systemic treatment as well. In the meantime, it will be necessary to compare the available methods and agents, and to choose the most appropriate ones. As well as the more usual Phase I goals regarding toxicity, minimal effective doses, which site(s) or types of tumours are appropriate, and pharmacokinetics, the additional goals of early trials of such agents are:

— to learn about the architecture of human tumours with respect to hypoxia;
— to determine whether a threshold of O_2 levels matters, or whether the average O_2 level will provide sufficient information. The latter would permit use of external detection by PET, MRI, etc.;
— to find the least invasive sampling method (*e.g.* FNA *vs.* core biopsy) which still gives enough information upon which to base the decisions for treatment;
— to compare with other alternative methods (comets, Eppendorf electrode etc.);
— to determine whether hypoxia is predictive of outcome.

Recent studies in this area includes development of new synthetic routes of the PET precursors [80], targeting EF5 to DNA [81] and quantitation of stain on cell by cell basis [82]. Comparison of two agents such as pimo and EF5 in one or more centres is recommended.

2.1.6. Polarographic microelectrode method to measure oxygen

The use of a polarographic oxygen microelectrode for laboratory and clinical assessment of tumour oxygenation status is now well established. The use of pO_2 measurements to predict radiation therapy response is fast becoming a reality in many clinical centers [2,3,5,34]. This method has also been used extensively to study the effects of hyperthermia [29–33]. Several

[2] **EF5** is 2-[2-nitro-1H-imidazol-1-yl]-N-(2,2,3,3,3-pentafluoropropyl) acetamide.

studies have found that the pO_2 measurement can be the most powerful predictor of radiation response and/or patient survival [83]. Some of the first clinical studies have shown that the majority of tumours with pO_2 reading below 2.5–10.0 mm Hg before treatment resulted in local failure while those with very few low pO_2 values before treatment resulted in higher local control rates [5,34]. The invasive nature of the probe has meant that most studies to date have been in breast, cervix, head and neck, and other accessible tumours. Finer probes have been developed [26,58], as well as less expensive probes.

The computer-controlled pO_2 measurement in multiple locations through tumour tissue has been found to be a valuable tool in predicting radiation response. It can be seen in experimental tumours that the measured pO_2 correlates to the radiation response using endpoints of cell survival and tumour growth delay. One of the most critical parameters in dictating radiation response appears to be the percentage of values below 5.0 mm Hg. In laboratory experiments, tumours with a higher portion of tumour with pO_2 below 5.0 mm Hg have higher cell survival and faster re-growth rates than those with less below 5.0 mm Hg — an indication of the relative amount of very hypoxic areas which exist in the tumours, where any viable cells will be highly resistant to ionising radiation. Many investigators have shown some degree of correlation between the pO_2 and radiation response. Sequential electrode measurements of pO_2 in a single or several tracks through a tumour are not able to directly determine the oxygenation status of the whole tumour mass. However, they can indicate extremely hypoxic areas, which may be sufficient for predicting an increased risk of radiotherapy failure. Although the electrode measures only a fraction of the total tumour volume, initial results indicate that the small cross-section of oxygenation values that can be obtained in therapy planning stages may allow more appropriate therapies to be used, depending on the relative amount of hypoxia believed to be present in the tumours in question. The pO_2 determined with this microelectrode method, however, is not always directly correlated to the radioresponsiveness of the tumours, although it can be used as a 'rule of thumb'. For example, carbogen breathing increases the pO_2 in tumours more than five-fold, but the increase in tumour response to radiotherapy was found to be only marginally significant. Nevertheless, the electrode method has been the only method to date which can be used in this fashion. This method is expected to be useful to elucidate whether an improved oxygenation in human tumour by carbogen, nicotinamide, or mild temperature hyperthermia improves the human tumour radioresponse. The response of human tumours in vivo with different pO_2 to fast neutron therapy is an interesting avenue of research which should be carried out.

2.1.7. Other considerations

Additional factors may need consideration in this area. One example is the radiation response of transient [10] *vs.* chronically hypoxic cells. There are a number of reports which refer to the effects of prolonged hypoxia. For instance, in HeLa cells under normal aeration and extreme hypoxia, a reduced oxygen effect was found for low dose-rate irradiation with cells maintained (and treated) in one of these atmosphere for up to 69 h [86]. In comparison with acutely hypoxic cells, those held under chronic hypoxia were 20–30% less resistant to high dose-rate irradiation [86]. Similar findings were reported for HeLa cells [87], V79 Chinese hamster lung fibroblasts [88], EMT6/SF mouse mammary tumour cells [89], and NHIK 3025 human cervical carcinoma cells [90]. The results of these studies supported the conclusion, first drawn by Berry et al. [87], that at least for certain types of tumour cells "the protection afforded by their hypoxic state is cancelled out by an increased radiosensitivity". Further studies are in progress [91].

Another example is low radiation doses. A hypersensitive region found in mammalian cells also exists in cells irradiated in hypoxia [92]. Thus, the OER is a complex function of dose in this region [93]. This is further complicated by the possibility that hypoxia primes (i.e. turns on the

increased resistance) which is being addressed [94]. Deliberations on the relevance of the possible low OER at very low doses to fractionation suggest that some tumours may not have hypoxic protection as suggested in previous paragraph [95].

2.1.8. Concluding remarks

The evidence for the existence and importance of hypoxia in human tumours is overwhelming [26, 28, 34, 40–57, 83]. Hypoxia is an important prognostic indicator, as well as a predictive indicator for radioresponsiveness. Many approaches are being applied to measure oxygen or its absence [8 ,9]. These methods still need thorough comparison with each other with respect to ease of use, accuracy, predictive/prognostic features, outcome, invasiveness, etc. [26, 59, 60, 84, 85], before it can be stated which is the best as a prognostic/predictive assay. Based on this information, modalities such as nimorazole [28], carbogen, higher LET, adjunct modalities, etc. [29–33], which appear to be successful in counteracting the hypoxic problem, can be used when indicated for a specific patient.

2.2. Thiols

It has been known for many years that the levels of oxygen affect the sensitivity of a cell to ionising radiation, particularly to low LET radiation such as x and γ rays. The effect of the oxygen concentration on endpoints, such as survival, is described by a sigmoidal response called the k-curve. On the other hand, agents containing thiols (RSH), particularly non-protein thiols (NPSH), have received considerable attention due to their protective nature. Two perspectives are:

— addition of thiols as protective agents for the normal well-oxygenated cells,
— removal of endogenous thiols, such as glutathione (GSH), to sensitize the tumour particularly in hypoxic conditions.

Clinical trials in both aspects have been carried out. In particular, in the second area, the competition between oxygen, which enhances fixation of the DNA damage, and thiols, which chemically repair the DNA damage caused by the radiation, has been studied by many researchers (e.g. Koch [96. 97]). Thus the k-curve can be shifted, and the response to radiation affected by thiol levels. These vary with cell line — and can also be manipulated by addition of agents such as N-acetyl cysteamine, which adds RSH; diamide which oxidises RSH; and buthionine sulphoximine (BSO), which inhibits GSH synthesis [97]. In considering the relevance in clinical outcome, attention has focused on GSH levels: e.g. the effect of thiol depletion or addition on the position of the k curves of various sensitizers, including oxygen.

2.2.1. Cysteine

During a study of BSO and etanidazole, it was found that BSO depleted GSH to approximately 1% of normal levels, but increased cysteine by approximately five-fold [98]. As cysteine (CySH) is expected to be a better protector, this may have a more significant impact on radioresponsiveness than GSH. Koch's group in Philadelphia subsequently found very high levels of cysteine in rodent tumours [97, 99]. This had eluded notice because of technical problems in measuring CySH levels — easy oxidation to the cystine form (S-S). However, if tumour samples are removed and immediately placed in cold acid with chelators, the CySH levels can be determined using HPLC and electrochemical detection[3]. While the initial studies were in rodent

[3] Details of the methodology can be found in the Koch papers. However, it seems to be a relevant factor which could be implemented in many centres, as HPLC is often available (Koch uses electrochemistry (Waters 460); C-18 columns Alltech "Adsorbosphere" C-18). The need for strong acid (*e.g.* 50 uM SSA (sulfosalicylic acid) leads to short column life and other technical problems; the chelators aid in preventing oxidation (diethyldithiocarbamate, final conc 50 uM); and biopsy must be placed immediately into cold acid. Standards (25–50uM) in 100 uM DDTC, 0.1 M formic acid, pH 3.5, then diluted in SSA & chelator.

tumours [99], human tumours are being examined in at least two centres. They also exhibit unexpectedly high CySH levels, and further detailed studies will indicate whether there is any predictive or prognostic value.

2.2.2. Glutathione

Glutathione (GSH) is an ubiquitous tripeptide, glutamyl-cysteinyl-glycine, present in most cells with diverse intracellular functions. It is of interest to the oncologist in two aspects: (1) its role in intracellular free radical scavenging and detoxification of xenobiotics, which can influence the sensitivity of normal and tumourous tissue to radiation and anticancer drugs; and (2) its role in DNA and protein synthesis which can potentially influence the growth of tumours. The actual mechanism by which GSH confers radioprotection is complicated. These include radical scavenging, restoration of damaged molecules by hydrogen donation, reduction of peroxides and maintenance of protein thiols in the reduced state. Of these mechanisms, hydrogen donation to DNA peroxyl radicals is probably the most important. GSH is a free radical scavenger both in the presence and absence of oxygen. GSH protects DNA against radiation damage by the same mechanism [100]. There are numerous reports on the relation of intracellular GSH content to radiosensitivity. It is seen that depletion of GSH increases sensitivity of cells to radiation [101]. The liver, lungs and kidneys are the main source of plasma GSH and circulating plasma GSH is an important source for production of intracellular GSH. Radioresistance may be related to the ability to synthesise GSH rather than intracellular GSH levels per se [102]. It is possible that elevated plasma GSH increases the supply of raw materials for GSH synthesis to tumours and elevates tumour tissue GSH. This may be due to active GSH synthesis/turnover in such tumours, and such tumours are likely to be more aggressive. A high plasma GSH content may supply greater raw material for intracellular GSH synthesis. GGT (gamma-glutamyl-transpeptidase), the enzyme intimately concerned in the synthesis and metabolism of glutathione through the gamma-glutamyl cycle, plays a role in the breakdown and intracellular transport of aminoacids. Hence it may be more appropriate to consider tumour GSH in conjunction with plasma GSH, i.e. as a ratio with plasma GSH. High plasma GSH may contribute to tumour resistance also because of other reasons. Radiation is known to induce cell kill by damaging not only the nuclei (i.e. the DNA), but also the cell membrane. The influence of GSH on radiation injury to the membrane is also documented. Glutathione and related enzymes form a part of the system which protects plasma membrane against radiation injury [103]. Exogenous GSH addition, even without increasing intracellular GSH concentration, affords protection against radiation. This suggests that interaction of exogenous GSH with the cell membrane (without penetrating) is sufficient to result in radioprotection [104]. This may be due to the fact that an external membrane component of damage is involved, long-range protection to DNA target radicals is possible from outside the cell (e.g. donation of electrons), and endogenous glutathione is not in a free or exchangeable state (e.g. bound) [105]. It had been observed that the severity of radiation mucositis in oral cancer patients is related to plasma GSH level [106]. GSH content had been evaluated in head and neck cancers and it was observed that advanced stage tumours had higher GSH content than adjacent mucosa [107]. GSH levels in primary breast cancers were found to be higher than in normal breast tissues but no correlation was found with tumour size or lymph node metastasis [108]. Plasma GSH had not been evaluated in either study.

In summary, estimation of thiols in tumour and plasma may provide an exciting opportunity for individualisation of treatment.

2.3. Kinetic factors

The proliferation of human tumours has been the subject of studies aimed at understanding its role in predicting clinical outcome of radiotherapy. However, progress in this area has been hampered by the limitation of measurement techniques and the complex nature of tumours.

Tumours are heterogeneous, in terms of the distribution of cells within the cell cycle. A high proportion of cells within a tumour are in G_0 which is considered to be a radioresistant portion of the cell cycle and the growth fraction of tumours can range between 30% and 80%. In addition, cells may be lost from the proliferative compartment (cell loss) and the degree and nature of cell loss varies between tumours. In order to fully describe the kinetics of tumour growth, estimation of the cell cycle time (Tc) and the duration of S- (Ts), G_2- and M-phase, the growth fraction and cell loss factor are important.

There has been increasing awareness of the fact that rapidly proliferating tumours should benefit from accelerated regimens. This has led to an interest in the assessment of the proliferative rate of tumour cells prior to radiotherapy. In the past, the most widely used method for cell kinetic measurements has been the technique of labelled mitosis which suffered from both technical and ethical drawbacks. The measurement of labelling index (LI), using tritiated thymidine and autoradiography, had the same drawbacks. The measurement of labelling index using halogenated pyrimidine (BUdR or IUdR) incorporation has recently been used in many clinical studies. This method has been shown to be predictive for the radiotherapy outcome in a number of sites.

Over the past ten years, there has been a considerable interest in measuring dynamic cytokinetic parameters of tumour cells as the potential doubling time (Tpot) [109]. Some studies have shown that this parameter can predict radiation response of human tumours [110, 111, 112], but a recent meta-analysis has suggested a limited role for the current method used for measuring Tpot in predicting clinical outcome [113, 114, 115]. Improvements in the existing flow cytometry methods may lead Tpot into a predictive method. In particular, there is no current consensus regarding sample preparation, data analysis and the effect of tumour heterogeneity [115].

There are other methods for measuring tumour cell proliferation. Many groups have considered the S-phase fraction (SPF), that is the number of cells synthesising DNA. Tumour growth fraction can be measured by identifying the fraction of cycling cells using antibodies which recognise important proteins such as Ki-67 (MIB1) or PCNA. The latter, however, are static markers and may not directly reflect active proliferation. Although these methods are useful in assessing tumour proliferation, their significance in predicting radiotherapy response is equivocal in the various human cancers that have been studied. The pMI (based on the ratio of mitotic index to growth fraction of the tumour indicated by MIB1) has also been reported to predict local control after radiation therapy and may have potential widespread clinical application [116].

The BUdR/IUdR technique for measuring LI and the assessment of growth fraction using the MIB1 antibody are currently the best available methods for studying human tumour proliferation in the clinical setting. There is also increasing awareness of the need to study the architecture of proliferating cells within tumours [117].

2.4. Molecular pathology

In general, there is wide variety of radiation response among human tumours of same histology, whereas in some tumours the histological subtype is correlated with the response. Recently, molecular biologic information was introduced to histopathology and cell cycle relating proteins, oncogenes as well as apoptosis relating proteins are known to have various association to radiation response. This molecular pathologic information is obtained mainly by immunohistochemical methods.

2.4.1. Immunohistochemical staining [118]

The S-phase fraction, growth fraction, various oncogene proteins (c-myc, c-erbB-2) and Mn-SOD (manganese superoxide dismutase) expression are correlated with radiation response in some human tumours. For clinical application, biopsy specimens with routine processing in 10% formaldehyde are desirable. Immunohistochemical staining with antibodies for cell cycle related proteins (Ki-67(MIB1), PC10, cyclin D1, p53, p21, p27) and apoptosis related proteins (bcl-2, bax), oncoproteins (c-myc, c-erbB-2), and Mn-SOD are applicable for radiation sensitivity study on human tumours.

2.4.2. Growth fraction

The growth fraction had long been difficult to measure in human tumours, but recently, it has become relatively easily assessed by using the Ki-67 antibody (MIB1). This method will provide much information on the radiobiological behaviour of G_0 cells which are resistant to radiation and are correlated with radiation sensitivity [116].

2.4.3. Cell cycle rate

Rapidly proliferating tumour cells are resistant to radiation. The mitotic index of tumour cells in vitro is usually proportional to the cell proliferation rate, but the index of tumour cells in vivo is biased by the large population of quiescent cells. The mitotic index, specific for the proliferating cell population (pMI) can express the relative cell cycle rate [116]. pMI can be calculated by the mitotic index divided by the Ki-67 growth fraction (Ki-GF) , which was described as:

$$\text{pMI} = \text{Mitotic index} / \text{Ki-GF}.$$

The pMI is strongly predictive for local control after radiation therapy. These methods have practical advantage since they are easy to carry out routinely in the clinic and the information can be obtained in a few days.

2.4.4. Apoptosis: In situ nick end labelling method

Apoptotic cells were detected on conventionally processed histological sections with the in situ nick end labelling method (ISEL®, Apoptag in situ apoptosis detection kit, Oncor [119]). The procedure is similar to the immunohistochemical method. Detection of apoptosis only by morphology using H&E stain is erroneous because of the leukocyte infiltration. To distinguish the apoptosis of tumour cells from the apoptosis of leukocytes, specific stainings for leukocytes, such as antibodies to LCA (Leukocyte Common Antigen) and vimentin etc. are necessary. Apoptosis may be a predictor of radiation sensitivity even though it is not largely involved in overall cell killing of human tumours (with some exceptions such as lymphoma, neuroblastoma etc.). Review of the many methods used to assess apoptosis is beyond the scope of this monograph.

2.5. Celluar radiosensitivity

The largest study looking at tumour cellular radiosensitivity in relation to radiotherapy outcome has been carried out in Manchester [120]. The work has used a soft agar clonogenic assay to examine radiosensitivity of cells derived from primary tumour biopsies. Results are generated within four weeks with a success rate of 70%. Some of the results from West's group are summarised within the proceedings. Briefly, her work has shown that in vitro measurement of tumour cell radiosensitivity is a highly significant prognostic factor for carcinoma of the cervix treated with radiotherapy. Several groups have studied tumour radiosensitivity using standard clonogenic assays on plastic using early passage cell strains. In Edmonton, Canada, a study was

made of the radiosensitivity of early passage strains from gynaecological tumours and malignant gliomas. For patients with carcinoma of the cervix and a minimum follow-up time of 13 months, tumours from 25 disease-free patients had pre-treatment SF_2 values that were not significantly different from values for 8 patients whose tumours recurred [121]. Applying the same method to head and neck tumours and a minimum follow-up of one year, the average D-bar (mean inactivation dose) for tumours from 21 patients with no evidence of disease was no different from that from 13 patients with in-field recurrences [122]. Finally, an analysis has been carried out on 50 malignant glioma cell lines derived from patients from a number of clinical centers and treated by surgery and postoperative radiotherapy with or without chemotherapy [123]. In this heterogeneous study, SF_2 did not predict clinical outcome.

The cell adhesive matrix (CAM) assay, developed at the M.D. Anderson Hospital [124], is the next most widely used in vitro test for measuring tumour radiosensitivity. With the CAM assay, tumour cell adhesion is optimised through the use of a matrix composed in part of fibronectin and fibrinopeptides, and cell growth through the use of hormone- and growth factor-supplemented culture medium. Growth is assessed 2–3 weeks after irradiation by quantitative image analysis of the absorbency of cultures stained with crystal violet. The advantages of this assay are that it is easier, more rapid, more automated, and requires fewer cells than standard clonogenic assays for primary tumour material. Two examples of the early studies using this approach are given here. In an analysis of 40 patients with head and neck cancers treated with radiotherapy and surgery with an average of 23 months of follow-up, cultures of cells from patients who had local recurrences tended to more radioresistant than the population as a whole but the difference was not statistically significant [124]. Girinsky and co-workers [125] determined the radiosensitivity of head and neck tumours treated predominantly with radiotherapy alone. In vitro radiosensitivity measured as α (from the linear quadratic fit) significantly reflected patient outcome when a high (above median) value was used to stratify data . A more recent analysis has been reported on 92 patients with a median follow-up of 68 months and using the quartiles of the value distribution to stratify data. Neither SF_2 or α were prognostic factors for local control or overall survival within univariate and multivariate analyses [126].

There have been other, smaller studies using a variety of assays that have suggested that patients who respond poorly to radiotherapy have tumours cells which are more radioresistant than those from patients who respond well. This has been shown using the MTT assay on early passage cell lines from gliomas [127], the DiSC assay applied to lymphocytes from leukaemia patients [128] and nucleoid light scatter on cells from bladder carcinomas [129]. Many other studies and approaches should be cited but limitation of space precludes a complete review.

2.6. Genetic factors

While cancer is believed to arise from an accumulation of somatic mutations affecting cell growth and behaviour, there are several inherited genes which enhance the susceptibility of individuals to malignancies by altering the cellular response to carcinogenic agents. Nearly 1% of all cancer patients are known to have such genes [130]. Cellular and molecular studies of the ”cancer susceptibility” genes responsible for the rare inherited cancer syndromes have produced a wealth of information regarding the mechanisms involved in the pathways of signal transduction, channelling of DNA lesions for repair, cell cycle regulation, abnormalities in cell growth and the origin of cancer. Mutations in some of these genes are often found to be associated with various tumour cells. An understanding of these properties of ”cancer susceptibility genes” not only helped us gain an insight into the causes of inherited cancer prone syndromes, but also provided very important clues regarding the origin of sporadic cancers. In addition, and of relevance to this

monograph, is the importance of genetic makeup in response to treatment such as radiotherapy [131, 132].

Although the occurrence of cancer susceptibility genes in homozygous states is rare, the (heterozygous) carriers of these genes may often be apparently asymptomatic. This large sector of the population has an increased cancer risk. If these gene carriers develop cancers, their normal and tumour cell response to cytotoxic agents (both chemical and physical) will be affected in a variety of ways. There are two classical examples to illustrate the issue; the cancer-prone inherited disorder Ataxia Telangiectasia caused by the gene now known as ATM, and the cancer susceptible Li-Fraumeni syndrome attributed to mutations in the p53 tumour suppresser genes. The carriers of ATM mutations may comprise as high as 1% of the population, while the frequency of mutant p53 carriers is not yet known. These two genes appear to be concerned with genomic instability via reduced DNA repair and a deregulation of cell cycle control mechanisms. The ATM gene is definitely associated with enhanced radiosensitivity and chromosomal instability, while mutations in p53 have been reported to reduce DNA repair and programmed cell death, to promote uncontrolled cell proliferation and genetic instability (gene amplification) while paradoxically increasing cellular radioresistance and resistance to therapy. An observation of increased radiosensitivity in some bladder cancer lines carrying p53 mutations, however, contradicts the findings of radioresistance attributed to p53 mutations. Nevertheless, both sets of data may suggest that p53 mutations and the level of p53 protein accumulation may have a relationship to radioresistance/radiosensitivity in different tumour cell types. Further studies are needed to clarify the role of p53 in radiotherapy as it will definitely affect the outcome of therapy by either leading to resistance to cell killing and recurrence of the disease, or increasing cell death and local control of tumours. In addition, the normal tissue damage often becomes a limiting factor in radiotherapeutic dose scheduling. If genes such as ATM and p53 which result in enhanced radiosensitivity are carried by some of the cancer patients, normal tissues around their irradiated tumours would show undesirable reactions requiring a modification of treatment protocol, thus, causing an interruption in therapy. As the primary objectives of radiotherapy are to achieve local control of the tumours and to prevent not only undesirable normal tissue reactions but also the occurrence of relapse and second malignancies, it is important to understand the radiobiological response of the patient's normal and tumour cells. Many investigators, therefore, use various radiobiological criteria correlating with specific radiotherapeutic outcome to develop predictive assays. Unfortunately, the results of all such studies only emphasise the need for further research to produce evidence of common denominators predicting therapeutic outcome.

Radiation and most genotoxic/cytotoxic agents induce diverse forms of damage in the cells, triggering a variety of cellular responses which may be controlled by several cancer susceptibility genes including ATM and p53. Two cellular characteristics which are most relevant to cell death and genetic instability after radiation treatments are increased radiosensitivity and cell cycle deregulation [133]. Thus, a molecular screening for genes such as ATM and p53 or their products responsible for such properties would be highly useful in predictive assays. However, many laboratories may not have the necessary personnel or facilities to screen mutations in all sites of the numerous genes known to alter cellular response to irradiation. As most cancer susceptibility genes including ATM and p53 appear to be concerned with either cell cycle deregulation or genetic instability resulting from defective DNA repair and apoptosis, bioassays based on cellular sensitivity to radiation and analysis of post-irradiation cell cycle regulation in normal and tumour cells would be very relevant and feasible for predicting radiotherapeutic outcome.

2.7. Response to treatment

The tumour suppresser gene p53, popularly know both as "the guardian of genome" and "the gatekeeper of growth and division", plays very important roles in determining cellular response to irradiation as well as to chemotherapeutic agents [134, 131, 135]. In the wild type

state, the p53 gene product is involved in the regulation of cell proliferation, DNA repair, programmed cell death and genomic stability. Overall, the significance of these functions of the p53 protein lies in its protection of the cells against genetic/carcinogenic damage. The role of p53 in carcinogenesis and tumour cell proliferation becomes more evident from the observations that mutations in this tumour suppresser gene are frequently associated with most human cancers, and that inherited p53 mutations have been linked to cancer susceptibility (Li-Fraumeni syndrome). Paradoxically, p53 mutations, which deregulate cell cycle by eliminating the G_1 checkpoint and reduce DNA repair processes, have been reported to be associated with increased cellular resistance to irradiation and tumour relapse after therapy [136]. However, data showing either an increase or no change in radiosensitivity of cells due to the loss of p53 have also been reported 137]. Whether or not these contradictory findings are due to an interaction between p53 and other tissue specific factors, the relationship between p53 and radiosensitivity/genomic instability remains an important factor in determining the outcome of radiotherapy. Functional loss of the p53 results not only from mutations in the gene but also the wild type protein can be negatively regulated or inactivated by various mechanisms, including an interaction with tumour viral proteins such as adenovirus E1B, SV40 large T antigen or HPV E6. The p53 protein is also known to be down-regulated by the ATM gene (responsible for Ataxia Telangiectasia) [131], while irradiation of cells enhances its expression, presumably due to an increase in the half-life. Thus, various factors may modulate the function of p53 and thus affect cell survival or death and protective mechanisms of cells against genetic injuries. In view of these roles, the p53 protein is often analysed in normal and tumour cells for its presence and functional quality in order to find its impact on therapeutic outcome. In case of a total abrogation of the protein or its functional loss, attempts are made to detect possible mutations in the p53 gene itself. In this TECDOC, however, only three methods for the detection p53 will be briefly described below. These methods are essentially based on the use of anti- p53 antibodies to detect the p53 protein levels. The methods can be modified based upon the cell types and goals of the studies.

2.7.1. Western blot analysis

Cells are grown to approx. 70% confluence and solubilsed in a lysis buffer (20mM Tris, pH7.4; 250mM Nacl; 1.0% NP40; 1mM EDTA; 50mg/mL leupeptin; 30mg/mL aprotinin and 1mM phenylmethylsulfonyl fluoride). The cells are kept on ice for 30min., harvested by scrapping, sonicated and clarified by centrifugation at 4°C. Protein quantitation is made using a suitable protein assay system. Samples are boiled in SDS Laemmli gels. The gels are transferred onto nitro-cellulose membrane, blocked with 10% milk and incubated for at least 2 hours. with primary anti- p53 antibody. After several washes, the appropriate horse radish peroxides-conjugated secondary antibody is added for 1hr. After final washes, immuno-reactivity of the blots is detected using the enhanced chemiluminescence system.

2.7.2. Immunocytochemical assay

Semi confluent cells grown on chamber slides are rinsed twice with PBS (pH 7.3). Cells are washed and fixed with ice cold acetone for 10min., rinsed 2 times with PBS for 10min. and then the slides are incubated with 0.3% H_20_2 in water at room temperature for 10min., washed again 2 times for 5 min. with PBS before incubating in 0.3% casein/0.05% Tween 20 in PBS (CPT buffer) for 30min. at room temperature. After removal of excess buffer, slides are incubated with anti- p53 antibody (DO-1, which reacts with amino terminal epitopes of p53: other such antibodies can be used) in 1% BSA/PBS at 4°C overnight in a humidifier. Slides are then washed with CPT at room temperature for 5min. and incubated for 30min. with biotinylated antimouse 1gG (1:1000 in 1% BSA/PBS). Slides are washed 2 times with PBS, incubated with 1:1000 dilution of avidin-biotinylated horse radish-peroxidase complex (ABC/HAP) in PBS reagent for 30min., washed 2 times with PBS buffer for 5min. (all at room temperature) and finally incubated with chromogen substrate (0.01% diaminobenizidene tetrahydrochloride). Colour development is

stopped after 5min. by adding water. Slides are then counterstained with haematoxylin, dehydrated in graded alcohol, cleared in xylene and mounted in Eukitt.

2.7.3. Flow cytometric assay

Cells grown to semi confluence are harvested by trypsinization, washed with PBS and re-suspended in 1 mL ice cold PBS. Cells are fixed by droporise addition of 70% methanol. Aliquots containing at least 0.5×10^6 cells are incubated on ice for 1hr. with 1μg/mL of the antibody DO-1 (or other suitable ones), washed 2 times and incubated on ice for 30min. with secondary FITC antibody. Cells are then washed and analysed in a FACScan (Becton-Dickinson). Levels of antigen (p53) expression is estimated by mean of fluorescence intensity.

In all three methods (Western Blot, Immunocytochemical, and Flow Cytometric Assay), a comparison is made between irradiated and unirradiated cells to determine whether the p53 is radioinducible and, thus, functionally active.

It should be pointed out that detection of p53 protein alone may not mean that it is functionally active. In fact, p53 mutations result in accumulation of inactive protein. Therefore, emphasis should be placed on the functional aspect of the protein.

2.8. Micronucleus assay

Micronuclei originate from acentric fragments or whole chromosomes and provide a measure of both chromosome breakage and loss, which is a somewhat different spectrum of damage from that obtained by chromosome analysis [138]. Enumeration of micronuclei in cells allows chromosome damage to be analysed in lymphocytes which have divided only once. This can be achieved in metaphase analysis only when bromodeoxyuridine uptake and differential staining is performed to distinguish between first and second division. Recently, cytokinesis-block (CB) method using Cytochalasin-B as a cytoplasmic division blocker, has been shown to be simple, reliable and very sensitive. This technique does not require highly specialised staff and should, therefore, be readily implemented for routine use [139, 140, 141, 142]. Furthermore, automated scoring of micronuclei would be relatively simpler than automated metaphase analysis and image analysis systems [143, 144].

The characteristics of the dose-response relationship obtained by the CB method for human lymphocytes exposed in vitro to ionising radiation have been evaluated by several researchers. The relationship of micronuclei in CB cells with X ray, γ ray, or β ray doses is linear up to about 2 Gy, which is in contrast to the linear-quadratic relationship usually observed in chromosome-type aberrations [140, 141, 145, 146]. Prosser et al. [146] have demonstrated that up to 1 Gy of exposure the frequency of induction of micronuclei per CB cell was very similar to that observed for the frequency of total aberrations, but beyond 1 Gy the level of aberrations became relatively higher. This could be explained by the fact that at low doses, damaged cells would not contain more than one acentric fragment, and this would have a finite probability of becoming a micronucleus. However, at higher doses, where a damaged cell might be expected to contain two or more acentric fragments, it becomes possible that more than one fragment will be incorporated in a micronucleus, especially if the fragments are very close to each other. This will have the effect of depressing the extent of observable damage, and thus may explain why beyond 1 Gy the micronuclei frequency dose-response remains virtually linear, while the chromosome-type aberrations dose-response shows an obvious quadratic component.

Kim et al. [147] have investigated the dose-response of the number of micronuclei in cytokinesis-blocked lymphocytes after *in vitro* irradiation with γ rays and fast neutrons. Measurements performed after irradiation showed a dose-dependent increase in micronuclei frequency in each of the donors studied. The dose-response curves coincided well with a linear-

quadratic equation and frequencies per 1000 CB cells were (0.31 ± 0.049)D + (0.0022 ± 0.0002)D^2 + (13.19 ± 1.854) following γ-irradiation and (0.99 ± 0.528)D + (0.0093 ± 0.0047)D^2 + (13.31 ± 7.309) following fast neutron irradiation. In the micronuclei frequency between 0.05 and 0.8 per cell, the RBE of neutrons was 2.37 ± 0.17 (Table I). Identification of kinetochores within micronucleus using anti-kinetochore antibodies [148] or centromeres using centromeric probes [149] provides a means of distinguishing micronuclei containing whole chromosomes from micronuclei containing acentric fragments, thus providing a better definition of the endpoint scored. The cytokinesis-blocked micronucleus assay may have the potential to complement metaphase analysis of chromosome for estimating chromosome damage in the tumour cells. Furthermore, it can be used for predicting the radiation response of tumour cells prior to irradiation as one of the predictive assays.

TABLE I. RELATIVE BIOLOGICAL EFFECTIVENESS OF NEUTRONS AND PHOTONS (γ) IN INDUCING MICRONUCLEI IN LYMPHOCYTES

MN per cell	Neutron dose (D_n) required (cGy)	Photon dose (D_r) required (cGy)	RBE (D_r/D_n)
0.05	29.14 ± 1.20	76.29 ± 2.09	2.62 ± 0.13
0.10	57.04 ± 1.47	139.48 ± 2.59	2.45 ± 0.08
0.20	98.13 ± 1.50	228.49 ± 2.76	2.33 ± 0.05
0.40	155.18 ± 3.55	353.80 ± 2.95	2.28 ± 0.06
0.80	242.44 ± 0.55	530.72 ± 3.08	2.19 ± 0.14

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3. SUMMARY TABLES

This publication covers a wide range of material, much of it infrequently used by practising radiation oncologists.

The contributors considered that the following tables would be useful in summarising the information in the text for the purpose of evaluating the current role of predictive assays.

Table II names the predictive assay and provides a brief description of its purpose. The final column indicates if this assay has entered clinical testing at the time of completion of this publication.

Table III addresses the practical constraints (technical difficulty and grade thereof), the delay between collecting a sample and obtaining a result and the costs involved in establishing such a test and variable costs incurred for each test performed.

TABLE II. CURRENT STATUS OF VARIOUS PREDICTIVE ASSAYS

Assay	**Brief description**	**Status (under study/clinical applicable)**
Tumour clonogenic survival (SF_2)	♦ Proof of reproductive integrity, usually in semi-solid agar supplemented with growth factors ♦ Assay of fresh tumour biopsies	Clinical
Tumour growth assay (CAM)	♦ Assay of fresh tumour biopsies for fibronectin- coated plates, using crystal violet	Clinical
Chromosome aberrations (PCC & FISH)	♦ Target cells fused with mitotic cells ♦ Assessment of interphase chromosome malformations	Study
Micronucleus assay	♦ Acentric fragments or aborted whole chromosomes detected by Cytokinesis-block method	Clinical
Apoptotic assay	♦ Quantitative index of radiation injury: Apoptotic body or fragments	Study
Oncogene expression	♦ Alteration in either expression or function of cellular genes like c-erb B-2, p53 expression, ras gene, p21 product, c-myc oncogene	Study/Clinical
BUdR labelling index	♦ Fresh tumour biopsy incubated with BUdR and analysed by flow cytometry	Clinical
Growth Fraction	♦ Heat processed immunostaining with MIB1	Clinical
pMI	♦ Ratio of the Mitotic cells to Ki-67 positive cells	Study/Clinical
Mn-SOD	♦ Paraffin section, Immunostaining with anti-Mn-SOD antibody	Study
Serial Cytology	♦ Real time assay; evaluation of nuclear changes (micro- or multinucleation)	Clinical
Lymphocyte clonogenic survival	♦ Separation of peripheral blood sample and lymphocyte cultured in medium supplemented with PHA and IL2	Clinical
Microvessel density (MVD)	♦ Evaluation of tumour specimens using a variety of stains (CD31, factor VIII)	Clinical
DNA dsb rejoining assay by Pulsed Field Gel Electro-Phoresis (PFGE)	♦ Estimation of amount of residual DNA double strand breaks	Clinical
Biochemical	Determination of thiols (GSH, CySH) in tissue and plasma	Study/Clinical
Polarographic pO_2 Measurement	♦ Microelectrode sequentially moved through tissue	Clinical
Markers	♦ Nitroimidazole binding in hypoxic cells, detected by immunohistochemistry or physical method (eg PET)	Clinical
Comets	♦ DNA breaks are enhanced by O_2	Study/Clinical

TABLE III. TECHNICAL ASPECTS AND COSTS

Method	**Technical difficulties**	**Grade of difficulties (high/low)**	**Time to obtain results (days)**	**Initial cost (US$)**	**Running cost per sample (US$)**
Tumour clonogenic survival (SF_2)	Poor PE	high	28	32,000	200
CAM assay	Success rate 70%	high	21	32,000	400
Lymphocyte clonogenic survival	Success rate 95%	high	14	32,000	80
Chromosome aberrations (PCC & FISH)	Difficulty of fusion	high	15	36,000	1,000
Micronucleus assay	Not automated	low	7	27,000	20
Apoptotic assay		low	5	27,000	100
Oncogene expression	Reproducibility	low	1- 5	30,000	500
Growth Fraction (MIB1)		low	3	32,000	100
pMI		low	3	22,000	50
Mn-SOD		low	3	20,000	50
DNA dsb rejoining assay by PFGE	Requires a large tumour sample. Quantitation is complicated.	high	6–7	20,000 in a well equipped lab	50
MVD	no, success rate 100%	low	1 hour	16,000	16
Polarographic pO_2 measurement	Probe consistency, sterilisation, calibration	high	1 hour	80,000	200

4. CURRENT AND FUTURE TRENDS

4.1. Intrinsic radiosensitivity

There are a variety of endpoints and assay methods that can be used to measure the cellular radiosensitivity of a tumour. Clonogenic assays are considered the gold standard method but currently the only work that has shown a significant correlation with clinical outcome following radiotherapy is the Manchester study. This assay is, however, too time-consuming and laborious for routine clinical application and so there is a need for a surrogate, potentially-more rapid assay showing either a good correlation with clinical outcome or with clonogenic data. Growth assays (e.g. CAM and MTT) are probably of limited use for a variety of reasons. There is an interest in cytogenetic endpoints. Currently only the micronucleus assay and a quantification of multinucleate (polykaryon) cells have shown a significant relationship with clinical outcome of radiotherapy. In addition, there are a number of other assays that may have future potential. These include the use of premature chromosome condensation (PCC) or fluorescence in situ hybridisation (FISH) methods to score chromosome aberrations or fragments. The prognostic value of DNA ploidy is equivocal and likely to be tumour type specific. Nevertheless with increasing use of flow cytometric methods on fresh material the detection of aneuploidy may show a clearer prognostic value. There are some preliminary data measuring DNA strand breaks that show promising correlation with clonogenic results in tumours. There are a variety of methods such as pulsed field gel electrophoresis, neutral elution and the comet assay that are all of future interest. radiosensitivity of cell s from blood of patients as surrogate predictors is also an area being examined.

4.1.1. Biochemical factors

Regarding biochemical factors as potential determinants of radiation response, there is an interest in protein and non-protein thiols, transitional metals, antioxidant enzymes and metallo-proteins such as catalases, ferritin, and superoxide dismutase (SOD). Of greatest interest are non-protein thiols such as glutathione (GSH) and cysteine. Preliminary studies suggest that GSH levels of tumour tissue and plasma may influence radiosensitivity and this too is an area of future interest.

4.1.2. Proliferation

Although accelerated re-population is clearly involved in failure to control disease following radiotherapy, recently published results show no correlation between pre-treatment measurements of Tpot and clinical outcome. Of the variety of other methods that exist for assessing tumour cell proliferation (e.g. SPF, PCNA, LI, Ki67, mitotic index of proliferating cells), determination of the ratio of labelling index (following both the in vivo and in vitro administration of halogenated pyrimidines), and growth fraction using Ki67 are of particular future interest. The organisation pattern of proliferating cells may also have potential as a prognostic factor for radiotherapy outcome.

4.1.3. Hypoxia

Measurement of tumour oxygenation using polarographic oxygen electrodes is currently of considerable interest and has been shown in several independent studies to be a determinant of radiation response. The Eppendorf histograph used in these studies is probably too expensive initially for widespread clinical use but there are a number of other methods that are likely to show promise in the future to be useful in determining the tumour oxygenation status. Among them are second generation, less expensive electrodes, the immunohistochemical/flow cytometric detection of nitroimidazoles (pimonidazole/EF5) following in vivo administration, the comet

assay (this may be limited due to the requirement of 3–4 Gy irradiation of tumours), the assessment of tumour vascularity, magnetic resonance imaging (MRI), and positron emission tomography (PET).

4.1.4. Apoptosis

There is an interest in evaluating both spontaneous and radiation-induced apoptosis. The prognostic value of scoring apoptosis is equivocal but future studies may clarify the usefulness of this endpoint.

4.1.5. Gene products

Most cancer susceptibility genes (e.g. ATM, BRCA1&2, p53, etc.) and oncogenes (e.g. c-erbB-2, bcl-2, myc, ras, etc.) discovered to date, disturb DNA repair and cell cycle progression. The detection of these genes, their products or their phenotypic characteristics (e.g. mutagen sensitivity and cell cycle defects) may have prognostic significance. Measurements on tumours can be made using immunohistochemistry or in situ hybridisation methods, both pre- and post radiation exposure. Correlation with clinical outcome is in general equivocal and the value of different genes is likely to be highly tumour-type specific. The most widely studied example is p53 which has yielded interesting but controversial prognostic information in relation to therapeutic outcome, but is still considered to warrant continuing investigation. The same is true for the other genes/products, making this an area that is worthy of future studies.

4.2. Conclusions

It was agreed that the most significant prognostic indicator for radioresponsiveness of human tumours, based on current information available, is the extent of hypoxia. It will be important to continue to develop assays for detection of hypoxia, and to compare them with respect to reliability and accuracy, ease of use, cost etc.

ABBREVIATIONS

ABC/HAP	avidin-biotinylated horse radish-peroxidase complex
A-T	ataxia telangiectasia
ATM	gene causing Ataxia Telangiectasia
BSO	buthionine sulphoximine
BUdR	bromodeoxyuridine
CAM	Cell Adhesive Matrix
CB	cytokinesis-block
CRP	Co-ordinated Research Project
CySH	cysteine
D-bar	mean inactivation dose
DiSC assay	differential staining cytotoxicity assay
EF5	2-[2-nitro-1H-imidazol-1-yl]-N-(2,2,3,3,3-pentafluoropropyl) acetamide
FITC	fluorescein isothiocyanate
FNA	Fine Needle Aspiration
FISH	Fluorescent In-Situ Hybridisation
GSH	glutathione
HRS	Hyperradiosensitivity
$ImNO_2$	nitroimidazoles and related nitroaromatics
IRR	induced or increased radioresistance
IUdR	halogenated pyrimidines iododeoxyuridine
LCA	Leukocyte Common Antigen
LET	Linear Energy Transfer
LI	Labelling Index
MI	Mitotic Index
Mn-SOD	manganese superoxide dismutase
MoAb	monoclonal antibody
MRI	Magnetic Resonance Imaging
MTT assay	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay
MVD	Microvessel density
NMR	Nuclear Magnetic Resonance
NPSH	non-protein thiols
OER	Oxygen Enhancement Ratio
PBS	Phosphate Buffer Saline
PCC	Premature Chromatine Condensation
PCNA	Proliferating Cell Nuclear Antigen
PET	Positron Emission Tomography
PFGE	Pulsed Field Gel Electro-Phoresis
pMI	mitotic index specific for the proliferating cell population
RCM	Research Co-ordination Meeting
RSH	agents containing thiols
SF_2	surviving fraction at 2 Gy
SOD	superoxide dismutase
SPECT	Single Photon Emission Computed Tomography
SPF	proportion of cells in S phase
S-S	cystine form
Tc	cell cycle time
Tpot	tumour potential doubling time
Ts	duration of S- phase

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