Oncogene (1999) 18, 3199–3204

http://www.stockton-press.co.uk/onc

An acidic environment leads to p53 dependent induction of apoptosis in human adenoma and carcinoma cell lines: implications for clonal selection during colorectal carcinogenesis

AC Williams*[,1], TJ Collard[1] and C Paraskeva[1]

[1]*CRC Colorectal Tumour Biology Research Group, Department of Pathology and Microbiology, School of Medical Sciences, University of Bristol, University Walk, Bristol BS8 1TD, UK*

As tumours are known to acidify their microenvironment and fluctuations in lumenal pH have been reported in a number of colonic disease conditions, we investigated whether loss of p53 function, commonly associated with the adenoma to carcinoma transition in human colorectal epithelium, was implicated in the cellular response to changes in extracellular pH. Human colonic adenoma and carcinoma derived cell lines were incubated at an inital pH range of 5.5–8.0 and the attached cell yield and apoptotic cell yield determined after 4 days. Exposure of all cell lines to an acidic growth environment was associated with a G1 arrest, down regulation of the retinoblastoma protein (pRb) protein and switch to the hypophosphorylated form of the protein, and increased expression of the p21 protein. However, induction of apoptosis, associated with increased p53 protein expression but not with changes in Bcl-2 expression, was only detected in the adenoma derived BH/C1 and AA/C1 cell lines which express wild type p53 activity. Furthermore, this induction of apoptosis was inhibited in the transfected cell line AA/273p53/B, in which the wild type p53 function has been abrogated. These results suggest that acidification of the microenvironment would provide a selective growth advantage for cells that have lost wild type p53 function, leading to clonal expansion of aberrant cell populations.

Keywords: pH; apoptosis; colon; p53; pRb

Introduction

The p53 gene product has been implicated in the cellular response to DNA damage leading to cell cycle arrest and/or apoptosis, through the transactivation of a number of down stream genes including the cyclin dependent kinase inhibitor p21 (reviewed by Levine 1997). Induction of wild type p53 function has also been observed in response to hypoxia (Graeber *et al*., 1996) and ribonucleotide depletion (Linke *et al*., 1996) suggesting a role for p53 as a 'sensor' of cellular stress.

In the current study we wished to address the question as to whether p53 function in colonic epithelium is implicated in response to stress conditions that may result from environmental changes in the colon, specifically changes in the extracellular pH. This is particularly relevant to colonic epithelium cells as, although the pH gradient in the normal colon has been shown to be small (from 6.8 in the right colon as compared to 7.2 in the left colon, Sasaki *et al*., 1997), in disease states this can be significantly altered. For example, in Crohns disease the overall mean luminal pH was 5.3, indicating that an extremely acidic colonic environment occurs either in active or inactive Crohns disease (Sasaki *et al*., 1997). In addition, luminal constituents such as short chain fatty acids may have significant effects on the intracellular pH and function of colonocytes, (Desoignie and Sellin, 1994; Zoran *et al*., 1997). Furthermore, acidification of the microenvironment by tumours has been widely reported, and any increase in the cell's ability to survive under acidic conditions could lead to selection and subsequent clonal expansion of aberrant cells.

In the current study changes in the extracellular pH were investigated as acidification of tumour tissues has been shown to be predominantly due to low extracellular pH (Griffiths, 1991), where as internal of malignant cells is similar or slightly more basic than that of normal cells (Gerweck *et al*., 1996). The aim of the study was to determine whether fluctuations in extracellular pH could play a role in the induction of apoptosis, a process known to be important in the maintenance of tissue homeostasis along the crypt (Gavrieli *et al*., 1992; Hall *et al*., 1994; Bedi *et al*., 1995). It was hypothesized that, as loss of p53 function is a relatively late event in the adenoma to carcinoma sequence, changes in the extracellular pH may have a significant effect on the survival of cells that had acquired p53 mutations, leading to the potential outgrowth of aberrant cells and hence clonal expansion of resistant cell populations. This would result in cells that acquired p53 mutations having a selective growth advantage over neighbouring cells with wild type p53 function. Therefore, using both human adenoma and carcinoma derived cell lines as a model of tumour progression, we wished to investigate whether an acidic environment would have a differential effect on the growth of human colonic epithelial cells dependent on their p53 status.

To determine cell survival in relation to external pH, three human colonic adenoma derived epithelial cell lines (AA/C1 and BH/C1 with wild type p53 expression and RG/C2 with a 282 (Arg→Trp) p53 mutation) and one carcinoma derived cell line (PC/JW2 which is null for p53 protein expression) were incubated at different external pH's for 4 days, the protein expression assessed and the attached and floating cell yields determined. As we and others have described pre-

*Correspondence: AC Williams
Received 24 June 1998; revised 18 December 1998; accepted 5 January 1999

viously, the level of apoptosis in cultured epithelial cell lines can be assessed by measuring the proportion of cells that have detached from the monolayer and are floating in the medium and by determining the fraction of these floating cells that are apoptotic (Hague *et al*., 1993; Elder *et al*., 1996; Tsuji and Dubois, 1995). Therefore after exposure to different external pH's the proportion of the total cell population that was floating was measured. To determine if the induction of floating cells was due to apoptosis, the attached and floating cell populations were stained with 5 μg/ml acridine orange in PBS, and analysed by flourescent microscopy for morphological features of apoptosis. The fraction of floating cells that were apoptotic did not significantly vary between treated and control untreated cell populations (for example 89–99% for the RG/C2 cell line), and therefore the number of floating cells could be used as a measure of the induction of apoptosis (refer to Materials and methods section). Exposure of all cell lines to an initial external pH of 6.5 and below was associated with a weak G1 arrest (data not shown), down regulation of the pRb protein and switch to the hypophosphorylated form of the protein (example shown in Figure 1). In addition there was increased expression of the p21 protein which was independent of the p53 status of the cell line (Figure 1). However, these changes alone were not sufficient to affect the attached cell yields over the 4 day experimental period (refer to Figure 3). Only the attached cell yields of the adenoma derived BH/C1 and AA/C1 cell lines (wild type p53 function) were significantly decreased in response to an initial external pH of 5.5–6.5, (Figure 2). The reduction in cell yield was shown to be accompanied by an induction of apoptosis (Figure 2), increased p53 protein expression (Figure 1), but not associated with changes in Bcl-2 expression, (which remained unchanged in all cell lines, data not shown). This was not simply due to a generalized cellular response to growth inhibition, as treating the cells with the inhibitory growth factor TGFβ has previously been shown to result in cell cycle arrest without the induction of apoptosis in these cell lines (Manning *et al*., 1991). In contrast the cell yield of the adenoma derived RG/C2 (282 Arg→Trp mutation) and the carcinoma derived cell line JW2 (null for p53 expression) were resistant to all initial pH's tested, no reduction in cell yield was observed and there was no significant induction of apoptosis under these growth conditions (Figure 3). Hence both cell lines that were growth inhibited by reduced external wild type p53 activity, whereas the resistant cell lines had no wild type p53 function.

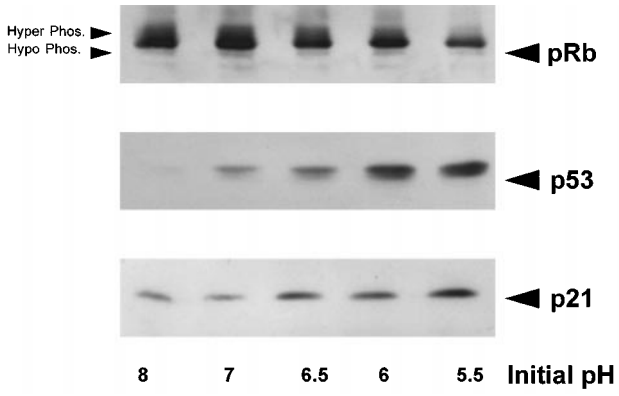

Figure 1 Western blot showing protein expression after 4 days in the AA/C1 cell line (wild type p53 expression, passage 83) after exposure to different initial extracellular pH

Therefore, to further determine whether induction of apoptosis by low external pH was dependent on the cells retaining functional wild type p53 activity, the response of a transfected derivative of the AA/C1 cell line, designated AA/273p53/B, and a vector control AA/PCMV/D were investigated. (The AA/273p53/B cells express a dominant negative 273 (Arg→His) p53 mutation, resulting in loss of wild type p53 activity as shown by loss of the G1 arrest after exposure to DNA damage, Williams *et al*., 1995.) Unlike the parental AA/C1 cell line and vector control, the AA/273p53/B cells were shown to be resistant to reduced pH (i.e. no inhibition of cell yield or induction of apoptosis, Figure 4), similar to the RG/C2 and JW2 cell lines (no wild type p53 function). In both the AA/273p53/B and vector control cell line, pRb protein was down regulated and hypophosphorylated and p21 protein expression increased as previously detected in all cell lines.

These results suggest that in human colonic epithelial cells, cell survival in an acidic growth environment is dependent on loss of wild type p53 function. We have shown that, when exposed to reduced extracellular pH, all cell lines investigated (independent of p53 status) exhibited decreased pRb expression, an accumulation of the remaining Rb protein in the hypophosphorylated form and a G1 arrest. However only cells expressing wild type p53 function were significantly growth inhibited by low external pH, and this was shown to be accompanied by the induction of p53 dependent apoptosis. In addition, loss of functional wild type p53 in the transfected cell line AA/273p53/B was shown to increase cell survival under these growth conditions.

The mechanism by which apoptosis is induced by an acidic environment remains unclear. Dephosphorylation of pRb has been reported to be a marker of an early event in the activation of apoptosis (Wolf *et al*., 1997), although in the current study dephosphorylation of pRb was observed in all cells, not only those undergoing apoptosis. DNA cleavage, an essential component of apoptosis, has been shown to occur via a pH dependent endonuclease present in the T24 human bladder cancer cell line (Shemtov *et al*., 1995). Intracellular acidification leading to endonuclease activation has been reported in a number of cell lines (Morana *et al*., 1994; Gottlieb *et al*., 1995; PerezSala *et al*., 1995; Collins *et al*., 1996). However induction of apoptosis is unlikely to be due to direct activation of pH dependent endonuclease due to intracellular buffering (Gerweck *et al*., 1996), and in the present study cells that had lost wild type p53 function remained resistant to induction of apoptosis by extracellular acidification.

Results from a study on CHO cells suggest that Bcl-2 and MCL-1 may regulate a pathway for intracellular pH homeostasis during apoptotic cell death (Reynolds *et al*., 1996). However the apoptosis observed in the adenoma derived cell lines with wild type p53 function

was not associated with changes in the Bcl-2 protein (data not shown). In addition bcl-2 expression does not protect the cells from pH induced apoptosis, as the BH/C1 cell line has high endogenous bcl-2 expression (Hague *et al*., 1998) and still undergoes p53 dependent apoptosis in response to an acidic environment. The most likely mechanism may involve p53 as a monitor of cellular stress: in acidic growth conditions, pRb expression is reduced and the protein is retained in the hypophosphorylated form resulting in a weak G1 arrest which was not found to be sufficient to significantly effect the cell yield over the experimental period. However if the cells retain wild type function, the p53 protein is upregulated and apoptosis is induced.

We propose that the lowering of the external pH by tumorigenic cells, as well as providing an environment for the selective activation of extracellular proteases (Van der Stappen *et al*., 1996), also provides a selective growth advantage for cells that have lost wild type p53 activity. This is highly relevant to the epithelial lining of the colon as fluctuations in the luminal pH have been reported in a number of disease states and therefore could provide a mechanism by which loss of wild type p53 function could lead to resistance and subsequent clonal expansion of aberrant cell populations. We suggest that, due to the low extracellular pH, loss of p53 function in tumorigenic progression would allow clonal selection of the effected cell population even in the absence of DNA damage, contributing to

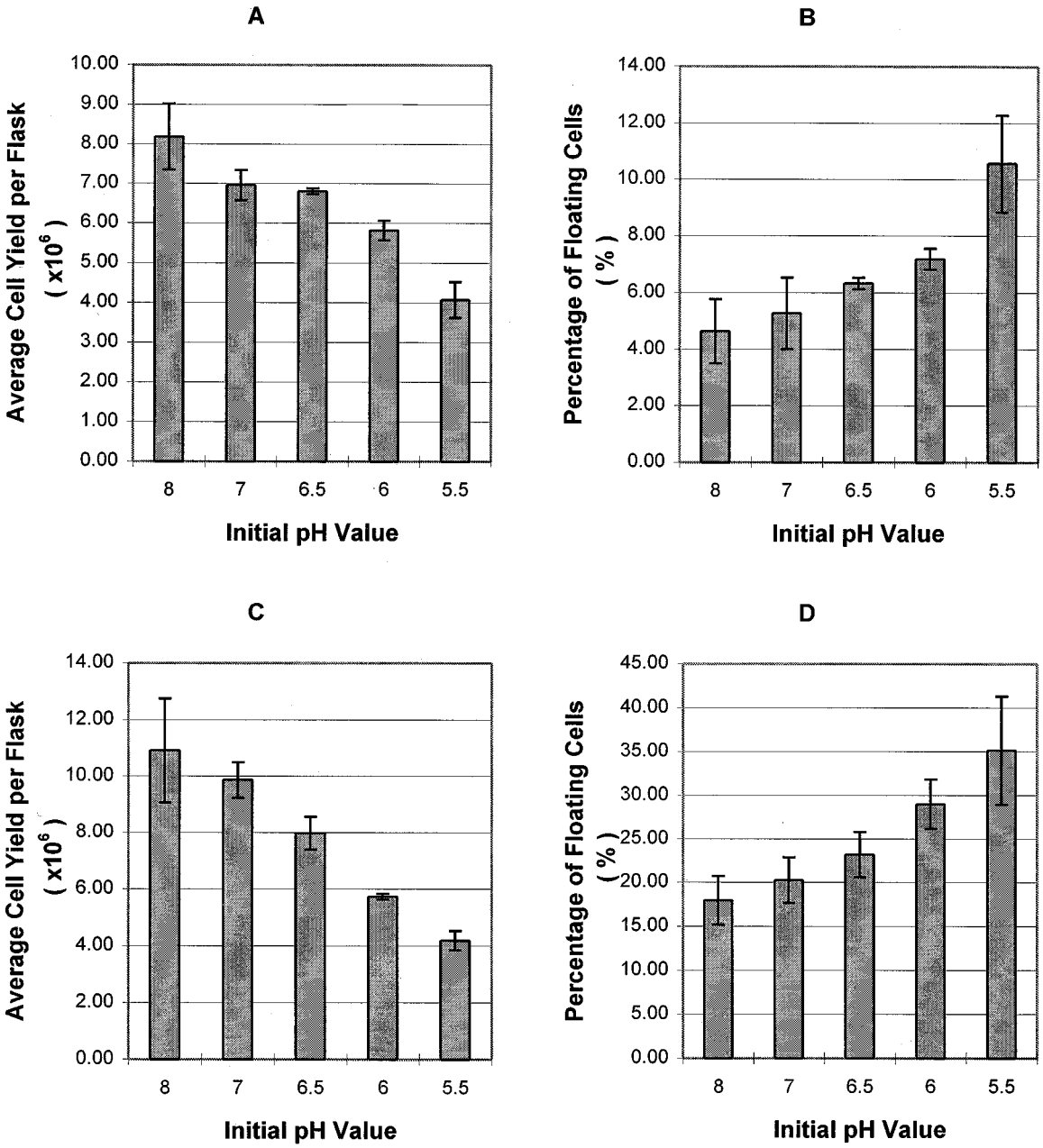

Figure 2 Cell survival of cells expressing wild type p53 function at 4 days after exposure to different initial extracellular pH as shown by: (1) Attached cell yield (**a**) AA/C1 cell line (passage 83–88), (**c**) BH/C1 cell line (passages 74–81). (2) Induction of apoptosis as shown by floating cells expressed as a percentage of the total cell population (**b**) AA/C1 cell line (passages 83–88), and (**d**) BH/C1 cell line (passages 74–81). The results shown are the means of triplicate experiments, ±s.d.

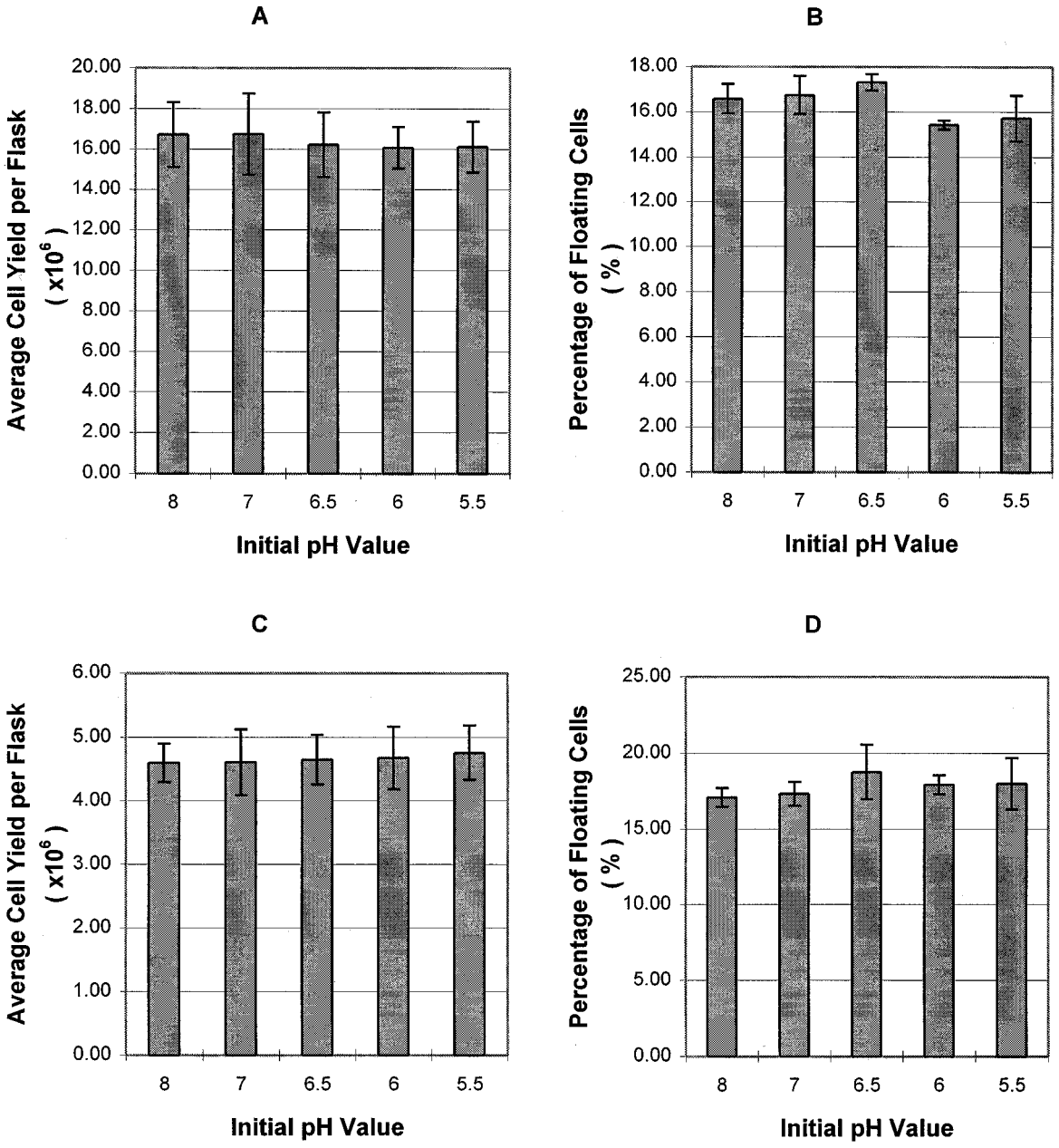

Figure 3 Cell survival in cell lines with mutant p53 (RG/C2 282Arg→Trp) or null for p53 protein expression (PC/JW2) at 4 days after exposure to different initial extracellular pH as shown by: (1) Attached cell yield (**a**) RG/C2 cell line (passages 39–41), (**c**) PC/JW2 cell line (passages 51–54). (2) Induction of apoptosis as shown by floating cells expressed as a percentage of the total cell population (**b**) RG/C2 cell line (passages 39–41), and (**d**) JW2 cell line (passages 51–54). The results shown are the means of triplicate experiments, ±s.d.

the selective pressure for loss of p53 in colorectal tumours (over 75% of human colonic tumours have lost functional wild type p53 activity).

Materials and methods

The cell lines used in this study have been derived from human colorectal adenomas. AA/C1 and BH/C1 are clonogenic, non-tumorigenic adenoma cell lines (Paraskeva *et al*., 1984), both of which express wild type p53 (Baker *et al*., 1990; Williams *et al*., 1993). RG/C2 is a clonogenic, non-tumorigenic adenoma derived cell line hemizygous for the p53 gene, with the remaining allele having a 282 (Arg→Trp) mutation (Baker *et al*., 1990). PC/JW2 is a carcinoma derived cell line that is null for p53 protein expression, Paraskeva *et al*. (1984). PC/AA/C1 cells transfected with, and stably expressing a vector containing mutant p53, AA/273p53/B (273 Arg→His, previously shown to act as a dominant negative abrogating wild type), and a vector control, AA/PCMV/D, were also used (Williams *et al*., 1995).

Cells were seeded at 2×10^6 per flask. At approximately 70% confluency duplicate flasks were treated with DMEM adjusted for pH using HCO_3/HCl (initial pH of 5.5, 6.0, 6.5, 7.0 and 8.0), 7–10 days after seeding. The medium was adjusted to the required pH before addition of 20% FBS. pH then was verified and medium added directly to the cells. The medium was left on the cells for 4 days, during which time the external pH of the medium was monitored. It was decided that rather than maintaining the pH of the medium with buffers (allowing the possibility of differential sensitivity

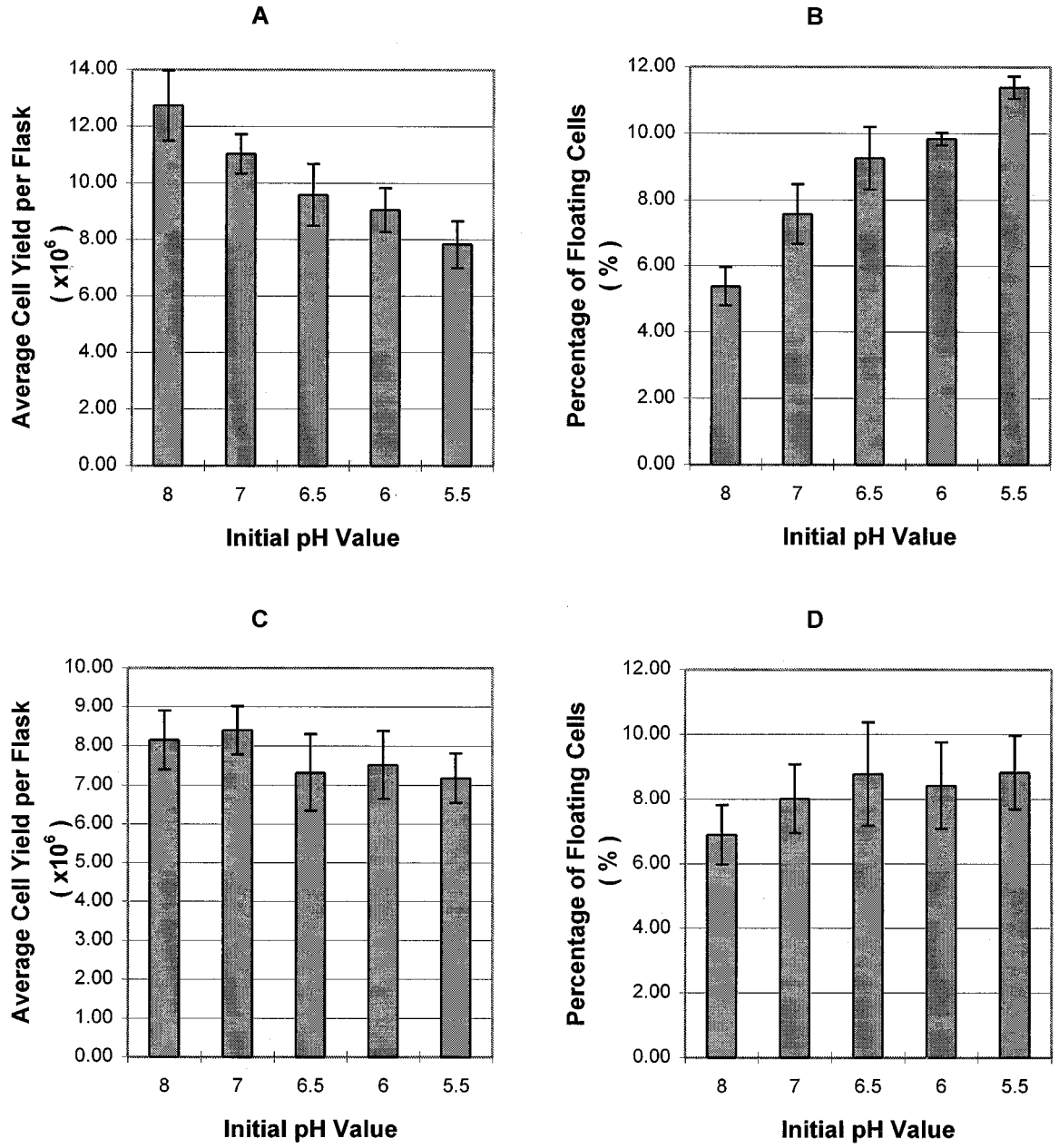

Figure 4 Cell survival of the transfected cell line AA/273p53/B [in which wild type p53 function has been knocked out] and the AA/PCMV/D vector control at 4 days after exposure to different initial extracellular pf as shown by: (1) Attached cell yield (**a**) AA/PCMV/D cell line (passages 84–85), (**c**) AA/273p53/A cell line (passages 83–87). (2) Induction of apoptosis as shown by floating cells expressed as a percentage of the total cell population (**b**) AA/PCMV/D cell line (passages 84–85), and (**d**) AA/273p53/A cell line (passages 83–87). The results shown are the means of a two or more experiments, ±s.d.

between adenoma and carcinoma derived cell lines to the presence of the buffers in the growth medium), we would allow the external pH to drift, thus mimicking potential pH fluctuations that may occur *in vivo*. Using this method we were able to detect significant changes in cell survival, despite the fact that the external pH remained at the defined pH for less than 24 h. Cell survival was determined after 4 days to encompass any delayed apoptotic response. After 4 days the floating and attached cells were counted. The level of apoptosis in cultured epithelial cell lines was assessed by measuring the proportion of cells that detached from the monolayer and were floating in the medium and by determining the fraction of these floating cells that were apoptotic. After exposure to different external pH's the proportion of the total cell population that was floating was measured. The attached and floating cell populations were stained with 5 μ g/ml acridine orange in PBS, and analysed by flourescent microscopy for morphological features of apoptosis (most obviously the characteristically condensed chromatin). Analysis was carried out by an experienced observer unaware of the cell type or treatment. The fraction of floating cells that were apoptotic did not significantly vary between treated and control untreated cell populations and therefore the number of floating cells could be used as a measure of the induction of apoptosis. Culturing the cells at pH 5.0 and below was found to cause cell death by necrosis.

Determination of cell cycle arrest

Cell cycle arrest after was determined by propidium iodine DNA content analysis on a FACScan (Becton Dickinson) with doublet discrimination as previously reported (Bracey *et al*., 1995), using a modified method described by O'Connor *et al*. (1993).

Protein expression by SDS–PAGE

Cell samples for Western blotting were prepared for p53 and p21 by the method described in Williams *et al*. (1995) and for pRb protein by the method decribed in Butt *et al*. (1997). p53 protein was detected by pAb 1801 (Banks *et al*., 1986), p21 protein was detected by the monoclonal Waf-1 antibody AB-1 (Oncogene Science), and pRb was detected using the monoclonal antibody 14001A (Pharminogen). All proteins were incubated with the horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Sigma) and bands were visualized using Amersham's enhanced chemiluminescence detection system following the manufacturer's protocol.

Acknowledgements
This research was funded by a programme grant from the Cancer Research Campaign of Great Britain

References

Baker SJ, Preisiner AC, Jessup JM, Paraskeva C, Markowitz S, Wilson JKV, Hamilton S and Vogelstein B. (1990). *Cancer Res.,* **50,** 7717–7722.
Banks L, Matlashewski G and Crawford L. (1986). *European J. Biochem.,* **159,** 529–534.
Bedi A, Pasricha PJ, Akhtar AJ, Barber JP, Bedi GC, Giardiello FM, Zehnbauer BA, Hamilton SR and Jones RJ. (1995). *Cancer Res.,* **55,** 1811–1816.
Bracey TS, Miller JC, Preece A and Paraskeva C. (1995). *Oncogene,* **10,** 2391–2396.
Butt AJ, Hague A and Paraskeva C. (1997). *Cell Death and Diff.,* **4,** 725–732.
Collins MKL, Furlong IJ, Malade P, Ascaso R, Oliver J and Lopez-Rigas A. (1996). *J. Cell Sci.,* **109,** 2393–2399.
Desoignie R and Sellin JH. (1994). *Gastroenterology,* **107,** 347–356.
Elder DJE, Hague A, Hicks DJ and Paraskeva C. (1996). *Cancer Res.,* **56,** 2273–2276.
Gavrieli Y, Sherman Y and Bensasson SA. (1992). *J. Cell Biol.,* **119,** 493–501.
Gerweck LE and Seetharaman K. (1996). *Cancer Res.,* **56,** 1194–1198.
Gottlieb RA, Giesing HA, Engler RL and Baboir BM. (1995). *Blood,* **86,** 2414–2418.
Graeber TG, Osmanian C, Jacks T, Housman DE, Kock CJ, Lowe SW and Giaccia AJ. (1996). *Nature,* **379,** 88–91.
Griffiths JR. (1991). *Br. J. Cancer,* **64,** 425–427.
Hague A, Bracey TS, Hicks DJ, Reed JC and Paraskeva C. (1998). *Carcinogenesis,* **19,** 1691–1695.
Hague A, Manning AM, Hanlon KA, Huschtscha LI, Hart D and Paraskeva C. (1993). *Int. J. Cancer,* **55,** 498–505.
Hall PA, Coates PJ, Ansari B and Hopwood D. (1994). *J. Cell Sci.,* **107,** 3569–3577.
Levine AJ. (1997). *Cell,* **88,** 323–331.
Linke SP, Clarkin KC, Dileonardo A, Tsou A and Wahl GM. (1996). *Gene Dev.,* **10,** 934–947.
Manning AM, Williams AC, Game SM and Paraskeva C. (1991). *Oncogene,* **6,** 1471–1476.
Morana S, Li J, Springer EW and Eastman A. (1994). *Int. J. Oncol.,* **5,** 153–158.
O'Connor PM, Jackman J, Jondle D, Bhatia K, Magrath I and Kohn KW. (1993). *Cancer Res.,* **53,** 4776–4780.
Paraskeva C, Buckle BG, Sheer D and Wigley CB. (1984). *Int. J. Cancer,* **34,** 49–56.
PerezSala D, ColladoEscobar D and Mollinedo F. (1995). *J. Biol. Chem.,* **270,** 6235–6242.
Reynolds JE, Li J, Craig RW and Eastman A. (1996). *Exp. Cell Res.,* **225,** 430–436.
Sasaki Y, Hada R, Nakajima H, Fukuda S and Munakata A. (1997). *Am. J. Gastro.,* **92,** 114–118.
Shemtov MM, Cheng DLW, Kong L, Shu WP, Sassaroli M, Droller and Lui BCS. (1995). *J. Urol.,* **154,** 269–274.
Tsuji M and Dubois RN. (1995). *Cell,* **83,** 493–501.
van der Stappen JWJ, Williams AC, Maciewicz RA and Paraskeva C. (1996). *Int. J. Cancer,* **67,** 547–554.
Williams AC, Browne SJ, Yeudal WA. Paterson IC, Marshall CJ, Lane DP and Paraskeva C. (1993). *Oncogene,* **8,** 3063–3072.
Williams AC, Miller JC, Collard TJ, Bracey TS, Cosulich S and Paraskeva C. (1995). *Oncogene,* **11,** 141–149.
Wolf CM, Morana SJ and Eastman A. (1997). *Cell Death and Diff.,* **4,** 125–129.
Zoran DL, Barhoumi R, Burghardt RC, Chapkin RS and Lupton JR. (1997). *Nutr. Cancer,* **27,** 222–230.