Programmed Cellular Response in Radiation Oncology

Basic and Translational Studies

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PROGRAMMED CELLULAR RESPONSE: AN INTRODUCTION

Summary

The following manuscript integrates various studies of cellular radiation response. This review gives a background to the original article in the accompanying edition of the journal *Acta Oncologica*. Every attempt has been made to avoid needless repetition of the text but to maintain the autonomy of both works. In most cases, figures or tables have been reproduced only once. The final aim of the studies was to understand better, and where possible, even improve on the success of cancer treatment using radiation therapy. From the start it was clear that qualitative aspects of cellular response to ionizing radiation could not have been predicted. Cells are normally exposed to very low levels of ionizing radiation. They are certainly not able to recognise this radiation, in spite of their rich spectrum of receptors. Cells can and do respond to the damage caused by radiation as it passes through, but the response to ionizing radiation is more complex than simply repair. Cellular programs are activated and processes are induced which interfere with the most fundamental aspects of biological life. The following is a tour-de-force to elucidate the principles behind programmed cellular response to ionizing radiation damage and to examine potential applications in the clinic.

Cellular response is initiated by the deposition of energy during passage of radiation through sensitive cellular targets and the production of macromolecular damage by ionization events and free radicals. At radiation doses employed for clinical purposes, or at doses of relevance for radiation protection, the cell responds in an active manner. Radiation damage is recognized by intra-cellular receptor molecules and signals are subsequently transduced throughout the cell which mobilize proteins involved in executing appropriate cellular responses. In many cell types, responses often involve an attempt to repair the damage. If the cells are replicating, transitory arrests also occur before or during critical phases of the cell cycle. However, probably because they are restricted to fewer growth controls than cells in solid tissues, lymphocytes preferentially respond to DNA damage by inducing a process of cellular suicide; apoptosis, which effectively eliminates this potential source of carcinogenic risk.

Cellular radiation response is the result of active molecular signalling and is not simply a passive physico-chemical process. The decision whether or not a cell should respond to radiation-induced damage either by induction of rescue systems, e.g. mobilization of repair proteins, or induction of suicide mechanisms, e.g. programmed cell death, is an expression of intricate cellular biochemistry. A cell must recognize damage in its genetic material and then activate an appropriate response. Cell type is important, the response of a fibroblast to radiation damage is both quantitatively and qualitatively different from a lymphocyte, and cellular transformation may also be accompanied by changes in radiosensitivity. The programmed component of radiation response is highly significant in radiation oncology and creates unique opportunities for enhanced treatment success.

The hallmark of an active cellular response is signal production; the encoding of some relevant piece of information in a form which can be handled by the cell in order to make a 'decision' at the molecular level (i.e. whether or not a repair mechanism should be activated).The cell cycle arrests induced either by radiation damage or staurosporine are closely related phenomena governed by a common kinase signalling pathway which recognizes DNA

damage and regulates the G2/M transition. Such signal pathways are believed to influence many of the programmed responses to radiation damage. Current estimates suggest that approximately 5 per cent of cancer patients receiving radiotherapy display adverse reactions due to hypersensitivity. These differences are often due to genetic defects which compromise programmed cellular response and result in abnormal radiosensitivity. Assays of radiation response have yielded much valuable information about the radiobiology of tumours. New developments in cellular and molecular biology increase our understanding of the programmed component of radiation response, helping us to take better advantage of this phenomenon in the clinical setting.

Barbara McClintock and programmed response, a historical perspective

Barbara McClintock was awarded the Nobel Prize in Physiology or Medicine for her most noted accomplishment; the discovery of transposable elements. This took place whilst she was working on the breakage-fusion-bridge cycle in maize during the 1930s and 1940s. Her Nobel lecture was introduced with the words: "*There are 'shocks' that a genome must face repeatedly, and for which it is prepared to respond in a programmed manner...but there are also responses of genomes to unanticipated challenges that are not so precisely programmed. The genome is unprepared for these shocks. Nevertheless, they are sensed, and the genome responds in a discernible but initially unforeseen manner.*" McClintock went on to describe her cytogenetic studies of Stadler's x-ray-induced recessive mutations in maize. Even then she noted that none of the x-ray-induced mutants arose from 'gene mutation', instead each reflected loss of a segment of a chromosome that carried the wild-type allele, and x-rays were responsible for inducing these deficiencies. Her studies lead her to conclude that broken ends of chromosomes are able to find each other and fuse; and any broken end with any other broken end. The cell is able to sense the broken ends, to direct them toward each other, and then to unite them so that the union of the two DNA strands is correctly oriented. The genomic challenge is met by a programmed response (1).

By introducing an abnormal chromosome 9, carrying an inverse duplication of its short arm, McClintock was able to produce a single broken chromosome end into maize pollen at meiosis. These single ends could not be repaired until the DNA was duplicated at S-phase. Then two broken ends were present which were joined together to produce a dicentric chromosome. At the next mitosis, the two centromeres of the dicentric were separated to opposite poles of the cell and during cytokinesis the dicentric was torn apart producing a single broken chromosome end in each of the daughter cells. The presence of the broken end was 'sensed' by the cell but nothing could be done until a second broken end became available. In the presence of this signal, the dormant transposable elements were activated and the genome became labile. The process of chromosome breakage and repair is called the breakage-fusion-bridge cycle. It continues in maize until fertilization occurs. In the zygote the broken end is healed. McClintock attributed this healing to the formation of a new telomere at the ruptured chromosome end (1).

These early studies of McClintock not only demonstrated the existence of transposable elements they also demonstrated that cells respond to radiation damage in a programmed manner. This program is activated by the presence of broken chromosome ends, can induce instability into the genome, and can be silenced by restoring a telomere to the broken end of the chromosome. Her discoveries in the 1930s and 1940s are now being applied in mammalian radiation biology and radiation oncology, accompanied by the fascinating realization that cellular radiation response is not simply a passive physico-chemical process but is the result of active molecular signalling. The decision whether or not a cell should respond to radiation-induced damage either by induction of rescue systems, e.g. mobilization of repair proteins; or induction of suicide mechanisms, e.g. programmed cell death, is an expression of intricate cellular biochemistry. A cell must recognize damage in its genetic material and then activate an appropriate response. The cell type is found to be important, the response of a fibroblast to radiation damage is both quantitatively and qualitatively different from a lymphocyte and cellular transformation may also be accompanied by changes in radiosensitivity. In radiation oncology, the programmed component of radiation response is highly significant and creates unique potentialities for enhanced treatment success.

Natural radiosensitivity and therapeutic radiation

Studies of the effects of low levels of ionizing radiation provide insights into the nature of the programmed response. Enhanced induction of 6-thioguanine resistant mutants in Chinese hamster V79-S lung fibroblasts at very low dose-rates has been observed (2, 3). In an attempt to understand the cause of the enhanced radiomutability, effects of exposure to dose-rates below 30 mGy/h on cell cycle duration, cell cycle distribution, plating efficiency, survival and mutation were examined. For none of these parameters was a change observed. It was concluded that the most likely cause for increased mutation induction was inactivity of a repair mechanism. This led to the hypothesis that an error-free repair mechanism, capable of reducing the frequency of mutation, is inactive under very low dose-rate exposure conditions and leaves damage to be repaired by a constitutive, more error-prone mechanism. Therefore, a biochemical program able to recognize damage and to activate response must exist in a cell. Below a critical level of damage, no recognition occurs and the error-free repair mechanism is not induced.

Numerous factors can alter the size of the programmed response. Reduction of dose-rate, for example, enhances survival since rescue mechanisms are time dependent. Genetic studies can be used to reveal details of some of the mechanisms involved. Cells from patients with the genetic disorder *ataxia telangiectasia* are distinctly more radiosensitive than corresponding cells from normal individuals. The difference in sensitivity between fibroblasts from *ataxia telangiectasia* patients and healthy individuals is about two orders of magnitude. The genetic disorder results from mutations in a single gene but gives rise to five different complementation groups (4). It appears that changes of molecular cell components and in the intracellular environment, associated with progression through the cell cycle, are sufficient to cause large variations in the size of the programmed response to radiation damage.

Another factor which has been found to influence radiation response is oncogenesis. Numerous accounts in the literature detail the changes in radiosensitivity associated with cellular transformation. The range of radiosensitivities of human tumour cell lines is orders of magnitude in size (5). Changes can be either greater or lesser radiosensitivity. In a few cases specific oncogenes, associated with development of the tumour, have been found to contribute to these changes in radiosensitivity (6–9). The spectrum of intracellular and extracellular signals, which interacts with the network of signalling pathways responsible for cellular homeostasis and activity, provides an almost inexhaustible source of possible responses. On the one hand this indicates the enormous complexity of the biological response; on the other hand it encourages directed research towards understanding principle factors which influence radiosensitivity, and not towards every minor alteration associated with perturbation of cell survival or response.

A further indication of the importance of the contribution of biological mechanisms to radiation response comes from studies of the cell cycle (10). Depending on the position of cells in the cell cycle, they display an increased or decreased radiosensitivity. Cells at the end of S phase tend to be most radioresistant; cells in G2 and especially in mitosis, are more radiosensitive. If the cells have an extensive G1, a second peak of radioresistance is usually observed. The difference in radiosensitivity between S phase and mitosis is about an order of magnitude. Differences in patient radiosensitivity are a challenge in the clinical situation, current estimates suggest 5 to 10 per cent of cancer patients receiving radiotherapy display adverse reaction due to hypersensitivity (11–13). These differences are often due to genetic abnormalities of natural radiosensitivity. We have developed an assay to estimate a patients' individual response to radiation treatment with a view to avoid severe acute reactions and as an initial baseline for the assessment of general response to radiotherapy. On the basis of the range of sensitivities displayed, by different leukocyte cell types using an apoptosis assay developed by MacDonald and Lees (14), the assay provides the clinician with a patient-specific radiosensitivity profile. Such profiles will assist the clinician in designing individualized radiotherapy protocols.

The fact that efficient repair mechanisms can be activated suggests that response to ionizing radiation is an active process rather than a passive one. An active response requires recognition of induced damage by some form of receptor complex and subsequent signal transduction in order to mobilize the elements of the cell which are responsible for the form the response takes; instead of being passive, where the cells' response is purely mechanical. The hallmark of an active cellular response is signal production; the encoding of some relevant piece of information in a form which can be handled by the cell in order to make a 'molecular' decision, (i.e. whether or not to activate specific repair mechanisms). In a series of studies investigating the role of the protein kinase inhibitor, staurosporine, in induction of cell cycle arrests, we have demonstrated the existence in mammalian cells of an active intracellular response mechanism to damage induced by ionizing radiation or its repair (15, 16). Both radiation and staurosporine were shown to interact with a kinase signalling pathway which governs various cell cycle arrests. The induced arrests were also shown to be equally sensitive to the arrest-release action of caffeine. It is expected that this intracellular response mechanism, will directly or indirectly affect many of the biological consequences of radiation exposure.

Programmed response to radiation damage is too complex to be readily manipulated. Nevertheless, this very complexity is a key to successful radiotherapy. Early and late responding tissues display a clear difference in the form of their linear-quadratic dose response curves. Fractionation regimes make use of these differences in order to maximize local tumour control without causing an intolerable load of late effects to normal tissues. Successful radiotherapy is a balance between tumour eradication and normal tissue tolerance. Enhancing the difference between the magnitude of the two effects by the optimal use of fractionation regimes significantly increases the likelihood of complication-free cure. With the modern developments in cellular and molecular biology, our understanding of the programmed component of radiation response is increasing rapidly and is helping us to apply its potential in the clinical setting.

AT THE LOWER LIMITS OF PROGRAM ACTIVATION

Programmed response requires reception of an activating stimulus. An observation of importance in understanding that radiation response is programmed is the finding that a threshold for activation of response exists. Dose response to low levels of radiation exposure was found to be higher

than expected in many studies of mutation induction, cell survival, and human cancer induction. The enhanced effectivity was observed in various experimental systems. It was observed at dose-rates between 3 and 30 mGy/h and doses between 0.02 and 0.6 Gy. It displayed dependence on intrinsic radiosensitivity, cell proliferation status and LET. The observations suggest that exposure to low levels of ionizing radiation may be insufficient to activate the spectrum of recovery processes typically observed after high level exposure.

Biological effects resulting from low level exposure of ionizing radiation are difficult to measure. Often the spontaneous frequency of these effects make it difficult to discriminate true radiation exposure effects from the natural background incidence (17). This is true for mutations, cell survival and cancer induction. These effects are studied in order to address important issues in risk assessment such as the predicted incidence of tumour induction after low level exposures. Recently, a number of advances have been made and techniques are now available which permit more precise study of the effects of low levels of ionizing radiation.

It is not possible to study the effects of exposure to very low dose-rate irradiation on survival. The impact of the few cells which die cannot be discerned amongst the natural fluctuations of the plating efficiency. However, it is possible to study the effects of very low dose-rates on mutation. This is because genetic effects are heritable and accumulated effects over many cell generations can be measured. In this manner the effects of long periods of low-level exposure can be monitored. Such long-term exposures required the development of suspension-culture techniques for monitoring mammalian cells lines. They also required the selection and establishment of cell lines capable of growth in suspension culture. With these developments, it was possible to study the effects of biologically significant doses of radiation (1–5 Gy) at dose-rates as low as 2.7 mGy/h (18).

To study the effects of low doses of radiation another approach has been taken. If the response of individual cells can be monitored, there is no statistical uncertainty about the number of cells plated. In the final assessment of plating efficiency this reduces the error, and thus the 'background noise', from Poisson to binomial. Using computerized microscopy (the Dynamic Microscope Image Processing Scanner: DMIPS) the response of individual cells to low doses of radiation can be followed. It has been possible to observe the effects of doses between 0.1 and 1 Gy. However, it is difficult to study mutation induction under these conditions as approximately one million cells have to be individually analysed for each data point. Flow cytometry may provide an approach to overcoming this difficulty.

Quantifying the risk of low levels of radiation on human populations is a formidable task. Large numbers of exposed individuals are required for such studies. Very often confounding circumstances prevail when such situations arise. Data are, therefore, limited. Recently, however, a few pertinent and useful clinical studies have appeared in the literature. Although these are limited in number they provide a basis for answering such questions and they suggest that under certain circumstances low levels of radiation exposure can cause an unexpectedly high frequency of secondary malignancies.

In a simplified radiation protection situation, a linear relationship would exist between the production of a biological effect and radiation dose. This relationship could then be extrapolated to low doses, and for any low level exposure a damage estimate made. However, as evidence accumulates indicating that response to radiation is a biologically complex event; requiring recognition of damage and recruitment of cellular defence and repair mechanisms, such oversimplifying assumptions about the consequences of low dose exposures require reappraisal. As a case in point: if recognition of cellular radiation damage has a threshold, below a certain low level of radiation, insufficient damage will be available to activate response.

The recovery pathways are expected to be energy demanding. Ideally, such pathways would only be activated after a minimum level of radiation damage had been produced. If true, a linear dose-response relationship would not be expected but rather a step function, radiation damage being reduced at the threshold dose where activation of high efficiency recovery pathways was induced. The very low dose-rate studies reported here, in the mGy/h range, are still 10^3–10^4 fold higher than natural background. Classification of dose rates is somewhat arbitrary and the proposed nomenclature of Hall is used here (19), with modifications: 'low' being 0.05–10 Gy/h and 'very low' being 0.1–50 mGy/h (1 cGy/min = 0.6 Gy/h). Evidence is reviewed demonstrating that a threshold for the induction of radio-protective mechanisms exists. This is based on data from mutation, survival and transformation studies. The results advise caution suggesting that unexpectedly high frequencies of secondary malignancies can result from very low dose-level treatment protocols.

Mutation studies

In 1985, Crompton et al. described increased mutation induction by very low dose-rate radiation in Chinese hamster cells (20). An inverse dose-rate effect for induction of 6-thioguanine resistant mutants in Chinese hamster V79-S lung fibroblasts was observed. The V79-S lines were adapted for growth in suspension culture from the V79-379 line. For a given dose, a decrease in mutation induction occurred as the dose rate was reduced from high to low. However, even more reduction in dose rate resulted in an increase in the frequency of mutants observed for any given dose. A 2 Gy isodosis curve was presented (20) in

order to evaluate the data in terms of the number of mutant cells per million survivors induced by the 2 Gy 'standard effective dose' (21) of ^{60}Co γ-rays, at dose-rates varying from 4.5 mGy/h to 84 Gy/h. Despite differences in magnitude, all three cell lines demonstrated a reduction in mutation frequency as the dose rate increased from the lowest dose-rates studied to between 30 and 50 mGy/h. This was followed by an increase in the mutation frequency at higher dose-rates. The effect is particularly pronounced in the S83 cells which may result from an additional mechanism, perhaps increased genetic instability (20, 22). At the very low dose-rates studied, the enhanced mutation induction could be attributed to none of the following: proliferation, plating efficiency, cell cycle phase distribution, and enhanced radio-sensitivity or radio-mutability to subsequent high dose-rate exposures (3). These low dose-rate studies with V79 cells have been reassessed independently at another laboratory, where enhanced mutation frequency was also observed at very low dose-rates: 6 and 19 mGy/h (B. Singh, Gray Laboratory, unpublished data).

Recent studies of another Chinese hamster cell line, PL61/1 examined the induction of resistance to 6-thioguanine after exposure to very low dose-rate (17.6 mGy/h) ^{137}Cs γ-rays (23). The PL61/1 cells were derived from Chinese hamster ovary (CHO) cells which were *hprt*-. These were transfected with a single copy plasmid carrying the *gpt* gene. The *gpt* gene is the bacterial (*E. coli*) gene encoding xanthine–guanine phosphoribosyltransferase. Its presence confers 6-thioguanine sensitivity on otherwise *hprt*-cells. Mutation to 6-thioguanine resistance in PL61/1 cells is 3–4 times as frequent as in V79 cells. During the irradiation period the cells were not maintained in suspension culture but in periodically trypsinized cultures: exponentially growing monolayers which were regularly passaged by harvesting the cells with trypsin. Enhanced mutation frequency was observed only at total doses below 2 Gy (24). It appeared that under these conditions a 1–2 Gy 'conditioning' dose was required to induce the enhanced premutational repair.

Confirmatory studies, if less extensive, were reported in L5178Y mouse leukaemia cells. Two different strains of L5178Y were studied; LY-R and a radiosensitive line derived from it, LY-S. Their general response to ionizing radiation (250 kVp x-rays) and UV light has been reported (25). The mutation studies performed with these cells investigated induction of 6-thioguanine resistant mutants. Although an early paper reported only a reduced mutation frequency at low dose-rates (20 mGy/h) (26), results presented later by the same group described enhanced mutation frequency at even lower dose-rates (3 mGy/h) in the LY-R strain. This increase was not observed in the radiation sensitive LY-S strain, see Table 1.

Furuno-Fukushi et al. (27) performed similar investigations of survival and mutation at 6.3 mGy/h (^{137}Cs γ-rays), 200 mGy/h (^{137}Cs γ-rays) and 30 Gy/h (^{60}Co γ-rays), in L5178Y cells. A decrease in survival was reported at both 200 mGy/h and 30 Gy/h but none at 6.3 mGy/h. The mutation frequency per dose unit was found to decrease as the dose-rate was increased from 6.3 mGy/h to 200 mGy/h and then to increase when the dose-rate was further increased to 30 Gy/h. The mutation frequency levels were similar for 6.3 mGy/h and 30 Gy/h. Cells previously exposed to various doses of radiation at 6.3 mGy/h displayed no enhanced radio-mutability when exposed to second doses of radiation at acute dose-rates. These results confirmed the reduction in mutation frequency at intermediate dose-rates, and at dose-rates similar to those for the Chinese hamster cells. Studies with a hypersensitive line, LX830, displayed a dose-rate independent mutation induction (28). As opposed to the parental L5178Y line, LX830 cells had no shoulder in the survival curve and were much more radiosensitive.

Furuno-Fukushi et al. (29) also reported studies of mutation induction in mouse m5S cells in plateau-phase culture and in exponentially growing, periodically trypsinized, cell culture; at dose rates of 13 mGy/h, 180 mGy/h and 30 Gy/h. The studies suggest that the inverse dose-rate effect may be related to cell proliferation. Plateau phase cultures at equal doses displayed an increasing mutation frequency with increasing dose rate. No enhanced mutation frequency at 13 mGy/h was observed. Exponentially growing, periodically trypsinized, cell cultures displayed a more complex response. At low total doses (below 5 Gy), the cells displayed a dose-rate independent mutation induction. However, at higher total doses the low dose-rate and very low dose-rate exposures resulted in an unexpected downward bending of the mutation induction curves. The authors described a tendency for an inverse dose-rate effect at lower dose-rates. Supplementary studies at other low and very low dose-rates would help to confirm these results.

Ongoing studies with human cell lines have revealed a similar pattern of dose-rate independence in hypersensitive

Table 1

Frequency of 6-thioguanine mutants per million cells induced by 0.5, 1.0 or 1.5 Gy x-rays administered at various dose-rates to two lines of mouse lymphoma L5178Y cells (338)

LY-R	0.5 Gy	1.0 Gy	1.5 Gy
3 mGy/h	13.2 ± 2.5	23.9 ± 10.7	45.5 ± 20.3
20 mGy/h	7.6 ± 2.5	14.7 ± 3.6	17.3 ± 2.6
53 Gy/h	6.5 ± 0.4	14.3 ± 2.1	25.4 ± 4.7
LY-S	0.5 Gy	1.0 Gy	1.5 Gy
3 mGy/h	1.8 ± 1.0	5.6 ± 2.1	7.0 ± 3.0
20 mGy/h	2.6 ± 1.1	8.0 ± 2.6	14.9 ± 4.0
53 Gy/h	5.4 ± 1.3	6.6 ± 2.4	11.4 ± 2.3

lines but dose-rate dependence in non-hypersensitive lines. Grosovsky and Little (30) reported studies using the TK6 human lymphoblastoid cell line. One advantage of using this cell line is that two mutation end points can be studied simultaneously: induction of resistance to 6-thioguanine and induction of resistance to trifluorothymidine. Daily doses of between 0.01 and 0.1 Gy (100 kVp x-rays) were administered at high dose-rate, rather than by continuous low dose-rate treatments. Mutation induction was found to be dose-rate independent for both end points. No effect of the size of the fractions (daily exposures) was observed, only the total dose administered effected the final mutation frequency. These experiments were repeated under conditions of continuous low dose-rate exposure by König and Kiefer (18). They also reported dose-rate independent induction of both types of mutations. In both studies only linear mutation induction curves were found. TK6 cells are hypersensitive to ionizing radiation. They have an exponential survival curve with a D_0 of 0.47 Gy. Like other blood and blood progenitor cells they display a radiosensitive interphase cell death (apoptosis) (31, 32).

Kohlpoth (33) studied dose-rate effects (^{60}Co γ-rays) in another human cell line with a non-linear mutation induction curve, referred to as SP3. The parental P3 cell line is a teratocarcinoma cell line, with epithelial morphology and a stable 46 chromosome karyotype (34). It has been successfully used in mutation studies (35). A sub-line was adapted for growth in suspension culture and named SP3 (33). The survival curve of the SP3 line has a prominent shoulder ($D_q = 1.5$ Gy) and final slope with a D_0 of 0.78 Gy. The mutation-induction curves of this human cell line are not linear but bend upward with increasing dose. Kohlpoth and Kiefer reported a fall in mutation frequency when the dose-rate was increased from 5 mGy/h to 50 mGy/h but an equivalent rise again in the mutation frequency when the dose rate was increased further from 50 mGy/h to 90 Gy/h. Once again, therefore, a reduced mutation frequency at intermediate dose-rates was observed. The influence of proliferative status on the mutation frequency in the parental P3 cells at very low dose-rates was investigated. Stationary monolayer cultures, which could be maintained over several weeks with occasional refeeding, were subjected to γ-rays at dose rates between 10 and 50 mGy/h. A continuous increase in mutation frequency with increasing dose rate was observed. This suggests that enhanced mutation frequency observed at very low dose-rates in exponentially-growing continuous cultures is linked to the active cellular proliferation (36). This was the same conclusion that had previously been drawn by Furuno-Fukushi et al. (29).

Survival and cancer studies

A widely used model to describe the decrease in survival of irradiated cells in vitro is the Linear-Quadratic (LQ) model. The LQ model has also been shown to predict the increase in total dose with decreasing dose per fraction needed for an isoeffect response to radiotherapy in normal tissues and tumours (37). This relationship reflects the gradual decrease in radiation effectiveness with lowered doses due to these doses being further and further back on the shoulder of the underlying survival curve for the cells at risk. Thus for doses higher than 1 Gy per fraction, the LQ model has been found to be a reasonable predictor.

The effect of low doses per fraction, less than 1 Gy and typically down to 0.1 Gy has been evaluated on lung, skin and kidney (38–40). These studies were possible utilising an experimental design in which only a part of the total underlying damage, equivalent to full tissue tolerance, was produced by the x-ray fractions of interest, and the balance was supplied by a 'top-up' dose of low-energy (high-LET) neutrons (38). The data obtained in mouse skin, lung and kidney all demonstrated an increased effect of small x-ray fractions, particularly skin and kidney. In the kidney, for example, the total curative dose was approximately 92 Gy at 1 Gy per fraction but decreased by a factor of 1.9 to about 49 Gy at 0.2 Gy per fraction.

One study on tumours irradiated in vivo assessed the response to doses per fraction less than 0.6 Gy. Beck-Bornholdt et al. (41) irradiated the R1H rhabdomyosarcoma of the rat with full-course schedules of 200 kVp x-rays administered in different numbers of fractions varying from 6 to 126. No top-up doses were used. The schedules were given in the same overall time of 6 weeks so that the influence of proliferation was expected to be the same. The periods between fractions were of at least 8 hours duration in all schedules to allow complete repair between the successive doses. Compared with the schedules using between 6 and 42 fractions (dose per fraction > 1 Gy), curative doses were lower, and net growth delay per Gray higher, for the 126-fraction schedule (dose per fraction < 0.5 Gy). The qualitative agreement of increased sensitivity at very low doses per fraction in both the R1H tumour and mouse skin, kidney and lung is interesting, suggesting that this reflects a phenomenon that is more fundamental and widespread. It indicates that the low dose radiosensitivity should be detectable in vitro given the right conditions.

Studies to compare the data obtained in vivo with direct measurements of survival made on established lines of mammalian cells in vitro exposed to very low doses of x-rays have been conducted at the Gray Laboratory. Accurate measurement of cell survival in the low dose, high survival region has been possible by using a computerized microscope, to locate and record the positions of single cells on a growth surface (DMIPS), and monitoring these cells individually to determine whether or not a colony, indicative of cell survival, grows (42–44). Survival response of the V79 hamster cell line (43) and of four radioresistant human tumour cell lines: HT29 (44), RT112 (45), MEWO (45), and Be11 (46), was measured after

irradiation with single doses of x-rays (0.05–5 Gy) using the DMIPS. The response over the x-ray dose range 2–5 Gy showed a good fit to the LQ model but, for x-ray doses below 1 Gy, an increased x-ray effectiveness was observed. Cell survival was found to be below that extrapolated from the LQ model based on high-dose data. This work has now been extended to L132 normal human lung epithelial cells (47). At doses between 0.15 and 1 Gy cell survival was significantly reduced compared to the predicted linear-quadratic response.

The low dose hypersensitivity was not seen after neutron irradiation of the V79 and HT29 cell lines. Neutron RBE's were calculated for each x-ray dose by taking the x-ray survival value and comparing it to the common LQ fit of the neutron data. Over the x-ray dose range 0.05–0.2 Gy, the RBE is close to 1 indicating that these very low doses of x-rays are of similar effectiveness to neutrons in killing cells. An increase in RBE with increasing dose over the range 0.05–1 Gy, and a slight decrease in RBE above 1 Gy, reflect changes in x-ray sensitivity over the dose range 0.05–5 Gy (44). Interestingly, low dose hypersensitivity of human tumour cells growing in vitro has been confirmed recently using another assay involving cell sorting. This method also improves on the resolution of cell survival data at low doses in a significant way (48).

The multiphasic form of the survival curve at low doses is less apparent in radiosensitive cell lines, such as the MEWO melanoma line, and no induced recovery was observed in the SW48 colo-rectal line (45). This and other theoretical arguments suggest that the phenomenon reflects induction of radioresistance mechanisms above a critical level of exposure. Confirmation of this also has come from a series of studies investigating split-dose exposures. If the first dose was capable of inducing radioresistance, the second dose was found to be far less cytotoxic than the first. However, this protective effect was not observed if the first dose (0.2 Gy) was below the inductive 'threshold' (49). All these data in vitro are consistent with the observations in vivo. Using low doses per fraction, the initial modest effect on the survival curve is successively duplicated and, therefore, the effect of the total dose administered during these treatments is magnified (50). Theoretical calculations based on in vivo and in vitro data suggest that the increased effectiveness of low doses might be turned to advantage in the treatment of very radioresistant tumours. These could be treated by hyperfractionation with doses per fraction less than 0.5 Gy or by continuous extended irradiation, e.g. brachytherapy (50).

The first studies that quantified the carcinogenic effect of ionizing radiations in humans appeared in the 1950s. They were focused mainly on the frequencies of leukaemia cases among survivors of the Hiroshima and Nagasaki bombings and in patients who had been irradiated for treatment of ankylosing spondylitis. However, most of these early studies did not accurately describe the whole dose-effect relationship, for two main reasons. Either, the irradiated populations and/or the increased incidence of cancers were small, or the range of doses given to critical tissues was narrow. Further, knowledge of the appropriate biostatistical techniques was inadequate in the 1950s, so that it was not possible to reliably estimate the true effects of low doses from the data.

One of the most important studies published recently on this topic is that of Boice et al. (51) entitled 'Leukaemia risk in patients treated for cancer of the cervix'. This work is a case-control study on about 150000 women. The study has four main features. First, the population was large (greater than that of the survivors of Hiroshima and Nagasaki). Second, the dose given could be calculated accurately (the physicist estimated that the dosimetry was high quality in more than 90% of the cases). Third, the range of doses given to the critical tissue namely the hematopoietic bone marrow was very wide (most values were between 1 Gy and 12.5 Gy). And lastly, the authors had a very suitable control population (the same type of patients, but treated by surgery only). The survival of these patients was evidently limited by the cancer itself and the age of the patients at the time of treatment. The 143 leukaemia cases actually studied were taken from a total of 195 cases confirmed by the pathologist. The remaining 52 patients were suffering from chronic lymphoid leukaemia, which is known not to be induced by ionizing radiations.

The dose-effect relationship was 'bell-shaped' with the greatest relative risk (RR) at a dose of 4 Gy (RR = 2.53), which then diminished at higher doses (RR = 1.42 for doses above 14 Gy). This type of 'bell-shaped' curve for the induction of leukaemia by ionizing radiations is exceptional in humans, but a similar type of relationship has been observed in mice (52). Boice et al. (51) suggest that this bell-shaped curve is the result of two competitive phenomena. One is the leukaemogenic effect, which dominates between 1 and 4 Gy, the other is the killing effect, which dominates above 4 Gy. This is the classical explanation for this type of dose-effect relationship which is very reminiscent of results obtained in vitro for rodent cells (53). The criteria used to analyse the rodent data were the survival rate and the transformation rate (54).

A second explanation for the relationship observed by Boice et al. has been proposed (55). The human leukaemia curve was obtained under irradiation conditions very different from those generally used experimentally. Under experimental conditions; a single dose, often of 1 Gy or greater, is normally used. The patients studied by Boice et al. (51) were treated either by external radiotherapy (usually 2 Gy per session to the target volume for 25 sessions), and/or by interstitial therapy whose total duration is not clear, but which was probably 3–6 days. Under these conditions, the most active leukaemogenic doses (1–4 Gy) were probably received by the bone marrow, at doses of 4–16 cGy per fraction (external beam radiotherapy) or

dose rates ranging from 7 to 55 mGy/h. These latter dose rates produced the highest mutation frequencies in in vitro mammalian cell culture experiments (3). Furthermore, a single dose of 16 cGy falls within the dose range per fraction that gives the highest relative rate of cell transformants per surviving cell in vitro (53). The bell-shaped curve obtained by Boice et al. could at least partly be due to a hypersensitivity to small doses (4–16 cGy) or to very low dose-rates (7–55 mGy/h).

Clearly, such comparisons of experimental and clinical data must be made cautiously. The same cell lines are not being compared, neither is cancer development an automatic consequence of transformation, although the latter appears to be a necessary first step in cancer development. Furthermore, to reconcile the Boice data with the rodent studies (53), it must be assumed that each small fraction (ranging from 4 to 16 cGy) produces the same effect per fraction until the final total dose has been administered. Nevertheless, the data reported by Boice et al. (51) on the induction of leukaemia in humans by radiation are not in conflict with the concept of hypersensitivity to low doses or very low dose-rates.

Chmelevsky et al. (56) recently reanalysed the incidence of bone sarcomas in patients who had been given injections of ^{224}Ra as treatment for bone tuberculosis or ankylosing spondylitis. While the group of patients was small (800 cases) the incidence of bone sarcomas in the group (56 cases: 14.3%) was considerable; less than 1 case would be expected in a non-treated group. The dose given to the bone surface was measured accurately, and displayed a wide range, from a few tens of cGy to tens of Gy. The simplest dose-effect relationship, a linear one, suggested that the incidence of this tumour is 1.8% per Gy. A later analysis, however, showed that a linear-quadratic fit provides a better description of the overall effects (56). The quadratic term corresponded mainly to the higher rate of bone sarcomas seen in patients who had been given the highest doses. But this explanation is not completely satisfactory. Several confounding factors, such as age, sex, original disease, dose fractionation, could all have interfered with the effects of irradiation.

There was a very good correlation between the total dose received by the bone and the total exposure time. Chmelevsky et al. (56) therefore examined the specific role of time (expressed as 'injection span') independently of the dose. The best fit was obtained using two coefficients: 'α' (0.0055/Gy), proportional to the dose 'D'; and 'γ' (0.18/month), proportional to the time 't'. This implies that the carcinogenic effect of a given dose of ^{224}Ra was greater if it was given oven a longer time. For example, reducing the dose rate three-fold doubled the carcinogenic effect. Addition of a quadratic-dose component 'β' to the two coefficients 'α' and 'γ' did not improve the fit, confirming that the apparent upward bending of the dose-effect curve depends more on exposure time than on the dose itself. The conclusion of this study seems to be particularly important. It shows that there is a reverse protraction factor for the carcinogenic effect of certain types of ionizing radiation in man. Such a reverse protraction factor has also been demonstrated in in vivo experiments (57). This type of phenomenon has seldom been described in man, except for some reports concerning tin and uranium miners (58, 59).

It is difficult to compare these clinical results with in vitro experimental data. As the half-life of ^{224}Ra is short (3.66 days) and the patients were given several injections of ^{224}Ra, the bone cells were clearly subjected to discontinuous low dose-rate irradiation. However, to a first approximation, an average dose rate can be calculated from the original data. The mean dose rate was 0.54 mGy/h (2.12 Gy/5.5 months) for 587 adults, and 1.36 mGy/h (10.71 Gy/10.9 months) for 192 children and adolescents. Thus Chmelevsky et al. (56) observed a reverse protraction factor below about 1 mGy/h. Crompton et al. (3) used in vitro mutagenicity as a criterion and found reverse protraction below a dose rate of about 1 cGy/h, which is ten times greater than that found by Chmelevsky et al. (56) This difference could perhaps be due to the fact that the RBE of α-particles from ^{224}Ra is about 20 times higher (60) than that of the γ-rays used by Crompton et al. (3) Although Chmelevsky et al. (56) give no biological explanation for their observation, it is not unreasonable to assume that it reflects hypersensitivity to very low dose-rates.

As stated previously, difficulties arise when comparing experimental results in vitro with radiation carcinogenesis in humans. This is particularly true when there is very little human epidemiological data. Nevertheless, several pieces of evidence suggest that carcinogenic effects in the low-dose range (10–60 cGy) and in the very low dose-rate range (about 1 cGy/h) are more severe than predicted by simple extrapolation of curves obtained at higher dose rates, a finding also supported by animal studies. Ullrich has described tumour induction in BALB/c mice following exposure to fission-spectrum neutrons (61). He studied induction of three tumour types: lung adenocarcinoma, mammary adenocarcinoma and ovarian tumours, in hundreds of mice. Low dose-rates (0.5–5 mGy/h) caused higher tumour incidence in lung and in mammary adenocarcinomas than high dose-rates (600–1500 mGy/h). The reverse was true for ovarian tumours. High dose-rates given as single doses or as two equally sized split doses, 24 hours or 30 days apart, were equally effective; with one exception. At the highest split-dose with a 30 days interval, lung and mammary adenocarcinoma induction was greater than after a single total dose. This was not the case for the ovarian tumours. Tumorigenic response varies depending on the tissue exposed.

Physiological explanations of enhanced response at low exposure levels

The dose-rates where a fall and rise in mutation induction frequency has been observed are similar in Chinese hamster fibroblasts, murine leukaemia cells and human teratocarcinoma cells. However, dose-rate dependent mutation induction has only been observed in cell lines where the survival curves are continuously bending and the mutation induction curves also bend upwards. In radio-sensitive cell lines displaying linear dose-response curves, mutation induction appears to be dose-rate independent. The results suggest that a component of recovery, responsible mainly for the linear component of dose response, is inducible and at very low dose-rates the damage produced in a cell is insufficient to activate this (or these) repair pathway(s). Another aspect of this enhanced recovery is its dependence on proliferation. The m5S mouse cell studies of Furuno-Fukushi et al. (29) and the P3 human cell studies (36) both indicate that enhanced mutation frequency at very low dose-rates may be linked to active cellular proliferation. Miller et al. (62) and Cao et al. (63) have suggested that inverse dose-rate phenomena may reflect fluctuations in dose response depending on position in the cell cycle. In this context a cell cycle 'window of sensitivity' has been hypothesized (62). However, at dose-rates where the 'inverse phenomena' reported here occurred, cells were already exposed for multiple generations and no changes in the duration of the whole cycle, or any specific stage, had been observed (3). Nevertheless, cell cycle arrests are dose dependent (64) and the possibility that such arrests are induced by the low exposure levels, but are insufficient to produce measurable changes, cannot be excluded.

Can the survival data, both in vivo and in vitro, be explained, alternatively, by induced repair mechanisms? One possibility is that the sensitive response at low doses is a consequence of a small subpopulation of cells (e.g. a particular position within the cell cycle) with exquisite radiosensitivity. When such a model is fitted to the in vivo data it predicts that this subpopulation would comprise about 7% of the cells and would have an increased sensitivity to x-rays, by a factor of about 13 compared to AT cells and 81 compared to human fibroblasts in general. This seems unlikely. Additionally, further experiments in vitro (43, 65) and further calculations (44) rule out the existence of a stable genetic subpopulation and also indicate that low-dose sensitivity occurs regardless of the phase of the cell cycle at the time of irradiation. It is also difficult to reconcile a mechanism based on perturbation of cell cycle progression with the data on mouse kidney, where estimates of labelling index are generally less than 0.3% (44, 66).

Induced radio-resistance is still the most likely explanation for the enhanced response at low exposure levels. It suggests that low-level radiation sensitivity represents a 'resting' response to radiation, and that higher dose levels become less effective at killing cells per unit dose because, although increased amounts of DNA damage are produced, this stimulates or induces repair systems, or stress proteins, or other protective mechanisms. Radiation-induced systems that protect against chromosomal damage are well known and have been documented in algae (67–69), plant cells in vitro (70), protozoan cells (71), hamster cells (72, 73), and human lymphocytes (74–76). Induced repair has been proposed to explain the shapes of highly radioresistant lepidopteran cell survival curves (77–79). Furthermore, ionizing radiation has been shown to induce expression of various genes, including for example the *gadd* genes (80).

It seems reasonable to assume similar mechanisms may be responsible for both the enhanced cell killing and mutation frequency after exposure to low levels of radiation. The two end points are not independent. Mutation frequency is a function of cell survival. If low doses of low LET radiations are more effective at killing cells than predicted, then low levels of radiation might be more effective at causing mutation (and by implication cancer induction). However, enhanced mutation frequencies are observed at dose rates where cell survival is virtually identical to that observed in non-irradiated controls. Although enhanced survival is expected to contribute to a reduction in the mutation frequency it is felt insufficient for this to be more than a minor contributing factor. An apparent difference between the two end-points is the dependence on cell proliferation. The mutation studies indicate that cell proliferation is necessary; however, the survival studies of murine kidney and lung were performed on essentially non-proliferating cells (kidney labelling index $< 0.3\%$), yet the low dose effect was still observed. The reason for this discrepancy is not immediately clear; it may be related to the ability of irradiated tissue to 'remember' radiation-induced lethal damage for years, even decades, before it is finally expressed. Thus, although damage is caused in non-proliferating cells, the lethal response may only be expressed when the cells proliferate.

Enhanced mutation, cell killing and transformation have all been observed at low levels of radiation exposure. These biological end points are thought to result from DNA damage, caused by radiation, which is either not repaired or repaired incorrectly. Do the radiation levels where this enhancement is observed correlate in the different test systems? Another way of expressing this question is: Does a common radiation threshold exist for the three phenomena? The threshold for induced radio-protection occurs at dose rates between 3 and 30 mGy/h or at doses between 0.02 and 0.6 Gy. Furthermore, the threshold appears to display an RBE dependence suggesting a specific type of damage may be involved.

Continuous, single, low dose-rate exposure to radiation may be thought of as a large number of small dose

fractions. This equivalence has been well known for many years and a formula for equating a low dose-rate exposure with a series of acute dose fractions to the same total dose giving the same effect, has been described (81). This formula was used to make comparisons with the low dose studies reported. This calculation requires the half-time for repair of sublethal radiation injury to be supplied; 1.5 h was used, as recommended by Marples and Joiner (43). The point below which a substantial increase in the mutational effectiveness of radiation occurs is equivalent to doses-per-fraction of the same order as was found for the increased cell killing effect in normal tissues and V79 cells. This suggests, although it does not prove, that a similar mechanism may be responsible for the increased mutational and lethal effects (per unit dose) of small radiation exposures.

Although the parallels predicted by the Liversage conversion are suggestive, the formula was intended to determine equally effective radiation regimes rather than 'thresholds' of response to low levels of radiation. An alternative approach to comparing low dose with low dose-rate exposures is based on equating the level of specific types of damage. Double strand breaks (DSB) are lesions potentially capable of activating recovery processes. Various cells respond demonstrably to the presence of single DSBs (1, 82). DSB levels after low dose exposures of 0.25 Gy, required to induce enhanced effect, would cause 7 DSB/V79 cell, based on current estimates of 26 DSB/Gy (83). DSB levels during low dose-rate exposures are more complex to determine. It is assumed that three factors contribute to the 'steady-state' level expected: rate of DSB production (P), rate of DSB rejoining (l_r), and DSB 'dilution' from cell division (l_d). DSB level $= P/(l_r + l_d)$. At dose rates of 50 mGy/h, approximately 1.3 DSB/h ($P = 1.3$/h) or 14.3 DSB/V79 cell cycle (11 h in suspension culture) are produced. Estimates (E. P. Malaise, personal communication, (83)) of $t_{1/2}$ for fast rejoining are 0.167 h ($l_{rfast} = 4.15$/h) and for slow rejoining are 3.5 h ($l_{rslow} = 0.198$/h). Under these assumptions, V79 cells have a doubling time of 11 h ($l_d = 0.063$/h). At dose rates of 50 mGy/h cells contain on average 0.6 DSB. However, if the fast component of rejoining is not active they contain on average 5 DSB (ranging from 3.5 DSB at the start of the cell cycle to 6.9 DSB at the end). These values are essentially identical to the numbers of DSB induced by the low dose exposures. If DSB are the inducing factors; the data suggest that more than one DSB (here between 6 and 7) is required to induce enhanced recovery (fast component of DSB rejoining).

Mammalian cells may mobilize additional repair mechanisms in the presence of more than one DSB in order to minimise misrepair or mis-rejoining of multiple DSB. However, if DNA DSBs are the initiating signal, it is difficult to account for the lack of induced resistance observed after low doses of neutrons. One could argue that the type of damage somehow effects induction. Enhanced neutron sensitivity has been attributed to the molecular severity of the inducing lesion (65), presumably making it more difficult to repair. Sister-chromatid exchanges (SCE) display very high neutron RBEs. SCE are difficult to detect using x-rays, a fact attributed to high-efficiency repair, but are readily induced by neutrons (84). In this type of scenario, neutrons still induce recovery processes but are unable to repair the damage, and enhanced cell killing is still observed. Another explanation for the lack of induced resistance observed after low doses of neutrons is irreversible activation of cell death mechanisms. This would make any subsequent repair spurious. These and other possibilities need to be examined experimentally.

If the enhanced radio-resistance results from accumulation of some form of radiation damage, what processes may be involved? Mobilization of efficient recovery mechanisms requires intracellular signalling between the damage site(s) and the elements of the recovery mechanism. Evidence for radiation induced intracellular signalling has been reported. Crompton et al. (85) have demonstrated that a signal kinase pathway which controls cell cycle arrests is activated following radiation exposure. Furthermore, damage typical of radiation exposure can induce such arrests. DSB produced by the topoisomerase II inhibitor, etoposide, induce cell cycle arrests (86). In yeast, elimination of a single telomere by a site-specific DSB is also sufficient to induce cell cycle arrest (82). In the yeast study, however, it is not clear if the initiating signal is the primary DSB, the associated chromosome break, or the prolonged presence of an unrepaired break (at either the DNA or chromosome level). Intracellular signal pathways can also be used to modify responses to signals. Thus non-proliferating cells may respond differently than proliferating cells, and display different recovery mechanisms. Cell cycle checkpoint controls sensitive to damage signals (87) are only expected to be functional in cycling cells. Induction of enhanced recovery may result from activation of an intracellular signalling pathway by the radiation damage. Strong preliminary evidence supporting this hypothesis based on dose-rate effects in murine leukaemia cells has recently been reported (88). These studies indicate a threshold dose-rate for induction of mitotic delay at 10–15 mGy/h for both the L5178Y-R and L5178Y-S cell lines. Above this threshold dose-rate the population doubling time is increased for these cells and also for the P388F mouse lymphoid neoplasm cells.

The studies described here provide evidence demonstrating that after low levels of exposure to ionizing radiation enhanced damage may be observed. Radiosensitive cell lines and some tissues do not display the phenomenon. However, when it is observed, a threshold for enhanced recovery is indicated. A possible explanation for the phenomenon predicts that more than one DSB will be required to activate some programmed recovery mechanisms. These results raise questions about dose lim-

its for radioprotection. However, the very low dose-rates and the low doses involved mean the total effect is small, which mitigates the severity of the hypersensitivity described.

MOLECULAR BASIS OF THE PROGRAMMED RESPONSE

Signal pathway control of the G2/M transition

The central role of signal transduction pathways in biological systems is gradually becoming clearer. Phenomenological evidence from studies on the effects of low levels of ionizing radiation demonstrate that radiation response is activated by a critical minimum level of damage, suggesting that molecular signalling is involved. Detailed investigations into cellular responses reveal complex protein systems within cells: molecular automata, which process incoming signals and subsequently effect appropriate response. A number of such systems are presently under intensive study; the control of cell cycling being of particular significance to radiation biology. Following a critical minimum dose of ionizing radiation, cells respond to the induced damage by delaying entry into mitosis until repair has taken place. A cellular receptor mechanism recognises the damage and a signal is passed on which induces G2 arrest. Caffeine can override this system, in which case no arrest occurs and excess unrepaired damage is present in the cells as they undergo mitosis resulting in increased frequencies of chromosomal aberrations, micronuclei and cellular lethality (89). We have been trying to elucidate the molecular mechanisms involved in this system (15, 90).

Eukaryotic cells in cycle respond to ionizing radiation by delaying entry into mitosis (91). Delay of entry into mitosis has been suggested to prevent fixation of a component of potentially lethal damage by extending the time period available for repair (92). This additional repair, in tumour cells, can impede radiotherapy and led to compromise of tumour control. Elucidation of the arrest mechanisms should permit the design of drugs to improve therapeutic ratios by mitigating tumour radioresistence and/or sparing neighbouring healthy tissue. We have been investigating the possible role of a signal transduction pathway in the induction of the G2 arrest. The mechanisms leading to the response and arrest are still not clear, although the initial event appear to be the presence of double strand breaks in the DNA (1, 93, 94).

Exposure to UV light also causes damage to cells, the repair of which is inhibited by caffeine. It has been demonstrated that caffeine intercalates into DNA (95, 96). Based on these findings and the results of the UV studies, Lang (97, 98) suggested that conformational changes in the DNA, induced by the clastogenic agent, prevent mitotic entry and that these are reversed by intercalation of caffeine into the DNA. Radiation damage (or its repair) induces DNA strand breaks which could prevent chromosome condensation and thus mitosis, and caffeine could intercalate into the DNA driving chromosome condensation and mitotic entry. An alternative model is that caffeine interferes with a signal pathway in the cell. Caffeine inhibits cAMP phosphodiesterase and it causes a rise in the levels of intracellular calcium; both these activities interfere with signal transduction pathways (99–102). It is important to answer the question whether cells respond passively or actively to radiation insult. An active response would involve activation of a program. Specific receptors would recognise some signal e.g. DNA damage (or its repair) and produce an intracellular signal. This would then be transduced to regulatory proteins which, in this case, control entry into mitosis. In such a scenario caffeine would either inhibit transduction of the signal or bypass the regulatory proteins by interfering with a biochemical component of the program.

The G2/M transition is regulated by a complex molecular program which integrates information about nutrient status, DNA integrity, cell size, etc. via specific receptors and couplers. Maturation promotion factor (MPF) is the principle switch of the G2/M transition (103). Its activation causes cells to enter mitosis and its subsequent inactivation causes cells to enter interphase (104–107). Under appropriate conditions MPF is activated (108–110). MPF is a multi-protein complex, two proteins in particular determine its activity (111–114). One of these proteins is p34, a protein kinase homologous to the *cdc2*$^+$ gene product of *S. pombe*; the other is a cyclin, homologous to the *cdc13*$^+$ gene product of *S. pombe* (115–117). A complex of protein kinases and phophatases assimilates information about nutrient status, DNA integrity, cell size, etc., and under appropriate conditions converts MPF from its interphase (G2) state to its mitotic state. This is achieved by phosphorylation and dephosphorlyation of p34, and by phosphorylation of the cyclin moiety (118, 119). Upon activation, MPF phosphorylates key proteins which causes those morphological features of a cell which are associated with mitosis: chromosome condensation, nuclear envelope breakdown, nucleolar disassembly, etc. (120). Previous work has demonstrated that x-rays cause a reduction in the phosphorylation levels of histone H1, a process which is inhibited in the presence of caffeine (91). Cyclin B protein binds to p34, resulting in histone kinase activity. Muschel et al. (121–123) have shown that G2 arrest is associated with both a delay in the accumulation of cyclin B mRNA, when cells are irradiated in S phase; and protracted low levels of the cyclin B protein despite high levels of mRNA, when cells are irradiated in G2.

The molecular program (which integrates incoming data as specified by receptor and coupler choice) for the G2/M transition checks DNA integrity (124) At least two checks are made: one monitors DNA replication using a protein homologous to the gene products of *cdc25*$^+$ (*S. pombe*) and *tsBN2* (BHK cells), the other monitors DNA repair

using a protein homologous to the gene product of RAD9 from *S. cerevisiae* (125–127). If DNA replication or repair is taking place, activation of MPF is inhibited. In both cases, millimolar concentrations of caffeine override the inhibitions, but it is not yet known if caffeine acts in a similar manner in both situations (128). Another aspect of the mitotic control program is cell size monitoring. In *S. pombe* the *wee1*$^+$ gene product plays the key role (129, 130). Mutants of the *wee1*$^+$ gene prematurely enter mitosis resulting in small cells: the wee phenotype. A second gene product, nim1, inhibits the *wee1*$^+$ function. Overexpression of either *nim1*$^+$ or *cdc25*$^+$ in an otherwise wild-type cell induces premature entry into mitosis and the wee phenotype (131). Importantly, there is no evidence indicating that caffeine can induce a wee phenotype, it only appears capable of overriding the actual cell cycle block but is not able to induce premature mitosis. *cdc25*$^+$ gene product is a protein phosphatase (120, 132), modified by a protein kinase (133–135). The products of the *cdc2*$^+$, *wee1*$^+$ and *nim1*$^+$ genes are all protein kinases. Protein kinases and their associated signal pathways play a prominent role in the G2/M transition and G2 arrest.

Artificial modulation of G2 arrest by protein kinase inhibition

Caffeine is a known inhibitor of the phospodiesterase breakdown of cyclic AMP, thereby, prolonging its effects. A number of workers have investigated the effects of cAMP on G2 arrest. They concluded that cAMP does not induce the cycle block (99, 100, 136, 101). cAMP is the second messenger released in the adenylate cyclase signal transfer pathway and its exclusion rules out protein kinase A as a mediator of G2 arrest. Caffeine also causes a rise in intracellular calcium (102). Investigations with this ion have also excluded it as being responsible for the arrest (137). We have investigated inositol triphosphate and diacylglycerol, the second messengers released in the phospholipase C signal transfer pathway, which activates protein kinase C (PKC) (138).

Specific signals are received by prescribed receptors and various G proteins transduce these signals into the PKC signalling pathway by activating phospholipase C. This is a diesterase which, upon activation, breaks down phosphatidyl-inositol biphosphate into two intracellular signal molecules; inositol triphosphate and diacylglycerol (DAG). DAG interacts directly with PKC; its effects are stimulated by an increase in intracellular calcium. Calcium release from the endoplasmic reticulum is induced via a receptor on its surface which responds to inositol triphosphate. PKC mediates its actions by phosphorylation of specific target proteins (139). Phorbol myristate acetate (PMA) mimics DAG in its action. Ionomycin is a calcium ionophore which artificially increases the intracellular concentration of calcium and thus mimics indirectly the action of inositol triphosphate.

These studies lead us to the following general working hypotheses:

1. Radiation-induced G2 arrest results from activation of a protein kinase signal pathway.
2. Caffeine overrides radiation-induced G2 arrest via interaction with this signal pathway.
3. A receptor for DNA damage exists and a kinase coupler for this receptor, which modulates control of mitotic entry.

They suggest the following specific working hypotheses for the phospholipase C signal transfer pathway:

1. Caffeine overrides G2 arrest by inhibiting the phosphodiesterase: phospholipase C.
2. Phorbol myristate acetate stimulates G2 arrest by mimicking diacylglycerol.
3. Ionomycin stimulates G2 arrest by raising intracellular calcium concentration.
4. Inhibitors of PKC mimic the effect of caffeine and override G2 arrest.

We examined various inhibitors and activators of PKC (138). Phorbol myristate acetate and ionomycin, both potentiators of PKC were found to induce a minor but caffeine-insensitive G2 arrest. The kinase inhibitors, H7 and staurosporine (which are known to inhibit a wide spectrum of kinases) had only a minor effect on G2 arrest at concentrations shown to block PKC. Sphingosine is chemically similar to DAG and PMA. At high concentrations it is toxic to the cells. Doses of sphingosine below 25 μM were used for the investigations to ensure that G2 arrest effects were related to PKC inhibition and not to any potential strand break production. Cells treated with 3 Gy x-rays and 0 μM or 10 μM sphingosine for 4 h and 6 h displayed a minor but significant reduction of the x-ray-induced G2 arrest.

The effects of caffeine on cells treated with both PMA and ionomycin in the presence of 0 mM and 2 mM caffeine for 4 h and 6 h were examined. There was no indication that the caffeine in any way rescued the minor effect. Despite the induced G2 arrest being small it could not be rescued by caffeine which suggests that caffeine acts upstream of PKC in the signal transduction pathway. Caffeine could be overriding the G2 arrest by direct inhibition of a repair enzyme and not via a signal pathway. The effects of caffeine on strand break damage induced by 10 Gy x-rays are displayed in Table 2. Neither the initial production of strand breaks nor their repair were significantly affected by the presence of 5 mM caffeine as has been previously reported (140, 141). This confirms that caffeine does not abrogate G2 arrest by inhibiting enzymes involved in any major DNA repair pathway (138).

Table 2

The first 20 minutes repair of DNA strand break damage following exposure to 10 Gy x-rays in the presence or absence of 5 mM caffeine

Repair time	No caffeine	Caffeine
Unexposed	0.967 ± 0.004	Not determined
0 min.	0.564 ± 0.007	0.597 ± 0.030
5 min.	0.776 ± 0.007	0.788 ± 0.001
10 min.	0.903 ± 0.004	0.902 ± 0.007
20 min.	0.933 ± 0.002	0.922 ± 0.007

The data is expressed as the ratio of double stranded undamaged DNA to total DNA. Standard errors are given.

The conclusions of these studies were:

1. Although PKC influences G2 arrest it is not the primary kinase involved in radiation-induced G2 block.
2. Caffeine does not appear to work via an effect on inositol triphosphate or diacylglycerol.
3. Protein kinase modulators affect G2 arrest, suggesting that a protein kinase signal pathway regulates G2 arrest and its inhibition by caffeine.

At concentrations of staurosporine significantly higher than those required to inhibit PKC, we and others observed that over a period of eight hours cells accumulate in the G2/M phase fraction (142, 143, 15). The kinase inhibitor induces a complete G2 arrest in both V79 Chinese hamster lung fibroblasts and TK6 human lymphoblastoid cells. The cells are equally sensitive to the inhibitor and the induced arrest is dependent upon its continued presence. At these higher concentrations staurosporine does not induce strand breaks, as determined by the method of alkaline elution, see Table 3. Whether staurosporine is administered at doses of 100 or 200 nM for either 2 or 4 hours, there is no indication of increased DNA strand break damage compared to the control (0 nM staurosporine).

If caffeine is present together with 200 nM staurosporine there is no effect on strand breakage either. However, millimolar concentrations of caffeine completely abrogated the staurosporine-induced arrest, release occurring at concentrations identical with those which abrogate radiation-induced arrest. The kinetics of caffeine-induced release and subsequent mitotic traversal are essentially identical for both types of G2 arrests. These results suggest that caffeine interacts with a signal transduction pathway which governs the G2/M transition and controls progression of the cell cycle. They demonstrate the existence in mammalian cells of a programmed intracellular response mechanism to ionizing radiation-induced damage, or its repair. This mechanism could affect many of the biological consequences of radiation exposure (15).

Table 3

Strand break damage to cellular DNA following exposure to staurosporine for two and four hours

Caffeine	Staurosporine	2 hours	4 hours
0 mM	0 nM	0.963 ± 0.001	0.963 ± 0.001
0 mM	100 nM	0.959 ± 0.004	0.963 ± 0.004
0 mM	200 nM	0.964 ± 0.001	0.962 ± 0.004
5 mM	200 nM	0.960 ± 0.004	0.959 ± 0.005

The data is expressed as the ratio of double strand undamaged) DNA to total DNA. Standard errors are given.

Cell division depends both upon the completion of DNA replication and the absence of radiation damage. If either condition is not satisfied, cells arrest prior to mitosis in G2. We have shown that the protein kinase inhibitor, staurosporine, is also capable of inducing this arrest. Furthermore, all three kinds of arrest display the same sensitivity to millimolar concentrations of caffeine. Despite these similarities some differences between the various types of arrest have been reported. The protein phosphatase cdc25 (named after the mutant in *S. pombe*) is necessary for the arrest induced by incomplete DNA replication, but not that induced by DNA damage (144). The reverse is true of the protein kinase *wee* (named after the mutant in *S. cerevisiae*). In this case, *wee* is required for damage arrest expression but *cdc25*$^+$ is not required (144). The two gene products both act directly on MPF. *wee* phosphorylates the kinase subunit p34, on tyrosine 15 which lies in the ATP binding site, rendering it inactive and inhibiting mitosis (145). cdc25 dephosphorylates the same site on p34, reactivating its ATP binding capacity, and permitting initiation of mitosis (146).

Studies examining MPF activity during radiation-induced and staurosporine-induced arrest, and their caffeine-induced release indicate that p34 kinase plays a crucial role (15, 90). The kinase is inhibited during induction of the arrests and re-activated during their release. The positions in the cell cycle where the two induced arrests and where p34 act, are closely associated (within minutes), as indicated by the rapid activation of p34 protein following caffeine administration. The positions of the two induced arrests are close to each other as indicated by the parallel nature of the increase in activity. It is not clear from the data how far upstream of the p34 protein, along the transduction pathway, radiation and staurosporine actually initiate the arrest signal. We cannot formally exclude the possibility that at least two arrest sites exist, closely associated within G2, and that these are the cause of the minor differences in entry time of cells into G1 following caffeine release. The p34 activation kinetics indicates that at most the two arrests can only be minutes apart, furthermore, they must both be equally sensitive to caffeine.

Double-arrest and staurosporine analogue studies

We continued these studies by examining consecutive double arrests followed by selective release of first arrest only.

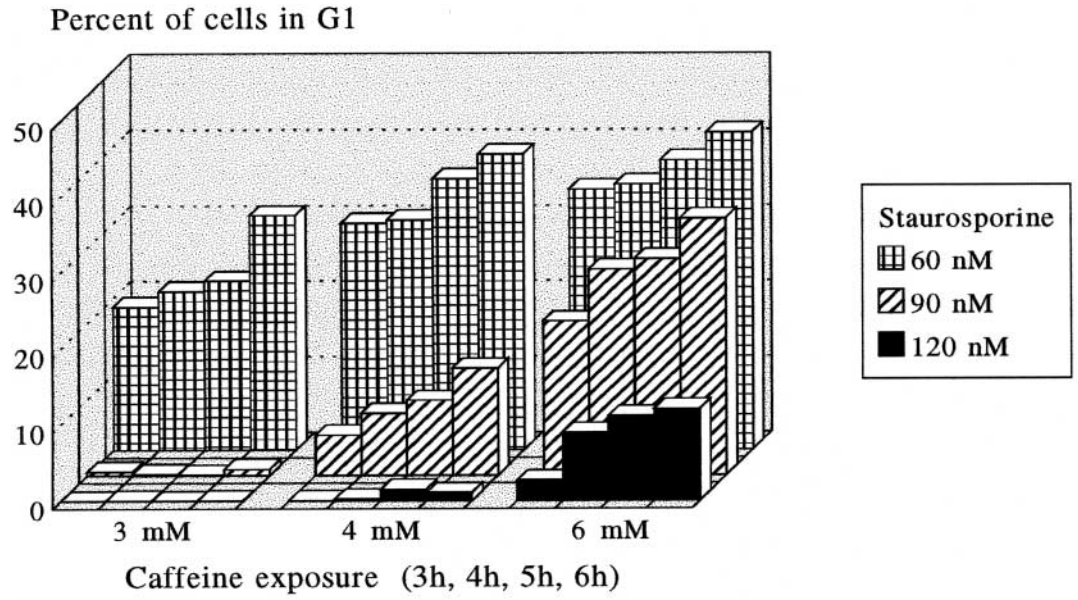

Fig. 1. Caffeine ameliorates the cell cycle arrests induced by staurosporine.

We also examined the competitive inhibition displayed between the block induced by staurosporine, and the release of the block induced by caffeine. Finally a number of analogues of staurosporine exist which made it possible to observe the effects of chemically modifying different regions of the drug on the induction of G2 arrest. The theory behind the double-arrest studies is as follows. In the presence of either inhibitor the cells stop cycling and are arrested prior to mitosis. If the earlier block is induced and then the latter, selective release of the first block results in no mitotic entry. If the latter block is induced and then the earlier, selective release of the first block results in release of the cells from the G2 arrest and mitotic entry.

In order to perform such experiments it was necessary to establish conditions where the different blocks could be selectively released. The G2 arrest induced by staurosporine could be released by addition of caffeine or simply by rinsing the medium to remove the drug. Rinsing the medium following exposure to ionizing radiation did not release the radiation-induced G2 arrest. It was, therefore, possible to selectively release the staurosporine-induced arrest. Selective release of the radiation-induced arrest was possible following observations on the caffeine sensitivity of the staurosporine-induced G2 arrest. Fig. 1 displays the competitive nature of the staurosporine-induced G2 arrest and its caffeine-induced release. At high doses of staurosporine, more caffeine is required to release the induced G2 arrest. At lower doses of staurosporine, less caffeine is required. Following a 90 nM dose of staurosporine, 3 mM caffeine is insufficient to release the induced arrest. However, 3 mM is sufficient to release 7 Gy x-ray-induced G2 arrest. It was, therefore, possible to selectively release the radiation-induced delay.

The first double-arrest studies were performed applying x-rays and subsequently staurosporine. Five hours after treating cells with 7 Gy of x-rays, the G1 phase was essentially empty. A small G1 peak became visible during the fifth hour, even though no caffeine was present, which is attributed to the consequence of cellular recovery from the radiation treatment and subsequent re-entry of cells into cycle (15). If during the fifth hour 3 mM caffeine was present, the G1 peak was significantly larger due to drug-induced release of the arrest. If during the last two hours 90 nM staurosporine was present, the G2 arrest was complete. No 'small peak' of cells in G1 appeared due to recovery from the radiation treatment. If during the last two hours 90 nM staurosporine was present and during the last hour 3 mM caffeine was present, none of the cells re-entered the cell cycle and no cells appeared in G1.

The second double-arrest studies were performed applying staurosporine and subsequently x-rays. Eight hours after addition of 60 nM staurosporine the G2 arrest was complete. If the cells were rinsed, one hour later a prominent G1 peak appeared due to removal of the blocking agent. If during the last two hours of the staurosporine treatment 7 Gy x-rays were administered, the G2 arrest remained complete. However, if the cells were then rinsed, one hour later, despite the x-ray treatment, the cells re-entered cycle and a G1 peak was observed. The G1 peak observed following rinsing of the double-arrested cells was much broader than the G1 peak observed following rinsing of the staurosporine-arrested cells. As expression of G2 arrest following exposure to x-rays may not have been immediate, the cells were tested for their response to rinsing for up to two hours following radiation exposure. There was no indication of induction of a rinse-resistant G2 arrest with time.

We were able to produce conditions which could selectively release either the radiation-induced G2 arrest or the staurosporine-induced G2 arrest without affecting the other. Addition of 3 mM caffeine to radiation-arrested cells released the block. Although staurosporine-arrested cells could also be released by caffeine treatment, 3 mM caffeine was insufficient to release 90 nM staurosporine-induced G2 arrest. No release of the double-arrest, caused by first x-ray exposure and then staurosporine treatment, was observed in the presence of 3 mM caffeine. This is consistent with the possibility that the x-ray-block precedes the staurosporine-block. It is also consistent with the possibility that the two agents together induce an equally or more caffeine-resistant block, than 90 nM staurosporine at the same point in the cell cycle. The result of reversing the order of administration of the arresting-agents was only consistent with the first explanation.

When the cells were blocked with staurosporine for eight hours, all the cells were found to be in the G2 phase. One hour after simply rinsing the cells, a clear synchronized release of the cells into the G1 phase was observed. The same level of G2 arrest was observed after an additional x-ray exposure, and one hour after simply rinsing these double-blocked cells a clear release of the cells into G1 phase was also observed, despite the induction of a rinse-resistant radiation-block. However, the width of the G1 peak demonstrates that the cells released from this double block had a heterogeneous quantity of DNA. This is consistent with the passage of radiation-damaged cells through mitosis which would cause high levels of chromosome aberrations and non-disjunction. The release of these doubly arrested cells supports the hypothesis that the x-ray-block precedes the staurosporine-block. Cells arrested at a time point subsequent to the radiation-induced block are radiation resistant till the next G2 phase. Cells first blocked by staurosporine, then treated with radiation, are free to pass through mitosis after selective release of the staurosporine block because they are blocked later in the cycle than the radiation-transition point.

Staurosporine induces a G2 arrest which can be released either by the simultaneous administration of caffeine, or by rinsing. The kinetics of release-induced G1 entry for both treatments is similar. There is evidence of a slightly more rapid release if staurosporine is simply rinsed off. However, the great similarity between the two types of release suggests that caffeine has an effect similar to rinsing, effectively eliminating staurosporine's action. This explanation is supported by the competitive antagonism displayed between the two compounds. If the concentration of either drug is increased so the concentration of the other has to be increased in order to still be effective; Fig. 1. Staurosporine has been shown to inhibit kinases by binding to the ATP binding site which presumably causes steric hindrance to ATP binding and prevents kinase activity. Gadbois et al. (147) have demonstrated in vitro that staurosporine, at concentrations which induce G2 arrest in cell culture, inhibits p34 activity.

The most parsimonious explanation for these observations is that staurosporine and caffeine compete for the same binding site on p34. If this is the case, it is not clear how caffeine binding simultaneously prevents staurosporine binding yet permits normal kinase function. Therefore, we studied the effects in a number of staurosporine analogues to examine if caffeine-like structures in these molecules are responsible for the G2 arrest phenomenon. The six analogues tested are shown in Fig. 2. The potent PKC inhibitor GF 109203X was incapable of inducing a G2 arrest, whereas, K252a induced an arrest between 30 and 100 nM. Three of the analogues induced an arrest, at the concentrations given (Fig. 2, upper panels) but three induced no arrest, even at the highest concentrations tested (Fig. 2, lower panels).

The analogue GF 109203X, a potent and selective inhibitor of PKC, induced no G2 arrest even when administered at 1000-fold higher concentrations than the staurosporine. Cyclization of the bisindolylmaleimide group to form indolocarbazole and reduction of the maleimide group, which reduce the activity and specificity of PKC inhibition, were always associated with inhibitors capable of inducing G2 arrest. However, KT 5720 and K252b both displayed these traits but induced no G2 arrest. These results confirm our previous findings that PKC is not a major kinase involved in the induction of G2 arrest (138, 15). K252a and KT 5926 were capable of inducing G2 arrest but KT 5720 and K252b were not. The minimal structural differences between K252a and KT 5720 and between KT 5926 and K252b indicate that the residual group is very important in conferring G2 arrest specificity. In particular the methyl ester group of the sugar residue is essential. If this is changed to a hexanyl ester or a free carboxyl group the ability to induce G2 arrest is lost. The shift from a five- to a six-member sugar ring results in a 10-fold greater specificity. If caffeine and staurosporine compete for the same binding site the indolyl group *cis* to the methyl ester group could be the region of the molecule which mimics caffeine. Another explanation for the antagonistic inhibition of the drugs could be their independent action on two competing enzymes. Staurosporine could inhibit the activity of a kinase, and caffeine the activity of a phosphatase; which phosphorylate and dephosphorylate, respectively, the same regulatory target molecule. The physiological target(s) of the two drugs still needs to be determined.

All three radiation-induced arrests: G1/S phase transition, S phase progression and G2/M transition are induced by staurosporine and equally sensitive to millimolar concentrations of caffeine (148). The site of action of the staurosporine remains undetermined. As all three arrests are similarly sensitive to caffeine, the most parsimonious explanation is that all three agents, independent of their

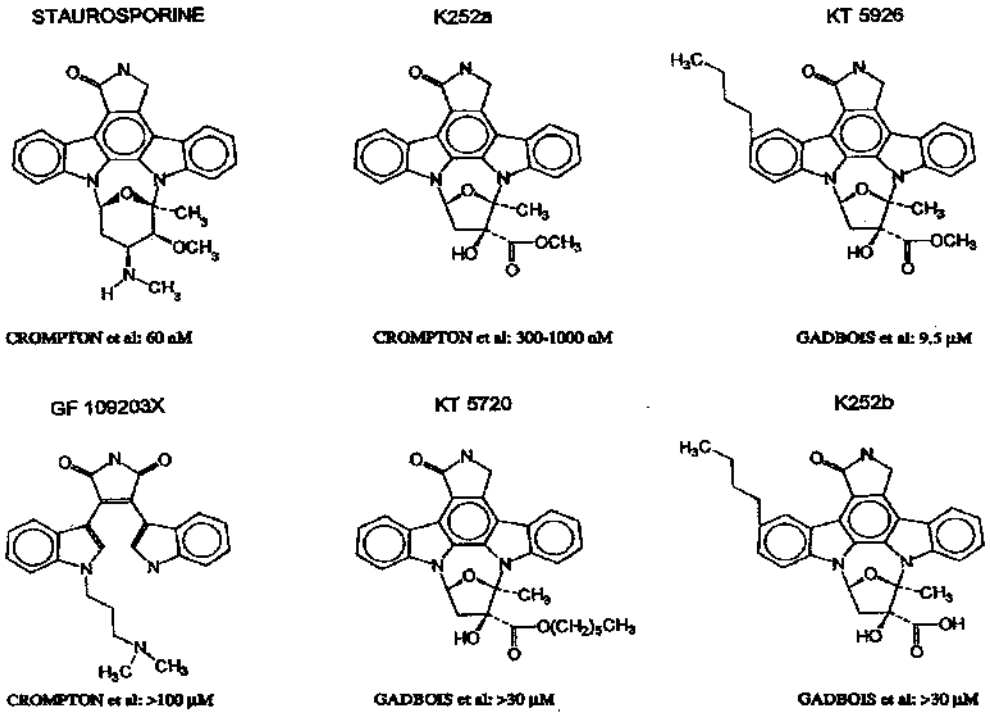

Fig. 2. Analogues of staurosporine. In the upper panels are analogues which could induce G2 arrest, and in the lower panels are those which could not, at the doses given.

primary site of action, eventually interfere with the action of cell cycle promoting factors via a common factor. A second explanation is based on the studies of Muschel et al. (121–123). These studies suggest that cyclin is involved in radiation-induced G2 arrest. In this scenario, radiation inhibits the availability of cyclin until the damage is repaired after which it associates with p34 and promotes mitosis, and staurosporine inhibits a kinase which phosphorylates cyclin in preparation for mitosis. This type of explanation is equally valid for any component necessary for G2/M transition capable of independent inhibition by both radiation and staurosporine. Although the biomolecular details of these pathways still require elucidation, these studies demonstrate that kinase signalling pathways are major components of the programmed response of cells to radiation.

INTRODUCTION OF A DEFECTIVE PROGRAM LOOP

Programmed response requires an adequate stimulus. After the stimulus has been removed either the program should automatically stop, or a subroutine should be initiated which causes its termination. In some situations, however, the response to radiation does not stop. Apparently a defective loop is induced in the program; not only does the response continue in the absence of the stimulus but the state of activation is transmitted to the daughter cells for many generations. The effect of this state of activation is a labile or unstable genome. That the genome can become labile has been evident since McClintock observed that the prolonged presence of a single broken chromosome end in a cell causes activation of dormant transposable elements (TE) (1). One of the consequences of this lability is an elevated probability of cellular transformation and carcinogenesis.

Radiation-induced genetic instability

The component cells of an irradiated normal-appearing survivor colony or clone do not have a proliferation capacity equivalent to that of unirradiated cells. It has long been known that regions of normal (healthy) tissue that have 'recovered' from irradiation will not tolerate anything like as much radiation as previously unirradiated tissue and will react unusually sensitively to subsequent exposures, or to cytotoxic drugs. Following irradiation, the cells have changed and this change is passed on to subsequent generations. Upon closer inspection it is found that the genome of these cells has become labile. The phenomenon is referred to as radiation-induced genetic instability (RIGI). Various types of RIGI have been observed. The types observed depend very much on the methods of observation used and they seem to be different expressions of the same phenomenon. The types include:

Delayed reproductive death
Microsatellite changes
Gene amplifications & chromosome aberrations

Increased mutation & transformation
Changes in ploidy

Delayed reproductive death was observed soon after assays based on cell death became available (149, 150). The progeny of irradiated cells were found to be generally less viable (151, 152). This reduced fecundity persisted in many cell types for 20–50 cell generations before, in some but not all, the fitness typical of unirradiated cells was restored (153–155). Microsatellites are di- tri- or tetra-nucleotide sequences which are repeated up to hundreds of thousands of times in the genome. Following irradiation the length of these sequences becomes highly variable, probably via a process of induced recombination (156, 157). Gene amplification is the abnormal multiplication of genes during the cell cycle. Not just the genes, but often a large DNA sequence in which the genes are located, is abnormally replicated. Once again the mechanism of this process could be associated with induced recombination. An increased incidence of chromosome aberrations is sometimes observed in the same cells, involving inversions, deletions, translocations and the production of polycentric chromosomes and chromosome fragments (158). These types of aberrations are usually detrimental to the cell and they are clear indicators of induced recombination. A normal cycling cell would not tolerate the existence of such chromosome abnormalities and would induce a process of programmed cell death. The fact that many cell lines do tolerate the damage indicates that they already have compromised genome-surveillance mechanisms (159).

Another type of RIGI is elevation of the rates of mutation (160, 161) and transformation (162, 163). Radiation is often thought to act directly on DNA when causing mutagenic damage. An alternative to this hypothesis is one where radiation causes some cryptic form of damage and mobilizes factors which influence the genome in an inappropriate manner and enhance spontaneous levels of mutation and transformation. These possibilities have been studied by irradiating very few cells and examining the consequences. The probability of causing a specific mutation event in only a few cells is very low. What is observed is that, independent of the number of cells irradiated, the frequency of transformation, and in some cases of gene mutation, is identical (164). This is best understood in terms of an induced genetic instability. Radiation-induced mutations have been sequenced. They are generally found to be caused by deletion or translocation events involving recombination, and not point mutations.

The frequency at which the various types of RIGI are induced is very high. Following even mild doses of radiation most of the cells demonstrate the RIGI phenotype (165). Therefore, the target cannot be a single gene. Much evidence suggests it is the presence of DSB which induce an elevated background of recombination events. This is not an unexpected finding. It has been known for some time that DSB and chromosome gaps are 'healed' using a repair mechanism which involves recombination. In this way the missing DNA sequences can be replaced. It is also interesting to note that all of the RIGI types are genomic changes which can be induced by recombination. A general observation is that although RIGI is induced in basically all cells irradiated, the severity of the genetic changes induced is very heterogeneous in the survivors and even in clones derived from irradiated cells.

McClintock demonstrated that chromosome breaks can cause genetic instability by activating at least two dormant transposable elements (TE) (166). The phenomenon of hybrid dysgenesis (a syndrome of related abnormalities caused by a TE becoming active after certain strains of *Drosophila melanogaster* cross-breed) is thought be directly related to this. Progeny from the dysgenic cross shows mobilization of at least four different TE at similar frequencies. The production of DSB caused by the mobilization of a single TE might induce a cellular response that releases other TE from repression (167). Introduction of non-physiological DSB into the genome may induce genetic instability. In the case of hybrid dysgenesis this is detrimental to the cell. Ionizing radiation can also cause DSB in the cell and much evidence has arisen to demonstrate that it also induces genetic instability. The genetic instability phenotype is a natural precursor to cellular transformation and carcinogenesis. Thus the production of non-physiological DSB is associated with detrimental alterations in the genetic material and the cancer-prone phenotype.

Instability in the genome causes many mutations. Organisms displaying these highly mutable phenotypes are called mutators. Cancer is a mutator phenotype which arises in somatic populations. Nowell (168) proposed the theory of clonal evolution of neoplasia. Development of cancer proceeds by sequential steps from a normal cell to an invasive, metastatic tumour. Multi-step carcinogenesis involves many cellular processes or defects including:

Induction of genetic instability
Compromised checkpoints (p53, pRb)
Loss of cell cycle control (G0/G1, G1/S, Contact inhibition)
Abrogation of induced apoptosis (*Bcl-1*, *Bax*, *Nbk*)
Evasion of the immune system
Induction of angiogenesis (loss of angiostatin)
Telomerase activation (observed in $>90\%$ of tumours)
Induction of the metastatic state
Multi-drug resistance (P-glycoprotein)

Based on epidemiological studies it has been estimated that the number of mutations necessary for cancer development can vary from as many as 7 in stomach cancer up to as many as 12 in the case of prostate cancer (169). Background mutation rate in normal cells can account for

only 2 to 3 mutations observed in tumours. In order to account for all the mutations observed; either during tumour growth the cells exhibit a mutator phenotype, or each successive mutation event can drive clonal repopulation, or both occur as they are not exclusive (170, 171). Hartwell (172) notes that defects in cell cycle checkpoints could contribute to genetic instability in cancer cells.

Genetic instability has been demonstrated to be a normal characteristic of tumours. Stepwise acquisition of mutations was originally suggested by Foulds (173). Cifone and Fidler (174) observed that highly metastatic cells display a greater mutation frequency rate than less metastatic cells. Seshadri (175) reported that leukaemia cells display mutation rates 100-fold greater than normal lymphocytes. Gene amplification rates in tumorigenic cells are 10^{-3}, whereas in normal cells this is below the detection rate of the experiment ($\sim 10^{-6}$) (176). Vogelstein et al. (177) demonstrated that multiple allele loss occurs in colon cancers. More recently Denko et al. (163) demonstrated that controlled mutation of Ha-*ras* in p53 negative cells resulted in genetic instability. Mao et al. (178) demonstrated that microsatellite alterations are detectable in $\sim 50\%$ of all human cancers. Inhibition of genetic instability would be an excellent means of preventing carcinogenesis. Some indication that this may be possible has arisen from studies with protease inhibitors (179). These can prevent the radiation-induced state associated with enhanced frequencies of transformation and have been demonstrated to inhibit carcinogenesis.

Radiation-induced ploidy instability

Radiation-induced transformation of C3H 10T1/2 mouse fibroblasts causes a number of morphologically visible alterations to the cells. We investigated various phenotypic characteristics of these cells to determine to what extent they are neoplastically transformed. C3H 10T1/2 cells at passage 13, which had never been allowed to grow to confluence, were plated in 75 cm^2 culture flasks. 24 h after plating they were exposed to 8 Gy x-rays at 0.9 Gy/min. Immediately after irradiation the cells were washed with PBS, harvested and plated in 9 cm dishes at a cell concentration of 2–3 survivors per cm^2. Cells were maintained in culture for more than eight weeks with weekly exchange of the medium. After 58 and 66 days foci were isolated from a total of eight dishes. They were isolated by trypsinization in a small isolation ring placed around the colony. The isolated focal lines were allowed to grow and from them cell clones were developed. Cells were harvested and then inoculated into 24-multiwell plates, at a concentration of one cell per well. The 24-multiwell plates were regularly monitored until colonies had grown. Cell clones were taken from those wells where only a single colony had been observed during the whole growth period. Any indication of adjacent satellite colonies or non-circularity of a colony resulted in its being excluded.

Two sets of radiation-induced morphological transformants were isolated with a period of approximately one year separating the two experiments. Foci were chosen based on their highly transformed type II/III appearance: darkly stained, piled-up foci with peripheral criss-cross growth (180). In both experiments a total of 19 foci were isolated from which a total of 63 clones were established. All of the clones retained a transformed appearance. All of the focal lines had individual characteristic morphologies. During the first experiment, six focal lines were isolated. Clones were established from these focal lines. Four clones were established from one of the focal lines. During the second experiment thirteen foci were isolated, only four of these lines were used to establish clones.

The survival curves of parental C3H 10T1/2 cells and two independent clones from the most morphologically transformed type III focus were compared. No significant difference in the radiosensitivity of the three lines was observed. The transformation frequency per survivor at 8 Gy was normal: $3.0 \times 10^{-3} \pm 1.7 \times 10^{-4}$. The DNA content of the radiation-induced morphologically transformed lines was examined. Both focal lines and clones of these lines were investigated. The control line was derived from a patch of irradiated but non-transformed monolayer surrounding the radiation-induced foci. Although the cells originate from mice which have a diploid chromosome count of 40, our parental C3H 10T1/2 cells have an average of 68 chromosomes reflecting a hypotetraploid state. The control line had the same DNA content as the 'normal' parental hypotetraploid line. Focal lines had varying DNA content (20). Giemsa staining demonstrated that hypooctaploid cells possess approximately 120 chromosomes confirming that the abnormal DNA content of the cell lines results from polyploidization (20).

Recently, it has been reported that transformation of cells, by transfection with various oncogenes, can increase their radioresistance in association with increased G2 delay (7, 181). We examined whether radiation-induced morphological transformation was also associated with an increase in radioresistance. The radiosensitivity of two clones from the focus with the most extreme type-III phenotype was compared to that of the parental C3H 10T1/2 line. No significant difference between the survival curves was observed. The estimated curve parameters obtained from the parental survival curve are typical for C3H 10T1/2 (for review see Reference (182)). The transformation frequency was also normal (183). The fact that no differences between the cell lines were observed suggests that the morphological transformation induced is an early stage in the development of neoplastic transformation.

Staurosporine induces a single, major, cell cycle arrest at the G2/M border in both transformed and non-transformed cells, however, it also produces a second major cell cycle arrest at the G1/S border in 'non-transformed' cells (143, 147, 15). We investigated whether this G1 arrest was

lost in the radiation-induced morphological transformants of C3H 10T1/2 cells. The ability to arrest in G1 was retained after exposure to either staurosporine or x-rays. This may reflect a normal status of the p53 genes in these cells because a 100% concordance between the status of the p53 gene and the ability of various mammalian cells to arrest in G1 following exposure to ionizing radiation has been reported (184–186). In the brain, low grade tumours (astrocytomas) progress to faster growing invasive high grade tumours (glioblastoma multiformae) in association with mutation of the p53 alleles (187). Furthermore, mutations at the p53 locus were found in 31% of x-ray transformed C3H 10T1/2 clones (188). Our transformed C3H 10T1/2 cells displayed both arrests suggesting that at least one of the p53 alleles is still functional.

Despite evidence of radiation-induced morphological transformation being an early stage in the neoplastic transformation pathway, the transformants display a clearly abnormal genetic instability. About 50% of the focal lines transformed with 8 Gy and their clones displayed this phenomenon. We have observed ploidies ranging from hypotriploid to hypododecaploid. The polyploid populations were not normally stable and in many cases reverted to their parental hypotetraploid state with time in culture, although exceptions were seen (15). Despite these changes in ploidy, all the clones retained their morphological transformed appearance throughout the course of the studies. Genomic rearrangements of minisatellite DNA have also been reported in x-ray transformants of C3H 10T1/2 cells (156). 40% of 6 Gy-induced transformants displayed this phenomenon. These authors found no correlation between the appearance of specific genomic abnormalities and tumorigenic potential of the x-ray transformants but they report perfect correlation between the genomic rearrangements and tumorigenicity of methylcholanthrene-induced transformants. The transformation event induced by x-rays produces an unstable state in the genetic constitution of C3H 10T1/2 cells.

Enhanced transformation in inhomogeneous irradiation fields

The previous data suggest that the transformation phenotype is frequently associated with genetic instability and in particular with a tendency to undergo polyploidization. Subsequent studies of C3H 10T1/2 cells in inhomogeneous fields of radiation suggest that the induction of an unstable state in the genome of a cell may not be a direct effect of the radiation (189). We developed an in vitro model-system employing a thin ß-emitting yttrium-90 wire, to produce extremely inhomogeneous radiation fields in order to investigate hot particle effects on the induction of cellular transformation (190). In the inhomogeneous radiation field surrounding beta-sources, non-lethally and superlethally irradiated cells are in close proximity permitting interaction via extracellular signals. This situation is typical of hot particles such as those released from Chernobyl. Beta-emitting yttrium-90 wires were employed to investigate radiation-induced cell transformation under these conditions. Integrated 24 hour-doses in the range from 0 to 1100 Gy across the field of exposure were produced. At equal levels of toxicity a 10-fold enhancement of transformation frequency in C3H 10T1/2 cells was observed in the presence of superlethally irradiated cells (189). Homogeneous fields of low dose-rate beta-irradiation produced transformation frequencies typical for photon at the same dose level.

C3H 10T1/2 cells within the SF_{65} dose region of the inhomogeneous irradiation field were isolated following exposure and examined by a standard transformation assay. Either superlethally irradiated cells were simultaneously present during exposure or they were excluded by localising the cells to the SF_{65} dose region alone by means of an agarose trench. The frequency of transformation observed in cells exposed to a background of superlethally irradiated cells was enhanced 10-fold at equivalent cytotoxicity. Homogeneous fields were made with an activated yttrium-90 foil at three different doses (4.5, 13 and 21 Gy). The results (4, 9, and 17×10^{-4} transformants per survivor, respectively) were in the range predicted for equivalent photon doses. Percent survival after inhomogeneous yttrium-irradiation in the investigated area was 66.1 ± 2.8 in the superlethal-background situation and 62.2 ± 12.3 in the non superlethal-background situation. These are not significantly different at the 0.01 level. Plating efficiencies were between 10 and 30%, which was somewhat lower than we observed in control experiments and is attributed to the additional handling, and/or growth in Petriperm dishes. Cells were also exposed to a homogeneous field of low dose-rate ^{90}Y-irradiation and examined for transformation. The exposures lasted 24 hours. 4.5 Gy (3.1 mGy min^{-1}) resulted in a transformation rate of 4.3×10^{-4}; 13 Gy (9 mGy min^{-1}) resulted in 9.4×10^{-4}; and 21 Gy (14.6 mGy min^{-1}) resulted in 17×10^{-4} transformants per survivor. These values are in the expected range for low LET radiations (191).

For the inhomogeneous exposures, the integrated dose over 24 hours fell sharply from approximately 1100 Gy directly above the wire to zero Gy at the edge of the dish. Proximal to the yttrium wire, high doses caused both cell death and cellular detachment. The transformation frequency per viable cell was 10.6×10^{-4} for cells exposed in a background of superlethally irradiated cells and 1×10^{-4} for cells in non superlethal-background conditions a ten-fold difference (189). In the non superlethal-background situation, the dose received by the cells varied no more than 30% across the 2 mm trench. The SF_{65} dose region under both conditions was identical with respect to dose, dose-rate and cell density. To exclude the possibility of artifacts caused by the experimental set-up, control experiments were conducted with 4 Gy high dose-rate (0.9

Gy min^{-1}) x-ray exposures. The resulting frequencies were 15.1×10^{-4} and 15.3×10^{-4}, for cells selected with aluminium frames or for cells in the agarose trenches, respectively. Such values were routinely obtained in our laboratory and have been reported in the literature (164).

These high dose-rate x-ray-experiments demonstrate that neither the agarose nor the method of isolating cells by means of aluminium frames had a measurable biasing effect on the transformation frequency. Following the 24 h exposure to ^{90}Y ß-irradiation, cells from both experimental set-ups were treated in exactly the same manner. The only obvious difference between the two set-ups was that in one case intercellular signalling arising from superlethally damaged cells was possible, either via cell–cell contact or via the medium; but in the other case, no superlethally damaged cells were present. It seems unlikely, but we cannot exclude the possibility, that radiation breakdown products in the media contribute to the enhanced transformation frequency (192). The transformation frequency after 4.5 Gy low dose-rate ß-irradiation is 3-fold lower than after high dose-rate x-ray irradiation. 13 Gy homogeneous ß-irradiation is about as effective as 6 Gy with superlethally damaged cells, corresponding to a 2-fold enhancement with respect to total dose.

Transformation has been demonstrated to be a multistep process. As the difference observed here was caused during the 24 h of exposure, it probably relates to the initiating event caused by radiation damage. The difference appears to be associated with the heavily damaged, superlethally irradiated cells. Some form of signal either passively or actively induces enhanced probability of transformation in non-lethally irradiated cells. We have not yet succeeded in isolating the signalling agent. The pioneering work of Kennedy et al. (164) demonstrated that a state of enhanced transformation is induced in cells by ionizing radiation. We examined foci and clones from colonies of radiation-induced C3H 10T1/2 transformed cells and observed a state of genetic instability causing multiple ploidy events (20). The molecular basis for these responses, associated with enhanced transformation frequencies, is not known. Its induction is not necessarily a direct effect of radiation. Studies of low-dose α-particle exposures revealed an unexpectedly high proportion of cells with sister chromatid exchanges (193, 194) and enhanced p53 gene expression (195). In these studies a bystander effect was indicated on microdosimetric grounds because more cells than those physically traversed by an alpha particle displayed features of radiation exposure.

Various forms of communication between differently damaged cells have been reported in the literature. Expression of DNA damage-activated genes is expected to be high in superlethally irradiated cells promoting enhanced release of a spectrum of damage-associated cytokines including TGFß and TNFα. Important DNA damage-activated genes that are induced by radiation are the protooncogenes c-*fos*, c-*jun*, *jun-B* and c-*myc*. They all code for nuclear transcription factors and have been discussed as critical target genes in radiation carcinogenesis (196). TGFß and c-*myc* have been found overexpressed in morphologically transformed C3H 10T1/2 cells (197), and transfection of untransformed cells with c-*myc* resulted in increased transformation (198). Another interesting DNA damage-activated gene codes for basic fibroblast growth factor (bFGF). It serves as an enhancer of potentially lethal damage repair via an extracellular autocrine loop and enhances DNA synthesis in untransformed C3H 10T1/2 cells (199). Dead and dying cells also release DNA fragments (200) which have demonstrable transformation activity. The ten-fold increase in transformation frequency is greater than differences observed in dose protraction experiments. It is as high as promoter-enhanced transformation, e.g. TPA (201). Superlethally irradiated cells appear to be acting like a tumour promoter.

PROGRAMMED CELL SUICIDE

Rupture of the plasmalemma is proof of cell death. It results in a collapse of physiological ionic gradients, irreversible loss of metabolic intermediates and cytosolic enzymes, and damage to the mitochondria. A number of techniques, measuring the permeability of such ruptured cells to various stains: nigrosine, trypan blue, and eosine, are commonly used to evaluate viability. Membrane rupture is also employed by the immune system to protect an organism from foreign or unwanted cells. A group of proteins collectively referred to as complement are targeted to the unwanted cells and open pores in the target membrane. Such rupture is also associated with the final stages of programmed cell suicide, apoptosis, which is a physiological process essential for morphogenesis during development (202, 203) and for tissue homeostasis in the adult. It has been extensively studied in the nematode *Caenorrhabditis elegans*. Cells undergoing cell death were observed to be engulfed by phagocytosis. This lead to the suggestion that the cells may actually be killed by phagocytosis, rather than suicide. Discovery of the *ced 1* and *ced 2* mutants of *C. elegans* helped to identify the mechanism of cell killing. These mutants are incapable of phagocytosis, nevertheless, the apoptotic cells still die at the appropriate time. This demonstrated that here death was an autonomic 'decision' made by the dying cells (204).

Programmed cell death is called apoptosis to distinguish it from necrosis (205). The word 'apoptosis' comes from the Greek and means 'falling off' as of petals from flowers or leaves from trees (206). Apoptosis appears to be the cell's way to die in a physiologically advantageous manner, assisting absorption of its debris by processing the intracellular contents, and thus avoiding the inflammatory response induced by the necrotic release of proteases and other cytolytic substances. Apoptosis occurs following gen-

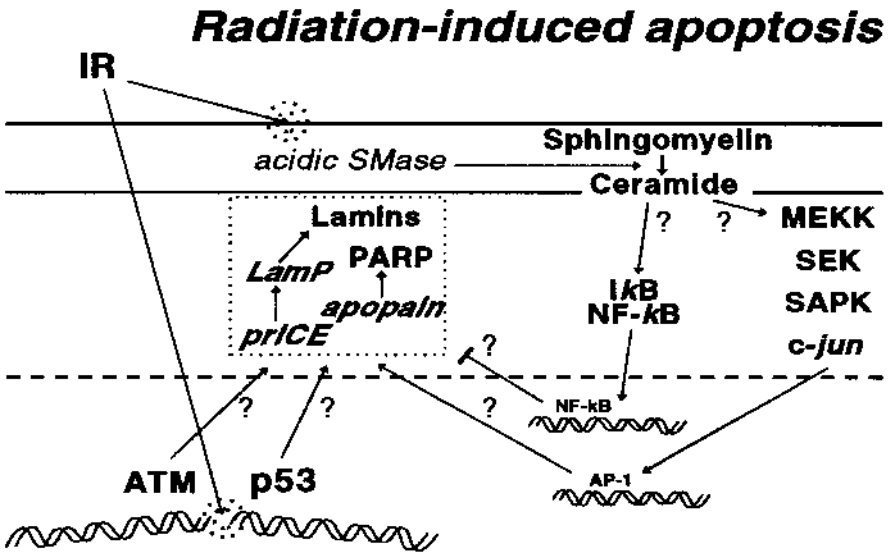

Fig. 3. Apoptosis can be induced through DNA or membrane damage.

eral injury and it is possible that it represents selective removal of cells whose survival would compromise that of the organism as a whole. Moderate doses of radiation have been found to cause apoptosis, rather than necrosis, in various tissues. A single small dose of ionizing radiation produces an extensive wave of apoptosis which peaks 3–6 h after irradiation in the highly radiosensitive mouse gut crypts (205). Apoptosis has a defined morphological pathogenesis. Its onset is sudden and is marked by a rounding up of attached cells. Then a period of cellular shrinkage follows. Often this is followed by violent pulsation and blebbing of the plasmalemma. Many of the surface protrusions detach in the form of spherical fragments called apoptotic bodies. Over the next few hours, if not phagocytosed, the residual cell mass and the apoptotic bodies undergo swelling and lysis: secondary necrosis. However, these details vary considerably from tissue to tissue (207). The mechanisms underlying apoptosis differ from those in necrosis in that: a) the process is frequently triggered by physiological stimuli, b) depletion of the cellular energy supply is not necessarily involved, and c) a step involving macromolecular synthesis is usually observed.

Many molecular studies of the signalling pathways involved in the induction of apoptosis have been performed. Studies of radiation-induced apoptosis indicate involvement of two different targets; Fig. 3. One pathway involved in apoptosis signalling is used when DNA is damaged. Enhanced expression of the p53 protein is a part of this pathway (208, 209). A second pathway involved in apoptosis signalling is taken when cellular membranes are damaged. Production of the second messenger, ceramide, from sphingosine is part of this pathway (210, 211). Subsequently, this pathway divides along the c-*jun* kinase pathway (212, 213) and the *NF-kB* pathway (214). Demonstration that apoptosis can be induced by radiation independently of chromosomal DNA damage was made possible by the development of cell systems devoid of nuclei.

The Leukocyte Apoptosis Assay

A rapid and simple assay for the estimation of a patient's individual response to radiation treatment with a view to avoiding severe acute reactions and as an initial baseline for the assessment of general response to radiotherapy was developed (215, 216). In 1985 the Lancet journal (ii, 23–25) published an editorial entitled 'Radiosensitivity and the clinician' examining the clinical relevance of tests of cellular radiosensitivity). It was recommended that a rapid, reproducible test based on lymphocyte response would be of value in radiotherapy. Individual variation of cancer patients in the susceptibility to skin damage during radiation treatment is considerable. Occasionally, the susceptibility is so profound as to interfere with the planned treatment protocol. It was such a clinical observation that led to the discovery of the molecular genetic defect in *ataxia telangiectasia*. A number of studies were begun, reviewed by Lewis (11). The need for such assays was re-emphasised as it became clear that individuals vary widely in the susceptibility of their tissues to ionizing radiation damage. Lewis summarised the situation by stating "*Current knowledge of individual variation is fragmentary; the problem has not been studied in depth and laboratory methods for assessing radiosensitivity are at present too laborious for large populations to be examined*".

Radiation-hypersensitive patients are still treated with standard doses. In some instances treatment continues until adverse reactions of the radiotherapy become highly acute. An assay which provides the physician with details of normal tissue radiosensitivity from a variety of cell

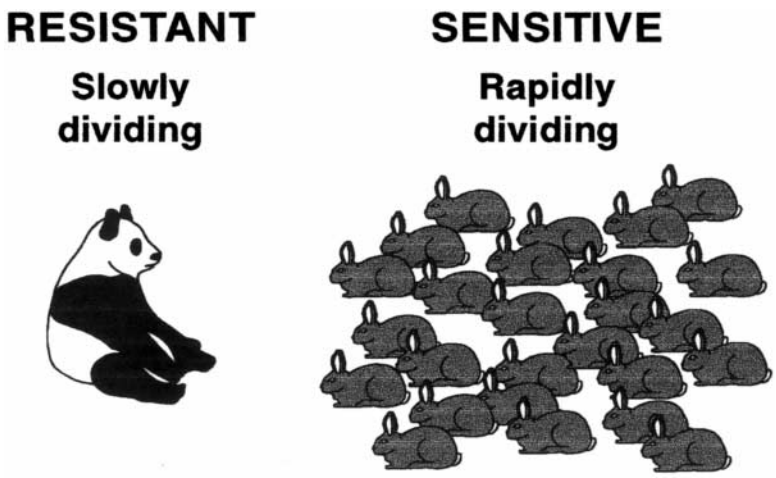

Fig. 4. A long-standing dogma: rapidly dividing cells are radiosensitive.

types would assist in the early recognition and appropriate handling of radio-hypersensitive patients. Current estimates suggest 5 to 10 per cent of cancer patients receiving radiotherapy display adverse reaction due to hypersensitivity (11). Development of the Leukocyte Apoptosis Assay involved the screening of large numbers of healthy persons in order to examine this value in the general population and also in radiotherapy patients.

In England patient groups are bringing litigation suites against radiotherapy clinics because standard doses, too high for hypersensitive patients, have been administered (P. D. Lewis, personal communication). With so much clinical evidence to suggest wide inter-patient radiosensitivity, this reaction is understandable, particularly as the routine assessment of radiosensitivity is now within our grasp. It has been estimated that if the most sensitive 5% of individuals diagnosed for radiotherapy could be identified and excluded from standard therapies, the remaining patients could be treated with an estimated 5% higher total dose. This would induce no greater total burden of complications but an estimated 20% increase in the frequency of local control due to the sigmoid response curve (217). This estimated percentage of radiosensitive patients may be too high. Nevertheless, there are various clinical situations where pre-treatment knowledge of a patient's healthy-tissue radiosensitivity would be welcome. Therefore, we have been developing an assay to determine normal tissue radiosensitivity based on the response of an individuals' different leukocyte cell types.

One of the long-standing dogmas of radiobiology is that slowly dividing tissues tend to be radioresistant but rapidly dividing tissues are radiosensitive; Fig. 4. However, in the clinic one often finds slowly growing tumours which are radiosensitive and rapidly growing tumours which are radioresistant (218). Cells respond to radiation via signals caused by the presence of the radiation-induced damage. Much indirect evidence points to damaged DNA as being the source of the signal. It is becoming increasingly clear that cell killing during fractionated radiotherapy is a programmed response sensitive to the integrity of the molecular components (219). The genetic component of normal tissue radiosensitivity has become a major theme in radiation oncology.

Radiation damage induces cell cycle arrests. There is a close link between these arrests and cell death. In transformed cells, a constant growth stimulus is present. This promotes cell cycling. Following genomic insult a second signal is induced, which inhibits cell cycling. When both occur simultaneously, the cell induces apoptosis (220, 221). Where positive signals promoting growth stimulation and negative signals inducing growth arrest conflict, the cell responds by apoptosis. A whole series of oncogenes is involved in this apoptotic response. Promoting apoptosis are the cellular oncogenes p53, *myc*, E2F and the viral oncogenes E1A, E1B19k and E7. Inhibiting apoptosis are the cellular oncogenes Rb, *ras* and *bcl-2* and the viral oncogenes E1B55k, E6 and MDM2. This list is in no way exhaustive, the role of some of the proteins is shown in Fig. 5. Perhaps the most intriguing aspect of this phenomenon is the fact that if proteins responsible for apoptosis are mutated or functionally inhibited, the cells become more radioresistant (222). Thus, radiation response is dependent on all of these different oncogenes as they work for and against the induction of apoptosis. There is also a strong genetic component. Genetic disorders associated with abnormal radiosensitivities include: *Ataxia telangiectasia*, Nijmegen's break syndrome, Huntington's disease, chromosome 13 abnormalities, immunodeficiency disease, neurofibromatosis, and a variety of reports of patients with severe radiotherapy reactions and demonstrably radiosensitive fibroblasts ((223) and citations therein). Neurofibromatosis is something of an exception to the rule, conferring radioresistance to the cells. This can be readily understood in terms of positive

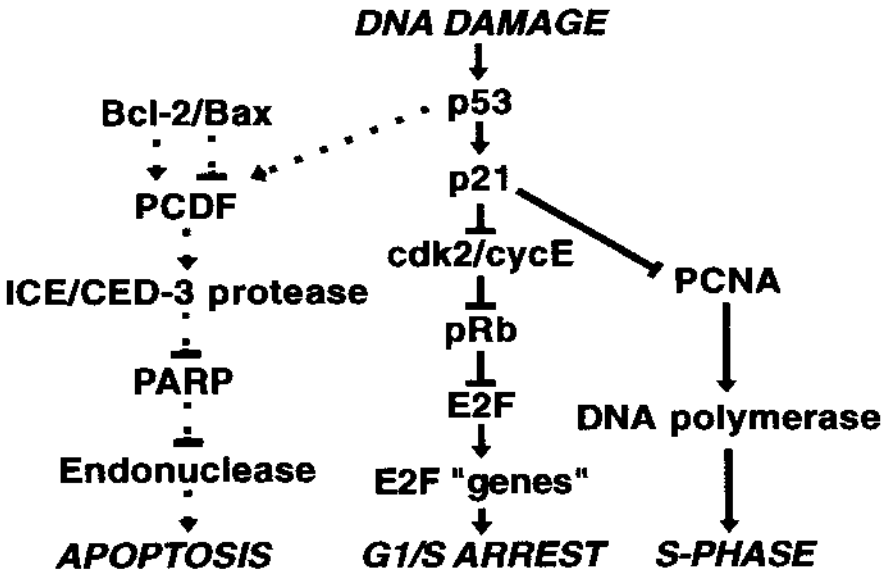

Fig. 5. DNA damage-activated pathways: p53 is an important component.

and negative signalling components. Indeed it has recently been demonstrated that the mutated gene causing neurofibromatosis, the *NF1* gene, is involved in cell signalling via the *ras* pathway. All of this leads to the conclusion: *At lower doses the cytotoxic action of many anticancer agents is largely determined by the genotype of the cell rather than the genotoxicity of the agent* (219).

Elyan et al. (12, 13) demonstrated that inter-donor differences were significantly larger than intra-experimental differences if low dose-rates were used to enhance differences in inter-donor recovery, and if an internal standard was used (in their case a store of lymphocytes from a single donor). Using a clonogenicity assay Stewart et al. (224) demonstrated differences in radiosensitivity up to one order of magnitude between lymphocyte cell types. If any shift in the relative frequencies of the various cell types occurs (as expected during the progression of a tumour), an unreliable estimate of radiosensitivity will result. Clear variation in the radiosensitivity of different leukocyte cell types was observed using the Leukocyte Apoptosis Assay (215). The assay also has the advantage that results become available to the clinician 24 h after receiving the sample and, because of the use of multiple leukocyte cell types, the need for an additional characterised donor is obviated.

A number of attempts by various laboratories to quantify intrinsic radiosensitivity have been described. The method of Elyan et al. (13) examines total peripheral blood lymphocyte survival using the limiting dilution assay method. The results become available after approximately 14 days. They are compared to an internal standard, the blood of a donor whose radiation response is already characterised in order to reduce experimental variation. Statistically significant differences between individuals can be obtained using this method. Another technique, that of Floyd and Cassoni (225) has also been described. This examines the induction of micronuclei using the cytochalasin B method. The results become available after approximately 3 days. They are compared to an internal standard, the blood of 9 other donors which was simultaneously examined. This provides a median value from which to determine radiosensitivity status. The Leukocyte Apoptosis Assay has the advantage that the use of multiple leukocyte types obviates the need for reference donors. Use of multiple leukocyte cell types takes into account the spectrum of radiosensitivities displayed by the cells rather than simply lumping the data together. It is possible that a sector of the variation between donors reported in previous lymphocyte radiosensitivity tests is due to shifts in the relative frequencies of the different blood types. The Leukocyte Apoptosis Assay was developed for a number of reasons:

(a) Development of techniques to assess the spectrum of radiosensitivities displayed amongst the various leukocyte sub-populations.
(b) Evaluation of the range of radiosensitivities observed in the general population and in particular in patients where radiotherapy is indicated.
(c) Correlation of these radiosensitivity spectrum to acute, subacute and late normal tissue responses induced by radiotherapy.
(d) Prediction of response to radiotherapy based on the spectrum of radiosensitivities displayed by the various leukocyte sub-populations.
(e) Patient-specific optimisation of radiotherapy treatment regimes based on the spectrum of radiosensitivities displayed by the various leukocyte sub-populations.

Development involved a two-pronged approach; first the development of the apoptosis assay using an established human leukocyte cell line, second the development of the techniques using freshly collected peripheral blood. TK6 human B lymphoblastoid cells were irradiated and left to incubate. The cells were then stained with propidium iodide and examined by flow cytometry. An essentially linear dose-response relationship was observed with increase in dose after 20 h incubation (226). The exquisitely sensitive cell cycle arrests in these cells are also readily observed. As the cell cycle arrests are induced by a kinase signal transduction pathway, the studies suggest the possibility of examining cell cycle arrest induced in PHA-stimulated peripheral blood lymphocytes as an additional indicator of radiation response. Radiation-induced apoptosis in these TK6 cells requires time to become fully expressed. Essentially only background levels of apoptosis were observed up to 12 h after irradiation. At 20 h and 24 h, up to 50% of the cells were found to be apoptotic, following a 6 Gy dose. These TK6 experiments demonstrate the utility of the assay in examining radiation-induced apoptosis (226).

A routine medical blood test which makes use of an antibody kit to inform the physician about the frequency of the various cell types and to compare these values to 'normal' frequencies as presented in Table 4 was used to recognise the various leukocyte cell types.

Those cell types examined for apoptotic response are highlighted. The mean frequency (cells/μl) of these cells as determined using the kit, and the antigen used for discrimination, are also included. Antigen CD45 is present on all human leukocytes and is used as a general marker. The three granulocyte sub-populations can be discriminated on the basis of their various light scattering properties. Eosinophils are the largest (display the greatest forward scatter) and most granular (display the greatest side scatter) of the three granulocytes, and the basophils are the smallest and least granular.

Table 4

The major leukocyte cell types

Cell type	Antigen	Frequency ($10^3/\mu$l)
Myeloid cells		
Granulocytes		4.2
Neutrophils		3.97
Eosinophils		0.18
Basophils		0.05
Monocytes	CD14	0.3
Lymphocytes		
B lymphocytes	CD19	0.3
T lymphocytes	CD3	1.4
Helper/Inducer	CD4	0.8
Cytotoxic/Suppressor	CD8	0.6
Activated T cells	CD3 and HLA-DR	0.15
Natural killer cells	CD16 and CD56	0.2

A major consideration was the development of a rapid radiosensitivity assay. Ideally, the physician should have data expeditiously about the radiosensitivity of a patient. The end-point for radiosensitivity, which is of most value when determining response of tissues to radiation therapy, is cell death. This is usually determined based on the colony forming ability of exposed cells. Most current radiosensitivity assays using primary fibroblast cultures require 5 or 6 weeks to perform. A technique using lymphocytes requiring one week of post-irradiation incubation has been developed (224). Radiation-induced death of cells in the immune system demonstrably results from apoptosis. Cells express features associated with this process within hours of exposure (14). Thus the Leukocyte Apoptosis Assay provides a rapid means of determining radiosensitivity (215). It can discriminate differences in radiation-induced cytotoxicity between individuals and can potentially be used for rapid screening for genetically hypersensitive patients. Correlations with existing methods of radiosensitivity testing are underway to confirm the reliability of the assay. Young donors display enhanced levels of programmed cell death following exposure to radiation as has been previously reported for neonatal blood (227). However, genetically abnormal patients, such as those with *ataxia telangiectasia* display a compromised ability to mount a programmed response to radiation and levels of induced apoptosis in these individuals are very low.

Induced apoptosis in radiation protection

Following radiation accidents, it is important to rapidly estimate the dose absorbed in persons exposed. The most thoroughly developed biological indicator currently available is quantification of chromosomal aberrations in peripheral blood lymphocytes from exposed individuals (228, 229). In vivo or in vitro irradiation of blood lymphocytes produces similar yields of chromosome damage per Gy, so that the observed levels of aberrations in exposed persons can be related to dose by comparison with dose response curves based on in vitro experiments. The most frequent type of chromosome aberration used for biological dosimetry following radiation accidents is the readily identified dicentric. Because of the longevity of some lymphocytes, chromosomal aberrations can be detected years after an accident. The equally reliable micronucleus (MN) assay has been suggested as an alternative method for determining radiation exposure (230–237). The method is easier and faster than scoring dicentric chromosomes, and permits screening of large numbers of cells. This, together with the possibilities of computerized image analysis (238), makes the MN assay more attractive for routine use. Theoretically it enables resolution of doses lower than the 50–100 mGy usually quoted as the limit for detection by scoring chromosome aberrations (228).

Table 5

Inter-donor variation of radiation-induced apoptosis in human T-lymphocytes among 5 donors at different independent times after irradiation. Means and standard deviations are given

	CD4 0.1 Gy	CD4 0.5 Gy	CD8 0.1 Gy	CD8 0.5 Gy	Mean CV
48 h inc.	$1.0 \pm 0.7\%$	$5.5 \pm 1.3\%$	$1.1 \pm 0.7\%$	$7.8 \pm 5.3\%$	0.57
72 h inc.	$1.5 \pm 1.3\%$	$11.3 \pm 3.3\%$	$2.7 \pm 1.4\%$	$15.8 \pm 3.5\%$	0.47
96 h inc.	$3.0 \pm 1.6\%$	$16.0 \pm 6.0\%$	$2.2 \pm 1.5\%$	$24.0 \pm 7.4\%$	0.47
120 h inc.	$4.1 \pm 1.4\%$	$23.2 \pm 2.5\%$	$7.7 \pm 5.0\%$	$40.9 \pm 6.9\%$	0.32
144 h inc.	$5.9 \pm 1.6\%$	$40.4 \pm 6.0\%$	$6.5 \pm 2.1\%$	$48.1 \pm 6.8\%$	0.22

We have developed a biological dosimeter based on the Leukocyte Apoptosis Assay (239). Blood samples were given 0.05 Gy, 0.1 Gy or 0.5 Gy. Radiation-induced apoptosis in CD4 and CD8 T-lymphocytes was examined every 6 hours up to 72 hours incubation time post-irradiation and after 4 and 5 days. Even at 50 mGy, radiation-induced apoptosis was consistently higher than the non-irradiated control values over the entire time range up to five days. The 0.5 Gy curve displayed a large increase in the frequency of apoptosis at incubation times greater than three days. This was observed both in the CD4 and the CD8 T-lymphocytes. The time dependence of apoptosis frequency indicates that by using longer incubation times the method can be used for investigating low-dose effects. Therefore, a 10 day time-course experiment was designed with analysis at 24 hour intervals. Due to deterioration of the quality of the blood sample, at the longer time intervals, it became difficult to differentiate apoptotic cells from normal cells during analysis. Following high doses, maximum radiation-induced apoptosis levels were reached within four days incubation. At low doses, 0.5 Gy and below, the time kinetics previously observed were reproduced. The time course experiment was performed several times in order to determine the intra-donor variation of radiation-induced apoptosis at various doses.

Intra-donor variation was defined as the variation in the apoptosis frequency measured from independent blood samples from the same donor. For each dose and incubation time the mean and standard deviation of the radiation-induced apoptosis frequencies were calculated. The uncertainty found in the intra-donor variation is much smaller than the separation of the two curves seen for the two doses used. In general, the error is larger for the higher dose. The kinetic experiments demonstrated that the optimal time for evaluating radiation-induced apoptosis is 4 to 5 days post-irradiation. Dose response curves were, therefore, determined four days after exposure. A linear function could be fitted to the curves. The slopes were 7.5% per 0.1 Gy and 8.6% per 0.1 Gy for CD4 and CD8 T-lymphocytes, respectively. The dose response was determined again after five days incubation and similar curves could be fit to the data. The slopes were: 7.8% per 0.1 Gy (CD4) and 8.0% per 0.1 Gy (CD8) (239).

Variation of the apoptotic response between different donors was determined by irradiating blood samples of 5 different donors at the same time with subsequent analysis after different incubation times. This experiment was repeated for five different incubation times. Mean values for all donors and the standard deviations were calculated. The results are shown in Table 5. The mean coefficients of variation (C.V.) are also displayed. C.V.s were found to decrease linearly with time. The reduced C.V.s at 4 and 5 days post-exposure improves the assay's reliability for biological dosimetry. At early time points, the high variability observed is made use of to distinguish between the radiosensitivities of donors, in order to stratify the patients for radiation therapy (216). The spontaneous frequency increases with time of incubation. Variation in the spontaneous frequency limits the sensitivity of the test. The data of the different time course experiments demonstrate that for doses up to 0.5 Gy, the radiation-induced apoptosis has similar time kinetics up to 5–6 days incubation time. At later times, variation in the apoptotic frequency is too high to evaluate the time kinetics reliably. At higher doses, the radiation-induced apoptosis frequency shows a steep increase even after short incubation times, rising to a maximum at about four days post-irradiation time and followed by a slight decrease at later times.

In order to quantify the low dose limit of this assay, all the data measured in the time course experiments at low doses up to 0.5 Gy, at the various post-irradiation times, were taken and using a t-test those doses where the apoptotic fraction was still significantly above the control, and which was the most significant post-irradiation incubation period, were checked. A significance level of 0.05 was used for the analysis. The low dose limit for this assay is about 0.1 Gy after an incubation time of 3 to 4 days. At shorter times, the radiation-induced apoptosis frequency is low, at longer times experimental variation is high. The delay before maximum values are observed results from time required for expression of radiation-induced apoptosis. The expression time is a function of dose and may also reflect differences in individual genetic constitution (216). The time kinetics curves of all the experiments show that for the whole range of incubation times the apoptotic values for the different doses are well separated, for both

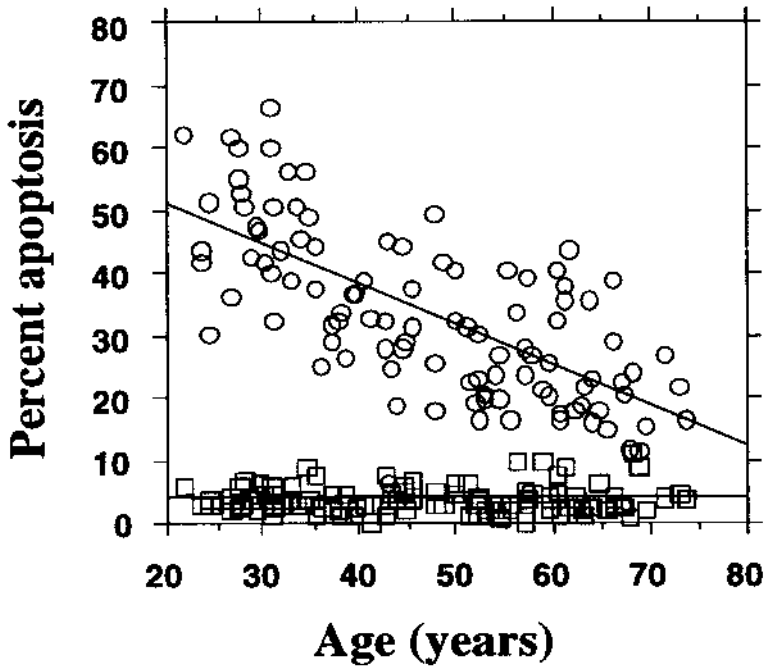

Fig. 6. A scattergram of donor age versus percent apoptotic CD4 T-lymphocytes 48 h after exposure to 300 kVp x-rays. The spontaneous levels of apoptosis (0 Gy) and radiation-induced levels of apoptosis (9–0 Gy, spontaneous levels subtracted) are displayed.

cell types chosen. If the incubation time chosen is in the range of 3–4 days, the assay is able to measure radiation-induced apoptosis significantly above control values at doses above about 100 mGy. This resolution is suitable in order to measure doses which may occur in accidents involving ionizing radiation. The magnitude of the radiation-induced apoptosis measured at 0.5 Gy after 3–4 days is more than sufficient for biological dosimetry at this dose.

Radiation-induced apoptosis, a biomarker of ageing

Radiation induces apoptosis in lymphocytes via a p53 signalling pathway (220). During the early development of the Leukocyte Apoptosis Assay, a weak correlation between apoptosis induced 24 h after irradiation and donor age was observed (for CD4 T-lymphocytes $R = 0.32$ and 0.30 after 2 and 9 Gy, respectively (216). With subsequent development of the assay as a sensitive biological dosimeter, the time dependency of apoptotic yield with dose was carefully investigated and in a subsequent double blind cross-sectional study we investigated T-lymphocytes from a cohort of 105 healthy blood donors (76 men and 29 women, between 21 and 73 years of age), 48 h after x-ray irradiation, to reexamine the relationship between radiation-induced apoptosis and age. A highly significant correlation between radiation-induced apoptosis and age in CD4 T-lymphocytes was observed ($r = 0.70$ and 0.67, $p < 0.0001$ and 0.0001, after 9 and 2 Gy x-rays, respectively; Fig. 6, no data are excluded). For CD8 T-lymphocytes a lower correlation was observed ($r = 0.50$ and 0.31, after 9 and 2 Gy, respectively). The smaller r-values may reflect the response of a mixed cell population recognised by the anti-CD8 antibodies employed. No correlation between spontaneous apoptosis and age was observed ($r = 0.014$, $p = 0.89$; Fig. 6, no data are excluded). Radiation-induced apoptosis in T-lymphocytes is dependent on genetic factors as demonstrated in a patient presenting with *immunodeficiency, centromeric instability and facial anomalies syndrome* (ICF) and a patient presenting with *Ataxia telangiectasia* (AT) (216). Studies of children's blood and preliminary studies of umbilical cord blood confirm high levels of radiation-induced apoptosis, 24 h after irradiation in young donors. If the age component of variation is subtracted, a significant correlation between CD4 and CD8 T-lymphocytes remains ($r = 0.69$ and 0.66, after 9 and 2 Gy, respectively) confirming that the assay continues to yield information relating to inter-donor differences in T-lymphocyte radiosensitivity. Apoptosis was confirmed by microscopy, electron microscopy, and by the use of commercially available apoptosis detection kits (in situ nick translation and annexin V).

Ageing leads to increases in the proportion of T-lymphocytes expressing the memory cell phenotype and declines in the proportion of naïve T-lymphocytes. There are several lines of evidence suggesting that this age-related transition from naive to memory T-cells contributes to

immunodeficiency in old age. The transition affects both the CD4 and CD8 pools and also affects T cells in the blood, lymph nodes, and spleen. In mice the transition leads to a 2- to 3-fold increase in the proportion of memory cells and to a 2- to 3-fold decline in the proportion of naive T-cells (240). However, memory T-cells in man are more radiosensitive than naive T-cells (241).The transition from naive to memory T cells does not explain the age response we report here. The ability of T-lymphocytes to mount an apoptotic response to ionizing radiation damage decreases linearly between 21 and 73 years of age: 3% every 5 years. The ability to repair DNA damage has also been shown to decrease with age (242–244). The reduced levels of apoptosis reported here could represent a reduced ability to repair DNA damage. However, reduced repair may itself be a consequence of a compromised ability to either recognise or respond to DNA damage with increasing age. Although the present studies were restricted to peripheral T-lymphocytes they indicate that estimates of apoptotic frequency for clinical purposes must take patient age into consideration.

A feature most obviously correlating with age-dependent changes in cells is telomere shortening. Telomeric length in T-lymphocytes decreases with age. Human hematopoietic cells purified from adult bone marrow have shorter telomeres than cells from foetal liver or umbilical cord blood (245). General reduction of telomere lengths is accompanied by increased cellular senescence. Senescence and checkpoint arrests are alternatives to apoptosis for preventing potentially detrimental cellular proliferation (246). With increasing age, accumulation of more and more senescent cells may be accompanied by a compromised ability to mount an apoptotic response to radiation, and perhaps other clastogenic agents, which could contribute to various aspects of the general ageing phenotype (247). Resistance to programmed cell death in senescent fibroblasts has been suggested to result from failure to repress *bcl-2* gene expression (248). Shifts in levels of *bcl-2* family members and their related antagonists with progressive cell divisions could provide a molecular explanation for the reduced apoptosis reported here. bcl-2 protein levels correlate with cellular sensitivity to x-rays and a variety of chemotherapeutic drugs. In addition to direct p53 regulation of *bcl-2* expression, *bcl-2* appears to be regulated at the protein level by another *bcl-2* family member, *bax*, which is a p53 immediate early response gene. The bcl-2 and bax proteins apparently compete with one another to control the relative susceptibility of cells to p53-mediated apoptosis (249). Telomere length or other measures of cellular senescence can predict the functional status of tissues and be considered 'biomarkers' of ageing. The data suggest that apoptotic response to ionizing radiation is a functional 'biomarker' of ageing.

INTERFERING WITH PHYSIOLOGICAL PROGRAMS IN THE CELL

Signalling pathways and biological processes

The programmed response of cells to the presence of radiation damage follows a number of principles which are common to all cellular phenomenon which have been characterised at the molecular level. An understanding of the principles involved provides insight into how cells function. From the point of view of information theory, a cell is a complex structure with a very high density of information. The macromolecules carry structural information stored in the form of nucleic acid sequences and from which it can be copied. The information is expressed in the three-dimensional structure and chemical properties of the cellular proteins. Programmed cellular response occurs when these proteins interact, when they recognise and process signals, and mobilize other proteins to execute an appropriate response. Biological response results from the activity of intricate cellular biochemistry forming signalling networks of communication and control. Wherever biological processes have been studied at the molecular level, elements of these signalling networks are evident. These molecular automata appear to be the building blocks, or 'bio-quanta', of life.

The G2/M transition is a process which involves mobilization of many of the cellular components (250). The process has been likened to a transitory differentiation, due to the total restructuring of the cytoskeleton and nucleus. A variety of both external and internal parameters are monitored which include cell size, completion of DNA synthesis, integrity of the genome, etc. They may also include factors such as nutrient status and absolute timing (87, 251). Experimental and genetic evidence is available to indicate that each of these parameters is monitored by specific signal transduction pathways. The pathways merge at a multi-protein complex where the executive protein, a kinase referred to as p34, is held in an inhibited state (252, 108, 253). When the requirements for mitosis are fulfilled, the kinase is activated and this, in turn, mobilizes the proteins involved in the execution of mitosis (254). If the requirements for mitosis are not fulfilled the kinase is maintained in its inhibited state.

Cells are capable of producing a great variety of receptors in order to respond to a great variety of stimuli. The spectrum of receptors produced by a differentiated cell-type is essentially fixed and determines which external stimuli are recognised amongst the many different signals with which the cell is constantly confronted. The range of receptors produced by any cell is strictly regulated by the activity of transcription factors active in, and specific for, that particular cell type. Subsequent integration of the various stimuli and activation of the appropriate response, however, appear to be performed by a limited number of signal transduction pathways which are common to many

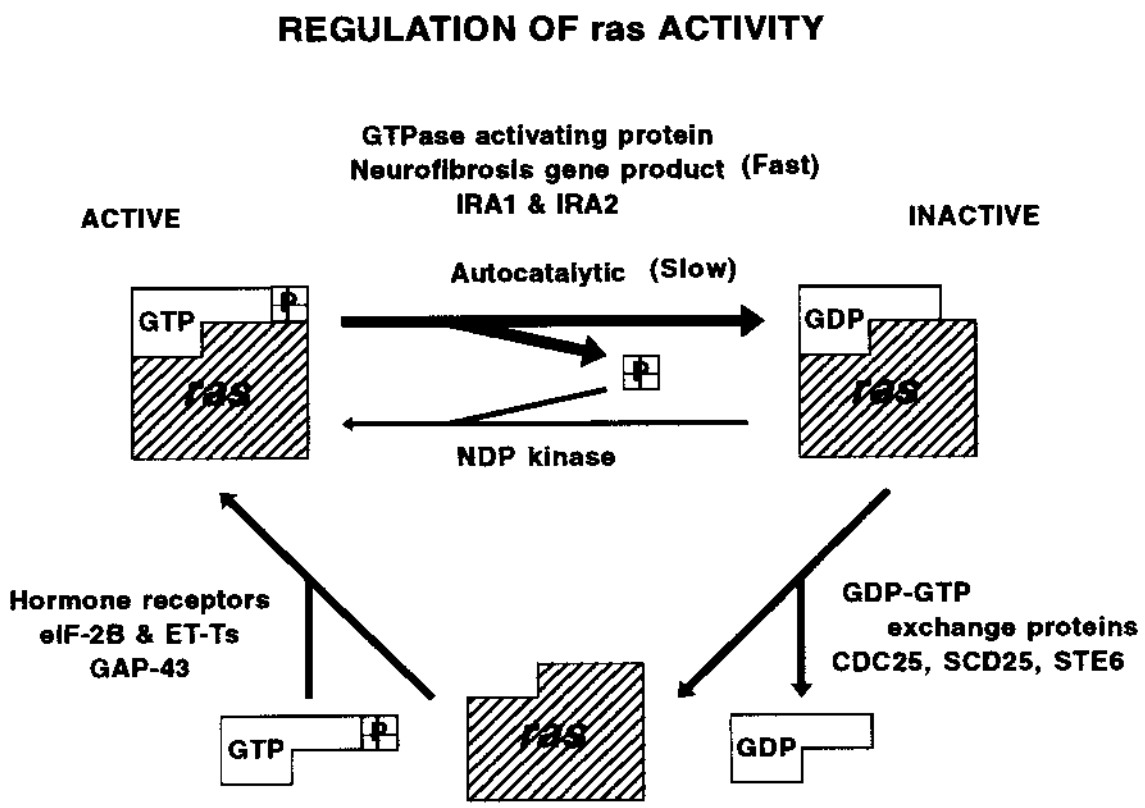

Fig. 7. Many factors fine tune the activity of the *ras* GTPase.

cell-types. The role of the receptors is two fold; first the recognition of specific signals, and second, the transduction of these signals to one or more of a limited number of intracellular transduction pathways (255).

Because, at the molecular level the cell is a large and complex structure, if a cell is to respond to a signal, either the signal must be of adequate magnitude or it has to be amplified. Furthermore, since the cell comprises various compartments, a signal must sometimes pass one or several barriers to reach the various target proteins required to execute the response. To deal with these problems the cell has a highly organised second-messenger system with integrated amplification cascades. Once the original reception of the message has occurred the next step is transduction of the message via molecular couplers to one or more second-messenger systems. Such couplers employed to transduce extracellular signals across the plasma membrane are G proteins. An important example of a molecular coupler in oncology is the *ras* protein. If the gene for this protein is mutated so that the protein is produced in a permanently activated state, the cell receives permanent instructions to divide and tumour growth can result. G proteins can process a number of incoming signals; Fig. 7.

What originally was considered to be a simple chain of events is now known to be a network of control elements permitting fine and well-tuned responses to complex external and internal stimuli (256).

Signals in cells are often transduced along the various links of the signal pathway as activated phosphorylation states. Protein kinases phosphorylate, and protein phosphatases dephosphorylate, substrate proteins. One typical transduction scenario is when a protein kinase I phosphorylates a protein kinase II which in turn phosphorylates substrate proteins. The pathway of transduction can have more kinase members. This type of control system has a number of interesting features. First, it is possible to transduce the signal through various cellular compartments in a controlled manner. Second, each protein kinase is capable of phosphorylating a large number of protein substrates permitting extensive amplification of the signal as it progresses through a number of phosphorylation steps. Thus a single signal event can eventually cause the activation of hundreds of millions of proteins. Third, each step can be tightly regulated by the activity of antagonistic protein phosphatases.

A typical example of the complexity of this system is observed in the control of the G2/M transition (255). p34 is a protein kinase whose activity governs entry into mitosis (104). p34 is a cyclin-dependent kinase (cdk), its primary regulator is a cyclin protein. p34 and cyclin B form the MPF kinase complex (107). Substrates of this kinase complex include: lamins which form the nuclear shell which supports the nuclear membrane, and histone H1 which contributes to chromatin superstructure and the

condensation state of chromosomes (257). MPF has two types of phosphorylation sites in its structure. One type inhibits its activity and prevents mitosis, the other enhances its activity and promotes mitosis. Both types of phosphorylation site have specific protein kinases which phosphorylate them and specific protein phosphatases which dephosphorylate them. These protein kinases and protein phosphatases are themselves under strict regulation, and their activities are sensitive to such factors as completion of DNA synthesis, damage to DNA, cell size, etc. These factors are those that have to be checked before a cell enters mitosis (15).

So far only post-translational examples of signal transduction have been described. These tend to be transitory in nature. The proteins involved are already present and operational in the cell. They are important dynamically. If a cell must react rapidly to the environment, it cannot wait for gene expression which requires RNA transcription, RNA processing and protein translation. Furthermore, the spectrum of stimuli which may be important for making decisions, and which can rapidly change, requires an extensive and flexible reception capacity which can best be achieved by a complex response network. However, more permanent decisions made by a cell, such as whether and in which direction it should differentiate, are predetermined in the genotype and phenotype of the cell. Only when an array of specific signals is received should the cell differentiate. These are 'decisions' at the molecular level with far-reaching consequences. They play a crucial role in the development and homeostasis of an organism and usually involve control of gene expression (256).

In front of the coding sequence of a gene, at its 5′ end (also but apparently to a lesser extent at its 3′ end) proteins bind to the DNA enhancing, promoting or inhibiting transcription of the gene. The proteins are referred to as transcription factors, their combined effect determines whether the gene is transcribed or not. The system can be superficially compared to a nerve cell which integrates many stimulatory and inhibitory signals from the numerous synapses on its surface, their combined effect determining whether the membrane should depolarise or not. The transcription factors are themselves under strict control, often via regulatory phosphorylations. For a few important genes, a great deal is known about the most immediate level of transcriptional regulation. The potential of these control systems is very large, permitting very precise expression of certain genes in specific cells in particular tissues, at defined places and times in an organism. These factors are responsible for pattern formation and the establishment of morphological gradients in the early embryo and play important roles in the continued development of the organism (258).

Cybersomes, semantics, and cell cycle arrest

Cellular response is controlled by complex biomolecular signalling pathways whose interactions follow the principles of cybernetic control systems. In addition to the stimulus itself, signal transduction in cells is characterised by four features (259, 260). Two of these are responsible for the recognition and processing of incoming information: the semantics of response. The other two are responsible for the execution of the appropriate response: the pragmatics of response, see Table 6. The semantic part consists of measuring devices (the receptors) and a processing system (the signal transduction network) e.g. the condemned phase of apoptosis (261). The pragmatic part consists of an actuator (often a protein kinase cascade, or transcription complex) and the motor (the protein kinase substrates, or the proteins of the transcribed genes) e.g. the execution phase of apoptosis (261). The role of the actuator is transduction of the execution signal from the signal transduction network to the elements of the motor. The motor once activated is the mechanism which executes the response. Typical examples of proteins involved in the pragmatics of a process include cytoskeletal proteins, such as actin and myosin which execute muscle contraction, the lamin proteins, which execute restructuring of the nuclear membrane, the different enzymes, which execute glycolysis, and the novel proteins which specify cell type during differentiation. These systems of intricate cellular biochemistry form molecular automata. They comprise a collection, or 'body' of proteins and other accessory cellular components which control and execute biological response and could be referred to as cybersomes.

Norbert Wiener (259) defined cybernetics as the science of control and communication in mechanisms, organisms, and society. It has been expanded to include general theories of control applied to any system (260). By 'system' is meant a group of elements of whatever kind considered as an interconnected whole. This definition includes proteins and biomolecules in cellular signalling pathways. Signals are a special form of information. As opposed to static, structural information, signals circulate in cybernetic systems (260). Five characteristics of signals are recognised: *statistics*; symbols and their frequency (262), *syntax*; signal coding and grammar (263), *semantics*; the meaning, *pragmatics*; execution of response, *apobetics*,

Table 6

Signal transduction and cybernetics

Information theory	Cybernetics	Cellular signal processing
Reception	Sensors	Receptors
Semantics	System control	Couplers & signal network
Pragmatics	Actuators	Kinase cascades & transcription
	Motors	Substrates & gene products

purpose of signal (264). All of these characteristics are displayed by intracellular signals. Exactly the same is true for signals in human communication; words in sentences and proteins in intracellular signal pathways play identical roles. Just as words in the linguistic sciences are recognised as possessing a three-sided character: concept (meaning), form (articulation or orthography), and syntax (relationships within a sentence) (265); proteins are also recognised as possessing this three-sided character: concept (function), form (amino acid structure), and syntax (precise location within the chain of interactions leading from a stimulus to a response).

In Miller's treatise on 'The Science of Words' he writes: *Each word is the synthesis of a concept, an utterance, and a syntactic role. A person who knows a word knows what it means, knows how to pronounce it, and knows the contexts in which it can be used. These are not three independent kinds of knowledge; they are different views of a single entity. Lines of research into all three of these aspects of language contribute to the science of words. The real wonder is the integrity of the word as a linguistic element. All the semantic, phonological, and syntactic complexity being assembled in such a small and handy package....There could not be a science of words if it were not possible to study complicated systems scientifically. Languages are systems. They are highly complex systems of sound and meanings that have their ultimate reality in the minds of the people who know and use them. The scientific way to understand complex systems is not to reduce them to patterns of physical energy exchange, but rather to describe their component parts and to characterise the functional relations within and among those parts* (265).

Information, signal transduction pathways and semantics are parts of complex systems. It is the description and investigation of complex systems which is the real challenge in modern biology. Physics, deservedly enjoying prominence amongst the sciences, has not yet risen to meet this challenge. One of its most brilliant contemporary leaders was Richard Feynman. In his celebrated 'Lectures on Physics' series he writes: *Everything is made of atoms. That is the key hypothesis. The most important hypothesis in all of biology, for example, is that everything that animals do, atoms do. In other words, there is nothing that living things do that cannot be understood from the point of view that they are made of atoms acting according to the laws of physics. This was not known from the beginning: it took some experimenting and theorising to suggest this hypothesis, but now it is accepted, and it is the most useful theory for producing new ideas in the field of biology* (266). With the greatest of respect, there are two sides to this coin. Although biology is most definitely based on atoms, semantics is also crucial. Reducing Shakespeare to a statistical analysis of English letters may be of some academic interest but it completely misses the point. Miller writes: *The difference, of course, is meaning. Phoneticians and telephone engineers may be able to ignore it, but to an average person meaning is crucial. It is meaning that makes language useful.... And meaning is not something subtle.... The difference between meaning and nonsense is—must be—a major dimension in any serious account of human experience* (265).

The goal of philosophical semantics is to formulate a general theory of meaning. Some of the best minds of Western civilisation have struggled with it. Yet any computer programmer or any molecular biologist working with signal pathways can immediately recognise semantics. Returning to Feynman's celebrated lecture series, during a discourse on the effects of gravity on celestial bodies he showed a spectacular photograph of a globular star cluster and commented: *If one cannot see gravitation acting here, he has no soul* (266). Using similar argumentation, if one is confronted with cellular signal pathways (267) and cannot see semantics, he has no soul. Cellular semantics is the cause of McClintock's (1984) 'unforseen'. Radiation response is 'unforseen' because cellular response is not based on static structures but on cybernetic systems of signalling pathways. Two practical consequences follow: a) physiological manipulation of radiation response is feasible, with associated therapeutic potentialities; and b) artificially-induced programmed response reflects interference with normal, physiological processes in the cell.

The transitory cell cycle arrests permit repair of damage before intracellular activities associated with progress through the cell cycle transform the potentially repairable damage into irreparable damage. Is this the *raison d'être* of these arrests? Various pieces of experimental evidence suggest that this may not be the case. Many studies have demonstrated that p53, the guardian of the genome, mediates both G1 cell cycle arrest and programmed cell death even in the same cell system; Fig. 5 (221). Why should a cell simultaneously induce recovery and destruction systems? Nagasawa et al. (268) examined the relationship between radiation-induced G2 arrest and cellular radiosensitivity in seven different cell lines. They observed that, for all the cell lines, equitoxic doses of x-rays produced equiextensive G2 arrests. However, the radiosensitive cell lines displayed long arrests and the radioresistant cell lines displayed short arrests. If G2 arrests significantly promote survival, those cells with long arrests should be more radioresistant and those cells with short arrests should be more radiosensitive.

The haploid yeast *S. pombe* exists in either of two mating types, which are capable of fusing to form a diploid cell. They can switch type via a precisely localised DSB. Sandell and Zakian (82) genetically manipulated a strain of yeast so that the site of this break was located close to the end of a chromosome. They then introduced a DSB repair deficiency into the yeast strain. By activating the switching they produced a yeast cell with an artificial broken chromosome end. As expected, the presence of this

end induced a programmed cell cycle arrest. Nevertheless, in spite of the continued presence of the break many cells eventually re-entered the cell cycle. Repair of the damage did not influence the duration of arrest. A similar observation was made in a study of Bae et al. (269) in human lymphoma cells. Radiation-induced genome damage enhances p53 activity, this in turn upregulates expression of the p21 gene, whose product induces G1 arrest; Fig. 5. The study showed that although initiation of the arrest depends on p53 and, therefore, on the presence of genome damage, the duration of arrest depended on the stability of the p21 protein, and not on the stability of the p53 protein. The duration of arrest was independent of the presence of genome damage. The conclusion from these and other studies is that the time available for repair of genome damage, resulting from cell cycle arrest, is a fortuitous side-effect and not the physiological reason for arrest. The main reason appears to be avoidance of cellular replication, possibly in association with cellular senescence. It should be noted that p21 protein levels are elevated during senescence (270).

Understanding the biological response to either physiological (e.g. hormones) or non-physiological (e.g. ionising radiation) stimuli requires characterisation of the particular biomolecular system activated by those stimuli. Although the magnitude of response to ionising radiation may be influenced by the frequency of broken chromosome ends, both size and type of response are determined by the signalling pathways activated. Therefore, radiation response does not necessarily reflect the cells attempt to simply repair damage. Some types of damage induced by radiation mimic structures which have physiological roles in the life of the cell and radiation response reflects programmed physiological response to these stimuli. If a broken chromosome end is mistakenly interpreted as the presence in the cell of two chromosome ends without telomeres, cellular senescence pathways are activated. Senescence pathways induce both cell cycle arrests and programmed cell death.

Cellular senescence and radiation response

The repertoire of signals to which a cell normally responds does not include damage caused by ionising radiation. Nevertheless, a number of programmed responses are induced including cell cycle arrests and apoptosis. McClintock recognised the signal source of this type of programmed response to be broken chromosome ends. Furthermore, the signal could be terminated either by joining the broken chromosome end to another broken chromosome end, or by adding a telomere. Her studies suggest that the physiological signals induced by radiation exposure are telomere-less chromosome ends. The normal physiological role for these telomere-less chromosome ends is in cellular senescence. McClintock's 'discernible but initially unforseen responses' of the cell, are cell cycle arrests, induction of genetic instability, and programmed cell death associated with an extreme type of cellular senescence, crisis (*vide infra*) which is part of a complex defence mechanism to protect an organism from cancer.

Recent research has demonstrated the presence of a mechanism of finite growth potential in cells. The mechanism has to be overcome in order for tumours to progress from the benign to the malignant state. At each cell division the ends of the chromosome arms (telomeres) shorten (271, 272). Upon reaching a critically short length a signal is generated and the cell enters senescence and eventually dies. This is considered the physiological basis for ageing. If senescent cells are forced through further rounds of division their telomeres become even shorter and programmed cell death is induced. To evade this physiological impasse a cell must make telomerase protein which prevents and can even reverse telomere shortening. This phenomenon is highly relevant to oncology and cellular radiation response. The molecular controls of the phenomenon are identical to those used by cells to induce cell cycle arrests and apoptosis after exposure to radiation, the telomerase activation status of cells is a highly predictive indicator of the malignant state of a cell, and the recently isolated *ataxia telangiectasia* gene (ATM) is significantly homologous to genes involved in the telomere control pathway (273).

There are a number of models of cellular senescence, amongst the more popular are those based on damage accumulation or programmed senescence. These are not necessarily exclusive. However, existence of immortal germ-lines, and the very fact that the so-called 'oldest old' generally appear fitter than those 20 years their junior, weighs against the damage-accumulation model. Many new insights into the molecular biology of ageing, support the programmed death hypothesis. According to this hypothesis after a relatively fixed or 'pre-programmed' number of divisions, cells enter senescence and eventually die. The strongest evidence for this phenomenon comes from studies of tissue explants. Tissue explants grown in culture display three growth phases. First those cells capable of growth in the extra-corporeal environment invade the culture dishes. Then follows a period of exponential cell growth. Finally, cell growth terminates with the arrest of cell division and culture senescence; Fig. 8. The duration of this senescence period is variable but eventually ends in death of the cells. The period of transition from exponential growth to senescence is referred to as the Hayflick limit after the discoverer of this phenomenon. Hayflick (274) observed that fibroblasts from tissue explants are capable of only a limited number of cell divisions before entering senescence, depending on the age of the tissue donor. Thus, cells from young donors are capable of more divisions than cells from old donors.

When cells enter senescence they take on an altered morphology (275). The cytoplasm of the senescent cells

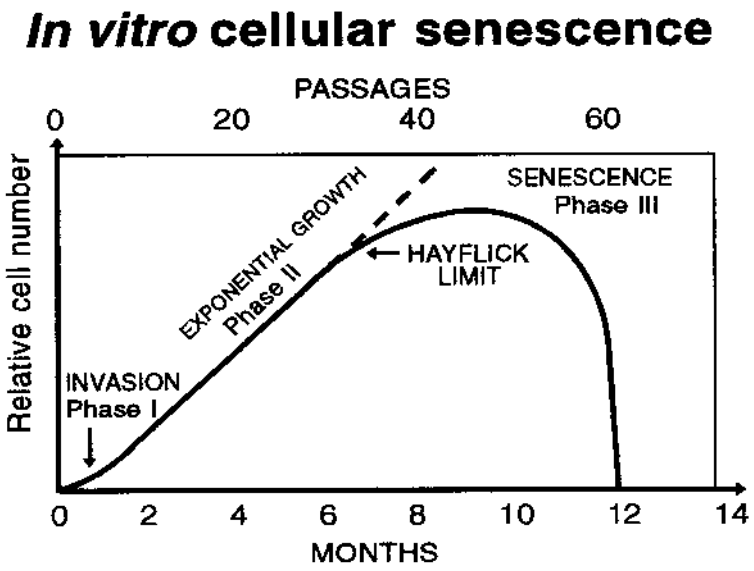

Fig. 8. Cells in culture have a limited division potential before entering senescence. Reproduced with permission (273).

flattens out and becomes more circular taking on an extended saucer-like appearance. This indicates that extensive re-modelling of the cytoplasm has taken place. The cells tend to arrest in the G0/G1 phase of the cell cycle. The cdk/cyclin complexes which drive the cell cycle are expressed abnormally in senescent cells (276, 277). Although senescent cells are eventually destined to die, they are resistant to apoptosis (248). When exposed to tumour viruses such as SV40 (which produce proteins that specifically bind to and inhibit the major cell cycle regulatory proteins, p53 and pRb) the senescent cells transform. The spindle-morphology characteristic of non-senescent cells returns and they reinitiate cell division but soon become irreversibly arrested and undergo programmed cell death, crisis (278). Those few cells which side-step this second arrest become immortal, continuing to proliferate indefinitely. Tumour-virus proteins which promote transformation of cells are referred to as viral oncogenes. Various cellular proteins, called proto-oncogenes, are also involved in regulating these processes. They can mutate into cellular oncogenes which promote uncontrolled division and carcinogenesis.

A number of studies have demonstrated a strong correlation between telomere length and senescence (279). In Tetrahymena cells, where the telomerase RNA has been mutated, abnormal telomeres are produced and the cells display features of senescence (280). The *est1* mutant of yeast is characterised by cells with ever shorter telomeres (*est*) and features typical of ageing and senescence (281). Further studies have demonstrated that the length of human fibroblast telomeres, like their proliferative potential, is directly related to age of the donor (271, 282). In progeria patients, who suffer from a disease causing premature ageing, fibroblasts display a greatly reduced division potential and enter senescence prematurely (283). Their chromosomes display abnormally short telomeres. Patients with *ataxia telangiectasia*, a multi-faceted syndrome who display abnormal radiation sensitivity show a less severe premature ageing and their cells are also found too have shorter telomeres.

H. J. Mueller (284, 285) first used the term, telomere, when investigating radiation-induced chromosome aberrations in Drosophila. The unexpectedly low frequency of terminal deletions indicated that special structures must be present on the end of chromosomes which protect them from damage. Telomeres shorten at each round of cell division due to problems associated with DNA replication at the end of the chromosome (286, 245). The DNA polymerase can ride along the 5′–3′ 'leading' strand to the very end of the chromosome replicating DNA the whole way. This is not possible along the 3′–5′ 'lagging' strand. Not only can the DNA polymerase not move in the 3′–5′ direction, it also requires a pre-existing 5′ DNA- or RNA-primer to start from. Along the lagging strand the DNA is replicated in pieces called Okazaki fragments. Each of these fragments is initiated with a RNA primer. After replication is finished, the RNA primers are removed and the short gaps left are filled by the activities of the polymerase. However, at the end of the chromosome, at the most distal gap, there is no pre-existing 5′ DNA for the DNA polymerase to bind to. Therefore, the end is not replicated and a short length of DNA is lost. After each division the chromosomes shorten at a rate of some tens of base pairs per cell cycle; Fig. 9.

There is a clear correlation between the number of divisions through which a cell has passed and the length of

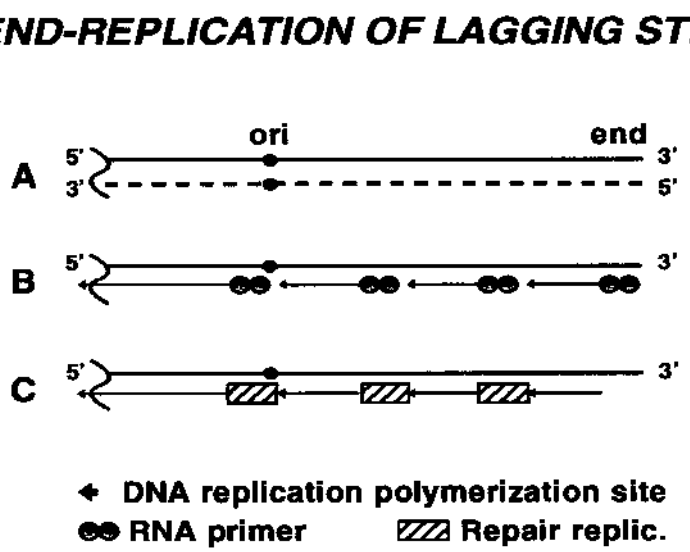

Fig. 9. DNA polymerases cannot replicate the end of the lagging strand. Reproduced with permission (273).

its telomeres. As cells divide their telomeres shorten. After reaching a critical length of about 4 kbp, the cells enter G0/G1 arrest and senesce (287). Cells remain in this state and eventually die. Cells enter senescence when a signal caused by telomere-attrition activates the p53 and pRb proteins which co-operate in inhibiting cell division and promote the senescent state (278, 221). When cells are irradiated they act in a similar manner. The chromosome breaks induce a signal causing the cell cycle machinery to activate cell cycle arrest. Arrest has been shown to involve the p53 protein, which promotes the activity of the potent cell cycle inhibitor p21 (269, 288). p21 interacts directly with the cyclin/kinase complexes which drive the cell through the division cycle; Fig. 5.

Senescence arrest can be overcome by the action of tumour viruses such as SV40, human papilloma viruses, etc. The viruses produce proteins such as the large T antigen (SV40), or E6 and E7 (human papilloma viruses) which bind to p53 and pRb (289, 290). This binding inhibits the normal inhibitory function of these proteins, permitting signals which promote cell growth to stimulate cell division. When cells are infected by tumour viruses they can escape the cell cycle arrest and continue to grow for a few more divisions, the telomeres getting even shorter. This extreme shortening is associated with genetic instability (291, 292). When, on average, the telomeres are about 1 kbp long, the cells enter a second arrest, referred to as crisis or M2, and die. However, a few cells circumvent crisis because the length of their telomeres stabilises due to the presence of a protein/RNA complex called telomerase. When this enzyme is active the cells are able to divide indefinitely, becoming immortal. In the germ line, telomerase is active so the line is capable of indefinite growth ensuring the preservation of the species; Fig. 10.

Somatic cells have only a finite division potential before they senesce. After each cell-division telomeres shorten and upon reaching a certain minimal length they signal to the cell to stop dividing and undergo senescence (271, 272). This marks the Hayflick limit, also referred to as M1. However, in cells of the germ line (responsible for the perpetuation of the species) a special ribonucleoparticle is active called telomerase. Telomerase maintains telomeres at a constant length so that cells do not senesce but are capable of unlimited division (293). Telomerase is a ribonucleoparticle; consisting of both protein subunits and an RNA molecule (293, 294). It is not yet known how many protein subunits the telomerase complex consists of. However, in a number of species the gene coding for the RNA molecule has been cloned and its sequence established. The RNA possesses a region of homology to the telomeric repeat. This region acts as a template for replication of the telomeric DNA. Because telomerase synthesizes new DNA from an RNA template it is a reverse transcriptase (295). The ability of telomerase to extend, or to hold constant, the length of the telomere over multiple cell divisions has been demonstrated to go hand-in-hand with unlimited growth potential and the development of malignancy (296). By modifying existing PCR technologies Kim et al. (297) developed a method to evaluate telomerase activity in single cells. They demonstrated that in essentially all immortalised tumour cell lines investigated (98 of 100) and in 90% of all tumours examined (90 of 101), telomerase activity had been reactivated. No existing tumour marker or oncogene is as frequently observed, in as many different tumours types, as telomerase activity. Cell immortalization requiring ectopic telomerase activity appears to be an almost universal step in carcinogenesis. Telomerase activity has high prognostic value and could provide the clinician with new alternative and supplemen-

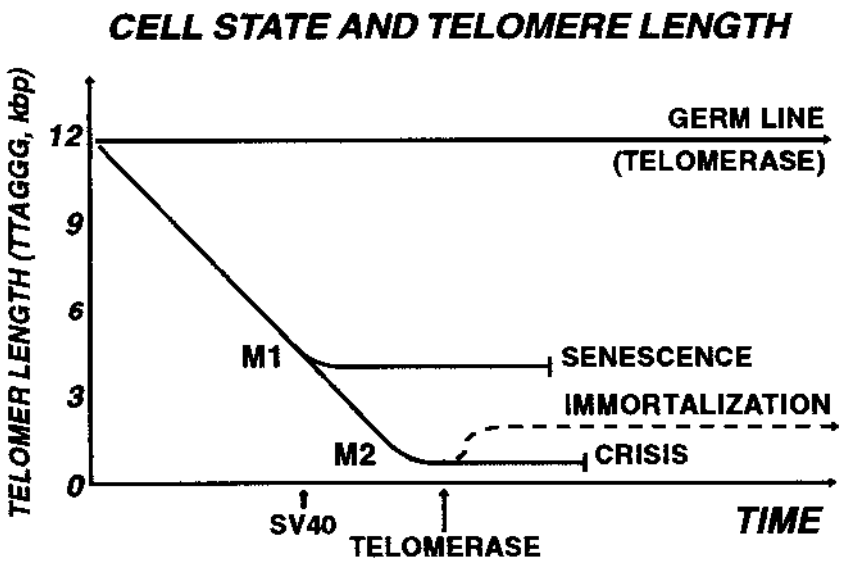

Fig. 10. Average telomere length depends on the 'age', type, and health of a cell. Reproduced with permission (273).

tary therapies. An intensive search for telomerase inhibitors is currently underway. These should re-initiate telomere shortening and restore finite division potential to the tumour cells, converting malignant to benign tumours (296, 298).

If transformed cells do not become immortal they tend to cause only benign tumours (299, 300). Thus cellular senescence, the physiological cause of ageing, can be considered the price paid to reduce the frequency of malignant tumours. Ageing is a natural consequence of an anti-oncogenesis mechanism. Longer-life resulting from an extension of the 'telomere-clock' would not mean being old for longer; rather the total process of ageing would be slowed down (282). However, the probability of cancer at the same time would be much greater because the probability of cancer inducing events increases with age and the probability of larger benign tumours becoming malignant increases with cell number. If it were possible to reset the telomere lengths via telomerase activation, specifically in non-tumorigenic cells, increased life-span might be feasible (301). As the process of ageing appears to be primarily a consequence of telomere-attrition driven cellular-senescence, such an increased life-span might be welcome, at least for the individual (273).

QUANTIFICATION OF PROGRAMMED RESPONSE FOR CLINICAL APPLICATION

Measurement of programmed radiation response has many practical applications in radiation therapy including:

(a) monitoring response to radiation therapy, especially when evaluating new modalities or treatment protocols.
(b) evaluating complementary therapy adjuvants, such as photodynamic therapy (PDT), neutron capture therapy (NCT), the use of cytokines in bone marrow transplantation, and the use of pharmaceuticals which modify radiation response.
(c) individualization of therapy protocols, tailoring them to the specific characteristics of the patient and his/her tumour.

Monitoring response to radiation therapy

One of the advantages of using protons for radiation therapy is the reduction of total dose made possible to the healthy tissues. Using comparative treatment planning techniques, it has been demonstrated that conform therapy using either protons or photons leads to similar total doses to target tissues and favourable dose fall-offs to therapy-limiting normal tissues surrounding the target. However, the integrated doses to more distal healthy tissues after conform photon irradiations are up to three times larger (30% of total therapy dose), than the proton integral dose (10–15% of total therapy dose) (302, 303). During photon treatments, large volumes of normal healthy tissue, 10 litres or more, may receive total doses greater than 20 Gy. Such exposed healthy tissues are many times larger than the actual target volume. Equivalent treatments using the advantageous physical properties of protons, permits reduction not only of the normal tissue volume irradiated but also the average dose, 6 Gy, received by these tissues.

Although therapy doses to normal tissues do not have a major impact on rate of patient survival, much evidence suggests that these tissues have been altered due to the radiation exposure. The tissues are hypersensitive to subsequent radiation treatment and display enhanced frequencies of various cytogenetic abnormalities and gene mutations. Radiation-induced damage acts in a manner similar to a co-carcinogen, see Chapter 4, and can cause transformation of the cells. Instability induced in the

genome, which accompanies transformation and which drives tumour progression, is induced by radiation in a high percentage of the cells. It can even be induced in cells indirectly; in cells adjacent to or near others which received the dose (193). Radiation-induced transformation is classified as a stochastic radiation effect, indicating that no threshold for dose induction is evident. Radiation protection in these cases recommends application of the ALARA principle; as low as reasonably achievable, in order to keep risk to a minimum. For the patient, however, Swiss law permits normal dose limits to be circumvented; 'Strahlenschutzgesetz, Art. 15'. Although justified in order to save lives, it is not meant to be a carte blanche for the radiotherapist. The same law refers to 'Artikel 9' which requires reduction of radiation exposures to those possible within the realms of 'contemporary technologies'. The ALARA principle needs to be applied in the clinic as well.

Proton conformal therapy is a typical 'contemporary technology' which can be used in practice to spare the patient unnecessary and harmful radiation exposures (304, 305). The ALARA principle in these cases is not always 'achievable' because of lack of availability of proton machines. In some instances of conformal therapy, reduction of dose to considerable volumes of normal healthy tissue is a major radiation protection issue. Using standard electrons or photons for conformal therapy, litres of otherwise healthy tissue are exposed to integral doses of 20 Gy or more, which is most unfortunate. This is not a negligible dose, even if it is fractionated. The average age of radiooncology patients is above 60 and life expectancy even after successful radiotherapy is not extensive. A shortsighted clinician can easily play down the risks involved. Unfortunately, it is young patients and children who are most endangered. They have risk values 10-fold greater or more than 'average-aged patients' (60, 306, 307). With the application of conformal proton irradiations, normal tissue doses could often be reduced at least 3-fold, with a commensurate reduction in secondary cancer risk and therapy-associated complications. In patients with extensive post-therapy life expectancies (e.g. patients with paediatric tumours) the preferential use of conformal proton therapies is clearly indicated. Previously it has been difficult to monitor the effects of radiation on normal healthy tissue in order to evaluate the severity of the radiation damage. The Leukocyte Apoptosis Assay, however, provides a means of monitoring the response of various blood cell types to fractionated therapy. With this assay, a detailed log of normal tissue response to radiation therapy is available and clinically relevant comparisons of biological response to proton and photon therapies can now be made.

Neutron capture therapy is another clinical modality whose therapeutic potential has been recognised for many years (308). Various technical limitations must still be overcome before it gains acceptance in clinical routine. Nevertheless, a fascinating therapeutic potentiality has arisen for treating rheumatoid arthritis (309), an autoimmune disease characterised by chronic inflammation of the joints. The body produces antibodies against its own antibodies, these build complexes together which are deposited in the synovia, the membranes lining the joints. These complexes activate the complement cascade and thus cause chronic inflammation of the synovia. If joints remain unresponsive to drug treatment, physical removal of inflamed synovia, synovectomy, becomes necessary. A non-invasive therapy using neutron capture therapy is being developed. First a boron-labelled compound is injected into the joints, where it is actively taken up by the synovial membranes. These membranes can then be selectively destroyed by irradiating the joints with neutrons (309). Use of the Leukocyte Apoptosis Assay will permit surveillance of normal tissue response for adverse reactions during development and application of this therapy.

Besides acting as a rapid screen for cancer patients hypersensitive to radiation, the Leukocyte Apoptosis Assay can assist the clinician in planning patient-specific treatment protocols. Correlations are being sought between the spectrum of radiosensitivities displayed by the leukocyte sub-populations and the levels of acute, subacute and late response of the normal tissue to photon and proton therapy. Preliminary studies of ocular melanoma patients treated at the OPTIS proton facility at the Paul Scherrer Institute display a correlation between radiation-induced apoptosis in the CD8 lymphocytes and the frequency of optical neuropathies observed 3.5 years after treatment. To date only 12 patients have been tested. Those patients (6) whose CD8 T-lymphocytes had less than the median frequency of apoptosis (15%) after an 8 Gy dose, displayed optical neuropathies with a frequency of 35%, and those patients (6) whose CD8 T-lymphocytes had more than the median frequency of apoptosis, displayed optical neuropathies with a frequency of 80%. None of the patients displayed extremely abnormal apoptotic responses. These preliminary findings suggest that individuals with a reduced capacity to respond to ionising radiation eventually develop fewer neuropathological complications.

Evaluating complementary therapy adjuvants

Photodynamic therapy. After accumulation of a light-sensitive substance (photosensitizer) in tumour cells, laser irradiation at a defined wavelength induces cell death. Since this therapy modality is relatively new, a number of characteristics, including the mode of cell death, still require evaluation. Using a number of cytotoxicity assays in various lymphoid cell types, we have demonstrated that cells are killed via a necrotic mechanism following treatment with PDT using mTHPC (meta tetra-hydroxylphenyl chlorin). Human blood from healthy donors, or TK6 human B-lymphoblastoid cells, were incubated in the pres-

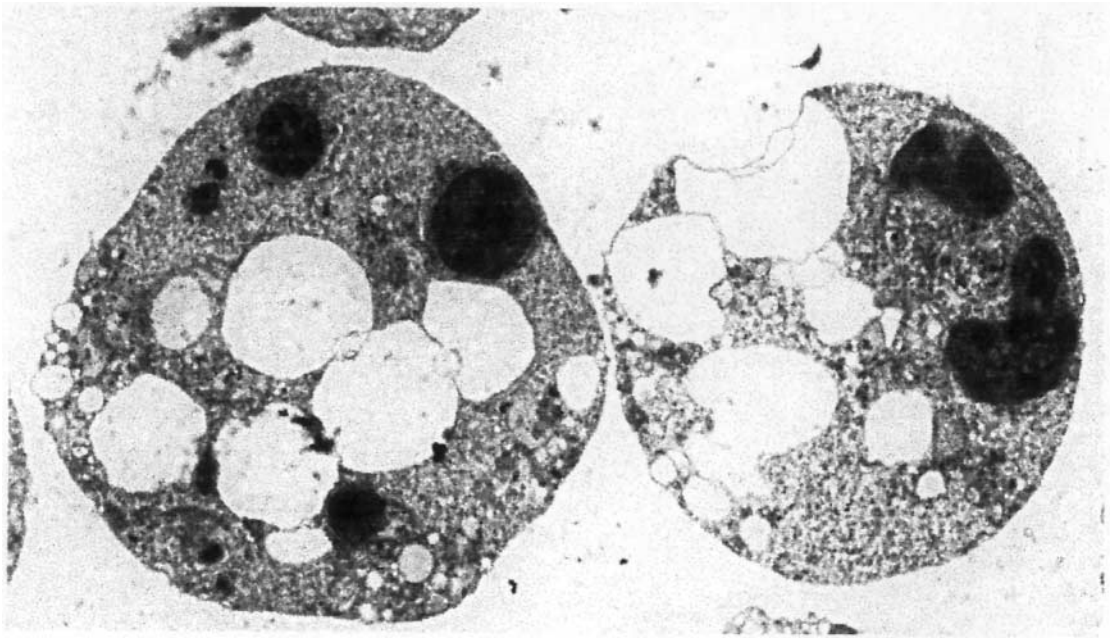

Fig. 11. Large vacuoles and apoptotic bodies induced in TK6 cells by Gy x-rays.

ence of the photosensitizer mTHPC for 24 h, subsequently exposed to 625 nm laserlight, and left to incubate for various periods up to 24 h to permit expression of cytotoxicity (310). PDT is a therapeutic modality well suited to the treatment of certain malignant tumours. The photosensitizer is critical and on its own should never be toxic to the patient. No genotoxic influences of the photosensitizer mTHPC have been observed (311). We have studied the mode of PDT-induced cell death. If PDT kills cells via a necrotic mechanism it would be a useful adjuvant for radiation therapy. If the programmed response of tumour cells is compromised due to mutation of components of the signalling pathways, tumour cells become resistant to radiation therapy. Alternative therapies which kill cells directly by causing necrosis are then desirable. We have examined cytotoxicity in various lymphoid cell types after PDT treatment.

The difference between the frequencies of necrotic and apoptotic cell death was determined using three separate assays. First, peripheral blood lymphocytes were examined for PDT-induced sub-G1 DNA peaks. No apoptotic cell populations were induced by PDT in either CD4 or CD8 T-lymphocytes, between 0.5 h or 72 h after treatment. Second, the time kinetics of PDT-induced cytotoxicity were compared to x-ray-induced apoptosis in TK6 lymphoblastoid cells. x-rays induce apoptosis in TK6 cells causing vital stain uptake in these cells starting 12 h after exposure, due to secondary necrosis which is the last phase of apoptotic death. However, TK6 cells treated with PDT displayed vital stain uptake within minutes after treatment and a maximum was observed within 8 h. Third, cells were examined with the electron microscope for evidence of apoptotic chromatin bodies. Although these were readily observed following exposure to x-rays no such bodies were observed at various doses or times post-PDT treatment; Figs. 11 and 12.

Based on the results from DNA and cell-size measurements in lymphocytes, loss of membrane integrity in TK6 lymphoblastoid cells, and electron microscopic examination of cytotoxic action, we conclude that PDT induces cell death in lymphoid cells by a necrotic mechanism. No evidence of PDT-induced apoptosis in CD4 and CD8 T-lymphocytes or TK6 B-lymphoblastoid cells was observed. This suggests that the success of PDT will be relatively independent of the mutational status of various oncogenes (e.g. p53, *myc*, *ras*) which have been shown to enhance resistance to radiotherapy (9, 6, 8, 220). Therefore, our experiments support the view that PDT should be a useful modality for salvaging radiotherapy failure. The study was performed with lymphoid cells, as these cells are generally most sensitive to induced apoptosis. Nevertheless, carcinomas and sarcomas may respond differently, and assessment of their specific reaction to PDT has yet to be established. We are presently developing a similar test system to examine solid tumour response.

Cytokines for bone marrow transplantations. Granulocyte colony-stimulating factor (G-CSF) not only increases the yield of polymorphonuclear neutrophils (PMN) in leukapheresis blood-donors but also protects the PMN from the harmful effects associated with harvesting and irradiation, and delays the onset of apoptosis in in vitro culture (312–314). We confirmed this observation. Samples of G-CSF-treated leukapheresis products tested 24 h after irradiation, using the Leukocyte Apoptosis Assay, showed

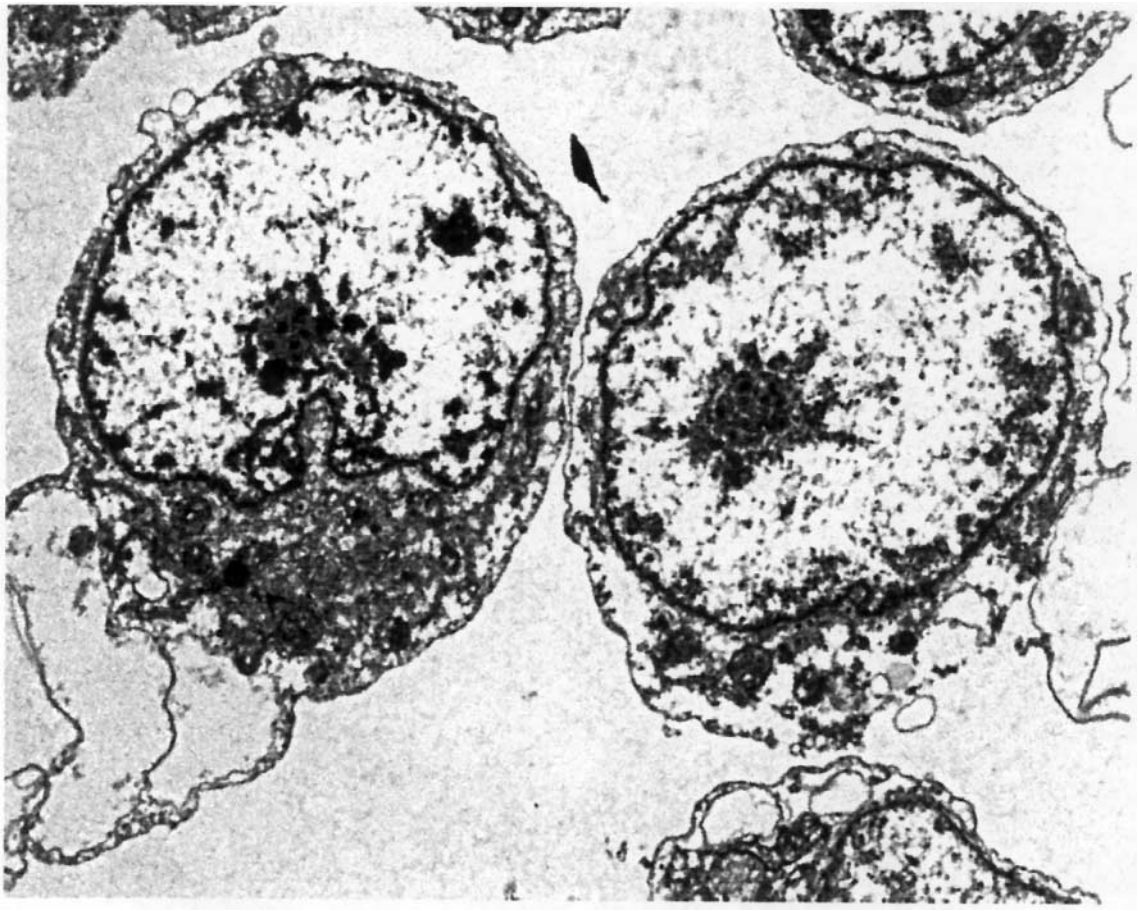

Fig. 12. Photodynamic therapy does not appear to affect the integrity of the nucleus, the damaged chromatin condenses onto the lamin scaffold and nucleolar structures.

the same levels of apoptosis as their non-irradiated, non G-CSF treated counterparts. Based on these data, a bone marrow transplantation (BMT) patient, the leukapheresis recipient, was also administered G-CSF at 10 μg/kg each day beginning with the first granulocyte transfusion. This method of G-CSF administration, to both donor and recipient, made successful passage through the aplasia period possible with only four transfusions of pre-activated, long-living granulocytes despite an aspergillus contraindication (315).

Chronic granulomatous disease (CGD) is a primary immunodeficiency resulting from complete absence or malfunction of the NADPH oxidase in the phagocytic cells, i.e. neutrophils, monocytes, macrophages and eosinophils. Deficiency of this oxidase causes a marked reduction or complete extinction of the phagocyte respiratory burst resulting in defective microbial killing. As a consequence, the patient is susceptible to recurrent bacterial and fungal infections. Based on the observation that granulocyte colonies, derived from the progenitor cells of CGD patients, display defective oxidative metabolism; bone marrow transplantation (BMT) appears to be an ideal method to replace the malfunctioning cell populations. The approach has been used on a dozen patients since 1976. Usually, BMT from a histocompatible donor results in cure of the disease, provided that the patient is transplanted in an infection-free interval. However, invasive aspergillosis has been a universal contraindication for BMT for fear of overwhelming aspergillus sepsis and death during aplasia (316). In order to overcome these problems, G-CSF mobilized, irradiated granulocytes from healthy donors were transfused during aplasia. G-CSF was administered to the donor to increase the quantity of granulocytes harvested 3- to 10-fold.

Granulocytes obtained from the leukapheresis process and granulocytes obtained from fresh blood samples from healthy volunteer donors were investigated. The leukapheresis granulocytes were given 0 or 25 Gy and examined 0, 24, and 48 h post-irradiation for vitality and apoptosis using the Leukocyte Apoptosis Assay. The granulocytes from fresh blood were separated from the lymphocyte fraction by density centrifugation. The samples were incubated with or without G-CSF for 24 h (312), irradiated at 0 or 25 Gy and examined at 0, 24, and 48 h post-irradiation for vitality and apoptosis (215). Flow-cytometric analysis of granulocytes confirmed previous findings

demonstrating protection against apoptosis by G-CSF treatment of PMN (315). Granulocyte transfusions from healthy donors mobilized with G-CSF provide about a four-fold increase in the PMN yield compared to steroid-mobilized leukapheresis products (317, 318). Moreover, G-CSF protects the PMN from deleterious effects of harvesting and irradiation, and delays the onset of apoptosis in in vitro systems (312–314).

Pharmaceutical manipulation of radiation response. Eukaryotic cells in cycle respond to ionising radiation by delaying entry into mitosis (91). Delay of entry into mitosis has been suggested to prevent the fixation of a component of potentially lethal damage by extending the repair period (92). This additional repair, in tumour cells, could impede radiotherapy and lead to compromise of tumour control. Elucidation of the arrest mechanism should permit the design of drugs to improve therapeutic ratios by mitigating tumour radioresistance and/or sparing neighbouring healthy tissue. Use of caffeine, as an adjuvant in radiotherapy due to the radiosensitizing effects of G2 arrest abrogation, is not yet feasible. Despite complete absorption following oral administration, the concentrations required are too toxic. Even a 2 mM caffeine dose (equivalent to approx. 4.5 litres of strong 'Robusta' expresso or more than 11 litres of 'Robusta' drip coffee) would cause emesis, seizure and death probably via the adenosine receptors of the central nervous system (319, 320). Maximum whole body doses of 0.2 mM may be tolerable but in our system these had no measurable effect on the duration of G2 delay. Means to localise millimolar caffeine concentrations are required.

Individualization of therapy protocols

Statistical testing of clinical assays. Measurement of programmed radiation response can be used to stratify patients for alternative clinical protocols (321). Based on the results, patients are given either conventional radiotherapy (CT) or an alternative (AT). The probability (P) of favourable outcome (O) given the alternative therapy must be greater than the probability of favourable outcome given the conventional therapy; $P(O|\,AT) > P(O|\,CT)$. Based on this stratification, the resultant increase in clinical gain provides a quantitative measure for the clinical relevance of the predictive assay in terms of percent gain in overall clinical outcome. The 'cut-off' value of the evidence (E), used for defining stratification, is usually the median. In this case 50% of the patients derive the benefits of the alternative treatment. However, it may be that only the upper (or lower) 10% of the patients significantly benefit from the alternative treatment and alternative 'cut-off' values can be used. The placing of the 'cut-off' is clearly critical, particularly for more aggressive alternative treatments. Clinical gain is a measure of average benefit to all patients tested. Here, the vital statistic is the proportion of patients stratified multiplied by percent gain per stratified patient. In a worst case scenario, predictive assay screening would result in too little improvement to justify its use. Patient inconvenience and assay overheads also play an important role in defining 'improvement'. The clinical attraction of normal-tissue radiosensitivity assays (215, 216) lies in their dual potential; both the high proportion of patients (95%) and the reasonable gain per patient (hypothetically 20%), given the alternative therapy.

Primary univariate analyses are rapid and simple tests of the ability of an assay to stratify patients based on standard Bayesian probabilities. Patients treated with a conventional therapy and demonstrating the clinical outcome of interest (O), are examined for the specific test results (E). Various test values are then determined: sensitivity, $P(E|\,O)$; specificity, $P(E'|\,O')$; predictive value, $P(O|\,E)$; false-positive rate, $P(O'|\,E)$; and false-negative rate, $P(O|\,E')$ (322). The efficiency of an assay can be measured in terms of the clinical outcome or in terms of the evidence. In terms of the clinical outcome; the sensitivity of an assay $P(E|\,O)$ is the proportion of evidence-positive individuals amongst the outcome-positive group (O); type I errors are caused by evidence-negative (E′) individuals amongst the outcome-positive group $P(E'|\,O)$. The specificity of an assay $P(E'|\,O')$ is the proportion of evidence-negative individuals amongst the outcome-negative group (O′); type II errors are caused by evidence-positive (E) individuals amongst the outcome-negative group $P(E|\,O')$. In terms of the evidence, the relationship $P(O|\,E) > P(O)$ is examined using contingency tests (chi-square, Fisher's, etc.), which concentrate on rejecting or not rejecting the hypothesis that the two probabilities are equal. Confidence intervals are even more illuminating; the z-test (difference of proportions) indicates both the reliability of the data and how to handle non-significant results.

More extensive univariate analyses make use of all the available data. The preferred method for such analyses uses logistic regression, defining a linear function between two outcome values and the evidence; Fig. 13 (323). The p-value obtained from this test defines the discriminatory power of the assay, and the slope of the function is the natural logarithm of relative risk. From such curves the sensitivity and specificity of the test at all cut-off (evidence) levels can be obtained and a receiver-operating characteristic (ROC) curve determined; Fig. 14 (324). ROC curves graphically display the discriminatory power of a predictive assay. In practice, the sensitivity of such a curve at the 95% specificity is given. However, standard levels of criticality are very restrictive for assay systems where both variables are subject to biological and experimental variation.

Using multiple tests it is possible to examine the relationship between multiple types of evidence and the chosen outcome (e.g. tumour cell survival can depend on SF2, CFE, oxic state, and Tpot). Multiple assays can be used to analyse the contribution of each parameter to a given

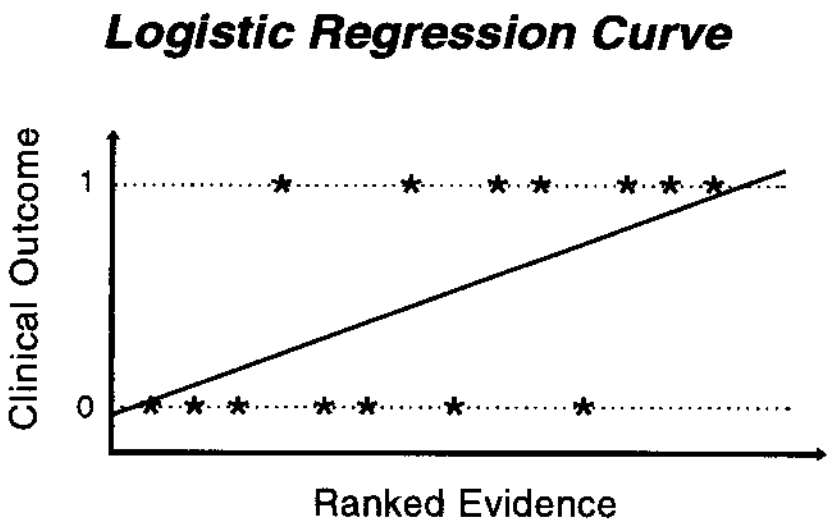

Fig. 13. Logistic regression curve, the illustrative data are purely fictitious.

outcome. Using multiple assays is equivalent to testing in series, and tends to increase specificity at the expense of sensitivity. Such methodologies, by decreasing the number of false positives, would have the advantage of increasing the expected therapeutic gain per patient and reducing the proportion of patients selected. Multivariate analysis can also be used to determine the independent contributions of each type of evidence to the outcome. These are given as relative risks, which can be used to provide better criteria for stratification. Furthermore, multiple predictive assays can give multiple types of evidence for more than just one type of clinically relevant factor (e.g. oxic state and Tpot are expected to have an influence on both radiosensitivity and tumour growth kinetics). Multivariate analysis can be used here to determine the impact of the various independent contributions which also provides better criteria for stratification. The appropriate test for stratification given multiple predictive assays is multiple logistic regression analysis. This yields a relative risk and confidence intervals for each factor, whose combined contributions are additive.

Evaluation of clinical outcome may often involve censored data (incomplete observations). In these cases, actuarial analyses should be conducted (323). Actuarial analyses are graphically displayed using Kaplan–Meier (325) or life-table methods. Curves are compared using the log-rank test (326), and regression models can be established according to the Cox proportional hazards regression model (327). Many assays predicting local cure pass through a peak of discriminatory power related to the confounding influence of recurrence. The true discriminatory power of a test, for any desired clinical outcome, only becomes apparent after some years. This is why retrospective studies are so useful when developing such assays. If statistical tests confirm the value of an assay, a formal two-armed, prospective, randomised trial is called for in order to confirm the usefulness and reliability of the assay in standard clinical practice. As all types of clinical outcome are normally censored (time dependent) data, only actuarial analyses are really appropriate (321).

Clinical assays of programmed radiation response. Individualization of therapy protocols is only practical if the response of the individual can be estimated in advance. Appropriate tests of radiation response must be made either on normal tissues from the patient or on biopsy specimens from the tumour. Previous responses from healthy donors or cancer patients can then be used as a basis for estimating the patients response and tailoring the therapy to his/her needs. Such tests evaluate many different aspects of normal tissue and tumour tissue response, including normal tissue radiosensitivity, tumour growth kinetics, tumour heterogeneity, and multi-parametric analyses. In the future, advances in molecular genetic techniques will provide new avenues for individualising patient therapy protocols.

When deciding upon an appropriate radiotherapy protocol, it is helpful for the clinician to know how quickly the tumour is growing. Using fluorescence labelled anti-BrdU antibodies and flow-cytometric methods it is possible to determine the growth rate of a tumour from BrdU-labelled tumour biopsies (328). We have also used this method to investigate radiation-induced cell cycle arrests in cell culture systems (148). The method is referred to as the Tpot method because it determines the potential doubling Time. We have been performing Tpot analyses on spontaneous-tumour biopsy samples from cats and dogs to test its application in veterinary radiation oncology (329–331). The animals were given bromodeoxyuridine intravenously 4–6 hours prior to tumour biopsy excision. Tumour cells in S-phase incorporate this thymidine analogue into their DNA. The Tpot technique involves determination of both the fraction of tumour cells replicating their DNA (la-

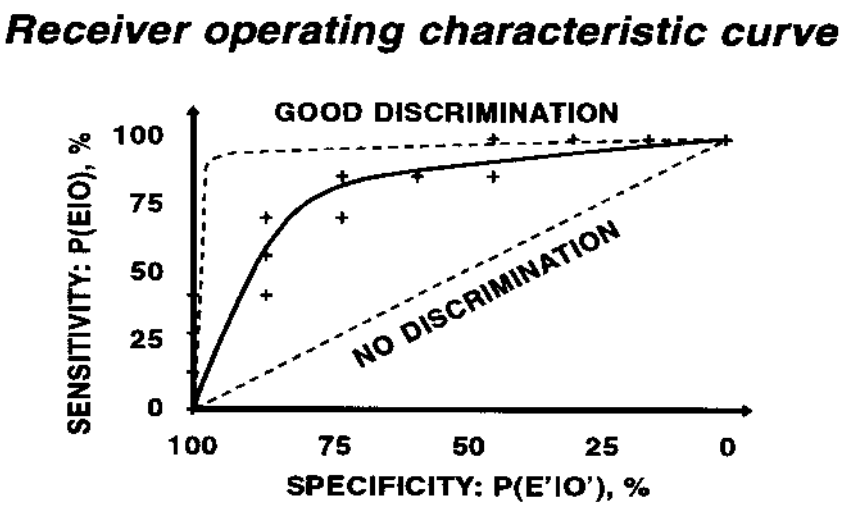

Fig. 14. Receiver operating characteristic curves, the data are from Fig. 13.

belling index) and the average length of the S phase (Ts). From these two parameters, the potential doubling time of the tumour can be determined (328). If the Tpot values indicate a rapidly growing tumour, a poor clinical response is also indicated. This is attributed to excessive tumour growth during the course of fractionated radiotherapy. We observed a large spectrum of Tpot values for epithelial and mesenchymal tumours both in cats and in dogs. A highly significant correlation between labelling index and Tpot was observed (331). This suggests that alternative approaches to tumour growth-rate determination are feasible based on non-invasive methods for measuring labelling index. Positron emission tomography can be used to evaluate labelling index based on tumour incorporation of positron-emitting radiolabelled thymidine analogues (332).

A major drawback of these existing techniques is their assessment of only one tumour parameter (333). Multiparametric analyses will have an important role in clinical radiotherapy in the 21st century. A technique for cell culture using palladium islands on a sea of agarose has been developed for multi-purpose applications in tumour-cell study. Petri dishes are layered with a thin film of agarose, and a pattern of 0.3 mm-diameter palladium islands are deposited onto the agarose. Cells are unable to grow on agarose but grow on the palladium islands to form colonies (334). Palladium islands are suitable for selecting cell cultures derived from tumour biopsies, in order to perform various types of radiosensitivity assay. Evaluation of single cells or their growth into colonies can then be conducted. Using this technique, survival curves of fibroblasts and tumour cells show good correlation with colony formation in dishes (335). We are currently developing compact cell cloning discs 10-cm in diameter, which carry more than 8000 'micropits' 0.8 mm in diameter, each holding a single palladium island (336). By FACS characterisation of preselected cells when seeding these discs, it will be possible to separate the various tumour-cell populations (tumour cells, fibroblasts, endothelial cells, etc.) from a patient biopsy. The cells can then be analysed on their islands for many types of programmed cellular response; e.g. colony forming ability, p53 transcription, etc. Confocal microscopy, antibody, and cytotoxicity studies can be performed; or the cells can be investigated with small-sample molecular biology technologies. The compact cell cloning discs provide many novel approaches for investigating tumour heterogeneity and, with further development, to establishing defined and reproducible conditions for live-biopsy archiving.

Exposure to ionising radiation causes the production of broken chromosome ends in a cell. The cell responds to these apparently telomere-less chromosome ends in a programmed manner by inducing cell cycle arrests or programmed cell death. With its experimental confirmation Barbara McClintock's remarkable insight that the genome responds in a 'discernible but unforseen manner' is being amply confirmed. The programmed component of radiation response is most significant in radiation oncology. The prescribed dose is actually determined by normal healthy tissue response rather than the type or size of the tumour being treated. The challenge to the therapist is finding methods of changing radiotherapy practice in ways that increase the probability of local cure whilst permitting isoeffective or reduced complication rates in normal tissues. Successful radiotherapy is synonymous with optimised sparing of healthy tissue. As our understanding of radiation response increases, the empirical advantages of dose fractionation, best achieved with low LET radiation; and dose conformation, best achieved with hadron radiation, take on a more mechanistic nature. Proton therapy which combines the advantages of both is a sophisticated and an astute approach to cancer treatment (337). One which will bring great benefits to the patient with its implementation in routine clinical practice.

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REFERENCES

1. McClintock B. The significance of responses of the genome to challenge. Science 1984; 226: 792–801.
2. Crompton NEA, Zoelzer F, Schneider E, Kiefer J. Increased mutant induction by very low dose-rate gamma irradiation. Naturwiss 1985; 72: 439–40.
3. Crompton NEA, Barth B, Kiefer J. Inverse dose-rate effect for the induction of 6-thioguanine-resistant mutants in chinese hamster V79-S cells by 60Co gamma rays. Radiat Res 1990; 124: 300–8.
4. Savitsky K, Bar-Shira A, Gilad S, et al. A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Nature 1995; 268: 1749–53.
5. Steel GG, Deacon JM, Duchesne GM, Horwich A, Kelland LR, Peacock JH. The dose-rate effect in human tumor cells. Radiother Oncol 1987; 9: 299–310.
6. Iliakis G, Metzger L, Muschel RJ, McKenna WG. Induction and repair of DNA double strand breaks in radiation-resistant cells obtained by transformation of primary rat embryo cells with the oncogenes H-ras and v-myc. Cancer Res 1990; 50: 6575–9.
7. McKenna WG, Weiss MC, Bakanauskas VJ, et al. The role of the H-ras oncogene in radiation resistance and metastasis. Int J Radiat Oncol Biol Phys 1990; 18: 849–59.
8. McKenna WG, Iliakis G, Weiss MC, Bernhard EJ, Muschel RJ. Increased G2 delay in radiation-resistant cells obtained by transformation of primary rat embryo cells with the oncogenes H-ras and v-myc. Radiat Res 1991; 125: 283–7.
9. Fitzgerald TJ, Daugherty C, Kase K, Rothstein LA, McKenna M. Activated human N-ras oncogene enhances x-irradiation repair of mammalian cells in vitro less effectively at low dose. Am J Clinic Oncol 1985; 8: 517–22.
10. Sinclair WK. Cyclic X-ray responses in mammalian cells in vitro. Radiat Res 1968; 33: 620–43.
11. Lewis PD. Variation in individual sensitivity to ionizing radiation. In: Jones RR, Southwood R, eds. Radiation and Health. New York: John Wiley and Sons, 1987: 167–77.
12. Elyan SAG, West CML, Roberts SA, Hunter RD. Use of low-dose rate irradiation to measure the intrinsic radiosensitivity of human T-lymphocytes. Int J Radiat Biol 1993; 64: 375–83.
13. Elyan SAG, West CML, Roberts SA, Hunter RD. Use of an internal standard in comparative measurements of the intrinsic radiosensitivities of human T-lymphocytes. Int J Radiat Biol 1993; 64: 385–91.
14. MacDonald HR, Lees RK. Programmed death of autoreactive thymocytes. Nature 1990; 343: 642–4.
15. Crompton NEA, Hain J, Jaussi R, Burkart W. Staurosporine and radiation induced G2 cell cycle blocks are equally released by caffeine. Radiat Res 1993; 135: 372–9.
16. Crompton NEA, Larsson B, Jaussi R. A signal transduction pathway controls radiation induced and staurosporine induced G2 arrest. In: Paliwal BR, Herbert D, Fowler JF, Kinsella TJ, eds. Prediction of Response in Radiation Therapy. New York, USA: American Association of Physicists in Medicine, 1993: 90–101.
17. Bedford JS, Griggs HG. The estimation of survival at low doses and the limits of resolution of the single-cell-plating technique. In: Alper T, ed. Cell Survival After Low Doses of Radiation: Theoretical and Clinical Implications. London: Wiley, 1975: 34–9.
18. Koenig F, Kiefer J. Lack of dose-rate effect for mutation induction by gamma rays in human TK6 cells. Int J Radiat Biol 1988; 54: 891–7.
19. Hall EJ. Radiation dose-rate: A factor of importance in radiobiology and radiotherapy. Br J Radiol 1972; 45: 81–97.
20. Crompton NEA, Sigg M, Jaussi R. Genome lability in radiation-induced transformants of C3H 10T1/2 mouse fibroblasts. Radiat Res 1994; 138: 105–8.
21. Yaes RJ. Using the LQ model in the clinic: definition of normalized total doses. Radiother Oncol 1993; 27: 172–3.
22. Crompton NEA, Emery GC, Shi Y-Q, Sigg M, Blattmann H. Radiation-induced genetic instability: no association with changes in radiosensitivity or cell cycle checkpoints in C3H 10T/2 mouse fibroblasts. Radiat Environ Biophys 1998; 37, in press.
23. Elkind MM, Xing Y, Liu J, et al. Mutation to 6-thioguanine resistance of Chinese hamster cells containing a bacterial gpt gene: Dose-rate dependence of 137Cs gamma-rays. In: Chadwick KH, Cox R, Leenhouts HP, Thacker J, eds. Molecular Mechanisms in Radiation-Induced Mutagenesis and Carcinogenesis. Luxemborg: European Commission Publishers, 1994: 79–84.
24. Xing X, Lindquist MS, Liu J, et al. Low dose rate Dependence of the Phenotypic and Genotypic Expressions of mutagenesis by 137Cs g-Rays. Radiat Oncol Invest 1995; 3: 17–28.
25. Beer JZ, Jacobson ED, Evans HH, Szumiel I. X-ray and UV mutagenesis in two L5178Y cell strains differing in tumorigenicity, radiosensitivity, and DNA repair. Br J Cancer 1984; 49: 107–11.
26. Evans HH, Horng M, Mencl J, Glazier KG, Beer JZ. The influence of dose rate on the lethal and mutagenic effects of X-rays in proliferating L5178Y cells differing in radiation sensitivity. Int J Radiat Biol 1985; 47: 553–62.
27. Furuno-Fukushi I, Ueno AK, Matsudaira H. Mutation induction by very low dose rate gamma rays in cultured mouse leukemia cells L5178Y. Radiat Res 1988; 115: 273–80.
28. Furuno-Fukushi I, Matsudaira H. Mutation induction by different dose rates of gamma rays in radiation-sensitive mutants of mouse leukemia cells. Radiat Res 1989; 120: 370–4.

29. Furuno-Fukushi I, Aoki K, Matsudaira H. Mutation induction by different dose rates of gamma rays in near-diploid mouse cells in plateau- and log-phase culture. Radiat Res 1993; 136: 97–102.
30. Grosovsky AJ, Little JB. Evidence for linear response for the induction of mutations in human cells by x-ray exposures below 10 rads. Proc Natl Acad Sci USA 1985; 82: 2092–5.
31. Kerr JFR, Searle J, Harmon BV, Bishop CJ. Apoptosis. In: Potten CS, ed. Perspectives on Cell Death. Oxford, UK: Oxford University Press, 1987: 93–128.
32. Yamada T, Ohyama H. Radiation-induced interphase death of rat thymocytes is internally programmed apoptosis). Int J Radiat Biol 1988; 53: 65–75.
33. Kohlpoth M. Untersuchungen zur Mutationsinduktion durch 60Co-Gamma-Strahlung in menschlichen Epithelzellen. Diss., Justus-Liebig-Universität, Giessen, Germany, 1991.
34. Zeuthen J, Norgaard JOR, Avner P, et al. Characterization of a human ovarian teratocarcinoma-derived cell line. Int J Cancer 1980; 25: 19–32.
35. Jones CA, Huberman E, Cunningham ML, Peak MJ. Mutagenesis and cytotoxicity in human epithelial cells by far-and near-ultraviolet radiations: action spectra. Radiat Res 1987; 110: 244–54.
36. Kiefer J. Dosisleistungsabhaengigkeit der Mutationsausloesung durch ionisierende Strahlen in Saeugerzellen. Molekulare und zellulaere Prozesse bei der Entstehung stochastischer Strahlenwirkungen. Veroeffentlichungen der Strahlenschutzkommssion, Band 33. Stuttgart: G. Fischer-Verlag, 1995: 103–16.
37. Thames HD, Withers HR, Peters LJ, Fletcher GH. Changes in early and late radiation responses with altered dose fractionation: implications for dose-survival relationships. Int J Radiat Oncol Biol Phys 1982; 8: 219–26.
38. Joiner MC, Denekamp J, Maughan RL. The use of 'top-up' experiments to investigate the effect of very small doses per fraction in mouse skin. Int J Radiat Biol 1986; 49: 565–80.
39. Joiner MC, Marples B, Johns H. The limitation of the linear-quadratic model at low doses per fraction. In: Beck-Bornholdt H-P, ed. Medical Radiology: Current Topics in Clinical Radiobiology of Tumors. Berlin: Springer-Verlag, 1993: 51–66.
40. Joiner MC, Johns H. Renal damage in the mouse: the response to very small doses per fraction. Radiat Res 1988; 114: 385–98.
41. Beck-Bornholdt HP, Maurer T, Becker S, Omnizynski M, Vogler H, Wurschmidt F. Radiotherapy of the rhabdomyosarcoma R1H of the rat: hyperfractionation-126 fractions applied within 6 weeks. Int J Radiat Oncol Biol Phys 1989; 16: 701–5.
42. Palcic B, Jaggi B. The use of solid state sensor technology to detect and characterize live mammalian cells growing in tissue culture. Int J Radiat Biol 1986; 50: 345–52.
43. Marples B, Joiner MC. The response of Chinese hamster V79 cells to low radiation doses: Evidence of enhanced sensitivity of the whole cell population. Radiat Res 1993; 133: 41–51.
44. Lambin P, Marples B, Malaise EP, Fertile B, Joiner MC. Hypersensitivity of a human tumour cell line to very low radiation doses. Int J Radiat Biol 1993; 63: 639–50.
45. Lambin P, Malaise EP, Joiner MC. Megafractionement: une methode pour agir sur les tumeurs intrinsequement radioresistantes? Bull Cancer Radiother 1993; 80: 417–23.
46. Lambin P, Fertil B, Malaise EP, Joiner MC. Multiphasic survival curve of human tumour cell lines: induced repair or hypersensitive subpopulations. Radiat Res 1994; 138: S32–6.
47. Singh B, Arrand JE, Joiner MC. Hypersensitive response of normal human lung epithelial cells at low radiation doses. Int J Radiat Biol 1994; 65: 457–64.
48. Wouters BG, Skarsgaard LD. The response of a human cell line to low radiation doses: evidence of enhanced sensitivity. Radiat Res 1994; 138: S76–80.
49. Singh B, Fahey J, Lambin P, Arrand JE, Joiner MC. Effect of dose and time on the induction of radioresistance in human cells exposed to low-doses of radiation. Proc 42nd Ann Meet Radiat Res Soc 1994; # P20–349: 194.
50. Lambin P, Marples B, Fertil B, Malaise EP, Joiner MC. Low dose radiation response of human tumour cell lines of different intrinsic radiosensitivity. In: Paliwal B, Herbert D, Fowler JF, Kinsella TJ, eds. Prediction of Response in Radiation Therapy. New York: American Association of Physicists in Medicine, 1993: 311–9.
51. Boice JD, Blettner M, Kleinerman RA, et al. Radiation dose and leukemia risk in patients treated for cancer of the cervix. JNCI 1993; 79: 1295–311.
52. Upton AC. The dose response relation in radiation induced cancer. Cancer Res 1961; 21: 717–29.
53. Hall EJ, Miller RC. The how and why of in vitro oncogenic transformation. Radiat Res 1981; 87: 208–23.
54. Hall EJ. Radiobiology for the Radiologist, 3rd edition. Philadelphia: J.B.Lippincott Company, 1988.
55. Malaise EP, Lambin P, Joiner MC. Radiosensitivity of human cell lines to small doses. Are there some clinical implications. Radiat Res 1994; 138: S25–7.
56. Chmelevsky D, Spiess H, Mays CW, Kellerer AM. The reverse protraction factor in the induction of bone sarcomas in radium-224 patients. Radiat Res 1990; 124: S69–79.
57. Hill CK, Carnes BA, Han A, Elkind MM. Neoplastic transformation is enhanced by multiple low doses of fission spectrum neutrons. Radiat Res 1985; 102: 404–10.
58. Sevc J, Kunz E, Tamasek L. Cancer in man after exposure to Radon daughters. Health Phys 1987; 54: 27–46.
59. Lubin JH, Qiao Y, Taylor PR, et al. Quantitative evaluation of the radon and lung cancer association in a case-control study of Chinese tin miners. Cancer Res 1985; 50: 174–80.
60. BEIR V. National Academy of Sciences, Committee on the Biological Effects of Ionizing Radiations. Health effects of exposure to low levels of ionizing radiation. National Academy Press, Washington D.C., USA, 1990.
61. Ullrich RL. Tumor induction in BALB/c mice after fractionated or protracted exposures to fission-spectrum neutrons. Radiat Res 1984; 97: 587–97.
62. Miller RC, Geard CR, Geard MJ, Hall EJ. Cell-cycle-dependent radiation-induced oncogenic transformation of C3H 10T1/2 cells. Radiat Res 1992; 130: 129–33.
63. Cao J, Wells RL, Elkind MM. Enhanced sensitivity to neoplastic transformation by ^{137}Cs gamma-rays of cells in the G_2-/M-phase age interval. Int J Radiat Biol 1992; 62: 191–9.
64. Schneiderman MH, Schneiderman GS. G2 cells: progression delay and survival. Radiat Res 1984; 98: 389–96.
65. Goodhead DT. The initial physical damage produced by ionizing radiations. Int J Radiat Biol 1989; 56: 623–34.
66. Deschavanne PJ, Debieu D, Fertil B, Malaise EP. Re-evaluation of in vitro radiosensitivity of human fibroblasts of different genetic origins. Int J Radiat Biol 1986; 50: 279–93.
67. Bryant PE. Changes in sensitivity of cells during exposure to radiation at low dose-rate. Int J Radiat Biol 1972; 22: 67–73.
68. Hendry JH. Radioresistance in fern spores by prior irradiation. Radiat Res 1986; 106: 396–400.
69. Santier S, Gilet R, Malaise EP. Induced radiation resistance during low-dose-rate gamma irradiation in plateau-phase Chlorella cells. Radiat Res 1985; 104: 224–33.

70. Leenhouts HP, Susma MJ, Litwiniszyn M, Broerties C, Chadwick KH. Radiation stimulated repair in Saintpaulia cells in vivo. In: Edwards HE, et al., eds. Radiation Biology and Chemistry. Amsterdam, Holland: Elsevier, 1979: 227–36.
71. Calkins J. An unusual form of response in X-irradiated protozoa and a hypothesis as to its origin. Int J Radiat Biol 1967; 12: 297–301.
72. Ikushima T. Chromosomal responses to ionizing radiation reminiscent of an adaptive 67 response in cultured Chinese hamster cells. Mutat Res 1987; 180: 215–21.
73. Ikushima T. Radio-adaptive response: characterization of a cytogenetic repair induced by low-level ionizing radiation in Chinese hamster cells. Mutat Res 1989; 227: 241–6.
74. Tuschl H, Altmann H, Kovac R, Topaloglou A, Egg D, Gunther R. Effects of low-dose radiation on repair processes in human lymphocytes. Radiat Res 1980; 81: 1–9.
75. Shadley JD, Wiencke JK. Induction of the adaptive response is dependent on radiation intensity. Int J Radiat Biol 1987; 56: 107–18.
76. Shadley JD, Wolff S. Very low dose of X-rays can cause human lymphocytes to become less susceptible to ionizing radiation. Mutagenesis 1989; 2: 95–6.
77. Koval TM. Multiphasic survival response of a radioresistant lepidopteran insect cell line. Radiat Res 1984; 98: 642–8.
78. Koval TM. Inducible repair of ionizing radiation damage in higher eukaryotic cells. Mutat Res 1986; 173: 291–3.
79. Koval TM. Enhanced recovery from ionizing radiation damage in a lepidopteran insect cell line. Radiat Res 1988; 115: 413–20.
80. Papathanasiou MA, Kerr NCK, Robbins JH, et al. Induction by ionizing radiation of the *gadd45* gene in cultured human cells: Lack of mediation by protein kinase C. Mol Cell Biol 1991; 11: 1009–16.
81. Liversage WE. A general formula for equating protracted and acute regimes of radiation. Br J Radiol 1969; 42: 432–40.
82. Sandell LL, Zakian VA. Loss of a yeast telomere: arrest, recovery, and chromosome loss. Cell 1993; 75: 729–39.
83. Foray N, Fertil B, Alsbeih MGA, Badie C, Chavaudra N, Iliakis G, Malaise EP. Dose-rate effect on radiation-induced DNA double-strand breaks in the human fibroblast HF19 cell line. Int J Radiat Biol 1995; 69: 241–9.
84. Savage JRK, Holloway M. Induction of sister-chromatid exchanges by d42 MeV) Be neutrons in unstimulated human blood lymphocytes. Br J Radiol 1988; 61: 231–4.
85. Crompton NEA, Hain J, Jaussi R, Burkart W. Staurosporine and radiation-induced G2 cell cycle blocks are equally released by caffeine. Radiat Res 1993; 135: 372–9.
86. Lock RB, Ross WE. Inhibition of p34cdc2 kinase activity by etoposide or irradiation as a mechanism of G2 arrest in Chinese hamster ovary cells. Cancer Res 1990; 50: 3761–6.
87. Hartwell LH, Weinert TA. Checkpoints: controls that ensure the order of cell cycle events. Science 1989; 246: 629–34.
88. Yi PN, Evans HH, Ricanati M, Mulvihill JW, Beer JZ. Threshold dose rate and sensitivity for mitotic delay. Proc 42nd Ann Meet Radiat Res Soc 1994; # P17–283: 177.
89. Rowley R. Comment on the paper by Grinfeld et al. Int J Radiat Biol 1987; 52: 87–90.
90. Hain J, Crompton NEA, Burkart W, Jaussi R. Caffeine release of radiation induced S and G2 phase arrest in V79 hamster cells: Increase of histone mRNA levels and p34cdc2 activation. Cancer Res 1993; 53: 1507–10.
91. Walters RA, Gurley LR, Tobey RA. Effects of caffeine on radiation-induced phenomena associated with cell-cycle traverse of mammalian cells. Biophys J 1974; 14: 99–118.
92. Painter RB, Young RB. Radiosensitivity in ataxia telangiectasia: a new explanation. Proc Natl Acad Sci USA 1980; 77: 7315–7.
93. Ward JF. The complexity of DNA damage: relevance to biological consequences. Int J Radiat Biol 1994; 66: 427–32.
94. Nelson WG, Kastan MB. DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. Mol Cell Biol 1994; 14: 1815–23.
95. Kan L-S, Borer PN, Cheng DM, Ts'o POP. H- and C-NMR studies of caffeine and its interaction with nucleic acids. Biopolymers 1980; 19: 1641–54.
96. Tornaletti S, Russo P, Parodi S, Pedrini AM. Biochim Biophys Acta 1989; 1007: 112–5.
97. Lang H. Model for repair inhibition by caffeine. Studia Biophys 1975; 50: 213–21.
98. Lang H. On the interaction between caffeine and nucleic acids I. The influence of caffeine on the secondary structure of native DNA and RNA. Studia Biophys 1976; 55: 137–56.
99. Stambrook PJ, Velez C. Reversible arrest of Chinese hamster V79 cells in G2 by dibutyryl cyclic AMP. Exp Cell Res 1976; 99: 57–62.
100. Oleinick NL, Brewer EN, Rustad RC. The reduction of radiation-induced mitotic delay by caffeine: A test for the cyclic AMP hypothesis. Int J Radiat Biol 1978; 33: 69–73.
101. Kimler BF, Leeper DB, Snyder MH, Rowley R, Schneiderman M. Modification of radiation-induced division delay by caffeine analogues and dibutyryl cyclic AMP. Int J Radiat Biol 1982; 41: 47–58.
102. Burgoyne RD, Cheek TR, Morgan A, O'Sullivan AJ. Distribution of two distinct Ca ATPase-like proteins. Nature 1989; 342: 72–4.
103. Draetta G. Cell cycle control in eukaryotes: molecular mechanisms of cdc2 activation. Trends Biochem Sci 1989; 15: 378–83.
104. Miake-Lye R, Newport JW, Kirschner MW. Maturation-promoting factor induces nuclear envelope breakdown in cycloheximide-arrested embryos of Xenopus laevis. J Cell Biol 1983; 97: 81–91.
105. Newport JW, Kirschner MW. Regulation of the cell cycle during early Xenopus development. Cell 1984; 37: 731–42.
106. Murray AW, Solomon MJ, Kirschner MW. The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. Nature 1989; 339: 280–6.
107. Hunt T, Luca FC, Ruderman JV. The requirements for protein synthesis and degradation, and the control of destruction of cyclins A and B in the meiotic and mitotic cell cycles of the clam embryo. J Cell Biol 1992; 116: 707–24.
108. Solomon MJ, Glotzer M, Lee TH, Philippe M, Kirschner MW. Cyclin activation of p34cdc2 Cell 1990; 63: 1013–24.
109. Broek D, Bartlett R, Crawford K, Nurse P. Involvement of p34cdc2 in establishing the dependency of S phase on mitosis. Nature 1991; 349: 388–93.
110. Lundgren K, Walworth N, Booher R, Dembski M, Kirschner M, Beach D. mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. Cell 1991; 64: 1111–22.
111. Draetta G, Beach D. Activation of cdc2 protein kinase during mitosis in human cells: cell cycle-dependent phosphorylation and subunit rearrangement. Cell 1988; 54: 17–26.
112. Brizuela L, Draetta G, Beach D. Activation of human CDC2 protein as a histone H1 kinase is associated with complex formation with the p62 subunit. Proc Natl Acad Sci USA 1989; 86: 4362–6.
113. Gautier J, Minshull J, Lohka M, Glotzer M, Hunt T, Maller JL. Cyclin is a component of maturation-promoting factor from Xenopus. Cell 1990; 60: 487–94.

114. Meikrantz W, Suprynowicz FA, Halleck MS, Schlegel RA. Identification of mitosis-specific p65 dimer as a component of human M phase-promoting factor. Proc Natl Acad Sci USA 1990; 87: 9600–4.
115. Dunphy WG, Newport JW. Fission yeast p13 blocks mitotic activation and tyrosine dephosphorylation of the Xenopus cdc2 protein kinase. Cell 1989; 58: 181–91.
116. Meijer L, Arion D, Golsteyn R, et al. Cyclin is a component of the sea urchin egg M-phase specific histone H1 kinase. EMBO J 1989; 8: 2275–82.
117. Meijer L, Azzi L, Wang JYJ. Cyclin B targets p34cdc2 for tyrosine phosphorylation. EMBO J 1991; 10: 1545–54.
118. Gould KL, Nurse P. Tyrosine phosphorylation of the fission yeast cdc2 protein kinase regulates entry into mitosis. Nature 1989; 342: 39–45.
119. Krek W, Nigg EA. Mutations of p34cdc2 phosphorylation sites induce premature mitotic events in HeLa cells: Evidence for a double block to p34cdc2 kinase activation in vertebrates. EMBO J 1991; 10: 3331–41.
120. Moreno S, Nurse P. Clues to action of cdc25 protein. Nature 1991; 351: 194.
121. Muschel RJ, Zhang HB, Iliakis G, McKenna WG. Cyclin B expression in HeLa cells during the G2 block induced by ionizing radiation. Cancer Res 1991; 51: 5113–7.
122. Muschel RJ, Zhang HB, Iliakis G, McKenna WG. Effects of ionizing radiation on cyclin expression in HeLa cells. Radiat Res 1992; 132: 153–7.
123. Muschel RJ, Zhang HB, McKenna WG. Differential effect of ionizing radiation on the expression of cyclin A and cyclin B in Hela cells. Cancer Res 1993; 53: 1128–35.
124. Enoch T, Carr A, Nurse P. Checkpoint check. Nature 1993; 361: 26.
125. Weinert TA, Hartwell LH. The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. Science 1988; 241: 317–22.
126. Weinert TA, Hartwell LH. Control of G2 delay by the rad9 gene of Saccharomyces cerevisiae. J Cell Sci Suppl 1989; 12: 145–8.
127. Weinert TA, Hartwell LH. Characterization of RAD9 of Saccharomyces cerevisiae and evidence that its function acts posttranslationally in cell cycle arrest after DNA damage. Mol Cell Biol 1990; 10: 6554–64.
128. Dasso M, Newport JW. Completion of DNA replication is monitored by a feedback system that controls the initiation of mitosis in vitro: studies in Xenopus. Cell 1990; 61: 811–23.
129. Heald R, McLoughlin M, McKeon F. Human wee1 maintains mitotic timing by protecting the nucleus from cytoplasmically activated Cdc2 kinase. Cell 1993; 74: 463–74.
130. McGowan CH, Russell P. Human Wee1 kinase inhibits cell division by phosphorylating p34cdc2 exclusively on Tyr15. EMBO J 1993; 12: 75–85.
131. Enoch T, Nurse P. Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. Cell 1990; 60: 665–73.
132. Honda R, Ohba Y, Nagata A, Okayama H, Yasuda H. Dephosphorylation of human p34cdc2 kinase on both Thr-14 and Tyr-15 by human cdc25B phosphatase. FEBS Lett 1993; 318: 331–4.
133. Dunphy WG, Kumagai A. The cdc25 protein contains an intrinsic phosphatase activity. Cell 1991; 67: 189–96.
134. Gautier J, Solomon MJ, Booher RN, Bazan JF, Kirschner MW. cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2. Cell 1991; 67: 197–211.
135. Millar JBA, Russell P. The cdc25 M-phase inducer: An unconventional protein phosphatase. Cell 1992; 68: 407–10.
136. Lehnert S. Relationship of cyclic AMP levels to duration of radiation-induced mitotic delay. Int J Radiat Biol 1979; 36: 417–22.
137. Rowley R, Zorch M, Leeper DB. Effect of caffeine on radiation-induced mitotic delay. Radiat Res 1984; 97: 178–85.
138. Crompton NEA, Michel C, Burkart W. The role of the phospholipase C signal transduction pathway in the expression of radiation induced G2 delay. In: Seymour CB, Mothershill C, eds. New Developments in Fundamental and Applied Radiobiology. London, UK: Taylor & Francis Ltd, 1991: 138–43.
139. Kikkawa U, Nishizuka Y. The role of protein kinase C in transmembrane signalling. Ann Rev Cell Biol 1986; 2: 149–78.
140. Lehmann AR. Effect of caffeine on DNA synthesis in mammalian cells. Biophys J 1972; 12: 1316–25.
141. Schroeder E, Magdon E. Influence of caffeine on the DNA synthesis and the sedimentation behaviour of normal and irradiated mammalian cells. Stud Biophys 1975; 51: 209–14.
142. Abe K, Yoshida M, Usui T, Horinouchi S, Beppu T. Highly synchronous culture of fibroblasts from G2 block caused by staurosporine, a potent inhibitor of protein kinases. Exp Cell Res 1991; 192: 122–7.
143. Crissman HA, Gadbois DM, Tobey RA, Bradbury EM. Transformed mammalian cells are deficient in kinase-mediated control of progression through the G1 phase of the cell cycle. Proc Natl Acad Sci USA 1991; 88: 7580–4.
144. Rowley R, Hudson J, Young PG. The wee1 protein kinase is required for radiation-induced mitotic delay. Nature 1992; 356: 353–5.
145. Parker LL, Piwnica-Worms H. Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase. Science 1992; 257: 1955–7.
146. Murray AW. Creative blocks: Cell-cycle checkpoints and feedback controls. Nature 1992; 359: 599–604.
147. Gadbois DM, Hamaguchi JR, Swank RA, Bradbury EM. Staurosporine is a potent inhibitor of p34cdc2 and p34cdc2-like kinases. Biochem Biophys Res Comm 1992; 184: 80–5.
148. Crompton NEA, Saydan N. Control of the cell cycle. J Neurooncol 1994; 22: 255–9.
149. Sinclair WK. X-ray-induced heritable damage small-colony formation) in cultured mammalian cells. Radiat Res 1964; 21: 584–611.
150. Beer JZ. Heritable lesions affecting proliferation of irradiated mammalian cells. Adv Radiat Biol 1979; 8: 363–417.
151. Seymour C, Mothersill C, Alper T. High yields of lethal mutations in somatic mammalian cells that survive ionizing radiation. Int J Radiat Biol 1986; 50: 167–79.
152. Seymour C, Mothersill C. All colonies of CHO-K1 cells surviving g-irradiation contain non-viable cells. Mutat Res 1992; 267: 19–30.
153. Chang WP, Little JB. Delayed reproductive death in X-irradiated Chinese hamster ovary cells. Int J Radiat Biol 1991; 60: 483–96.
154. Chang WP, Little JB. Evidence that DNA double-strand breaks initiate the phenotype of delayed reproductive death in Chinese hamster ovary cells. Radiat Res 1992; 131: 53–9.
155. Hendry JH, West CML. Implications of delayed reproductive death lethal mutations/genomic instability for the interpretation of tissue responses. Int J Radiat Biol 1995; 68: 363–7.
156. Paquette B, Little JB. Genomic rearrangements in mouse C3H/10T1/2 cells transformed by X-rays, UV-C, and 3-methylcholanthrene detected by a DNA fingerprint assay. Cancer Res 1992; 52: 5788–93.

157. Paquette B, Little JB. In vivo enhancement of genomic instability in minisatellite sequences of mouse C3H/10T1/2 cells transformed in vitro by X-rays. Cancer Res 1994; 54: 3173–8.
158. Tlsty TD, White A, Sanchez J. Suppression of gene amplification in human cell hybrids. Science 1992; 255: 1425–7.
159. Livingstone LR, White A, Sprouse J, Livanos E, Jacks T, Tlsty TD. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell 1992; 70: 923–35.
160. Stamato T, Weinstein R, Peters B, Hu J, Doherty B, Giaccia A. Delayed mutation in Chinese hamster cells. Somat Cell Mol Genet 1987; 13: 57–66.
161. Little JB, Gorgojo L, Vetrovs H. Delayed appearance of lethal and specific gene mutations in irradiated mammalian cells. Int J Radiat Oncol Biol Phys 1989; 19: 1425–9.
162. Mendonca MS, Antoniono RJ, Redpath JL. Delayed heritable damage and epigenetics in radiation-induced neoplastic transformation of human hybrid cells. Radiat Res 1993; 134: 209–16.
163. Denko NC, Giaccia AJ, Stringer JR, Stambrrok PJ. The human Ha-ras oncogene induces genomic instability in murine fibroblasts within one cell cycle. Proc Natl Acad Sci USA 1994; 91: 5124–8.
164. Kennedy AR, Fox M, Murphy G, Little JB. Relationship between x-ray exposure and malignant transformation in C3H 10T1/2 cells. Proc Natl Acad Sci USA 1980; 77: 7262–6.
165. Kennedy AR, Cairns J, Little JB. Timing of the steps in transformation of C3H 10T1/2 cells by X-irradiation. Nature 1984; 307: 85–7.
166. McClintock B. Chromosome organization and genic expression. CSH Symp Quant Biol 1951; 16: 13–47.
167. Petrov DA, Schutzman JL, Hartl DL, Lozovskaya ER. Diverse transposable elements are mobilized in hybrid dysgenesis in Drosophila virilis. Proc Natl Acad Sci USA 1995; 92: 8050–4.
168. Nowell PC. The clonal evolution of tumour cell populations. Science 1976; 194: 23–8.
169. Renan MJ. How many mutations are required for tumorigenesis? Implications from human cancer data. Mol Carcinogen 1993; 7: 139–46.
170. Loeb LA. Mutator phenotype may be required for multistage carcinogenesis. Cancer Res 1991; 51: 3075–9.
171. Loeb LA. Microsatellite instability: marker of a mutator phenotype in cancer. Cancer Res 1994; 54: 5059–63.
172. Hartwell LH. Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. Cell 1992; 71: 543–6.
173. Foulds L. The experimental study of tumor progression: a review. Cancer Res 1954; 14: 317–39.
174. Cifone MA, Fidler IJ. Increasing metastatic potential is associated with increasing genetic instability of clones isolated from muring neoplasms. Proc Natl Acad Sci USA 1981; 78: 6949–52.
175. Seshadri R, Kutlaca RJ, Trainor K, Matthews C, Morley AA. Mutation rate of normal and malignant human lymphocytes. Cancer Res 1987; 47: 407–9.
176. Tlsty TD, Margolin BH, Lum K. Differences in the rates of gene amplification in nontumorigenic and tumorigeneic cell lines as measured by Luria-Delbrueck fluctuation analysis. Proc Natl Acad Sci USA 1989; 86: 9441–5.
177. Vogelstein B, Fearson ER, Kern SE, et al. Allelotype of colorectal carcinomas. Science 1989; 241: 207–11.
178. Mao L, Lee DJ, Tockman MS, Erozan YS, Askin F, Sidransky D. Microsatellite alterations as clonal markers for the detection of human cancer. Proc Natl Acad Sci USA 1994; 91: 9871–5.
179. Kennedy AR. Is a mutagenic event involved in radiation-induced malignant transformation? Mutat Res 1996; 350: 81–91.
180. Reznikoff CA, Bertram J, Brankow DW, Heidelberger C. Quantitative and qualitative studies of chemical transformation of clonal C3H mouse embryo cells sensitive to post confluence inhibitin of cell division. Cancer Res 1973; 33: 3239–49.
181. Su L-N, Little JB. Prolonged cell cycle delay in radioresistant human cell lines transfected with activated ras oncogene and/or Simian virus 40 T-antigen. Radiat Res 1993; 133: 73–9.
182. Bettega D, Calzolari P, Ottolenghi A, Tallone Lombadi L. Criteria and techniques for analysing cell survival data. Radiat Environ Biophys 1991; 30: 53–70.
183. Terzaghi M, Little JB. X-radiation-induced transformation in a C3H mouse embryo-derived cell line. Cancer Res 1976; 36: 1367–74.
184. Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. Participation of p53 protein in the cellular response to DNA damage. Cancer Res 1991; 51: 6304–11.
185. Inui K, Yamamoto M, Saito H. Transepithelial transport of oral cephalosporins by monolayers of intestinal epithelial cell line Caco-2: Specific transport systems in apical and basolateral membranes. J Pharmacol Exp Therap 1992; 261: 195–201.
186. Kastan MB, Zhan Q, El-Deiry WS, et al. A mammalian cell cycle checkpoint pathway utilizing p53 and *GADD45* is defective in ataxia-telangiectasia. Cell 1992; 71: 587–97.
187. Sidransky D, Mikkelsen T, Schwechheimer K, Rosenblum ML, Cavanee W, Vogelstein B. Clonal expansion of p53 mutant cells is associated with brain tumor progression. Nature 1992; 355: 846–7.
188. Krolewski B, Little JB. Application of denaturing gradient gel blots to detect p53 mutations in X-ray-transformed mouse C3H 10T1/2 clones. Mol Carcinog 1993; 7: 190–6.
189. Sigg M, Crompton NEA, Burkart W. Enhanced transformation in an inhomogeneous radiation field: an effect of exposure to superlethally damaged cells. Radiat Res 1997; 148: 543–7.
190. Sigg M, Crompton NEA, Burkart W. A pure beta line source to assess hot particles in vitro. Health Phys 1996; 71: 135–41.
191. Han A, Hill CK, Elkind MM. Repair of cell killing and neoplastic transformation at reduced dose rates of Co-60 gamma-rays. Cancer Res 1980; 40: 3328–32.
192. Jacobson MD. Reactive oxygen species and programmed cell death. TIBS 1996; 21: 83–6.
193. Nagasawa H, Little JB. Induction of sister chromatid exchanges by extremely low doses of alpha-particles. Cancer Res 1992; 52: 6394–6.
194. Deshpand A, Goodwin EH, Bailey SM, Marrone DL, Lehnert BE. Alpha-particle-induced sister chromatid exchanges in normal human lung fibroblasts: Evidence for an extranuclear target. Rad Res 1996; 145: 260–7.
195. Hickman AW, Jaramillo RJ, Lechner JF, Johnson NF. Alpha-particle-induced p53 protein expression in a rat lung epithelial cell strain. Cancer Res 1994; 54: 5797–800.
196. Weichselbaum RR, Hallahan DE, Sukhatme V, Dritschilo A, Sherman ML, Kufe DW. Biological consequences of gene regulation after ionizing radiation exposure. J Natl Cancer Inst 1991; 83: 480–4.
197. Coleman WB, Throneburg DB, Grisham JW, Smith GW. Overexpression of c-K-ras, c-N-ras and transforming growth

factor beta co-segregate with tumorigenicity in morphologically transformed C3H 10T1/2 cell lines. Carcinogenesis 1994; 15: 1005–12.
198. Sorrentino V, Drozdoff V, Zeitz L, Fleissner E. Increased radiation-induced transformation in C3H/10T1/2 cells after transfer of an exogeneous c-myc gene. Proc Natl Acad Sci USA 1987; 84: 4131–4.
199. Schwarz LC, Damen JE, Greenberg AH, Wright JA. Altered responsiveness of metastatic versus non-metastatic fibroblasts to heparin-binding growth factors. Cancer Lett 1988; 42: 193–7.
200. Van Bekkum DW, Bentvelzen P. The concept of gene transfer-misrepair mechanism of radiation carcinogenesis may challenge the linear extrapolation model of risk estimation for low radiation doses. Health Phys 1982; 43: 231–7.
201. Hill CK, Han A, Elkind MM. Promoter-enhanced neoplastic transformation after gamma-ray exposure at 10 cGy/day. Radiat Res 1989; 199: 348–55.
202. Milligan CE, Schwartz LM. Programmed cell death during development of animals. Cellular Aging and Cell Death. New York, USA: Wiley-Liss, Inc, 1996: 181–208.
203. Zou H, Niswander L. Requirement for BMP signaling in interdigital apoptosis and scale formation. Science 1996; 272: 738–41.
204. Hedgecock EM, Sulston JE, Thomson JN. Mutations affecting programmed cell deaths in the nematode Caenorhabditis elegans. Science 1983; 220: 1277–9.
205. Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol 1981; 68: 251–306.
206. Hall EJ. Radiobiology for the Radiologist, 4th edition. Philadelphia: J.B.Lippincott Company, 1994.
207. Gorman A, McCarthy J, Finucane D, Reville W, Cotter T. Morphological assessment of apoptosis. In: Cotter TG, Martin SJ, eds. Techniques in Apoptosis. London, UK: Portland Press Ltd, 1994: 1–20.
208. Tanaka N, Ishihara M, Lamphier MS, et al. Cooperation of the tumour suppressors IRF-1 and p53 in response to DNA damage. Nature 1996; 382: 816–8.
209. Hale AJ, Smith CA, Sutherland LC, et al. Apoptosis: molecular regulation of cell death. Eur J Biochem 1996; 236: 1–26.
210. Cuvillier O, Pirianov G, Kleuser B, et al. Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. Nature 1996; 381: 800–3.
211. Santana P, Pena LA, Haimovitz-Friedman A, et al. Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation-induced apoptosis. Cell 1996; 86: 189–99.
212. Verheij M, Bose R, Lin XH, et al. Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature 1996; 380: 75–9.
213. Kassid U, Suy S, Dent P, Ray S, Whiteside TL, Sturgill TW. Activation of Raf by ionizing radiation. Nature 1996; 382: 813–6.
214. Wang C-Y, Mayo MW, Baldwin AS. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kB. Science 1996; 274: 784–7.
215. Crompton NEA, Ozsahin M. A versatile and rapid radiosensitivity assay of peripheral blood leukocytes based on DNA and surface marker assessment of cytotoxicity. Radiat Res 1997; 147: 55–60.
216. Ozsahin M, Ozsahin H, Shi Y, Larsson B, Würgler FE, Crompton NEA. A rapid assay of intrinsic radiosensitivity based on apoptosis in human CD4 and CD8 T-lymphocytes. Int J Radiat Oncol Biol Phys 1997; 38: 427–40.
217. Peters LJ. Significance of genetic variability in radiosensitivity in clinical radiotherapy. J Jpn Soc Ther Radiol Oncol 1990; 2: 247–53.
218. Fisher DE. Apoptosis in cancer therapy: crossing the threshold. Cell 1994; 78: 539–42.
219. Lowe S, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993; 74: 957–67.
220. Lowe S, Schmitt EM, Smith SW, Osborne BA, Jacks T. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 1993; 362: 847–9.
221. White E. p53, guardian of Rb. Nature 1994; 371: 21–2.
222. McIlwrath AJ, Vasey PA, Ross GM, Brown R. Cell cycle arrests and radiosensitivity of human tumour cell lines: dependence on wild-type p53 for radiosensitivity. Cancer Res 1994; 54: 3718–22.
223. Begg AC, Russell NS, Knaken H, Lebesque JV. Lack of correlation of human fibroblast radiosensitivity in vitro with early skin reactions in patients undergoing radiotherapy. Int J Radiat Biol 1993; 64: 393–405.
224. Stewart CC, Stevenson AP, Habbersett RC. The effect of low-dose irradiation on unstimulated and PHA-stimulated human lymphocyte subsets. Int J Radiat Biol 1988; 53: 77–87.
225. Floyd DN, Cassoni AM. Intrinsic radiosensitivity of adult and cord blood lymphocytes as determined by the micronucleus assay. Eur J Cancer 1994; 30A: 615–20.
226. Crompton NEA, Ozsahin M, Melicharova L, Larsson B. A rapid assay of patient radiosensitivity. In: Germond J-F, Haefliger J-M, eds. Proc of the Journées Scientifiques de la Sociéte Suisse de Radiobiologie et Physique Médicale. La Chaux-de-Fonds, Switzerland: Verlag Hengg S.A, 1994: 157–62.
227. Waugh APW, Beare DM, Arlett CF, Green MHL, Cole J. Comparative human cellular radiosensitivity: IV. The increased sensitivity of human neonatal cord blood lymphocytes to gamma-irradiation compared with lymphocytes from children and adults. Int J Radiat Biol 1991; 59: 767–76.
228. Natarajan AT, Beninson D, Lloyd DC, Obe G, Preston RJ, Sasaki MS. In: Biological Dosimetry: Chromosomal aberration analysis for dose assessments. IAEA Technical reports series no. 260, Vienna, Austria, 1986.
229. Lloyd DC, Purrott RJ. Chromosome aberration analysis in radiological protection dosimetry. Radiat Protect Dosimetry 1981; 1: 19–28.
230. Fenech M, Morley A. Measurement of micronuclei in lymphocytes. Mutat Res 1985; 147: 29–36.
231. Mill AJ, Wells J, Hall SC, Butler A. Micronucleus induction in human lymphocytes: comparative effects of x-rays, alpha-particles, beta-particles and neutrons and implications for biological dosimetry. Radiat Res 1986; 145: 575–85.
232. Almassey Z, Krepinsky AB, Bianco A, Koeteles GJ. The present state and perspectives of micronucleus assay in radiation protection. A review. Appl Radiat Isot 1987; 38: 241–9.
233. Ramalho A, Sunjevaric I, Natarajan AT. Use of the frequencies of micronuclei as quantitative indicators of x-ray induced chromosomal aberrations in human peripheral blood lymphocytes: comparison of two methods. Radiat Res 1988; 207: 141–6.
234. Littlefield LG, Sayer AM, Frome EL. Comparisons of dose-response parameters for radiation induced acentric fragments and micronuclei observed in cytokinesis-arrested lymphocytes. Mutagenesis 1989; 4: 265–70.
235. Fenech M, Denham J, Francis W, Morley A. Micronuclei in cytokinesis-blocked lymphocytes of cancer patients following fractionated partial-body radiotherapy. Int J Radiat Biol 1990; 57: 373–83.

236. Gantenberg HW, Wuttke K, Streffer C, Muller WU. Micronuclei in human lymphocytes irradiated in vitro or in vivo. Radiat Res 1991; 128: 276–81.
237. Thierens H, Vral A, de Ridder L. Biological dosimetry using the micronucleus assay for lymphocytes: inter-individual differences in dose response. Health Phys 1991; 61: 623–30.
238. Castelain P, van Hummelen P, Deleener A, Kirsch-Volders M. Automated detection of cytochalasin-B blocked binucleated lymphocytes for scoring micronuclei. Mutagenesis 1993; 8: 285–93.
239. Menz R, Andres R, Larsson B, Ozsahin M, Trott K, Crompton NEA. Biological dosimetry: the potential use of radiation-induced apoptosis in human T-lymphocytes. Radiat Environ Biophys 1997; 36: 175–81.
240. Miller RA. Aging and the immune response. In: Schneider and Rowe, eds. Handbook of the Biology of Aging, 4th edition, New York: Academic Press, 1996:355–392.
241. Uzawa A, Suzuki G, Nakata Y, Akashi M, Ohyama H, Akanuma A. Radiosensitivity of CD45RO + memory and CD45RO − naive T cells in culture. Radiat Res 1994; 137: 25–33.
242. Harris G, Holmes A, Sabovljev SA, et al. Sensitivity to X-irradiation of peripheral blood lymphocytes from ageing donors. Int J Radiat Biol 1986; 50: 685–94.
243. Wei Q, Matanoski GM, Farmer ER, Hedayati MA, Grossman L. DNA repair and aging in basal cell carcinoma: a molecular epidemiology study. Proc Natl Acad Sci USA 1993; 90: 1614–8.
244. Kruk PA, Rampino NJ, Bihr VA. DNA damage and repair in telomeres: relation to aging. Proc Natl Acad Sci USA 1995; 92: 258–62.
245. Vaziri H, Dragowska W, Allsopp RC, Thomas TE, Harley CB, Lansdorp PM. Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric DNA with age. Proc Natl Acad Sci USA 1994; 91: 9857–60.
246. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16. Cell 1997; 88: 593–602.
247. Campisi J. Repicative senescence: an old lives' tale. Cell 1996; 84: 497–500.
248. Wang E. Senescent human fibroblasts resist programmed cell death, and failure to suppress bcl2 is involved. Cancer Res 1995; 55: 2284–92.
249. Nagel JE, Chrest FJ, Lal P, Adlker WH. Immune function, cell death, and aging. In: Holbrook NJ, Martin GR, Lockshin RA, eds. Cellular Aging and Cell Death. New York: Wiley-Liss, Inc, 1996: 51–65.
250. Tyson JJ. Modeling the cell division cycle: cdc2 and cyclin interactions. Proc Natl Acad Sci USA 1991; 88: 7328–32.
251. Norbury C, Nurse P. Animal cell cycles and their control. Ann Rev Biochem 1992; 61: 441–70.
252. Pines J, Hunter T. Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. Cell 1989; 58: 833–46.
253. Solomon MJ, Gautier J, Lee TH, Kirschner MW. Control of p34cdc2 activation. Cold Spring Habour Symp Quant Biol 1991; 56: 427–35.
254. Jessus C, Beach D. Oscillation of MPF is accompanied by periodic association between cdc25 and cdc2-cyclin B. Cell 1992; 68: 323–32.
255. Lewin B. Genes V. Oxford, UK: Oxford Uni. Press, 1994.
256. Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Molecular Biology of the Cell, 3rd edition. New York, USA: Garland Pub. Inc, 1994.
257. Bradbury EM. Reversible histone modifications and the chromosome cell cycle. BioEssays 1992; 14: 9–16.
258. Gilbert S. Developmental Biology, 3rd edition. Sunderland, Mass., USA: Sinauer Assoc. Inc, 1991.
259. Wiener N. Cybernetics or Control and Communication in the Animal and Machine. Cambridge, USA: MIT Press, 1948.
260. Lerner AY. Fundamentals of Cybernetics. New York, USA: Plenum, 1972.
261. Earnshaw WC. Nuclear changes in apoptosis. Curr Opin Cell Biol 1995; 7: 337–43.
262. Shannon CE, Weaver W. The Mathematical Theory of Communication. Urbana, USA: Uni. Illinois Press, 1949.
263. Chomsky N. Aspects of the Theory of Syntax. Cambridge, USA: MIT Press, 1965.
264. Gitt W. Information: the third fundamental quantity. Siemens Rev 1989; 56 (6): 2–7.
265. Miller GA. The Science of Words. New York, USA: Scientific American Library, Freeman and Co, 1991.
266. Feynman R, Lieghton RB, Sands ML. The Feynman Lectures on Physics: Commemorative Issue. Reading, Mass. USA: Addison-Wesley Publishing Co., Inc, 1989.
267. Nurse P. Checkpoint pathways come of age. Cell 1997; 91: 865–7.
268. Nagasawa H, Keng P, Harley R, Dahlberg W, Little JB. Relationship between gamma ray-induced G2/M delay and cellular radiosensitivity. Int J Radiat Biol 1994; 66: 373–9.
269. Bae I, Fan S, Bhatia K, Kohn KW, Fornace AJ, O'Connor PM. Relationships between G1 arrest and stability of the p53 and p21 proteins following gamma irradiation of human lymphoma cells. Cancer Res 1995; 55: 2387–93.
270. Noda A, Ning Y, Venable SF, Pereira-Smith OM, Smith JR. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. Exp Cell Res 1994; 211: 90–8.
271. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature 1990; 345: 458–60.
272. Lindsey J, McGill NI, Lindsey LA, Green DK, Cooke HJ. In vivo loss of telomeric repeats with age in humans. Mutat Res 1991; 256: 45–8.
273. Crompton NEA. Telomeres, senescence and cellular radiation response. Cell Mol Life Sci 1997; 53: 568–75.
274. Hayflick L. The limited in vitro lifetime of human diploid cell strains. Exp Cell Res 1965; 37: 614–36.
275. Matsumara T, Zerrudo Z, Hayflick L. Senescent human diploid cells in culture: survival, DNA synthesis and morphology. J Gerontol 1979; 34: 328–34.
276. Stein GH, Drullinger LF, Robetorye RS, Pereira-Smith OM, Smith JR. Senescent cells fail to express cdc2, cycA and cycB in response to mitogen stimulation. Proc Natl Acad Sci USA 1991; 88: 11012–6.
277. Dulic V, Drullinger LF, Lees E, Reed SI, Stein GH. Altered regulation of G1 cyclins in senescent human diploid fibroblasts: accumulation of inactive cyclin E-cdk2 and cyclin D1-cdk2 complexes. Proc Natl Acad Sci USA 1993; 90: 11304–8.
278. Hara E, Tsurui H, Shinozaki A, Nakada S, Oda K. Cooperative effects of antisense-Rb and antisense-p53 oligomers on the extension of life span in human diploid fibroblasts, TIG-1. Biochem Biophys Res Comm 1991; 179: 528–34.
279. Allsopp RC, Vaziri H, Patterson C, et al. Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acad Sci USA 1992; 89: 10114–8.
280. Yu G-L, Bradley JD, Attardi LD, Blackburn EH. In vivo alteration of telomere sequences and senescence caused by mutated Tetrahymena telomerase RNAs. Nature 1990; 344: 126–32.

281. Lundblad V, Blackburn EH. An alternative pathway for yeast telomere maintenance rescues est1- senescence. Cell 1993; 73: 347–60.
282. Harley CB. Telomeres and aging: fact, fancy, and the future. J NIH Res 1995; 7: 64–8.
283. Salk D, Au K, Hoehn H, Stenchver MR, Martin GM. Evidence of clonal attenuation, clonal succession, and clonal expansion in mass cultures of aging Werner's syndrome skin fibroblasts. Cytogenet Cell Genet 1981; 30: 108–17.
284. Mueller HJ. The remaking of chromosomes. Collect Net 1938; 13: 181–98.
285. Mueller HJ, Herskowitz IH. Concerning the healing of chromosome ends produced by breakage in Drosophila melanogater. Am Nat 1954; 88: 177–208.
286. Levy MZ, Allsopp RC, Futcher AB, Greider CW, Harley CB. Telomere end-replication problem and cell aging. J Mol Biol 1992; 225: 951–60.
287. Goldstein S. Replicative senescence: the human fibroblast comes of age. Science 1990; 249: 1129–33.
288. Tahara H, Sato E, Noda A, Ide T. Increase in expression level of p21 with increasing division age in both normal and SV40-trabsformed human fibroblasts. Oncogene 1995; 10: 835–40.
289. Shay JW, Wright WE, Brasiskyte D, van der Haegen BA. E6 of human papillomavirus type 16 can overcome the M1 stage of immortalization in human mammary epithelial cells but not in human fibroblasts. Oncogene 1993; 8: 1407–13.
290. Klingelhutz AJ, Barber SA, Smith PP, Dyer K, McDougall JK. Restoration of telomeres in human papillomavirus-immortalized humananogenital epithelial cells. Mol Cell Biol 1994; 14: 961–9.
291. Harley CB, Vaziri H, Counter CM, Allsopp RC. The telomere hypothesis of cellular aging. Exp Gerontol 1992; 27: 375–82.
292. Counter CM, Avilion AA, LeFeuvre CE, et al. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J 1992; 11: 1921–9.
293. Blackburn EH. Telomerases. Ann Rev Biochem 1992; 61: 113–29.
294. Cech TC. Chromosome end games. Science 1994; 266: 387–8.
295. Greider CW. Telomeres, telomerase and senescence. Bioassays 1990; 12: 363–9.
296. de Lange T. Activation of telomerase in a human tumour. Proc Natl Acad Sci USA 1994; 91: 2882–5.
297. Kim NW, Piatyszek MA, Prowse KR, et al. Specific association of human telomerase activity with immortal cells and cancer. Science 1994; 266: 2011–4.
298. Haber DA. Telomeres, cancer, and immortality. New Engl J Med 1995; 332: 955–6.
299. Marx J. Chromosome ends catch fire. Science 1994; 265: 1656–8.
300. Rennie J. Immortal's enzyme. Sci Am July, 8–10, 1994.
301. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu C-P, Morin GB, Harley CB, Shaw JW, Lichtsteiner S, Wright WE. Extension of life-span by introduction of telomerase into normal human cells. Science 1998; 279: 349–52.
302. Lomax A, Munkel G, Blattmann H, Pedroni E. Comparative treatment planning as a tool for determining indications and the efficacy of proton therapy. PSI Ann Report. Annex II. 1994:16–18.
303. Scheib S, Pedroni E, Lomax A, et al. Spot scanning with protons at PSI: experimental results and treatment planning. In: Amaldi U, Larsson B, eds. Hadrontherapy in Oncology. Amsterdam, Holland: Elsevier, 1994: 471–80.
304. Blattmann H, Munkel G, Pedroni E, et al. The Swiss prototherapy program. In: Amaldi U, Larsson B, eds. Hadrontherapy in Oncology. Amsterdam, Holland: Elsevier, 1994: 122–9.
305. Larsson B. The new radiations in cancer therapy. In: Amaldi U, Larsson B, eds. Hadrontherapy in Oncology. Amsterdam, Holland: Elsevier, 1994: 33–44.
306. Pierce DA, Shimizu Y, Preston DL, Vaeth M, Mabuchi K. Studies of the mortality of atomic bomb survivors. Report 12, part I. cancer: 1950–1990. Radiat Res 1996; 146: 1–27.
307. Delongchamp RR, Mabuchi K, Yoshimoto Y, Preston DL. Cancer mortality among atomic bomb survivors exposed in utero or as young children, October 1950–May 1992. Radiat Res 1997; 147: 385–95.
308. Locher GL. Biological effects and therapeutic possibilities with neutrons. Am J Roentgenol Radium Ther 1936; 36: 1–13.
309. Binello E, Shortkroff S, Jones A et al. In vitro analysis of 10B uptake for boron neutron capture synovectomy. In: Larsson B, Crawford J, Weinreich R, eds. Advances in Neutron Capture Therapy. Volume 2, Chemistry and Biology. Amsterdam: Elsevier Science, 1997: 609–613.
310. Hornung R, Walt H, Jentsch B, Crompton NEA, Haller U. In vitro effects of localisation of the photosensitizers m-THPC and m-THPC MD on carcinoma cells of the human breasts MCF-7) and Chinese hamster fibroblasts V-79). Lasers Surg Med 1997; 20: 443–50.
311. Schwiezer P, Walt H, Fehr M, Haller U. Basic research on the biology of m-THPC for photodynamic therapy in Gynecology: Preclinical tests for genotoxicity and somatic mutagenicity with Drosophila melanogaster. Lasers Med Sci 1997; 12: 280–4.
312. Colotta F, Fabio R, Polentarutti N, Sozzani S, Mantovani A. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. Blood 1992; 80: 2012–20.
313. Rex JH, Bhala SC, Cohen DM, Hester JP, Vartivarian SE, Anaissie EJ. Protection of human polymorphonuclear leukocyte function from the deleterious effects of isolation, irradiation, and storage by interferon-gamma and granulocyte-colony-stimulating factor. Transfusion 1995; 35: 605–11.
314. Gottlieb RA, Giesing HA, Zhu JY, Engler RL, Baboir BM. Cell acidification in apoptosis: granulocyte colony-stimulating factor delays programmed cell death in neutrophils by upregulating the vacuolar H-ATPase. Proc Natl Acad Sci USA 1995; 92: 5965–8.
315. Ozsahin H, von Planta M, Mueller I et al. Successful treatment of 72invasive aspergillosis in chronic granulomatous disease by emergency bone marrow transplantation, G-CSF mobilized granulocytes, and liposomal amphotericin-B. 1998, Blood, in press.
316. Ribaud P, Esperou-Bourdeau H, Devergie A, Gluckman E. Aspergillose invasive et allograffe de moelle. Path Biol 1994; 42: 652–5.
317. Bensinger WI, Price TH, Dale DC. The effects of daily recombinant human granulocyte colony-stimulating factor administration on normal granulocyte donors undergoing leukapheresis. Blood 1993; 81: 1883–8.
318. Caspar CB, Seger RA, Burger J, Gmuer J. Effective stimulation of donors for granulocyte transfusions with recombinant methionly granulocyte colony-stimulating factor. Blood 1993; 81: 2866–71.
319. Stavric B. Methylxanthines: toxicity to humans. 2. Caffeine. Food Chem Toxicol 1988; 26: 645–62.

320. IARC monographs on the evaluation of carcinogenic risks to humans: Coffee, Tea, Mate, Methylxanthines and Methylglyoxal. IARC monographs 51, 1991.
321. Crompton NEA, Ozsahin M, Schweizer P, Larsson B, Luetolf UM. Theory and practise of predictive assays in radiation therapy. Strahlenther Onkol 1997; 173: 58–67.
322. Daly LE, Bourke GJ, McGilvray J. Interpretation and Uses of Medical Statistics, 4th edition. Oxford: Blackwell Scientific Publications, 1991.
323. Matthews DE, Farewell VT. Using and Understanding Medical Statistics, 2nd edition. Basel: S. Karger AG, 1988.
324. Sieber P, Fateh-Moghadam A. Tumormarker und ihr sinvoller Einsatz. Klin Lab 1993; 39: 291–306.
325. Kaplan EL, Meier P. Non-parametric estimation from incomplete observations. J Am Stat Assoc 1958; 53: 457–81.
326. Peto P, Pike MC, Armitage P, et al. Design and analysis of randomised clinical trials requiring prolonged observation of each patient: Part II. Br J Cancer 1977; 35: 1–39.
327. Cox DR. Regression models and life tables. J Royal Stat Soc 1972; 34: 187–220.
328. Begg AC, McNally NJ, Shrieve DC. A method to measure the duration of DNA synthesis and the potential doubling time from a single sample. Cytometry 1985; 6: 620–6.
329. Kaser-Hotz B, Blattmann H, Böhringer T et al. Canine tumours as a model to develop proton conformation radiotherapy for humans. In: Manenti L, ed. Spontaneous Animal Tumours, 1997, in press.
330. Schwyn U, Kaser-Hotz B, Hauser B, Fodor G, Ruckstuhl H, Crompton NEA. Bestimmung der potentiellen Verdopplungszeit von Tumoren mittels Bromodeoxyuridin und Durchflusszytometrie. Schweiz Arch Tierheil 1997; 139: 441–8.
331. Schwyn U, Crompton NEA, Blattmann N et al. Potential tumour doubling time: determination of Tpot for various canine and feline tumours. Vet Res Comm 1998, in press.
332. Roelcke U, Blasberg R, von Ammon K, et al. In vivo assessment of brain tumours using positron emission tomography and I-124 iodo-dexyuridine. J Neurooncol 1996; 30: 97.
333. Tucker SL, Thames HD. The effect of patient-to-patient variability on the accuracy of predictive assays of tumor response to radiotherapy: a theoretical analysis. Int J Radiat Oncol Biol Phys 1989; 17: 145–57.
334. Nilsson S, Carlsson J, Larsson B, Ponten J. Survival of irradiated glia and glioma cells studied with a new cloning technique. Int J Radiat Biol 1980; 37: 267–79.
335. Burkard W, Larsson B. Towards individualization and optimization of radiation therapy: biological behaviours of tumor cells derived from human tumor biopsies. In: Amaldi U, Larsson B, eds. Hadron Therapy in Oncology. Excerpta Medica: Int Congress Series Vol 1077. Amsterdam, NL: Elsevier Science B.V, 1994: 735–41.
336. Larsson B. Kompaktzellkulturscheibe. International Patent Application PCT/CH96/00332, 1996.
337. Larsson B. Pretherapeutic physical experiments with high energy protons. Br J Radiol 1961; 34: 143–51.
338. Beer JZ, Mencl J, Horng M, Evans HH. Dose rate dependence of the cytotoxic and mutagenic effects during chronic X irradiation of radioresistant and radiosensitive L5178Y cells. Proc Ann Meet Radiat Res Soc 1993; 34: 115.