Differentiation (1999) 64:185–193

ORIGINAL ARTICLE

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Bromodeoxyuridine induces keratin protein synthesis at a posttranscriptional level in human lung tumour cell lines

Accepted in revised form: 3 November 1998

Abstract Keratin intermediate filaments are formed in epithelial cells in a cell- and tissue-specific manner, but much remains unknown regarding the mechanisms which control the synthesis of these proteins. We examined the effect of the differentiation modulation agent, bromodeoxyuridine (BrdU), on two human keratin-negative (by immunocytochemistry) lung cell lines, DLKP and H82, and showed immunohistochemically that treatment with 10 μ*M* BrdU over 7 days induced K8 and K18 protein synthesis in both lines. Immunoprecipitation and Western blot analyses revealed low levels of K8 and K18 proteins in untreated cell homogenates. These levels increased following treatment with BrdU for 7 days. K8 and K18 mRNAs were detected by Northern blot and reverse transcriptase polymerase chain reaction analyses in both lines before BrdU treatment, but no increase in mRNA levels was observed in either cell line over 21 days of treatment. This suggests, firstly, that keratin synthesis is normally blocked at a posttranscriptional level in DLKP and H82 cells, and secondly, that BrdU can reverse this block. A549 is a human lung cell line which contains K8 and K18 proteins. Treatment with BrdU increased K8 and K18 protein levels in these cells. No corresponding increase in K8 mRNA levels occurred, while an apparent increase in K18 mRNA levels was detected. HL-60 is a leukaemic cell line of haematopoietic rather than epithelial lineage which contains K8 and K18 mRNA transcripts prior to BrdU treatment, but does not contain keratin proteins. Again, K8 and K18 mRNA levels remained unchanged during BrdU treatment. However, neither K8 nor K18 proteins were detected following treatment, although BrdU is known to alter expression of other genes in HL-60 cells. BrdU thus appears to act at a posttranscriptional level and in an epithelial-specific manner to reverse a block in keratin synthesis in keratin-negative lung cancer cells and increase synthesis in keratin-positive lung cancer cells. This may represent a regulatory step in early lung development or a mechanism whereby tumour cells downregulate expression of a differentiated phenotype.

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Introduction

Intermediate filaments (IFs) form a family of tissue-specific cytoskeletal proteins. These include keratins, which are mainly found in epithelia; vimentin, a mesenchymal protein; desmin, which is specific for muscle cells; glial fibrillary acidic protein (GFAP), found in glial cells and astrocytes; neurofilament proteins, which are expressed in nerve cells; and lamins, which comprise the nuclear lamina (reviewed in [36]). Keratin intermediate filaments are among the most differentiation-specific proteins synthesized in epithelial cells. More than 20 different cellular keratins (known as cytokeratins to distinguish them from keratins present in nails and hair) have been identified [27, 28], each of which appears to have a distinctive pattern of synthesis in normal epithelia [35]. The keratin family of proteins is generally subdivided into basic type II keratins (K1–K8) and acidic type I keratins (K9–K20) [27], which form heteropolymers consisting of at least one type I and one type II chain [19]. For example, K8 and K18 are partner molecules which dimerise to form keratin filaments.

During foetal development, keratins are the first intermediate filament proteins detectable, with the simple epithelial keratins K8 and K18 appearing first [14]. Gene sequence analysis indicates that the more specialised keratins, which are expressed at later stages of development and differentiation, probably evolved from K8 and K18 [4, 37]. An elaborate pattern of keratin proteins is displayed, first in developing, and then in adult epithelia. For example, both embryonic and adult epidermal basal cells initially synthesise K5 and K14 proteins [30]. As these basal cells differentiate, they down-regulate K5 and

K14 and begin to express K1 and K10 proteins [36]. In addition, during wound healing and in diseases of hyperproliferation, K6 and K16 can be present in suprabasal epidermal cells [26, 36]. Thus, epithelial cells possess specific keratin profiles characteristic of their cell type, stage of development, disease state and degree of differentiation. It is believed that intermediate filaments organise cellular internal structure and provide mechanical support [17], and human genetic mutations which result in disruption of IF cellular networks give rise to often debilitating skin-blistering disorders where stress causes cytolysis [16].

While many epithelial tumours retain a profile of keratin synthesis characteristic of their differentiation state [6], alterations in keratin synthesis during malignancy have been observed [42, 46]. Variant small cell lung carcinoma (SCLC-V) cells in particular, despite being epithelial in origin, often contain no detectable amounts of keratin [5] and can instead synthesize neurofilaments or vimentin. DLKP is a cell line which was established in our laboratory from a tumour histologically diagnosed as a poorly differentiated lung carcinoma. DLKP cells have properties which suggest they could be classified as either SLCL-V or non-small-cell lung carcinoma with neuroendocrine differentiation (NSCLC-NE) [8, 25], as these tumour types have a similar profile of protein markers. DLKP contains neurofilament and vimentin proteins, but no keratin proteins are detectable at the immunocytochemical level (nor are other epithelial markers such as epithelial membrane antigen, epithelial specific antigen, EP16 or desmosomal proteins).

BrdU is a thymidine analogue capable of inducing epitheloid morphology and altering the expression of neuroendocrine markers in SCLC cell lines [13]. The ability of BrdU to alter differentiation in neuronal, muscle and haematopoietic lineages is also well established [12, 40, 48]. BrdU competes with thymidine for incorporation into DNA, a property which has led to its widespread use in cell proliferation assays [10]. While it appears that BrdU must be incorporated into DNA in order to modulate differentiation [18], relatively little is known regarding the mechanism(s) of action involved. There is evidence that in some systems altered conformation of BrdU-containing DNA may affect interactions with specific transcription factors, leading to either inhibition or induction of differentiation, depending on the cell type [24]. In order to investigate the mechanisms resulting in the absence of keratin (and other epithelial-specific proteins) from DLKP cells and a SCLC-V cell line, H82, we examined the effects of BrdU on keratin synthesis in these cells.

Methods

Cell lines

The DLKP cell line was derived from a lymph node metastasis of a human lung carcinoma which was histologically defined as a poorly differentiated, squamous cell carcinoma [23]. However, more recent immunohistochemical analysis carried out in this laboratory indicates that the DLKP cell line expresses many features typical of SCLC-V or NSCLC-NE [25]. These cells were routinely cultured in a 1:1 mixture of DMEM:Ham's F12, which was supplemented with 5% foetal calf serum and 2 m*M* L-glutamine. All other cell lines used in this study were obtained from the American Type Culture Collection. H82, a human SCLC-V cell line and HL-60, a human promyelocytic cell line, both of which grow in suspension, were cultured in RPMI 1640 medium, supplemented with 10% foetal calf serum and 2 m*M* L-glutamine. A549, an adherent human adenocarcinoma cell line, was cultured in DMEM:Hams F12 (1:1), supplemented with 5% foetal calf serum and 2 m*M* L-glutamine. All lines were grown in sealed tissue culture flasks at 37° C except HL-60, which was maintained in vented flasks at 37° C in 5% CO_2.

BrdU treatment of cells for immunocytochemistry

Stock solutions of 10 m*M* BrdU (Sigma, Poole, UK) were prepared in sterile phosphate-buffered saline (PBS) and stored at –20° C. Cells were seeded at densities of 1×10^3 cells per well on sterile multiwell Nunc slides (Life Technologies, Paisley, UK) and incubated for 24 h at 37° C in humidified 5% CO_2. Appropriate dilutions of BrdU were made in normal growth medium and added to the cells at a final concentration of 10 μ*M*. Control wells contained growth medium without BrdU. Slides were incubated at 37° C in 5% CO_2 for up to 21 days, during which time the medium was replaced every 2–3 days.

Immunocytochemical analyses

For immunocytochemical analyses, adherent cells growing on multi-well slides were rinsed three times with PBS and fixed at –20° C for 5 min in methanol, which had been prechilled to –20° C. Cells growing in suspension were harvested by centrifugation, rinsed three times with PBS, cytocentrifuged onto clean glass slides, and fixed with methanol as above.

Immunocytochemical staining was performed employing either the avidin-biotin-peroxidase (AB-HRP) or avidin-biotin-alkaline phosphatase (AB-AP) technique using DAKO Labs ABC visualising kits (DAKO, High Wycombe, UK) according to the manufacturer's instructions. Briefly, nonspecific binding was blocked using dilute rabbit serum. For AB-HRP, endogenous peroxidase activity was quenched prior to blocking using 3% H_2O_2. Primary antibodies were then incubated for 2 h at 37° C. Secondary antibodies were incubated for 30 min at room temperature, and following 30 min of incubation with a streptABComplex/HRP or/AP solution, either HRP reactivity was visualised using a 3,3-diaminobenzidine tetrahydrochloride (DAB) chromogen, which resulted in a brown-coloured precipitate, or AP reactivity was visualised using Vector red substrate (Vector Labs, Peterborough, UK), which resulted in a red stain. Cells were counterstained with a 2% methyl green solution or haematoxylin. Primary antibodies used were as follows: anti-cytokeratin 8 (Sigma, clone no. M20), anit-cytokeratin 10 (DAKO, clone no. DE-K10), anti-cytokeratin 14 (Sigma, clone no. CKB1), anti-cytokeratin 18 (Sigma, clone no. CY-90) and anti-CD14 (a monoclonal antibody produced at Edinburgh University, clone no. UCMH1).

Immunoprecipitation and Western blots

For A549 Western blots, cells were harvested by trypsinization and washed three times with PBS. Equal cell numbers were lysed in 2.5× loading buffer, which was 2.5 ml 1.25 *M* Tris-HCl, pH 6.8; 1.0 g sodium dodecyl sulfate (SDS); 5.8 ml glycerol; 1.4 ml H_2O, and separated on 12% polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes and blocked for 4 h at room temperature with 5% semi-skimmed dried milk in TBS/0.5% Tween 20. Membranes were then probed overnight with keratin

antibodies, washed and probed with an anti-mouse peroxidase-labelled secondary antibody for 1 h and visualised by chemiluminescence using ECL reagents (Amersham, Dublin, Ireland). Immunoprecipitations were performed using a biotin labelling kit (Boehringer Mannheim, Lewes, UK). Briefly, cells were harvested as above, resuspended in lysis buffer of 50 m*M* sodium borate, 150 m*M* NaCl, 0.1 mg/ml phenyl methyl sulfonyl furate (PMSF), 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1% Nonidet P-40, 0.5% sodium deoxycholate, sonicated until lysed, as determined microscopically, and incubated on ice for 30 min. Lysates were then centrifuged at 12,000 *g* for 10 min. Supernatants were transferred to clean tubes and biotinylation reactions were carried out according to the manufacturer's instructions. Lysates were precleared by incubating with 20 µl protein-G beads for 1 h at 4° C, centrifuged at 12,000 *g* for 40 s at 4° C. Supernatants were then incubated with 25 µg keratin antibody overnight at 4° C. Then 30 µl protein-G beads was added to the lysates and incubated for a further 24 h at 4° C. Beads were washed according to labelling kit instructions, pelleted at 12,000 *g* for 20 s, resuspended in 5× loading buffer (see above) and run on 12% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and probed with a biotin antibody (Sigma). Bands were visualised by chemiluminescence. For the low-intensity signals in DLKP, H82 and HL-60 blots, Supersignal reagents (Pierce Labs, Dublin, Ireland) were used. The same keratin antibodies were used in the immunocytochemical, immunoprecipitation and Western blot studies.

RNA isolation and Northern blot analyses

For RNA isolation and Northern blot analyses, cells were grown in 175-cm^2 tissue culture flasks in medium as above and treated with 10 µ*M* BrdU. Medium was replaced every 2–3 days. Cells were harvested at various time points for up to 21 days. Total RNA from pelleted cells was isolated by extraction with TriReagent (Sigma) according to manufacturer's instructions. RNA (20 µg) was separated by electrophoresis on 1% agarose-formaldehyde gels and transferred onto Hybond N+ membranes (Amersham). Equal loading of gels was confirmed by ethidium bromide (EtBr) staining of ribosomal RNA bands on gels. Filters were prehybridised and hybridised at 42° C for 16–18 h in 50% formamide, 6×SSC, 5×Denharts solution, 0.5% SDS and 100 µg/ml salmon sperm DNA. Probes were ^{32}P-labelled with a random primer labelling kit (Promega). Filters were washed once in 2×SSC (5 min), twice in 0.5×SSC/0.1% SDS (30 min) and twice in 0.1×SSC/0.1% SDS (30 min) at 65° C and exposed to autoradiographic film. The K8 probe was obtained from the ATCC and the K18 probe (pK 189) was kindly supplied by Dr. Robert Oshima, The Burnham Institute, La Jolla, Calif., USA. Both probes are full-length cDNA probes.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated as described above. First-strand cDNA was synthesised from 1 µg RNA using oligo (dT) primers (Promega, Dublin, Ireland). Then 5 µl cDNA was amplified in a 50 µl PCR reaction solution of 1×Promega Taq polymerase buffer containing 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTPs, 20 µ*M* oligonucleotide primers and 2.5 U Taq polymerase. The PCR program was as follows: an initial denaturation step of 94° C for 1.5 min, followed by 30 cycles of 94° C for 1.5 min, 55° C for 1 min and 72° C for 3 min, followed by 72° C for 10 min. RT-PCR products were separated on a 3% agarose gel by electrophoresis. Sense and antisense primers, respectively, used for cDNA amplification were as follows; K8, AACAACCTTAGGCGGCAGCT (cDNA position +449 to +468) and GCCTGAGGAAGTTGATCTCG (cDNA position +673 to +692), product size 244 bp[1]; K18, CAAGATCATCGAGGACCTG (cDNA position +436 to +454) and CTCTCCTCAATCTGCTGAGA (cDNA position +860 to +879), product size 444 bp[2]; β-actin, TGGACATCCGCAAAGACCTGTAC (cDNA position +901 to +923) and TCAGGAGGAGCAATGATCTTGA (cDNA position +1021 to +1042), product size 142 bp[3].

[1] Primer sequences published [7]

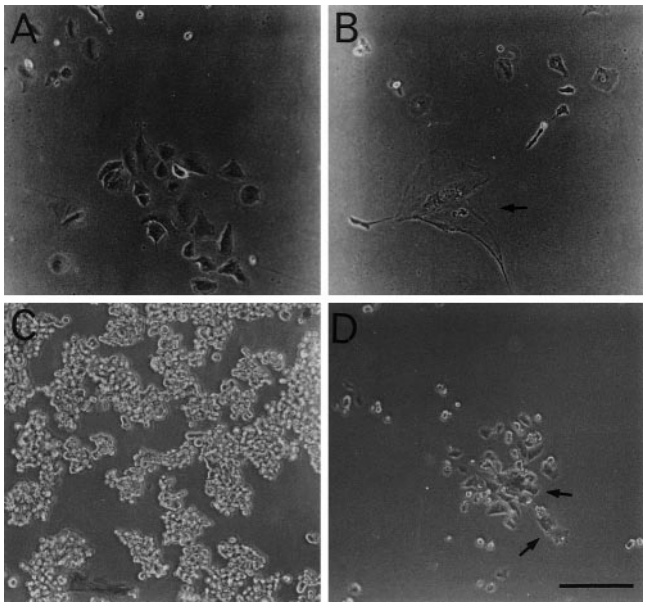


Fig. 1A–D Effects of bromodeoxyuridine (BrdU) on morphologies of DLKP and H82 cells showing untreated DLKP cells (**A**), DLKP cells cultured in the presence of 10 µ*M* BrdU for 7 days (**B**), *arrow* indicating at least a 10-fold increase in surface area of some cells. Untreated H82 cells (**C**) and H82 cells cultured in the presence of 10 µ*M* BrdU for 7 days (**D**), *arrows* showing attached and spread cells. (*Bar* 20 µm)

Results

Morphological effects of BrdU

The morphologies of DLKP, H82 and A549 were altered when grown in the presence of 10 µ*M* BrdU. In the case of DLKP (Fig. 1A, B) and A549, an approximately 5- to 10-fold increase in cell surface area occurred, with most cells acquiring a stretched, flattened appearance. H82 cells grow in suspension under normal conditions with a very small proportion (<5%) of cells spontaneously attaching gradually over 5–7 days. Following 5 days of treatment with BrdU, approximately 80%–90% of H82 cells had attached (Fig. 1C, D). Similar effects on morphology and adherence have been reported following BrdU treatment of other cell types [13, 39, 45]. At 1 µ*M* and 5 µ*M*, BrdU had similar, but less pronounced, effects on DLKP and H82, i.e., fewer cells had altered morphologies or were keratin positive at the lower BrdU concentrations. No effect on the morphology of HL-60 cells was apparent following treatment, with cells remaining in suspension before and after 10 µ*M* BrdU exposure.

[2] designed from corresponding rat K18 primers published [15]
[3] designed at NCTCC, Dublin City University, Ireland

Cell densities were reduced by approximately 30%–50% in all four cell lines following treatment with 10 µ*M* BrdU for 7 days. This was probably due to both inhibition of proliferation and some toxicity.

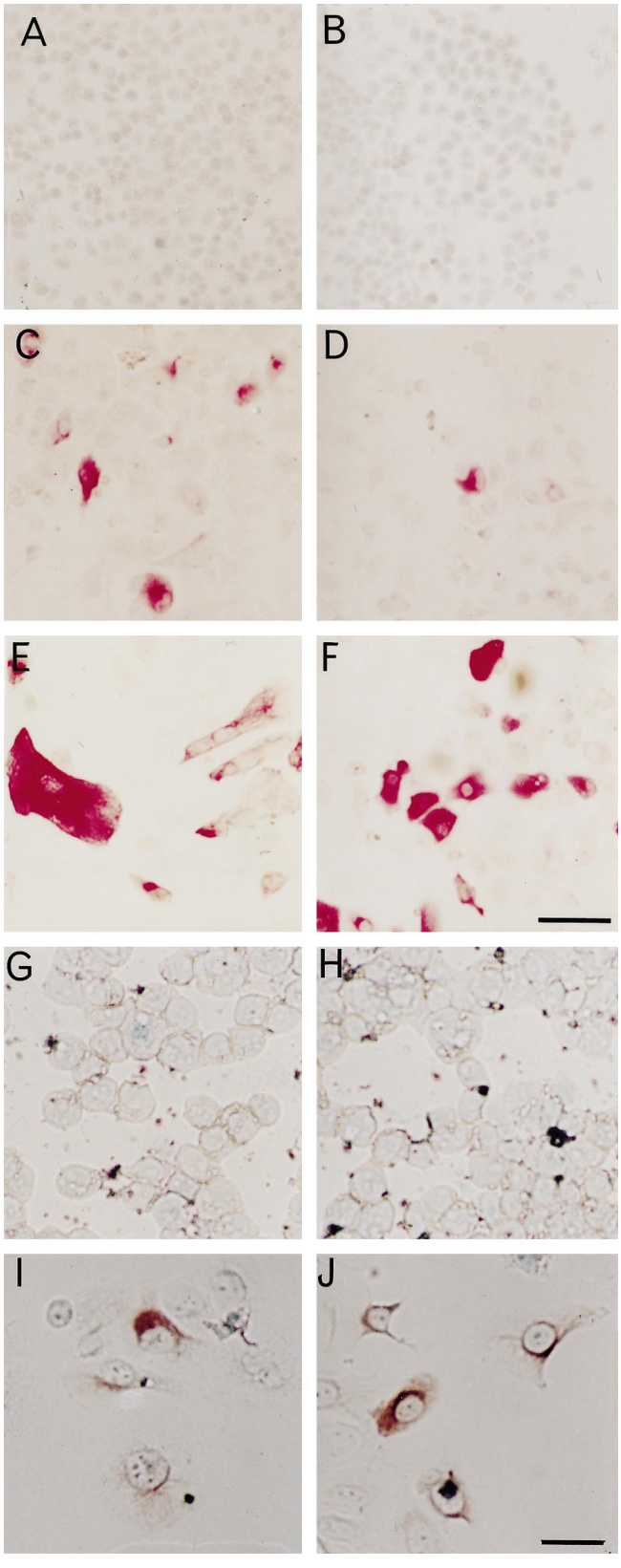


Fig. 2A–J Immunocytochemical detection of keratin (K) proteins in BrdU-treated DLKP and H82 cells. Untreated DLKP cells did not stain for K8 (**A**) or K18 (**B**) on day 7. DLKP cells cultured for 7 days in the presence of 10 µ*M* BrdU expressed K8 (**C**) and K18 (**D**) proteins. K8 (**E**) and K18 (**F**) staining increased further in DLKP cells treated for 21 days. Similarly, untreated H82 cells were negative for K8 (**G**) and K18 (**H**) on day 7 while K8 (**I**) and K18 (**J**) proteins were expressed in H82 cells following BrdU treatment for 7 days. (*Bars* **A–F** 20 µm; **G–J** 10 µm)

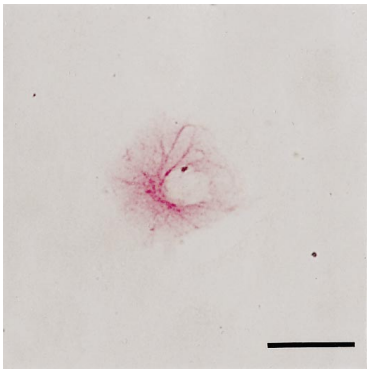


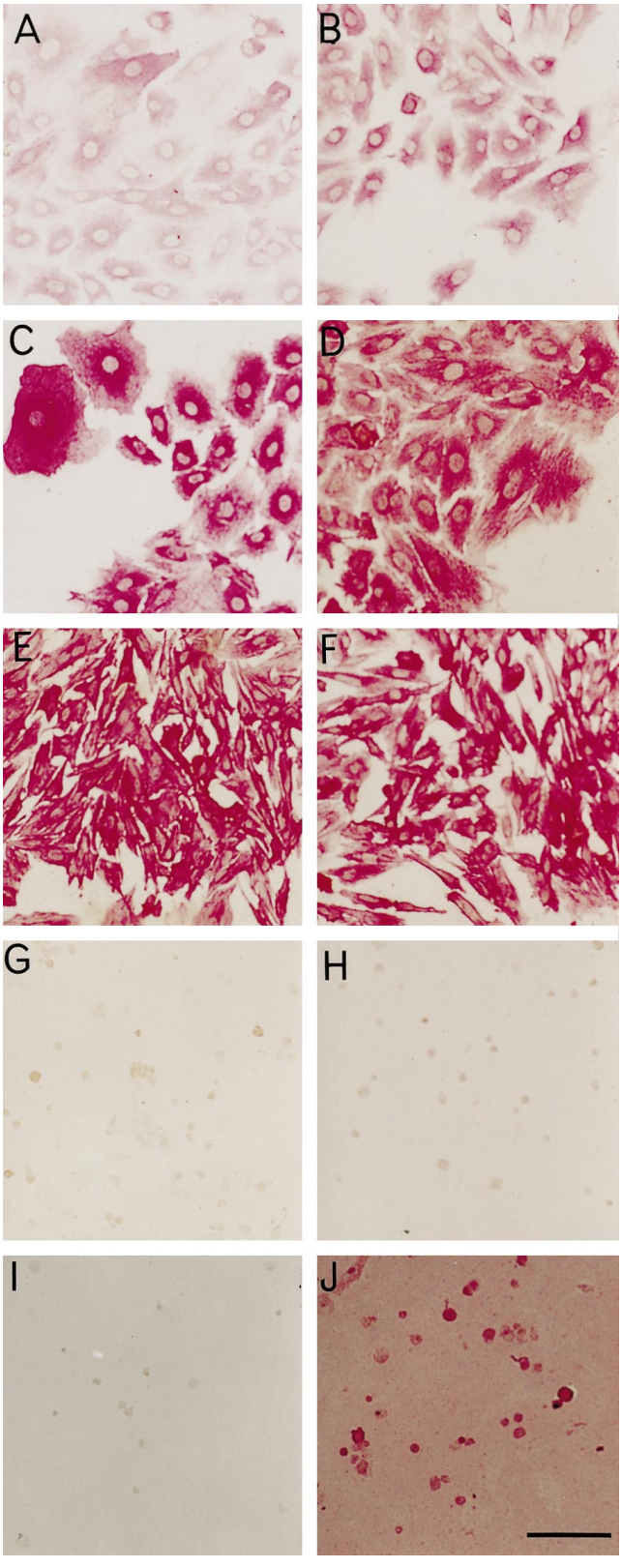
Fig. 3 Immunocytochemical demonstration of K18 filament assembly in BrdU-treated DLKP cells. Filaments were seen to radiate from the nucleus, throughout the cytoplasm, to the cell membrane. (*Bar* 6 µm). Similar filaments were seen in cells stained with K8 antibody (not shown)

Induction of keratin protein synthesis

Each cell line was examined immunocytochemically for the presence of keratin filaments before and after exposure to 10 µ*M* BrdU. K8, K10, K14 and K18 were selected for initial studies, as these keratins are likely to be expressed in lung epithelial cells similar to those in our study [35]. K8 and K18 were not detected by immunocytochemistry or Western blot analysis in DLKP or H82 cells before treatment with BrdU, but following treatment, K8- and K18-containing filaments were present in approximately 10% of DLKP and 50% of H82 cells (Fig. 2). Increased numbers of K8- and K18-positive cells and increased intensity of staining was evident in DLKP cells treated with BrdU for 21 days (Fig. 2E, F). The keratin filaments extended from the nuclear lamina out to the cell membrane (Fig. 3) indicating that K8 and K18 proteins induced by BrdU were functional and capable of dimerisation and filament assembly. Subsequent immunoprecipitation and Western blot studies revealed low levels of K8 and K18 proteins in DLKP and H82 prior to treatment and increased levels of both proteins in each cell line after 7 days of BrdU exposure (Fig. 5). Neither K10 nor K14 proteins were detected in either cell line before or after treatment (results not shown).

K8 and K18 filaments were present in A549 cells prior to BrdU treatment (Fig. 4A, B). Increased K8 and K18 protein synthesis was detected immunocytochemically following treatment (Fig. 4C–F), and this was confirmed by Western blotting (Fig. 5). Unlike DLKP, H82

and HL-60, immunoprecipitation prior to Western blotting was not required to detect K8 and K18 proteins in A549 cell lysates as signals were sufficiently strong. Again, K10 and K14 proteins were absent both before and after treatment (results not shown). None of the keratin proteins examined here were detected immunohistochemically or by Western blotting or by immunoprecipitation in HL-60 cells before or after exposure to BrdU (Figs. 4G–J and 5). CD 14 was used as a positive control in HL-60 immunohistochemistry experiments.



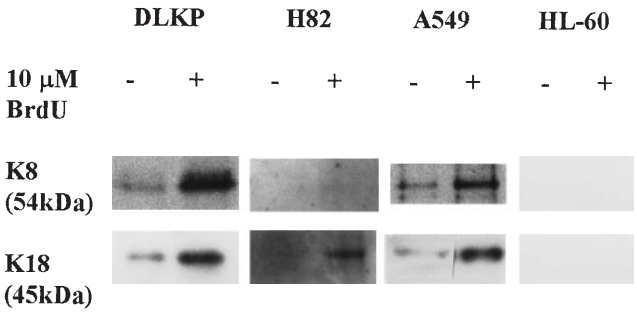


Fig. 5 Immunoprecipitation and Western blot analyses of K8 and K18 proteins in each cell line following treatment with 10 μ*M* BrdU for 7 days. For DLKP, H82 and HL-60, 1×10^7 cells per immunprecipitation reaction were used. H82 cells contained very low levels of K8 and K18 proteins prior to treatment, resulting in barely visible signals. Stronger signals, though still relatively weak, were present after treatment. No K8 and K18 proteins were detected in HL-60 lysates before or after BrdU treatment. Immunoprecipitation was not required to detect A549 K8 or K18 proteins by Western blot and 2×10^4 cells were run per lane

The possibility that keratin synthesis occurred as a result of toxic stress induced by BrdU, rather than a specific differentiation effect, was investigated by exposing each cell line to the chemotherapeutic drug adriamycin. Exposure to 0.02 μg/ml adriamycin for 7 days resulted in approximately 75% cell death. However, none of the BrdU-induced effects such as keratin synthesis, increased cell size or increased adherence occurred in response to adriamycin exposure (results not shown).

Keratin mRNA levels following BrdU treatment

To determine if the induction and upregulation of keratin protein synthesis observed in these lung cell lines reflected alterations in transcription of their respective genes, Northern blot analyses were carried out. K8 and K18 mRNA levels were examined before and after BrdU treatment. K8 mRNA was present in all four cell lines prior to treatment with BrdU, although, as was also the case for K18, A549 produced the strongest signals (Fig. 6). K8 mRNA levels did not increase in any cell line over 21 days of BrdU treatment. K18 mRNA was present in

Fig. 4A–J Immunocytochemical analysis of keratin protein expression in A549 and HL-60 cells. Untreated A549 cells were positive for K8 (**A**) and K18 (**B**) on day 7. K8 (**C**) and K 18 (**D**) expression appeared upregulated in 10 μ*M* BrdU-treated A549 cells on day 7. Expression of K8 (**E**) and K18 (**F**) remained upregulated after 21 days of BrdU treatment, although cultures were approaching confluency at this time. Untreated HL-60 cells were negative for K8 (**G**) and for K18 (not shown) on day 7. HL-60 cells treated with BrdU for 7 days were also negative for K8 (**H**) and K18 (**I**), but positive for CD14 (**J**), a positive control. (*Bar* 20 μm)

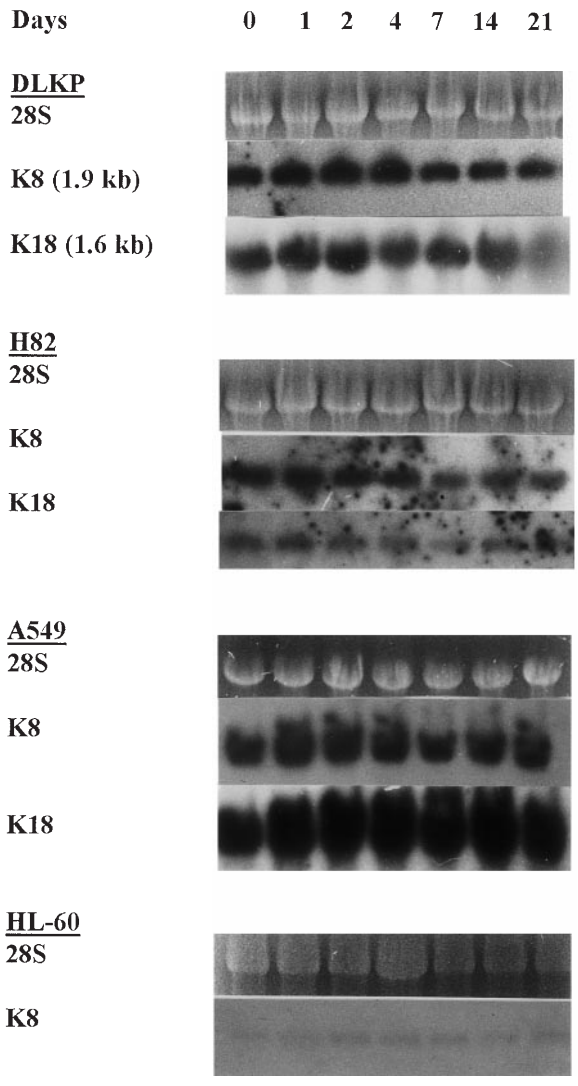


Fig. 6 Northern blot analysis of K8 and K18 mRNA in each cell line. Ethidium bromide staining of 28S rRNA is indicated to demonstrate equal loading. A K18 signal was not detectable in HL-60 cells. A549 blots were exposed at −70° C for 16 h and all others for 10 days

each cell line with the exception of HL-60, where no K18 mRNA was detectable in Northern blots before or after BrdU treatment. K18 levels remained unchanged in DLKP and H82 following treatment. However, it is possible that BrdU induced an increase in A549 K18 mRNA.

RT-PCR was carried out using K18-specific primers to determine if low levels of mRNA which were undetectable by Northern blot analysis were present in HL-60 cells. K18 mRNA was subsequently detected in HL-60 cells both before and after BrdU treatment (Fig. 7). The RT-PCR data are not quantitative but specifically demonstrate the presence of K18 mRNA transcripts. RT-PCR was also carried out on total RNA from DLKP, H82, and A549, and K8 and K18 transcripts were again shown to be present in each cell line prior to and during BrdU treatment, confirming the Northern blot results (results not shown).

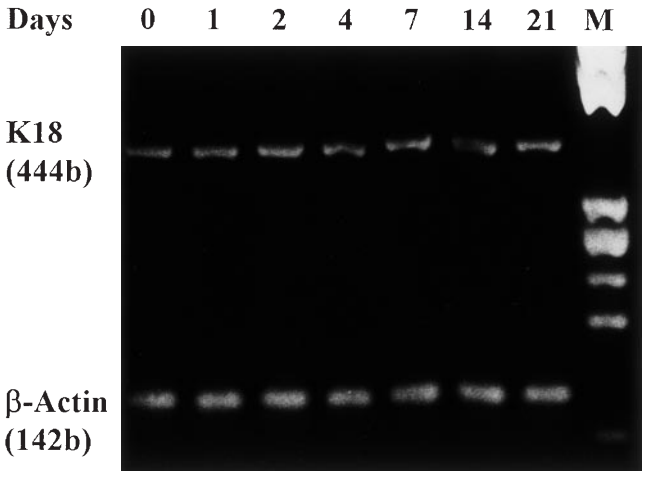


Fig. 7 Reverse transcriptase polymerase chain reaction analysis of K18 mRNA in HL-60 cells treated with 10 μ*M* BrdU for 21 days. β-Actin was used as an internal control. *Lane M* contains molecular-weight markers

Discussion

Relatively little is known concerning the mechanisms which control the complex patterns of keratin protein synthesis in epithelial cells. While posttranscriptional [3, 9, 22, 32, 38] and, less frequently, translational [38, 44] and posttranslational [2, 17] regulation of keratin synthesis have been reported, most studies carried out to date have demonstrated regulation to be at the mRNA level [3, 34, 36]. In vitro cell culture systems have indicated that agents as varied as retinoids [20], thyroid hormone [41], calcium [49], nickel [3] and transformation by SV40 [29] can alter keratin protein synthesis.

We have investigated the effect of the differentiation modulation agent, BrdU, on keratin synthesis in keratin-negative human lung tumour cell lines. Only one other report of a related study appears in the literature, where Pampinella et al. [33] observed K8 and K18 protein synthesis in rabbit serosal cells following BrdU treatment, while untreated cells contained only K18. Messenger RNA levels were not examined in their study. Such findings have led to speculation that genes encoding so-called 'epithelial-specific' proteins such as keratins are not as strictly regulated as was once believed [21]. Several reports describe the detection of keratin mRNA transcripts in cells which do not contain the corresponding keratin proteins. K8 mRNA is present in rat liver epithelial cell lines which do not express K8 protein [2], and K8 and K18 mRNAs have been detected in normal bone marrow, normal lymph node and normal peripheral blood cells, cultured endothelial cells and cultured skin fibroblasts [7, 43]. Similarly, each of the three keratin-negative cell lines used in our study was found to contain keratin mRNA transcripts prior to treatment with BrdU, demonstrating that controls other than those at the gene-transcriptional level are involved in keratin synthesis in these cells.

Tyner and Fuchs [44] have also demonstrated the presence of K6 and K14 mRNA in skin in the absence of corresponding proteins. The authors propose that an unidentified physiological block in translation prevents K6 protein synthesis until required during episodes of hyperproliferation, and that deregulation of this block could be associated with some disease states. Understanding the mechanism of such differentiation blocks has led to developments in differentiation therapy. Most notably, in the case of acute promyelocytic leukaemia (APL), all-*trans* retinoic acid successfully reverses the arrest in maturation of blood cells which leads to APL [31]. Identification of other blocks in cancer cell differentiation should reveal possibilities for similar strategies.

Most epithelial tumours synthesise keratin proteins, although it is not clear if the structure and function of these keratin filaments is identical to that of normal cells. An exception is SCLC-V, where keratin proteins are often absent. Alterations in cell adhesion molecules have also been reported in these cells [8]. In general, in vivo, these poorly-differentiated tumours exhibit increased invasiveness and have a poorer prognosis compared to well-differentiated tumours [1]. The loss of function or alteration of epithelial features such as keratins and proteins involved in cell-cell and cell-matrix contacts appears to facilitate cell migration and metastasis. However, the molecular mechanisms controlling these blocks in differentiation are unclear. We have demonstrated that a block in K8 and K18 protein synthesis and filament formation occurs at a posttranscriptional level in DLKP and H82 cells and that BrdU exposure results in the removal of this block. Whether this is by positive or negative regulation is unclear at the moment. Although K8 and K18 proteins were not immunocytochemically detectable in untreated DLKP or H82 cells, the same keratin antibodies could detect low levels of these proteins in the more-sensitive immunoprecipitation and Western-blot studies. This may be due to a basal level of translation activity, unstable translation products or soluble forms of keratin molecules which cannot dimerise and form filaments. In three of the four cell lines examined here, BrdU did not alter keratin mRNA levels, suggesting its effect was directly or indirectly on protein translation or stabilisation of translation products already prsent, or both. The increase in keratin protein levels observed in keratin-positive A549 cells could also reflect an increase in either translation or stability of translation products, although K18 mRNA levels appeared elevated following BrdU treatment, indicating control at the transcriptional and/or posttranscriptional level may also occur in this cell line.

Keratin protein synthesis in response to BrdU was limited to a proportion of DLKP and H82 cells. Double-labelling in situ hybridisation and immunocytochemical studies will be required to determine whether keratin mRNA transcripts are present only in those cells which proceed to synthesise keratin protein, or whether keratin mRNA is universally present but translated only in a subpopulation of permissive cells. Heterogeneity in keratin protein synthesis may reflect the presence of different subpopulations within the tumour cell lines. DLKP contains at least three such populations [25], but each has been found to express K8 and K18 in response to BrdU treatment (results not shown). Heterogeneous responses have been previously reported using calcium as a differentiating agent for normal mouse epidermal cells, where keratin synthesis was shown only in a minority (10%–50%) of cells [34, 49]. Further studies examining a range of culture conditions including various differentiating agents may reveal circumstances under which keratin protein synthesis is inducible in a higher proportion of cells.

It has been proposed that BrdU selectively modulates the expression of a class of regulatory genes which are important in the differentiation processes of various cell lineages [40]. The effects of BrdU on HL-60 haematopoietic cell differentiation are well documented [47, 48]. However, we found that BrdU does not induce keratin protein synthesis in these cells as it does in epithelial cells, despite the presence of the corresponding keratin mRNA. This may indicate that an alternative mechanism which BrdU does not affect prevents keratin protein synthesis in haematopoietic cells, in contrast to lung epithelial cells. Alterations in cellular morphology were also apparent in DLKP, H82 and A549 cells treated with BrdU. H82 cells were induced to adhere and spread, indicating that changes in the expression of cell adhesion molecules may have occurred in addition to alterations in cytoskeletal proteins. An increase in cell surface area was also evident in DLKP and A549 cells. No such morphological effects were apparent in BrdU-treated HL-60 cells. This indicates that BrdU may be affecting these cell lines in a lineage-specific manner, altering the synthesis of epithelial cytoskeletal proteins and adhesion molecules in DLKP, H82 and A549, but affecting a different set of haematopoietic-related proteins in HL-60, as has previously been determined [47, 48]. We are currently investigating possible alterations in the synthesis of adhesion molecules and other epithelial-specific proteins in BrdU-treated cells in order to more fully characterise the differentiation pathways involved.

In conclusion, there appears to be a posttranscriptional block on K8 and K18 synthesis in DLKP and H82 cells which can be reversed by exposure to BrdU. In addition, BrdU-induced keratin protein synthesis seems to be epithelial cell-specific. While the extent to which this process corresponds to epithelial differentiation in vivo remains to be determined, it should nonetheless prove to be a useful system for examining mechanisms which lead to blocks in epithelial differentiation and subsequent tumour formation. It is also possible that this regulatory step is involved in normal physiological maturation of primitive or stem cell-stage lung epithelial cells [11].

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