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p53 expression in cultured cells following radioisotope labelling

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SUMMARY

p53 inhibits division following cellular damage. Cultured cells were found to express p53 protein following pulse labelling with radioisotopes, even at low doses normally used for growth and metabolic labelling studies. Some stem cells are exquisitely sensitive to radiation and thus p53 may have evolved as a major regulator of stem cell function. Therefore *any* genetic damage may be able to induce p53 expression, which in turn will affect the biochemical outcome of many experiments by both cell cycle arrest and other mechanisms. In some cases the use of radioisotopes may directly change the results of the experiment. This will require a careful re-evaluation of the current literature and experimental protocols utilising radioisotopes.

Key words: p53, radioisotope, proliferation

INTRODUCTION

Modern biology and medicine relies heavily on the use of radiolabelled tracer molecules. Their use is routine in many standard techniques in laboratories world-wide. We were concerned that their use involving living cells or tissues may be invalid or misleading in certain circumstances. In experiments using radioisotopes (with the exception of those specifically designed to cause radiation-induced death) the assumption is *always* made that the radiation has no effect on the physiology of the test material. We felt that the recent literature pointed towards a fundamental role for p53 in the growth control of renewing tissues and to the fact that our methods of studying growth may *in themselves* affect the physiology of the cells and, in some cases, therefore invalidate the observations.

Recently p53 was found to be induced in normal skin in vivo and in skin cells in vitro by simulated solar radiation (Hall et al., 1992). High levels of p53 protein can block cycle progression (Kastan et al., 1991; Lin et al., 1992; Fritsche et al., 1993; Kuerbitz et al., 1992). These data suggest that p53 plays a role in the detection and response to genotoxic insult.

Whilst cell cycle delay has been reported following isotope incorporation (Beck, 1982; Beck and Omnyczinski, 1981) this has generally been ignored or considered to be of little importance by most workers. We have investigated whether doses of DNA labelling agents typically used to measure proliferation and isotopes commonly used for metabolic labelling could induce higher intracellular levels of p53.

MATERIALS AND METHODS

Cell culture and labelling

Normal human skin fibroblasts (3rd-5th passage) were plated onto glass coverslips and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS for two days prior to use. Keratinocyte cultures were established from human foreskins using the Rheinwald and Green method (1975) in DMEM 10% FCS with hydrocortisone and insulin and were plated onto glass coverslips for treatment (passage 2-4). All cells were used at between 30% and 60% confluence. Cells were treated with either (a) 1 or 10 μCi/ml [^{3}H]thymidine ([^{3}H]TdR), (25 Ci/mMol, Amersham International) for one hour, or (b) with [^{35}S]methionine for 2 hours. Cells were washed and re-incubated for 3-24 hours in unlabelled medium (3 hours illustrated). Control cells were treated identically but received no isotope or were fed unlabelled thymidine (4×10^{-7} mM). Cells were fixed in acetone:methanol (1:1) at –20°C for 10 minutes then air dried. p53 was detected by immunocytochemistry using DO7 (Dako, UK) at 1:1000 dilution. Control cells were negative for p53 as were those fed unlabelled thymidine. Controls with no primary antibody were consistently negative.

p53 null mouse fibroblasts were grown in DMEM with 15% FCS.

Western blots

Polyacrylamide gel electrophoresis of cell extracts was followed by electroblotting and immunodetection. Lanes were loaded with equal amounts of protein (20 μg/lane). Human dermal fibroblasts were grown in 175 cm^2 flasks. They were treated when 60-70% confluent. Cells were lysed in 100 mM Tris-HCl, pH 7.4, 10 mM dithiothreitol, 0.1% SDS (w/v). The extract was briefly sonicated, centrifuged and added to Laemmli loading buffer.

Immunoblotting

Proteins were separated by SDS-polyacrylamide gel electrophoresis (18% w/v) and transferred to nitrocellulose (0.45 μm, Schleicher & Schuell, FRG). The nitrocellulose sheet was blocked using 3% FCS in PBS for 1 hour, washed 5 times in PBS/Tween-20 (0.1% v/v) and incubated for 2 hours at room temperature with DO7 antibody (1:600). Following 5 washes with PBS/Tween, the sheet was incubated with peroxidase-conjugated rabbit anti-mouse Ig antibody (Dako UK) (1:600) for 1 hour. It was then subjected to five further PBS/Tween washes and developed using 3,3′-diaminobenzidine as a chromogen.

Northern blots

Normal human fibroblasts were treated with 1 μCi/ml tritiated thymidine for 1 hour and then washed 3 times in 'cold' medium and re-incubated for either 4 or 24 hours, respectively. Control cells were treated identically but were not thymidine-treated. RNA was extracted using guanidine isothiocyanate and resolved on agarose-formaldehyde gels using 5 μg per lane. The RNA was transferred to nylon membrane and hybridised using a cDNA probe (a gift from D. Lane), which had been ^{32}P-labelled by random priming. Hybridisation of probe to filters was at 42°C in 50% formamide, 5× SSC, 2× Denhardt's solution, 0.1% SDS, 0.1 mg/ml salmon sperm DNA. Final washes were in 0.1× SSC, 0.1% SDS, 65°C. Blots were exposed on Kodak XAR film with an intensifying screen. Controls for equal loading were performed using β-actin and confirmed equal loading (data not shown).

Cytometry

T22 fibroblasts were grown in 60 mm dishes (Falcon) in DMEM 10% FCS. Cells were used when 50% confluent. Cells were treated with either; fresh medium (controls), 1 μCi/ml [^{3}H]TdR for 1 hour or [^{35}S]methionine 50 μCi/ml for 2 hours, then washed and refed and fixed at various times up to 24 hours. Cells were stained with propidium iodide and analysed using a Becton Dickinson FACScan plus. These experiments were repeated a number of times on separate occasions. The degree of confluence of the cells obviously has an effect on the results as maximal changes can be observed when the cells are growing exponentially. Data from one experiment where the cells were in early log phase is presented.

Some experiments were also performed under similar conditions using p53 null cells.

RESULTS AND DISCUSSION

When cultured cells were pulse-chased with either tritiated thymidine ([^{3}H]TdR) or [^{35}S]methionine, p53 protein was detected in the treated population but not in controls. Cells expressed p53 following even 1 μCi/ml of [^{3}H]TdR for one hour (Fig. 1). At higher doses (10 μCi/ml) the expression appeared to be even stronger as shown by more intense immuno-reaction product deposition. When cells were treated for 2 hours with 50 μCi/ml [^{35}S]methionine then chased for 4 hours prior to fixing, p53 expression was again elevated above control levels. Unlabelled bromodeoxyuridine (10^{-6} M) did not induce immunodetectable p53 expression following a 1 hour pulse and up to 24 hour chase in control medium.

Western blotting confirmed that protein levels rose markedly following radioisotope labelling, even after a single hour-long thymidine pulse (see Fig. 2A). Northern blots show that expression of p53 mRNA increased with time after labelling (Fig. 2B).

To demonstrate a biological response to the induction of p53 we harvested log phase cultured mouse T22 fibroblasts at intervals for flow cytometry. A reduction in the proportion of S phase cells was seen in radioisotope-treated compared with control cells (Fig. 3). Cultured cells from p53 null mice do not show this response to labelling agents. Thus p53 expression is required for the cycle delay induced by the radiolabels reported here.

Thymidine-based methods have long been known to have methodological problems associated with their use. [^{3}H]TdR was found to affect circadian variation in the mitotic index of epidermis (Olsson, 1976) and oral epithelium (Möller, 1978;

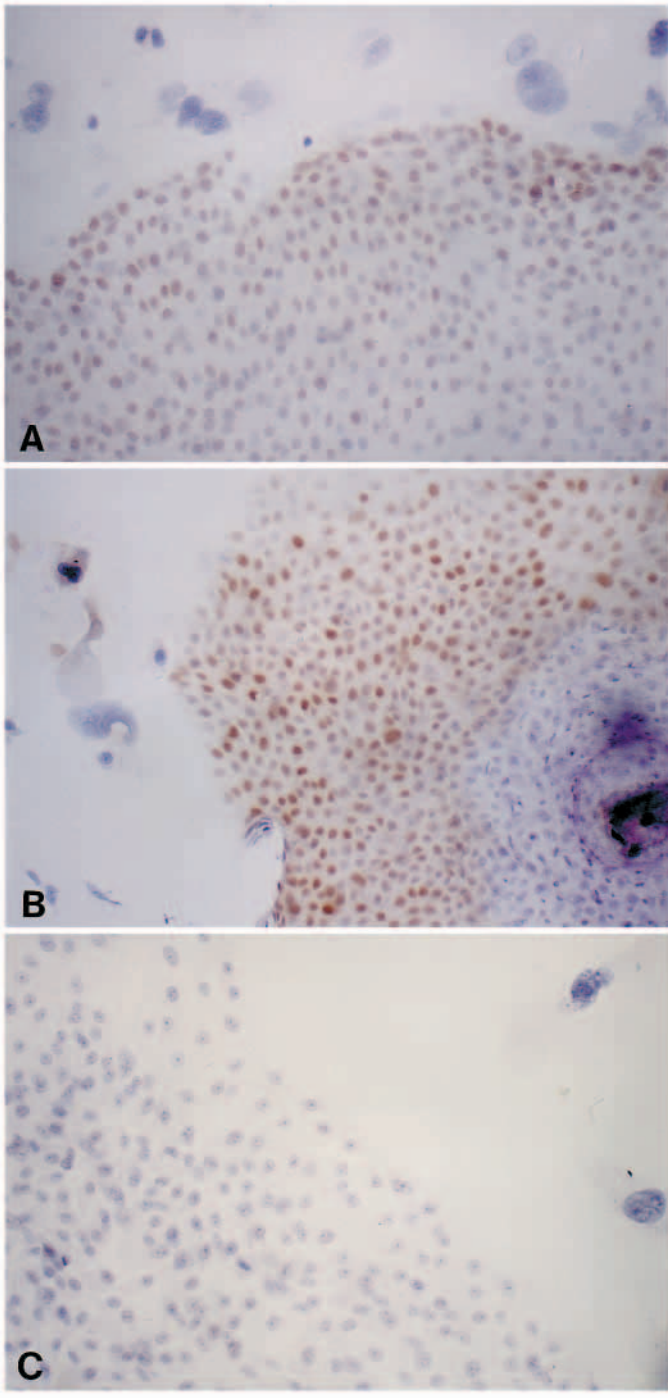

Fig. 1. (A) p53 immunopositive cells at the edge of a keratinocyte colony labelled with 1 μCi/ml tritiated thymidine ([^{3}H]TdR). The distribution of p53-positive cells is similar to that of cells that take up [^{3}H]TdR, i.e. largely, but not exclusively, peripheral. Remnants of the 3T3 feeder layer remain unstained. (B) A keratinocyte colony labelled with [^{35}S]methionine. Here p53 immunopositive cells are more evenly distributed through the colony and many more cells show positivity but at a range of different intensities. Fibroblast cells produced similar results but without obvious geographic variation. (C) The edge of an untreated control keratinocyte colony stained for p53 only occasional weakly positive cells are visible under the conditions employed here.

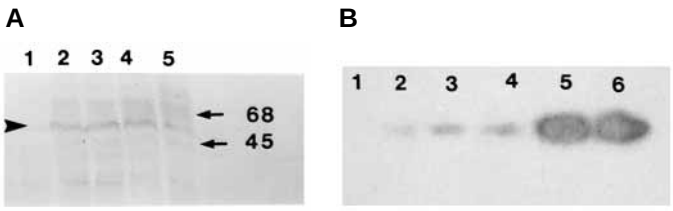

Fig. 2. (A) Detection of p53 by western blotting. Lane 1, control, untreated cells. Lane 2, an extract from cells pulsed for 1 hour with 1 μCi/ml [^{3}H]TdR and then washed and re-incubated in 'cold medium' for 3 hours prior to extraction. Lane 3, an extract from cells labelled for 1 hour with 1 μCi/ml [^{3}H]TdR then chased for 24 hours in unlabelled medium prior to extraction. Lane 4, an extract from cells pulsed for 1 hour with 10 μCi/ml [^{3}H]TdR then chased for 24 hours. Lane 5, an extract from cells incubated for 2 hours with [^{35}S]methionine (50 μCi/ml) in methionine-free medium, then re-incubated in cold methionine-containing medium for 24 hours. Arrowhead, position of p53; 68, 45, size standards (in kDa). (B) Northern blot. Lanes 1 and 2, duplicated control cells; lanes 3 and 4, from cells extracted at 4 hours post-thymidine labelling; lanes 5 and 6, from cells extracted at 24 hours post-thymidine labelling.

Möller et al., 1974). Mitotic delay was observed by Beck (1982). These effects may be attributable to p53 expression.

There is evidence of heterogeneity in the response to genotoxic insult by cells in renewing tissues. Overall growth control is achieved principally by the regulation of stem cells. Because of their key position in tissue homeostasis stem cells are also thought to be the most important targets for carcinogenesis. There are a number of experimental observations that suggest that stem cells differ in their response to a number of external agents and treatments. These may have evolved as protective mechanisms to ensure the integrity of, firstly, the stem cells then, failing that, the integrity of the tissue and ultimately of the individual. The role of p53 in these populations may be crucially important for tissue integrity. A sub-population of cells within intestinal crypts was found to be sensitive to low dose radiation from ^{3}[H]TdR (Potten, 1977). These cells, which are in the stem cell zone, responded by apoptosis. Stem cells may be exquisitely sensitive to DNA damaging agents; we predict that this sensitivity would not be found in p53 null mice. The phenomenon of late labelling (Hume and Potten, 1982) occurs in a subpopulation of cells thought to be the stem cells of the tongue papillae. Radiolabelled thymidine given hours earlier is held in an intracellular pool within stem cells and then incorporated into DNA. The increase in labelled cells was not prevented by treatment with drugs that block mitosis, and therefore is not simply the result of division of a labelled cell to generate two new labelled cells. The mechanism behind such behaviour is unknown. Unusual fraction labelled mitoses (FLM) curves were found for epithelia both in vivo and in vitro (Dover and Potten, 1983; Potten et al., 1982). Taken with other evidence these data were

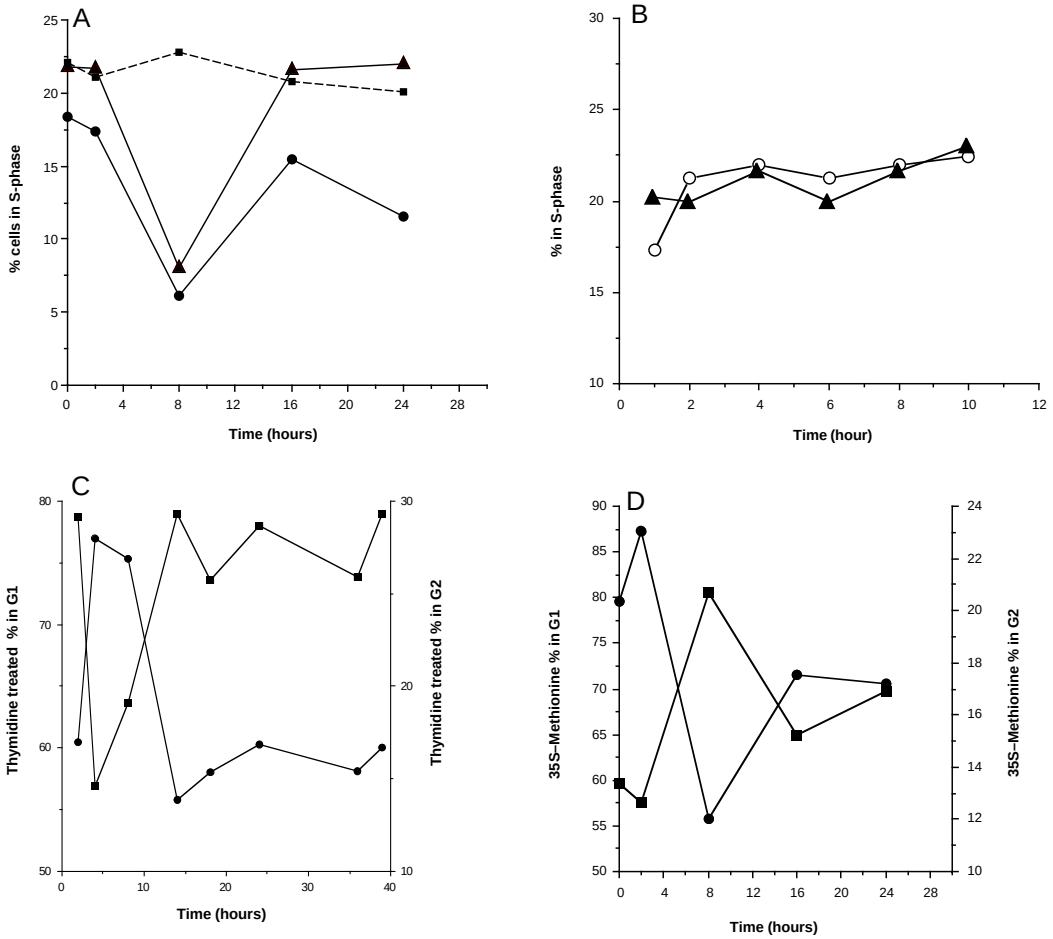

Fig. 3. (A) The percentage of cells in S phase at different times after treatment, ■ control, ▲ [^{3}H]TdR-treated (1 μCi/ml, 1 hour), ● [^{35}S]methionine-treated (50 μCi/ml, 2 hours). (B) The % of p53 null cells in S phase following a 1 μCi/ml [^{3}H]TdR pulse label ▲, or untreated control ○. (C) The percentage of [^{3}H]TdR-treated cells in either G_1 ■ or G_2/M ●. (D) The percentage of [^{35}S]methionine-treated cells in either G_1 ■ or G_2/M ●.

interpreted as indicating the presence of at least two populations of cells with different cycle times. The curves consisted of a first peak, followed by a small second peak and a third higher peak of labelled mitoses. This is unexpected as the third peak would be expected to show even more severe damping, rather than an increase, compared to the second peak. An alternative possibility is that the small second peak represents only those cells *not* delayed in the cycle by radiation-induced p53 expression. The third peak might then consist of a combination of delayed cells re-entering the cycle semi-synchronously and non-delayed cells in their *second* cycle, thus explaining the unexpected height of this peak. Is the phenomenon of selective sensitivity of stem cells, and hence cycle delay, mediated by p53 in other experimental and physiological situations?

There is controversy as to whether low level radiation is hazardous to man. The dose from incorporated [^{3}H]TdR is difficult to measure and estimates vary greatly; a single ^{3}H decay has been estimated as being equivalent to 1.68Gy (Kisieleski et al., 1964), 0.01 Gy (Goodheart, 1961), and 0.0048 Gy (Cleaver et al., 1972). Thus it is difficult to compare our data with radiation from external sources. However, it is important that the effect, if any, on p53 expression from medical or industrial exposure to radiation should be investigated and the short and long term effects considered. Whilst p53 expression may be an adaptive response to environmental agents such as ultraviolet radiation in exposed tissues like the skin, what are the consequences of elevated p53 on, for example, bone marrow, spermatogonia and during embryonic development?

The p53 response to DNA damage appears to involve both new protein synthesis and protein stabilisation (Tishler et al., 1993). We report evidence of increased mRNA following labelling. Kastan et al. (1992) presented evidence for at least two pathways for G_1 growth arrest, but both involve the *GADD45* gene, p53 being involved in only one pathway. Our data suggest that p53 is required for the cell cycle delay induced by tritiated thymidine incorporation as p53 deficient cells do not display cycle delay when challenged with [^{3}H]TdR. There is urgent need for further studies to reveal if there are differences in the sensitivity of sub-populations of renewing tissues to the induction of p53. Our results may invalidate some experiments using radiolabelling for cell kinetic studies or metabolic labelling as in many cases the method used to study an effect itself perturbs the experiment - a biological Schrödinger's cat. New strategies will be required for a number of experimental protocols and much of the current literature may need to be critically re-evaluated in the light of our findings.

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