Normal Tissue Response to Low Doses of Radiotherapy Assessed by Molecular Markers

A Study of Skin in Patients Treated for Prostate Cancer

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Acta Oncologica Vol. 40, No. 8, pp. 941–951, 2001

The aim of this study was to evaluate normal tissue response by molecular markers to multifraction low doses of ionizing radiation, with the focus on changes in repopulation, estimated using Ki-67 as the proliferation marker, and on expressions of the p53 and p21 proteins, identified as key proteins in the DNA damage checkpoint. Repeated skin biopsies were taken from patients treated for prostate cancer with radiotherapy. The expressions of Ki-67, p53 and p21 of the keratinocytes in the basal cell layer of the epidermis were quantified immunohistochemically. The dose to the basal layer was 1.1 Gy per fraction, given five times per week for seven weeks. The indices of the three markers were determined over the whole period. A significant suppression of the Ki-67 index was observed during the first weeks, followed by a significant gradual increase in the Ki-67 index over the last weeks. The p53 and p21 protein levels were almost zero in the unirradiated skin. Upon irradiation, both the p53 and p21 index increased in a pattern very congruent to the Ki-67 index. In conclusion, daily fractions of about 1 Gy to the skin resulted in, for the keratinocytes in the basal layer, a cell growth arrest for a couple of weeks and a subsequent acceleration in repopulation during the following weeks of irradiation. The present findings also provided novel insights into the role of the p53/p21 pathway in the response of a normal epithelium to ionizing radiation as it is applied in radiotherapy.

Received 31 October 2001
Accepted 7 November 2001

The increasing implementation of more sophisticated 3D conformal treatment planning of radiotherapy with photon beams including intensity-modulated radiotherapy (IMRT) means a very different dose distribution to the surrounding normal tissues in comparison with the conventional 3D treatment plans used over the past decades (1). Generally, the consequences of this development are large volume irradiation with small total doses and very low doses per fraction instead of limited volume irradiation to fairly large doses and doses per fraction closer to those prescribed to the tumour target. The question then arises about the biological impact of giving a small dose to a large area instead of a high dose to a small area concerning various side effects, including the risk of induced malignancies.

This scenario should be considered in the light of recent knowledge from in vitro and in vivo studies about the phenomenon of hyperradiosensitivity (HRS) to very low doses, below 0.5 Gy, followed by induced radioresistance (IRR) in the dose range 0.5 to 1 Gy (2–6). A large body of these studies has emerged from the Gray Laboratory and the research group of M. Joiner (3–6). However, it is worth emphasizing that the HRS/IRR phenomenon has also been established in comprehensive studies by Wouters et al. and Skarsgard et al. (7–9). The current status of low-dose hyperradiosensitivity was recently reviewed from both an experimental and a clinical point of view (10).

The HRS/IRR features are seen following low-LET radiation but not following high LET. Various human tumour and non-malignant cell lines have been studied in addition to the immense studies on V79 hamster fibroblasts. HRS/IRR has been detected in about 80% of the cell lines assessed so far. No relationships between the existence of HRS and histological type, p53 status, apoptosis, cell-cycle time or G1 arrest have been observed (Joiner, personal communication). Importantly, the radiosensitivity

 ISSN 0284-186X

in the HRS region of the cell survival curve was similar for all cell lines regardless of their response above 1 Gy. Recently, it was also shown for a glioblastoma cell line that HRS occurred in fractionated irradiation using three doses of 0.4 Gy per day, 4 h apart. This schedule resulted in significantly increased cell killing compared with 1.2 Gy given once daily (11). These findings suggest that ultrafractionation should have the potential to increase the therapeutic ratio for tumours that are radioresistant to conventional fractionation.

There is evidence that HRS in vitro translates into additional effectiveness of fractionated radiotherapy given in very small doses per fraction in vivo. This has been confirmed for mice, in skin, kidney and lung (12–14). In fact, these tissues showed a 'reverse fractionation effect' below 1 Gy. Additionally, clinical evidence is now accumulating on the existence of the HRS/IRR phenomenon in skin from our own group (10) and from the Mount Vernon Hospital (N. Shah, personal communication), and also for the salivary function, shown by the group of P. Lambin (10).

Evidence now emerges that the mechanism for IRR is related to induced DNA damage repair, and that the level of radiation-induced injury produced by doses above 0.3–0.5 Gy is sufficient to activate the cellular repair systems. The establishment of HRS/IRR suggests that both cancer cells and normal cells respond to radiation injury by triggering repair processes, and that the activation is dictated by the amount of DNA damage received (15, 16).

In our ongoing project, the aim is to establish dose-response relationships to low doses per fraction for various normal cell populations in skin for patients receiving standard radiotherapy. Repeated skin biopsies are taken during the treatment course. This enables us separately to study the dose response of keratinocytes, melanocytes and Langerhan's cells in the epidermis by counting the reduction in cell density versus dose for fraction doses in the range of 0.1 to 1.1 Gy. In a second part of the project the aim is to investigate the expression and dose response of various molecular markers in sections from the same biopsies as used for cell counting. We expect that such a mapping will give a deeper knowledge of various mechanisms behind the cellular response observed in situ.

Our work focuses on molecular markers recently identified as main actors in the network of signalling pathways in response to DNA damage by ionizing irradiation. In this report we present the first results of the expression of Ki-67, p53 and p21 assessed by immunohistochemistry for daily fractions of 1.1 Gy over a period of 7 weeks. The purpose is to illustrate the usefulness of this approach in achieving a deeper understanding of the response of the various cellular components of a normal tissue.

Why assessment of Ki-67?

There is much evidence to show that accelerated repopulation is a feature of many normal epithelial cell populations in response to a certain amount of cell depletion during a radiotherapy course with conventional fractionation. The mechanisms driving this effect are largely unknown. Whether threshold damage is needed and when accelerated repopulation is likely to start are therefore still highly obscure (17–19). This issue is continuously being debated concerning epithelial tumours in particular.

To our knowledge there is no clinical study exploring cell proliferation disturbances to daily fractions of low doses. If significant cell proliferation changes occur this might be an important factor determining the dose-response relationship seen in the low-dose region.

In order to measure the epidermal cell proliferation activity during a 7-week-long course of radiotherapy, we used Ki-67 as a molecular marker. Ki-67 reacts with a nuclear antigen expressed by cycling cells, i.e. in all cycle phases G1, S, G2 and M. G0 or quiescent cells do not express this antigen (20). There is also evidence that the Ki-67 protein expression is an absolute requirement for cell proliferation and the maintaining of the cell cycle. Therefore, it is possible to perform a rapid and reproducible determination of the growth fraction of a particular cell population immunohistochemically (21).

Why assessment of p53 and p21?

The p53 tumour suppressor gene is activated by many diverse stress signals including ionizing radiation (IR) through mechanisms that result in stabilization and accumulation of the p53 protein (22–27). p53 protein levels in normal unstressed cells are maintained at low levels by rapid degradation through ubiquitin-dependent proteolysis. Importantly, p53 has a remarkable sensitivity to DNA damage. Just one double-strand break in the DNA molecule may be sufficient to trigger a rise in levels of the p53 protein (28). IR induces activation of p53 through site-specific phosphorylations within the N-terminus of p53, which to a large extent is mediated through the ATM protein upon double-strand breaks in DNA (29–34). In turn, activated p53 exerts various activities leading to growth arrest, DNA repair or apoptosis and senescence (see Fig. 1 (35–39)).

A second mechanism for stabilization and accumulation of p53 is related to the proliferative response of a tissue (Fig. 1). This is obtained through the ARF protein in response to abnormal proliferative signals mediated by oncogenes such as *myc* and *ras* or by aberrant E2F1 activity (40–42). In situations where accelerated repopulation takes place, the ARF-p53 pathway will probably regulate the appropriateness of this process. It is noteworthy, that the ARF-induced p53 stabilization does not require phosphorylation of p53 and may occur without

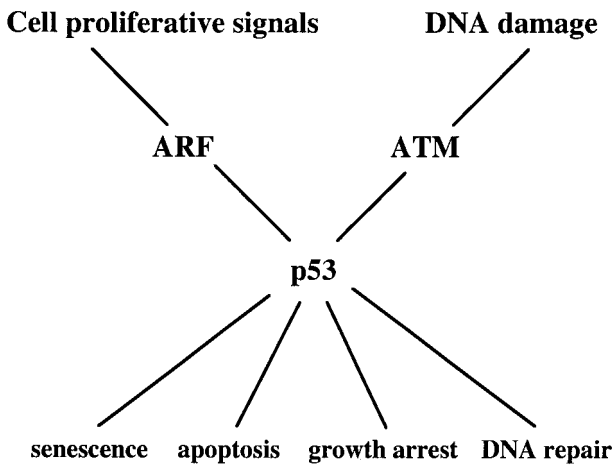

Fig. 1. Main pathways for cellular/tissue response to IR.

cellular DNA damage. Therefore, during a radiotherapy course wild-type p53 may have dual control of the cell behaviour related to the amount of induced DNA damage and the degree of compensatory accelerated cell proliferation.

Activated p53 exerts its function through transcription, translation and subcellular localization. Nuclear localization of activated p53 is essential for its normal function. One of the first effects of p53 activation in nearly all mammalian cell types is a block in the cell-division cycle. The ability of p53 to inhibit cell growth is related to its function as a transcription factor. P21 (WAF1) is the principal mediator of p53-dependent cell-cycle arrest. P21 is a potent inhibitor of cyclin-dependent kinases. Its upregulation therefore induces cell-cycle arrest in the transitions of G1–S and G2–M (43–47). P21 has greatest affinity to G1-cyclin-CDK complexes, allowing hypophosphorylation of the retinoblastoma protein, which appears to be required for G1 arrest following DNA damage by IR. Moreover the p21 protein has the capacity to inhibit the DNA replication and cell-cycle progression into the S-phase, without affecting DNA repair. Furthermore, the ARF-induced growth control of stimulated cell proliferation is mediated through p21 (Fig. 1). The cell-cycle inhibitory activity of p21 is correlated with its nuclear localization.

Novel and broader biological functions of p21 have recently been identified (46). Importantly, p21 may also be able to control epithelial self-renewal and commitment to differentiation; this has been elucidated particularly for keratinocyte stem cells (48, 49). Therefore, p21 may be the critical mediator not only in the regulation of cell-cycle arrest but also in the process of compensatory accelerated cell proliferation in the response of normal epithelium to radiotherapy.

The target genes and pathways that mediate p53-dependent apoptosis are less well understood despite comprehensive research on this topic. However, evidence exists that apoptosis is mediated by p53 through both the mitochondrial and the death receptor pathways (50–55). P21 appears to be a modulator of the apoptotic process, and primarily protects cells from p53-mediated apoptosis (46). The prevailing view is that severe DNA damage is needed for induction of p53-regulated apoptotic cell killing (56). Further penetration of p53-induced apoptosis is beyond the scope of this presentation.

MATERIAL AND METHODS

Clinical assay

Patients with prostate cancer treated with radiotherapy with curative intent who gave informed consent to repeated skin biopsies were recruited for this study. The prescribed dose to the tumour target was either 35 fractions of 2 Gy in 7 weeks (Groups 1–3, see below) or 25 fractions of 2 Gy divided into two parts, allowing two high-dose-rate brachytherapy treatments of 10 Gy in between (Group 4). Eleven or 15 MV photons were applied in a 3-field technique with an anterior field and opposed lateral fields with wedges. A 5 mm bolus was added on the left lateral field. Careful consideration was given to the dosimetry of the skin. Comprehensive dosimetric measurements were performed in a phantom, for standard geometries and also for typical patient field configurations. Dose determination to the skin with TLD was used in most of the patients on five occasions at the beginning of the treatment course. The dose per fraction at a depth of 0.1 mm below the skin, relevant for the biological endpoints used in this study, was estimated individually for each patient at specified points from which biopsies were taken.

In this report we present the results from skin biopsies taken from the left lateral field, for which the daily dose per fraction was 1.10 ± 0.07 (SD) Gy at a depth of 0.1 mm, below the skin surface.

Skin biopsies

Punch biopsies 3 mm in diameter were taken under local anaesthesia, using lidocaine hydrochloride 5 mg/ml without adrenaline. All biopsies were coded. One control biopsy was taken within the field and one from an area outside but close to the field. Biopsies were taken at regular intervals during the radiotherapy course according to following schedules:

Group 1. Biopsies were taken after 1, 3, 5 and 7 weeks of radiotherapy in 10 patients.
Group 2. Biopsies were taken after 3, 4, 5 and 6 weeks of radiotherapy in 13 patients.
Group 3. Biopsies were taken after 4, 5, 6 and 7 weeks of radiotherapy in 8 patients.

Group 4. One biopsy was taken after 2.5 weeks of radiotherapy in 16 patients.

Immunohistochemistry

Three 4 μm sections from each formalin-fixed and paraffin-embedded tissue biopsy were cut and mounted on a Superfrost plus slide (Menzel-Gläser, Germany). The three sections were taken from various levels in the biopsies according to a prescribed pattern. The slides were dried in 37°C overnight. Deparaffinized sections were first incubated in 3% H_2O_2/PBS pH 7.4 (phosphate-buffered saline) for 10 min to block endogenous peroxidase activity and subsequently boiled twice for 5 min in citrate buffer (pH6.0) in microwave oven at 750W, and cooled to room temperature. Then the sections were exposed to 1% BSA (bovine serum albumin, Sigma) for 10 min, incubated overnight with the relevant primary antibody (see Table 1) and washed 3 times for 5 min in PBS. Sections were incubated for 30 min with biotinylated goat anti-mouse/rabbit Ig, washed 3 times for 5 min and incubated for 30 min in ABC-complex (DAKO). Staining was developed using 0.03% 3,3-diaminobenzidin-tetrahydro-chloride (Sigma-Aldrich) in TRIS-buffer. Sections were counterstained with haematoxylin and mounted.

Endpoints

The epidermis is built up of various characteristic cell layers of keratinocytes. The basal layer, closest to the dermis, is orderly covered by the spinous—granular and cornified layers. The basal cell layer resides stem cells, transient amplifying cells and terminal differentiating cells. Transient amplifying cells are daughters of stem cells that undergo a finite number of divisions and are committed to terminal differentiation. Terminal differentiating cells, also named post-mitotic cells, are the progeny of transient amplifying cells that are destined to move upwards from the basal cell layer (57, 58). Quantitative estimates of the indices of Ki-67, p53 and p21-stained cells established in this work were restricted to cell counting in the basal layer of the interfollicular epidermis. Only cells that were attached to the underlying basement membrane were included.

The total number of basal cells per millimetre of interfollicular epidermis was determined previously for all biopsies on five separate sections stained in haematoxylin-eosin and PAS (Periodic Acid Schiff). The mean of basal cells in controls was found to be 167 ± 3 (SE) per millimetre (10).

Table 1

Description of primary antibodies

Antibody	Clone	Working dilution	Source
Ki-67	MIB1	1/100	Immunotech
P53	DO-7	1/50	DAKO
P21	WAF1	1/50	Calbiochem

In total, about 1500 basal cells were counted in the control biopsies. About 50% of the basal cells were still in the basal cell layer at the end of the 7-week treatment with daily fractions of 1.1 Gy, allowing 700–800 cells to be counted (10). The basal cell density assay of epidermis has already been described (59).

Melanocytes are also located at fairly regular intervals in the basal cell layer of the interfollicular epidermis. These can be distinguished fairly well through their morphological characteristics in a magnification of 400. In the controls the mean was found to be 16 ± 1 (SE) per millimetre. Thus, in control biopsies, the melanocytes constitute 10% of the cells in the basal layer. The number stayed approximately constant during the 7 weeks' treatment with 1.1 Gy per fraction. However, details of the melanocyte dose response will be omitted in this presentation. The few Langerhan's cells and Merkel cells that usually exist in the basal cell layer were neglected in the present analysis.

From the immunohistochemically stained sections, the number of cells in the basal cell layer per millimetre of interfollicular epidermis with clear-cut and strong nuclear staining was counted in a magnification of 400. As nuclear localization is highly relevant to the biological functions of Ki-67, p53 and p21, particular attention was given to staining of the nucleus. This was a requirement in order to classify the cell as positive. The indices of Ki-67, p53 and p21 were calculated by dividing the number of stained cells per millimetre by the total number of basal cells per millimetre. It should be noted that the non-keratinocytes in the basal layer were included in the total number. All patients were evaluated for all three markers.

RESULTS

Immunohistochemistry of Ki-67

Nuclear Ki-67 staining was restricted to keratinocytes in the basal cell layer and the suprabasal cell layer of the interfollicular epidermis. In this analysis we counted only Ki-67-positive cells in the basal cell layer. We did not observe any Ki-67 nuclear staining of the melanocytes or the Langerhan's cells.

The mean of Ki-67-stained cells in the controls was 4.6 ± 0.6 (SE) per millimetre. At 1 week and 2.5 weeks of radiotherapy, i.e. after 5 and 12 fractions, respectively, the Ki-67 values were significantly reduced to 1.6 ± 0.5 and 1.6 ± 0.3 per mm. From 3 weeks and onwards there was a steady increase in the number of Ki-67-stained cells. As early as 5 weeks and 25 fractions a substantial increase above the control value was seen. The number of stained cells reached a value of 7.5 ± 1.5 per mm at the end of the 7 weeks of treatment.

The Ki-67 index determination over the 7 weeks is presented in Fig. 2. In the controls, the Ki-67 index was determined to $2.8 \pm 0.3\%$. A clear-cut reduction in the index to about 1% was observed during the first 2.5 weeks,

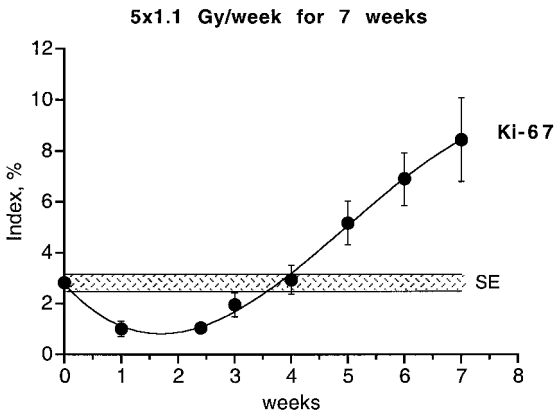

Fig. 2. The Ki-67 index ± (SE) during daily fractions of 1.1 Gy, totalling 35 fractions over 7 weeks.

followed by a gradual increase. At 5 weeks the index was about 5% and significantly higher than that in the controls. During the last 3 weeks of treatment there was acceleration in repopulation among basal cell keratinocytes. The Ki-67 index determination was 8% at the end of treatment. Thus, daily fractions of 1.1 Gy with 5 fractions per week cause significant disturbances in the cell proliferation pattern of the basal keratinocyte cell population. An induced suppression of the proliferation from the start, lasting over several weeks, was followed by an accelerated proliferation over the last 3 weeks of treatment.

Immunohistochemistry of p53

None or very few cells with positive nuclear p53 staining could be observed in the control biopsies. Upon radiation, p53-stained cells were seen in keratinocytes not only in the basal cell layer but also dispersed in the upper cell layers of the epidermis. As for Ki-67, we counted only p53-positive cells in the basal cell layer. We did not observe any p53 nuclear staining of melanocytes or Langerhan's cells.

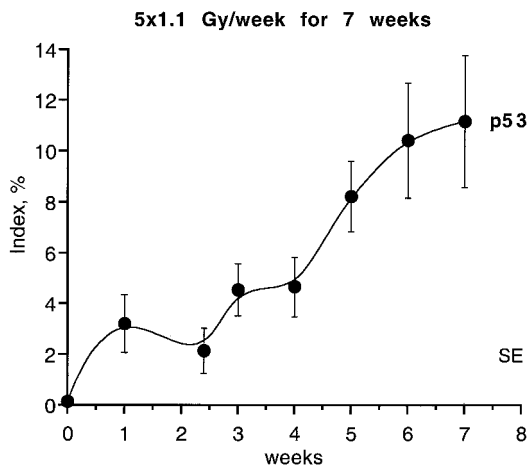

Fig. 3. The p53 index ± (SE) during daily fractions of 1.1 Gy, totalling 35 fractions over 7 weeks.

The p53 index determinations over the 7 weeks are presented in Fig. 3. In the controls the p53 index was determined to 0.14 ± 0.04%. During the first 2.5 weeks a plateau was seen with an index between 2 and 3%, which was significantly higher than that found in control biopsies. This was followed by a gradual increase to about 10% at the end of 7 weeks' treatment. It is noteworthy, that the pattern of the p53 index to a large extent mirrored the pattern of the Ki-67 index over the treatment course. The p53 index was marginally higher than the Ki-67 index over the whole period.

Immunohistochemistry of p21

None or very few positive nuclear p21 positive cells could be detected in the control biopsies, as was the case for p53. Upon radiotherapy, p21-stained cells appeared among the keratinocytes in the basal cell layer and occasionally in the suprabasal cell layer but not elsewhere. Only p21-positive cells in the basal cell layer were counted. As for Ki-67 and p53, we did not observe any p21 nuclear staining of melanocytes or Langerhan's cells.

The p21 index determinations over the 7-week period of radiotherapy are presented in Fig. 4. In the controls the p21 index was determined to 0.15 ± 0.06%. During the first weeks, the index was slightly higher than that in the control biopsies. At 3 weeks there was a significant increase, although the p21 index was as low as about 1%. From this time onwards the p21 index rose gradually and reached 4% at the end of the 7 weeks' treatment. The pattern of the p21 index reflected the pattern of the Ki-67 index. However, the p21 index was lower than the Ki-67 index over the whole period.

DISCUSSION

In the design of this project we emphasized quantification of the cell numbers of positively stained cells for the molecular markers, Ki-67, p53 and p21 in a well-defined tissue subcompartment. The idea was numerically to deter-

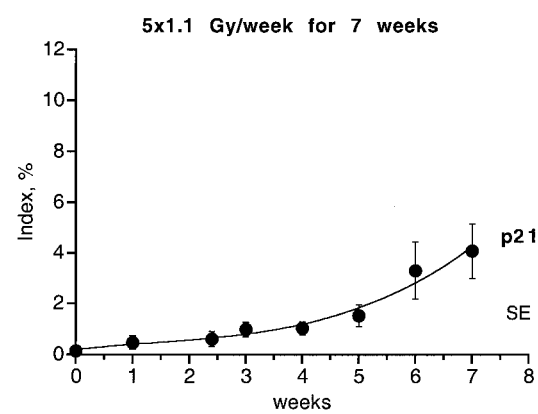

Fig. 4. The p21 index ± (SE) during daily fractions of 1.1 Gy, totalling 35 fractions over 7 weeks.

mine the expression of various markers for different low doses per fraction over the whole period of a standard radiotherapy course. Therefore we considered it necessary to place restrictions on the cell compartment to be analysed. The most logical choice for the skin epithelium is the basal cell layer, because it resides the stem cells and early progenitors of the keratinocytes, and is the most easily recognized cell layer. Moreover, the basal cell layer is the main location for the melanocytes in the skin and will be the subject of separate analyses. Secondly, we sought to utilize the advantage of the morphological picture of the tissue by using histological sections and we spent considerable effort in establishing how the different markers were expressed in the various cell types and cell layers of the epidermis. These descriptive patterns were very informative, among other things, about the quality of immunohistochemical staining and made it possible to sort out sections with a pattern of unspecifically stained cells.

Changes in the rate of repopulation during daily low-dose fractions

In the literature there are no similar data for comparison, with the exception of some estimates of the Ki-67 index for keratinocytes in normal skin. The values found by others were mostly in the range of 2 to 5%, when strongly nuclear-stained cells were counted. However, these estimates were based upon a limited number of patients (60–63). In our study, the Ki-67 index for keratinocytes in the controls fell into this range. The value was $2.8 \pm 0.3\%$, and after correction for the number of melanocytes in the basal layer the growth fraction for keratinocytes was equal to 3.1%. We did not find any Ki-67-stained melanocytes, which is in line with the slow proliferation of this cell type.

The results of the determination of the keratinocyte proliferation in terms of the absolute number of Ki-67-stained cells per millimetre and the Ki-67 index during daily fractions of 1.1 Gy were surprising from two aspects. First, early in the treatment an obvious suppression of the number of cycling cells lasting for more than 2 weeks was observed. Secondly, subsequent to the period of suppression, we observed a gradual increase in the number of cycling cells that generated Ki-67 index values substantially higher than those in unirradiated skin over the last 2 to 3 weeks of treatment. Intuitively, low doses per fraction given once a day were not expected to induce such remarkable disturbances in the proliferation kinetics of epithelial tissues. To our knowledge, there have been no previous experimental and clinical data published indicating that such effects might occur.

Almost 30 years ago, Juliana Denekamp (64) demonstrated in a skilfully designed experiment the phenomenon of accelerated repopulation in the skin of the mouse. She established that the onset of a compensatory accelerated proliferation was delayed by about one and a half weeks from the start of daily fractions of 3 Gy. Since that time, this has been a feature recognized in different normal epithelial tissues (65–70). Today, the understanding is that the potential of cell repopulation is a most important issue that should be taken into account in the evaluation of the response of acutely reacting tissues to various dose-fractionation schedules. In particular, the CHART trial is one of the most convincing illustrations of the clinical impact of repopulation on the early mucosa reaction in the radiotherapy of head and neck cancer (71).

During the seventies the phenomenon of compensatory accelerated proliferation was also the basis, derived empirically in the clinic, for allowing a gap in the radiotherapy course in order to increase the tolerance of the acute morbidity to high tumour doses given with curative intent. It was not until more than a decade later that it was realized that this manoeuvre and, equally, a prolonged overall treatment time could be a disaster for the curability of various epithelial tumours (72). Certain tumours might keep the capacity of accelerated repopulation like their normal tissue of origin, or increase their growth fraction for other reasons. Recently, a review of this issue was written by Jack Fowler (73). The onset time of rapid proliferation, extracted by careful analysis of clinical data of the curability of head and neck tumours, was estimated to be between 21 and 35 days. Intriguingly, this was in fully agreement with the findings in this report.

Some years ago Klaus Trott published an exposé about probable mechanisms regulating accelerated repopulation in squamous epithelia during daily fractionation (18). He emphasized that the reduction of the total number of cells below a threshold in the epithelium might be the probable trigger at the cellular level. Experimental studies in rodent models confirmed the conclusion from clinical studies that accelerated repopulation takes place after a delay and was consistently found in squamous cell carcinomas. The pattern of compensatory proliferation in experimental differentiated squamous cell carcinomas was very similar to that seen in normal epithelium such as skin and oral mucosa (74). Therefore, it was suggested that acceleration of repopulation might be a characteristic response of normal and malignant squamous epithelia to the progressive radiation damage caused by fractionated radiotherapy, and thus might share some important features of response regulation.

The findings, shown in Fig. 2, are the first and only documentation of the induced changes in the growth fraction of the germinal cell layer of an epithelium during a whole course of daily fractions of radiotherapy. A significant accelerated repopulation occurred subsequent to a period of several weeks of almost no proliferation, when 1.1 Gy once a day, five times per week, was applied for 7 weeks. Daily dose fractions of less than 0.5 Gy caused a similar suppression in cell proliferation, which remained a little longer than that for 1.1 Gy, but did not promote any overshoot in the growth fraction compared to the unirradiated skin (unpublished data).

Dose fractions of 1.1 Gy belong to the upper end of the dose range for the putative existence of IRR. However, in working up accumulated data for dose fractions of 0.1, 0.2 and 0.4 Gy, it was obvious that there was a significant suppression in the number of cycling cells also for these very low daily doses. The inhibition of the proliferation was to some extent dose dependent for both the degree and the length of the period (unpublished data). Such an antiproliferative response of the keratinocyte germinal cells may dominate the reduction rate of the basal cell density in the epidermis, which in our project was used as the endpoint to establish whether the HRS/IRR phenomenon exists in clinical radiotherapy (10). An abrupt delay in the progression of cells from G0 to G1 into the cell cycle upon induced DNA damage may be an important mechanism of the tissue to escape from apoptotic cell kill, which preferentially occurs in cycling cells. At the same time, a G0–G1 block will allow time for recognition and repair of genomic insult. At tissue level, this condition can be considered a prerequisite for induced repair upon DNA damage. Our findings suggest a block in the G0 to G1 entry, as hardly any Ki-67-positive cells were noticed during the first weeks of radiotherapy (Fig. 2). Furthermore, the very rare occurrence of p21-positive cells during this period (Fig. 4) supports this conclusion, as will be discussed below.

During the growth inhibition period, depletion of cells from the basal cell layers occurred to some extent by apoptosis, premature senescence and displacement of cells through the upper cell layers of the epidermis (75), accompanied by a severely diminished cell production. This would result in a decreasing basal cell density and in an increasing hypoplasia of the epidermis, which at a certain point might promote a mitogenic response and subsequent accelerated proliferation in the germinal cell layer. The trigger could be related to changes in intercellular keratinocyte communication induced by the decreased cell density as well as from signalling pathways between the effector keratinocytes and non-keratinocytes, such as dendritic cells and mesenchymal cells in the irradiated skin (18).

The present finding raises another important aspect i.e., what impact do the periods of suppressive and accelerated repopulation have upon cellular radioresponsiveness of keratinocytic stem cells and early progenitor cells in normal epithelia, respectively. Proliferating cells were generally expected to be more radiosensitive than quiescent G0 cells or cells arrested in the G0–G1 border. In ongoing analyses of HRS/IRR we are using mathematical modelling to determine the impact of primary growth suppression and secondary growth stimulation on tissue radiosensitivity during accelerated repopulation.

P53 and p21 as markers of tissue response to daily low-dose fractions

The wild-type p53 protein is almost undetectable with immunohistochemistry in normal unstressed cells because the half-life of the protein is very short. In addition to this low protein concentration, in some cells, p53 probably also exists in a latent form, inactive for transcription. The situation is different after DNA damage. IR induces single- and double-strand breaks that cause accumulation of p53, mainly due to reduced degradation and by way of dramatically increasing the half-life of the protein. The enhancement in p53 levels is known to be proportional to the extent of DNA damage. Thereby, it is possible to visualize the wild-type p53 protein immunohistochemically and screen the potential overexpression of the p53 protein induced in the germinal cell layer of the skin by daily low-dose fractions.

In the assessment of the p53 expression we had to consider the fact that exposure to sun may induce clusters of keratinocytes stained with p53 in the skin (76–78). These clusters emanate from acquired p53 mutations. Characterization of these clones was consistent with the founder cell being a stem or transit-amplifying cell (77). The present study was based on skin biopsies taken from the pelvic area, which generally is less directly exposed to UV radiation than most other parts of the body. Therefore, clones of p53-stained keratinocytes were only rarely observed in the unirradiated skin of our patients.

In unirradiated skin, as expected, we observed very few cells that expressed immunostaining for p53 and p21. During the first weeks of daily fractions a small increase in the number of both p53 and p21 positive cells was seen (Figs. 3 and 4). This was a reflection of the few cycling cells in this period (Fig. 2). The reason will be explained later. From 3 weeks onwards there was a gradual increase in the p53 index and the p21 index. Although the p21 index was substantially lower than the p53 index, both were fairly parallel, and again markedly parallel to the Ki-67 index. Recordings of the expressions of the three markers from the same biopsies certainly contributed to the congruent findings during the whole treatment period.

Recently, the expressions of p53 and p21 in all cell layers of the skin were evaluated immunohistochemically after a single dose of 2 Gy and 54 Gy in daily fractions of 2 Gy. The results indicated a dose response and a clear-cut correlation between p53 and p21 (79).

The most intensively investigated pathway to p53 activation is that initiated by DNA damage. Mammalian cells have evolved surveillance mechanisms that monitor the structure of chromosomes and coordinate DNA repair and cell-cycle progression (43). The main functions of DNA damage checkpoints are to control cell-cycle arrest, DNA repair pathways and induction of apoptosis. DNA damage is sensed by checkpoints that retard progress through the cell cycle until the damage is repaired (37). Importantly, the cell growth inhibition allows time for the crucial checkpoint decision-making to promote life or death of the

cell upon the inflicting and potentially mutagenic damage (80).

ATM is a recently identified checkpoint protein that senses and signals DNA damage (Fig. 1). Upon IR, ATM induces several specific phosphorylations of p53, among which the residues serine 15 and serine 20 are identified as crucial for p53 activation (29, 30, 81–83). With immunohistochemistry using phospho-specific antibodies to serine 15 and serine 20, it may be possible to detect this step in tissues. In the present work we used the DO-7 antibody, which is active to epitopes 19–26, and not phospho-specific, as a measure of the total amount of the p53 protein. However, attempts are currently under way to investigate whether DNA damage-induced phosphorylation occurs at serine 15 by using a phospho-specific antibody. During the first period of the radiotherapy course, when the cell proliferation was limited, the p53–serine 15 index was similar to that for DO-7 (unpublished data). This indicates that the p53 stabilization observed by DO-7 was related to DNA-induced damage.

A comprehensive study of whole-body irradiated mice showed that the p53-mediated effects were tissue specific (84). The basal level of p53 varied considerably among tissues. Generally, radiation-resistant tissues expressed low amounts, while sensitive tissues expressed high amounts of p53 protein, and the same association was found for the p53 mRNA level. The pre-existing basal level of the p53 protein in a particular tissue determined the amount of p53 protein accumulated after genotoxic stress. It was concluded that p53 regulation at the mRNA level constitutively was a determinant of cellular sensitivity to IR-induced genotoxic stress (84).

Furthermore, there is evidence that only cells in a certain phase of the cell cycle or differentiation status respond to DNA damage by overexpressing p53. Within the proliferative basal layer of epidermis, stem cells give rise to transit amplifying cells. These initiate terminal differentiation after a few rounds of proliferation when mitosis is blocked (58). p53 is mainly expressed in the proliferating cells, stem cells and transit-amplifying cells, of the human epidermis, and barely detectable in terminal-differentiating cells (85). The p53 expression was tightly regulated by HDM2 during the transition from proliferation to terminal differentiation. The HDM2 expression was inversely correlated to the p53 expression. Both loss of p53 and transient induction of HDM2 occurred as basal keratinocytes lose their proliferative potential (85).

Knowledge about the distribution of the constitutive levels of p53 among the keratinocytes in the basal layer in unirradiated skin and also that the pre-existing levels determine the amount of accumulated p53 upon radiation are extremely relevant for the interpretation of our results presented in Figs. 2–4. This means that the immunostaining of Ki-67 and p53 that was detected probably appeared from the same cell population, because the p53 protein is expected to be substantially higher in proliferating cells compared to post-mitotic cells. To some extent, this was confirmed by comparing adjacent sections stained for p53 and Ki-67, respectively. However, double staining will be necessary to ensure whether this is the case.

The p53-dependent transcription of p21 has bearing on the function of the protein in the control of the cell cycle and DNA synthesis upon DNA damage. To ensure that the expression of p21 in Fig. 4 was a reflection of the p53-p21 pathway induced by the DNA-damage checkpoint, the p21-stained cells had to be p53 stained. Moreover, the p21 cells had to be Ki-67-positive as evidence of being in the cell cycle. Again we obtained certain support for both conditions by comparing positively stained cells on adjacent sections. However, this needs to be confirmed by double staining procedures.

By comparing the expression of Ki-67 and p21 during the first weeks of radiation (Figs. 2 and 4), we found evidence of cell-cycle arrest in the G0/G1 border, as there was no sign of any accumulation of cells either in G1/S or G2/M. There are several known possible pathways that might be regulators of a G0/G1 block on DNA damage. One possibility is another p53-dependent pathway, which acts through a specific membrane protein (43). Other candidates are Bcl-2 (86, 87), and TGFβ (88). Further investigation of the mechanisms responsible for this very sensitive tissue effect upon DNA damage is highly justified.

In using skin as a clinical assay, a reliable identification of the p21-positive cells is important because, apart from the function of p21 in the cell-cycle arrest upon DNA damage, it plays a complex role in differentiation. In skin, expression of p21 is induced in post-mitotic cells immediately adjacent to the proliferative compartment, but is decreased in cells further along the differentiation pathway (48). However, as described above, p21 positive post-mitotic cells are expected to be unstained for p53. Therefore, with the appropriate methods, it should be possible to distinguish the origin of the p21-expressing cells in Fig. 4.

A recent discovery, particularly relevant for the present results, is that p21 is involved in the control of stem self-renewal in keratinocytes. p21 has an essential function in restricting the self-renewal potential of the keratinocyte stem cell population through promoting these cells to irreversible commitment to differentiation (49). During stimulated repopulation, as in Fig. 2, such a function of p21 might be crucial for the control of homeostasis in the epidermis.

In this way, p21 probably mediates its function in the ARF-p53 pathway, which is activated by hyperproliferative signals (Fig. 1). An abnormal threshold of mitogenic signals, i.e. through *c-myc* or E2F1, activates ARF. Intriguingly, a bridge was recently discovered between the ATM and ARF pathways upstream of p53 (Fig. 1), through E2F1, which was found to be stabilized, and

activated by ATM upon DNA damage (89). Therefore, further studies of the induced expression of ARF and E2F1 in our clinical assay will elucidate the mechanisms behind the significant cell proliferation disturbances established in the present work.

Concerning p53 and p21, a vast array of possible interactions and regulatory networks has to be considered. Despite a clear and coherent picture of the expressions of the p53 and p21 proteins related to the growth fraction, estimated by means of Ki-67, was established in the present work. This was possible through comprehensive quantifications of the cellular response in the well-defined germinal cell layer of the skin to daily low doses of radiation.

In conclusion, we report that daily fractions of 1 Gy induce significant changes in proliferation of basal keratinocytes in the epidermis. Over a period of 2.5 weeks there is a depression in proliferation, which is followed by accelerated repopulation for up to 7 weeks. Moreover, consistent expressions are observed for p53 and p21, which to some extent mirror the proliferative pattern shown by Ki-67. Our findings strongly support, and so far are the only evidence, that the recently identified biochemical and molecular pathways regulating DNA damage and abnormal proliferation, with p53 as the node in a complex network, take place in the normal skin epithelium of patients receiving a standard radiotherapy course. At the moment we cannot separate the DNA damage response from the proliferative response, which both involve the p53–p21 pathway. However, this is a highly relevant issue that we are giving the highest priority to resolve.

ACKNOWLEDGEMENTS

This work was supported by the Swedish Cancer Society, the Research Foundation of the Department of Oncology at the University of Uppsala, the Lions Cancer Research Foundation in Uppsala, and the King Gustav V Jubilee Clinic Cancer Research Foundation in Gothenburg.

REFERENCES

1. Tubiana M, Eschwege F. Conformal radiotherapy and intensity-modulated radiotherapy—clinical data. Acta Oncol 2000; 39: 555–67.
2. Turesson I, Joiner MC. Clinical evidence of hypersensitivity to low doses in radiotherapy. Radiother Oncol 1996; 40: 1–3.
3. 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.
4. Lambin P, Malaise EP, Joiner MC. Might intrinsic radioresistance of human tumour cells be induced by radiation? Int J Radiat Biol 1996; 69: 279–90.
5. Marples B, Lambin P, Skov KA, Joiner MC. Low dose hyper-radiosensitivity and increased radioresistance in mammalian cells. Int J Radiat Biol 1997; 71: 721–35.
6. Joiner MC, Lambin P, Marples B. Adaptive response and induced resistance. C R Acad Sci III 1999; 322: 167–75.
7. Wouters BG, Sy AM, Skarsgard LD. Low-dose hypersensitivity and increased radioresistance in a panel of human tumor cell lines with different radiosensitivity. Radiat Res 1996; 146: 399–413.
8. Wouters BG, Skarsgard LD. Low-dose radiation sensitivity and induced radioresistance to cell killing in HT-29 cells is distinct from the 'adaptive response' and cannot be explained by a subpopulation of sensitive cells. Radiat Res 1997; 148: 435–42.
9. Skarsgard LD, Hill AA, Acheson DK. Evidence for two forms of substructure in the cell survival curve—mechanisms and clinical consequences. Acta Oncol 1999; 38: 895–902.
10. Joiner MC, Marples B, Lambin P, Short SC, Turesson I. Low-dose hypersensitivity: current status and possible mechanisms. Int J Radiat Oncol Biol Phys 2001; 49: 379–89.
11. Short SC, Kelly J, Mayes CR, Woodcock M, Joiner MC. Low-dose hypersensitivity after fractionated low-dose irradiation in vitro. Int J Radiat Biol 2001; 77: 655–64.
12. 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 Relat Stud Phys Chem Med 1986; 49: 565–80.
13. Joiner MC, Johns H. Renal damage in the mouse: the response to very small doses per fraction. Radiat Res 1988; 114: 385–98.
14. Parkins CS, Fowler JF. The linear quadratic fit for lung function after irradiation with x-rays at smaller doses per fraction than 2 Gy. Br J Cancer 1986; 7 (Suppl): 320–3.
15. Robson T, Joiner MC, Wilson GD, et al. A novel human stress response-related gene with a potential role in induced radioresistance. Radiat Res 1999; 152: 451–61.
16. Marples B, Joiner MC. Modification of survival by DNA repair modifiers: a probable explanation for the phenomenon of increased radioresistance. Int J Radiat Biol 2000; 76: 305–12.
17. Dorr W. Three A's of repopulation during fractionated irradiation of squamous epithelia: asymmetry loss, acceleration of stem-cell divisions and abortive divisions. Int J Radiat Biol 1997; 72: 635–43.
18. Trott KR. The mechanisms of acceleration of repopulation in squamous epithelia during daily irradiation. Acta Oncol 1999; 38: 153–7.
19. Dorr W, Brankovic K, Hartmann B. Repopulation in mouse oral mucosa: changes in the effect of dose fractionation. Int J Radiat Biol 2000; 76: 383–90.
20. Duchrow M, Gerdes J, Schluter C. The proliferation-associated Ki-67 protein: definition in molecular terms. Cell Prolif 1994; 27: 235–42.
21. McCormick D, Chong H, Hobbs C, Datta C, Hall PA. Detection of the Ki-67 antigen in fixed and wax-embedded sections with the monoclonal antibody MIB1. Histopathology 1993; 22: 355–60.
22. Morgan SE, Kastan MB. p53 and ATM: cell cycle, cell death, and cancer. Adv Cancer Res 1997; 71: 1–25.
23. el-Deiry WS. Regulation of p53 downstream genes. Semin Cancer Biol 1998; 8: 345–57.
24. Amundson SA, Do KT, Fornace AJ Jr. Induction of stress genes by low doses of gamma rays. Radiat Res 1999; 152: 225–31.
25. Prives C, Hall PA. The p53 pathway. J Pathol 1999; 187: 112–26.
26. Appella E, Anderson CW. Signaling to p53: breaking the posttranslational modification code. Pathol Biol (Paris) 2000; 48: 227–45.
27. Ashcroft M, Taya Y, Vousden KH. Stress signals utilize multiple pathways to stabilize p53. Mol Cell Biol 2000; 20: 3224–33.

28. Huang LC, Clarkin KC, Wahl GM. Sensitivity and selectivity of the DNA damage sensor responsible for activating p53-dependent G1 arrest. Proc Natl Acad Sci USA 1996; 93: 4827–32.
29. Banin S, Moyal L, Shieh S, et al. Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science 1998; 281: 1674–7.
30. Canman CE, Lim DS, Cimprich KA, et al. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science 1998; 281: 1677–9.
31. Pandita TK, Lieberman HB, Lim DS, et al. Ionizing radiation activates the ATM kinase throughout the cell cycle. Oncogene 2000; 19: 1386–91.
32. Durocher D, Jackson SP. DNA-PK, ATM and ATR as sensors of DNA damage: variations on a theme? Curr Opin Cell Biol 2001; 13: 225–31.
33. Fang ZM, Lee CS, Sarris M, et al. Rapid radiation-induction of ATM protein levels in situ. Pathology 2001; 33: 30–6.
34. Shiloh Y. ATM and ATR: networking cellular responses to DNA damage. Curr Opin Genet Dev 2001; 11: 71–7.
35. Sherr CJ, DePinho RA. Cellular senescence: mitotic clock or culture shock? Cell 2000; 102: 407–10.
36. Tanaka H, Arakawa H, Yamaguchi T, et al. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. Nature 2000; 404: 42–9.
37. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000; 408: 307–10.
38. Ryan KM, Phillips AC, Vousden KH. Regulation and function of the p53 tumor suppressor protein. Curr Opin Cell Biol 2001; 13: 332–7.
39. Woods DB, Vousden KH. Regulation of p53 function. Exp Cell Res 2001; 264: 56–66.
40. Sherr CJ, Weber JD. The ARF/p53 pathway. Curr Opin Genet Dev 2000; 10: 94–9.
41. Sherr CJ. Parsing Ink4a/Arf: 'pure' p16-null mice. Cell 2001; 106: 531–4.
42. Sherr CJ. The ink4a/arf network in tumour suppression. Nat Rev Mol Cell Biol 2001; 2: 731–7.
43. Levine AJ. p53, the cellular gatekeeper for growth and division. Cell 1997; 88: 323–31.
44. Bunz F, Dutriaux A, Lengauer C, et al. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science 1998; 282: 1497–501.
45. Gartel AL, Tyner AL. Transcriptional regulation of the p21((WAF1/CIP1)) gene. Exp Cell Res 1999; 246: 280–9.
46. Dotto GP. p21(WAF1/Cip1): more than a break to the cell cycle? Biochim Biophys Acta 2000; 1471: M43–56.
47. Sherr CJ. The Pezcoller lecture: cancer cell cycles revisited. Cancer Res 2000; 60: 3689–95.
48. Di Cunto F, Topley G, Calautti E, et al. Inhibitory function of p21Cip1/WAF1 in differentiation of primary mouse keratinocytes independent of cell cycle control. Science 1998; 280: 1069–72.
49. Topley GI, Okuyama R, Gonzales JG, Conti C, Dotto GP. p21(WAF1/Cip1) functions as a suppressor of malignant skin tumor formation and a determinant of keratinocyte stem-cell potential. Proc Natl Acad Sci USA 1999; 96: 9089–94.
50. Bennett M, Macdonald K, Chan SW, Luzio JP, Simari R, Weissberg P. Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. Science 1998; 282: 290–3.
51. Bates S, Vousden KH. Mechanisms of p53-mediated apoptosis. Cell Mol Life Sci 1999; 55: 28–37.
52. Burns TF, El-Deiry WS. The p53 pathway and apoptosis. J Cell Physiol 1999; 181: 231–9.
53. Marchenko ND, Zaika A, Moll UM. Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. J Biol Chem 2000; 275: 16202–12.
54. Vousden KH. p53: death star. Cell 2000; 103: 691–4.
55. Burns TF, Bernhard EJ, El-Deiry WS. Tissue specific expression of p53 target genes suggests a key role for KILLER/DR5 in p53-dependent apoptosis in vivo. Oncogene 2001; 20: 4601–12.
56. Oda K, Arakawa H, Tanaka T, et al. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell 2000; 102: 849–62.
57. Watt FM. Epidermal stem cells: markers, patterning and the control of stem cell fate. Philos Trans R Soc Lond B Biol Sci 1998; 353: 831–7.
58. Watt FM. Stem cell fate and patterning in mammalian epidermis. Curr Opin Genet Dev 2001; 11: 410–7.
59. Nyman J, Turesson I. Basal cell density in human skin for various fractionation schedules in radiotherapy. Radiother Oncol 1994; 33: 117–24.
60. Heenen M, Thiriar S, Noel JC, Galand P. Ki-67 immunostaining of normal human epidermis: comparison with 3H-thymidine labelling and PCNA immunostaining. Dermatology 1998; 197: 123–6.
61. Pablos JL, Santiago B, Galindo M, Carreira PE, Ballestin C, Gomez-Reino JJ. Keratinocyte apoptosis and p53 expression in cutaneous lupus and dermatomyositis. J Pathol 1999; 188: 63–8.
62. Han KH, Huh CH, Cho KH. Proliferation and differentiation of the keratinocytes in hyperplastic epidermis overlying dermatofibroma: immunohistochemical characterization. Am J Dermatopathol 2001; 23: 90–8.
63. Onuma H, Mastui C, Morohashi M. Quantitative analysis of the proliferation of epidermal cells using a human skin organ culture system and the effect of DbcAMP using markers of proliferation (BrdU, Ki-67, PCNA). Arch Dermatol Res 2001; 293: 133–8.
64. Denekamp J. Changes in the rate of repopulation during multifraction irradiation of mouse skin. Br J Radiol 1973; 46: 381–7.
65. Dorr W, Kummermehr J. Accelerated repopulation of mouse tongue epithelium during fractionated irradiations or following single doses. Radiother Oncol 1990; 17: 249–59.
66. Potten CS, Owen G, Roberts SA. The temporal and spatial changes in cell proliferation within the irradiated crypts of the murine small intestine. Int J Radiat Biol 1990; 57: 185–99.
67. Taylor JM, Withers HR, Mason KA, Davis CA. Repopulation of mouse jejunal crypt cells. Radiother Oncol 1991; 20: 181–90.
68. Dorr W, Emmendorfer H, Haide E, Kummermehr J. Proliferation equivalent of 'accelerated repopulation' in mouse oral mucosa. Int J Radiat Biol 1994; 66: 157–67.
69. Shirazi A, Liu K, Trott KR. Epidermal morphology, cell proliferation and repopulation in mouse skin during daily fractionated irradiation. Int J Radiat Biol 1995; 68: 215–21.
70. Evans SC, Mack DC, Mason KA, Thames HD. The proliferative response of mouse jejunal crypt cells to radiation-induced cell depletion is not mediated exclusively by transforming growth factor alpha. Radiat Res 2001; 155: 866–9.
71. Bentzen SM, Saunders MI, Dische S, Bond SJ. Radiotherapy-related early morbidity in head and neck cancer: quantitative clinical radiobiology as deduced from the CHART trial. Radiother Oncol 2001; 60: 123–35.
72. Withers HR, Taylor JM, Maciejewski B. The hazard of accelerated tumor clonogen repopulation during radiotherapy. Acta Oncol 1988; 27: 131–46.
73. Fowler JF. Biological factors influencing optimum fractionation in radiation therapy. Acta Oncol 2001; 40: 712–7.

74. Trott KR, Kummermehr J. Rapid repopulation in radiotherapy: a debate on mechanism. Accelerated repopulation in tumours and normal tissues. Radiother Oncol 1991; 22: 159–60.
75. Gandarillas A, Goldsmith LA, Gschmeissner S, Leigh IM, Watt FM. Evidence that apoptosis and terminal differentiation of epidermal keratinocytes are distinct processes. Exp Dermatol 1999; 8: 71–9.
76. Ren ZP, Ponten F, Nister M, Ponten J. Two distinct p53 immunohistochemical patterns in human squamous-cell skin cancer, precursors and normal epidermis. Int J Cancer 1996; 69: 174–9.
77. Jensen UB, Lowell S, Watt FM. The spatial relationship between stem cells and their progeny in the basal layer of human epidermis: a new view based on whole-mount labelling and lineage analysis. Development 1999; 126: 2409–18.
78. Ling G, Persson A, Berne B, Uhlen M, Lundeberg J, Ponten F. Persistent p53 mutations in single cells from normal human skin. Am J Pathol 2001; 159: 1247–53.
79. Ponten F, Lindman H, Bostrom A, Berne B, Bergh J. Induction of p53 expression in skin by radiotherapy and UV radiation: a randomized study. J Natl Cancer Inst 2001; 93: 128–33.
80. Rich T, Allen RL, Wyllie AH. Defying death after DNA damage. Nature 2000; 407: 777–83.
81. Chaturvedi P, Eng WK, Zhu Y, et al. Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway. Oncogene 1999; 18: 4047–54.
82. Matsuoka S, Rotman G, Ogawa A, Shiloh Y, Tamai K, Elledge SJ. Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. Proc Natl Acad Sci U S A 2000; 97: 10389–94.
83. Turenne GA, Paul P, Laflair L, Price BD. Activation of p53 transcriptional activity requires ATM's kinase domain and multiple N-terminal serine residues of p53. Oncogene 2001; 20: 5100–10.
84. Komarova EA, Christov K, Faerman AI, Gudkov AV. Different impact of p53 and p21 on the radiation response of mouse tissues. Oncogene 2000; 19: 3791–8.
85. Dazard JE, Piette J, Basset-Seguin N, Blanchard JM, Gandarillas A. Switch from p53 to MDM2 as differentiating human keratinocytes lose their proliferative potential and increase in cellular size. Oncogene 2000; 19: 3693–705.
86. Borner C. Diminished cell proliferation associated with the death-protective activity of Bcl-2. J Biol Chem 1996; 271: 12695–8.
87. Vairo G, Soos TJ, Upton TM, et al. Bcl-2 retards cell cycle entry through p27(Kip1), pRB relative p130, and altered E2F regulation. Mol Cell Biol 2000; 20: 4745–53.
88. Hata A. TGF beta signaling and cancer. Exp Cell Res 2001; 264: 111–6.
89. Lin WC, Lin FT, Nevins JR. Selective induction of E2F1 in response to DNA damage, mediated by ATM-dependent phosphorylation. Genes Dev 2001; 15: 1833–44.