Vol. 197, No. 1, 1993
November 30, 1993
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 46-54

REGULATION OF PROLIFERATION-SPECIFIC AND DIFFERENTIATION-SPECIFIC GENES DURING SENESCENCE OF HUMAN EPIDERMAL KERATINOCYTE AND MAMMARY EPITHELIAL CELLS

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Received October 6, 1993

SUMMARY: The expression of proliferation-associated genes, cdc2 and E2F-1 and squamous-specific genes transglutaminase type I and cornifin were examined in senescing human epidermal keratinocytes. Cultured keratinocytes underwent 34 population doublings before senescing. Senescence in keratinocytes was characterised by reduced thymidine incorporation, a change in morphology and the inability of cells to undergo mitosis. This was accompanied by downregulation of cdc2 and E2F-1 mRNA's. In addition, senescing keratinocytes started to express genes such as cornifin, that are specific for squamous differentiation. These changes were similar to those observed in keratinocytes induced to differentiate with phorbol ester or by confluence. E2F-1, cdc2 and cornifin were similarly altered in senescing human mammary epithelial cells. Our data suggest that events regulating senescence may also be linked to squamous differentiation.

Primary cultures of eukaryotic cells have a finite replicative potential. Termination of replication is characterised by an irreversible withdrawal from the cell cycle whilst maintaining viability and protein/RNA synthesising capability. This process is referred to as replicative senescence. It has been suggested that senescence is a genetically programmed event (1,2). Several observations support this contention. For instance, the introduction of human chromosome 1 induces replicative senescence in immortal syrian hamster cells suggesting specific gene(s) are involved in senescence (3). Furthermore, genes such as cdc2 (4,5), cyclin A and cyclin B (5) have been reported to be downregulated in senescing fibroblasts. A lack of reponsiveness to mitogenic stimuli such as serum (5), PDGF, EGF or IGF-I has also been reported in senescent cells (1).

Since many neoplastic cells have lost the ability to senesce, an understanding of the mechanisms controlling senescence has obvious application to the study of its dysregulation during carcinogenesis. To date most studies of senescence have used fibroblasts. However, the appearance of tumors in such cell types is 100 fold less frequent than in epithelia (6). Our interest in senescence centers on senescence as a naturally-occurring growth inhibitory pathway.

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0006-291X/93 $4.00

Keratinocytes are an excellent model to study this process since primary cultures of human epidermal keratinocytes undergo replicative senescence (7) and are a common site of tumour occurrence. Since these earlier studies, specific genes have been identified that are closely associated with keratinocyte proliferation such as cdc2 (8,9) and E2F-1 (9). In addition, several specific markers have been identified that are induced both *in vivo* and *in vitro* during squamous differentiation of keratinocytes (10). The squamous differentiation pathway in keratinocytes can be manipulated by diverse stimuli such as treatment with the phorbol ester, 12-0-tetradecanoylphorbol-13-acetate (TPA), or treatment with interferon-γ (9,11) which result in irreversible growth arrest and expression of squamous-specific genes. Other agents such as transforming growth factor-β1 induce reversible growth arrest whilst suppressing the expression of the squamous-specific marker genes (9).

In the present study we analyze the expression of proliferation markers (E2F-1 and cdc2) and differentiation markers (cornifin and transglutaminase type I) during senescence of human keratinocytes and compare them to those induced by stimuli known to induce growth arrest and/or differentiation. This strategy would allow us to determine whether senescence of keratinocytes senescence resembles terminal differentiation or another type of irreversible growth arrest. We demonstrate that senescence of keratinocytes is accompanied by the downregulation of proliferation associated genes E2F-1 and cdc2 and the induction of squamous-specific marker genes cornifin (SQ37) and transglutaminase type I. Our studies indicate a link between the signalling pathway leading to senescence with that leading to squamous differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture: Second passage normal human epidermal keratinocytes (NHEK's) and sixth passage human mammary epithelial cells (MEC's) were obtained and cultured in keratinocyte growth medium or mammary epithelial cell growth medium (Clonetics; San Diego, CA) respectively. Growth data for early passage keratinocytes were generously provided by Clonetics. Similar data for MEC's were unavailable due to the method of selection of the MEC's. In all experiments cells were plated at a density of 2 - 5.5 x 10^3 cells / cm^2. 16 hours later the plating efficiency was determined to estimate the initial cell number plated. Cells were harvested and thymidine incorporation determined when cultures reached approximately 40% confluence. Population doublings of serially passaged cultures were estimated using the following equation: $n = (\log_{10}F - \log_{10}I) / 0.301$ where n = the population doublings, F = cell number at the end of the cell culture passage and I = the attached cell number at the beginning of the culture passage.

12-0-tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma Chemical Co. (St Louis, MO). Human recombinant transforming growth factor-beta1 (TGF-β1) was obtained from R & D Systems (Minneapolis, MN).

RNA Isolation and Northern Analysis: RNA isolation and preparation of northern blots has been previously described (11). Blots were probed for c-myc, cdc2, E2F-1, RBAP2, cornifin (SQ37), transglutaminase type I (TGase I) and chicken glyceraldehyde phosphate dehydrogenase (GAD). Probe preparation for E2F-1[1], cdc2, transglutaminase type I, c-myc, SQ37 and GAD has been described (9). A probe for RBAP2 was prepared from human fetal lung mRNA (9) using primers to the RBAP2 sequence (12; 5' primer CAAACATGCCGTATGGCTTA; 3' primer TCTACTTCATGGCAAACCAA). All probes were gel purified and labeled by random primer

[1] Saunders,NA.,and Jetten,AM, submitted for publication.

labeling (BRL; Gaithersburg, MD) to a specific activity of 0.5 - 2 x 10^9 cpm [^{32}P] / μg DNA . Prehybridization, hybridization and washing conditions were previously described (11).

Protein and Western Blotting: Cells were rinsed twice with PBS and scraped into sample buffer (7% glycerol, 0.5 mM ethylene diamine tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 2% sodium dodecyl sulfate, 10 mM dithiothreitol, 60 mM Tris.HCl, pH 6.8). Total cellular protein was isolated and equal amounts loaded onto 10/20 % gradient SDS PAGE gels. Following fractionation samples were transferred to Nitroplus 2000 nitrocellulose membranes (Integrated Separation Systems; Natick, MA) as described (13). Immunodetection of cornifin has been described (9). Immunodetection of cdc2 used a commercially available antibody (Upstate Biotechnology Inc; Lake Placid, NY; #05-161) and was detected using the ECL immunodetection kit (Amersham: Arlington Heights, IL).

DNA Synthesis Assay: The proliferative capacity of the senescing cultures was estimated by their ability to incorporate thymidine into their DNA. Cells were incubated with 2.5 μCi / ml of [^{3}H] methyl thymidine (5.0 Ci / mmole; Amersham) for 3 hours at 37°C and thymidine incorporation estimated (9).

RESULTS

Growth Characteristics and Morphology During Keratinocyte Senescence: Proliferating NHEK's grew as small colonies of polygonal shaped cells (Fig. 1A). As the NHEK cultures senesced the cells aquired a flattened appearance with frequent binucleated cells and a vacuolated cytoplasm (Fig. 1B). Figure 2 demonstrates that in the particular donor studied replicative senescence was attained after 34 population doublings. The senescent state of these cultures was confirmed by the minimal [^{3}H] thymidine incorporation and the observation that these cultures could be maintained in an apparently viable state in the absence of mitosis (Fig. 2).

Expression of Proliferation- and Differentiation-Specific Marker Genes: By examining changes in gene expression at various times during keratinocyte senescence we hoped to identify genes that play a role in senescence. Using this strategy it was also possible to compare the time of changes in gene expression relative to one another. Figure 3 shows that the expression of E2F-1 and cdc2 mRNA was downregulated progressively as the cultures approached senescence. Moreover, the decrease in mRNA levels for these genes correlated very closely with the growth curve shown in Fig. 2 and the thymidine incorporation data (Fig. 2). Conversely, the differentiation marker cornifin (SQ37) was induced in predominantly senescent cultures. The downregulation of cdc2 and E2F-1 mRNA preceded that of the induction of cornifin by approximately 5 doublings (Fig. 3). The expression of cdc2 protein was downregulated prior to cornifin protein induction (Fig. 4). The protein levels of cdc2 and cornifin also appeared to be more responsive to senescence (compare Fig. 3 with Fig. 4).

We compared the expression of proliferation-associated and differentiation-specific genes during senescence with the changes that accompany treatment with TPA or TGFβ1. Figure 5 indicates that mRNA for the proliferation associated genes E2F-1 and cdc2 are reduced by TGFβ1 (100 pM), TPA (80 nM), confluence (differentiation) and senescence. As in senescent cells, expression of mRNA for the squamous-specific marker, cornifin, was induced in TPA treated and differentiated cultures but not significantly in TGFβ1 treated cultures (Fig. 5).

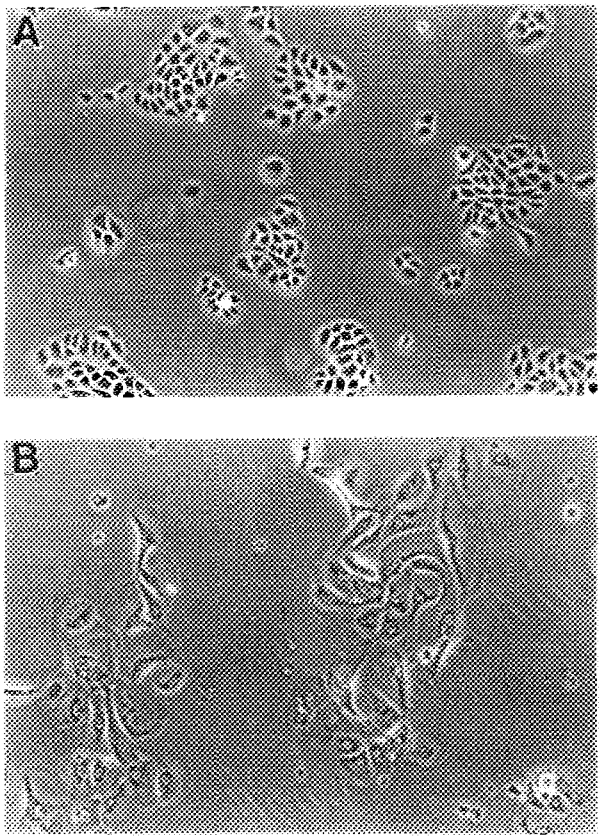

FIGURE 1. Morphology of Senescing Human Kerationocytes. Subconfluent cultures of human epidermal keratinocytes following 14 doublings (A) or 34 doublings (B).

However, c-myc mRNA was not altered in senescing keratinocytes (Fig. 3) whereas it is dramatically reduced by other growth-inhibitory stimuli such as TGFβ1 (9) and TPA (11). These data clearly show that senescing keratinocytes exhibit some, but not all, of the changes in gene expression that occur during keratinocyte differentiation.

Senescence in MEC's: To determine whether the characteristics of senescence in NHEK's are common to other epithelial cell types we examined gene expression in senescing mammary epithelial cells. MEC's were purchased as passage 7 cultures and the growth curve for all further doublings (approximately 36 doublings) is presented in figure 6A. Similar to NHEK's, MEC's downregulated the proliferation-specific genes cdc2 and E2F-1 (Fig. 6B). In addition, senescent MEC's expressed the squamous-specific marker, cornifin (Fig. 6B). However, the squamous-specific gene transglutaminase type I was not induced during MEC senescence (not shown). The downregulation of the proliferation markers and the induction of a squamous-specific marker correlated with an 8 fold decrease in thymidine incorporation of the early passage cultures (4641 ± 585 dpm μg / protein) compared with the late passage senescent cultures (561 ± 49 dpm μg /

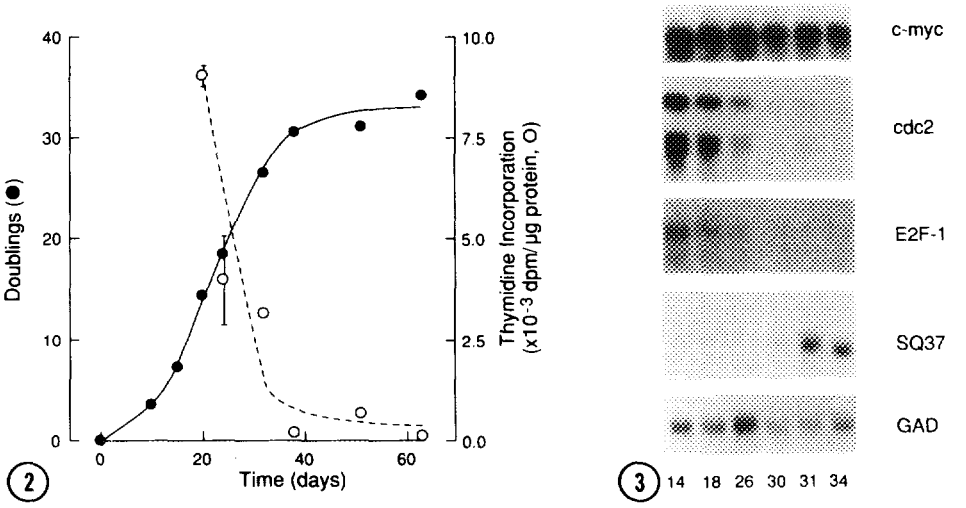

FIGURE 2. Growth Curve and [^{3}H] Thymidine Incorporation in Senescing Human Keratinocytes. Subconfluent cultures of human epidermal keratinocytes were assayed for their ability to incorporate tritiated-thymidine at various intervals during their replicative lifespan (o; dashed line). Data presented as mean ± sem of triplicate determinations normalised for cellular protein. The cumulative number of cell doublings is also shown (●; solid line).

FIGURE 3. Expression of mRNA's for Proliferation- and Differentiation-Specific Genes During Human Keratinocyte Senescence. Subconfluent cultures of NHEK's were collected at the various population doublings shown and 20 µg total RNA blotted and probed for the proliferation-associated genes c-myc, cdc2 and E2F-1 or the differentiation-specific gene cornifin (SQ37). GAD levels are shown to compare for loading inequalities. Population doublings are shown below the lanes.

protein; Fig. 6B). mRNA expression for other possible proliferation markers such as c-myc or the E2F family member RBAP2 were not altered (Fig. 6B). RBAP2 mRNA was not downregulated in senescing NHEK's either (not shown). The senescent state of the cultures was confirmed by the low thymidine incorporation, the expression of the differentiation markers and the attainment of a stable plateau phase on the growth curve (Fig. 6A & B).

DISCUSSION

The present study is a clear example of programmed differentiation accompanying that of replicative senescence in epithelia. This is in contrast to some other cell systems in which senescence is accompanied by loss of differentiated functions. For instance, changes in differentiation-specific functions have been reported for senescing adrenocortical cells in which the cells lose the ability to induce 17α-hydroxylase expression in response to cAMP (14). Similarly, trabecular meshwork cells also display changes in differentiated functions when they senesce (15). The cell type specificity of the senescence program is aptly demonstrated by the loss of differentiated function in senescing adrenocortical cells and the induction of differentiation in senescing keratinocytes.

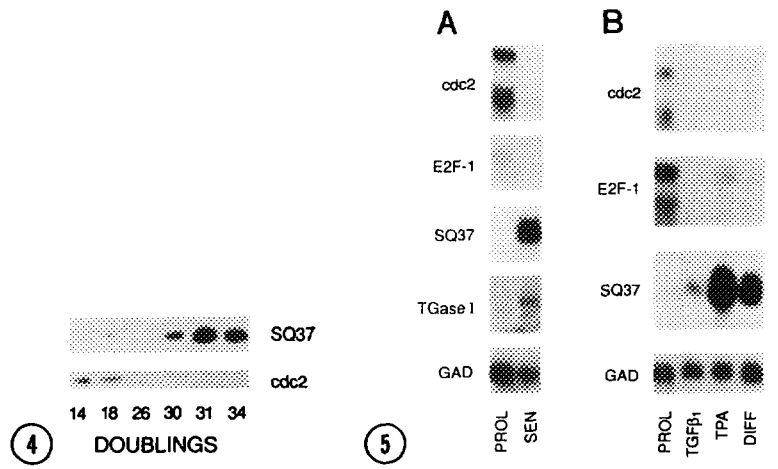

FIGURE 4. Protein Levels for Proliferation- and Differentiation-Specific Genes in Senescing NHEK's. Proteins were isolated from the same cultures described in Fig. 3. 20 μg of protein / lane was loaded and immunodetection of cornifin (SQ37) and cdc2 performed as described in "Experimental Procedures".

FIGURE 5. Comparison of The Effects of Growth Arrest and Differentiation Stimuli with Senescence. Total RNA (20 μg / lane) from subconfluent cultures of NHEK's were isolated, blotted and probed for GAD and the proliferation associated (cdc2 and E2F-1) and the differentiation-specific genes cornifin (SQ37) and transglutaminase type I (TGase I). A) Cells were treated with vehicle alone (PROL) or allowed to senesce (SEN). B) Cells were treated with vehicle alone (PROL), 100 pM TGF-β1 (TGFβ1), 80 nM TPA (TPA) as indicated or allowed to differentiate (DIFF).

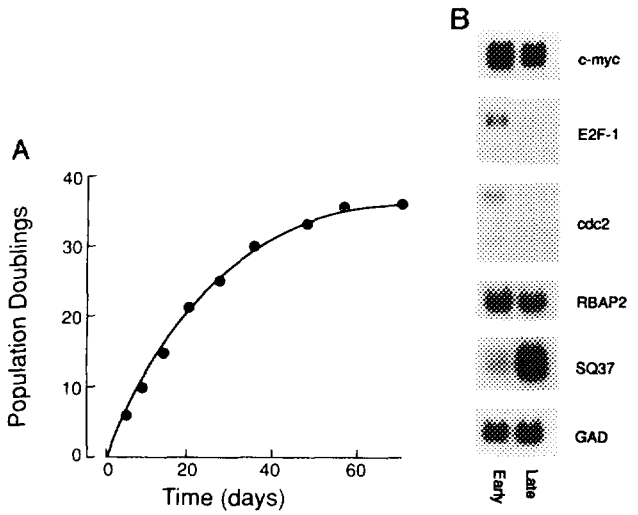

FIGURE 6. Growth Curve and Marker Gene Expression in early Passage and Senescent Mammary Epithelial Cells. Subconfluent cultures of MEC's were serially passaged and A) the cumulative cell doublings estimated (●). B) 20 μg total RNA / lane from cells at early passage and late passage was probed with proliferation-associated (cdc2 and E2F-1) and the squamous-specific gene cornifin (SQ37). In addition, MEC total RNA was also probed for RBAP2, c-myc and GAD gene expression.

The lag between the downregulation of proliferation-associated genes and the expression of squamous-specific marker genes in senescing keratinocytes support the previously proposed multistep pathway of squamous differentiation in keratinocytes. For instance, the induction of keratinocyte differentiation by phorbol ester or IFNγ treatment or the attainment of confluence is characterised by decreases in cdc2 and E2F-1 mRNA levels and the induction of cornifin and transglutaminase type I mRNA (9). The actions of phorbol esters (11) and IFNγ (9) are mediated by different signal transduction pathways which share the ability to induce irreversible growth arrest and squamous differentiation. Senescence clearly shares these properties. Whether growth arrest and differentiation induced during senescence shares the same signalling pathway as IFNγ or TPA has yet to be determined. The existence of different transcription factors being associated with the control of either the growth arrest-genes or the various differentiation-marker genes in keratinocytes has been previously proposed (16) and is supported by reports indicating a specific PKC requirement for spinous to granular differentiation in the intact epidermis (17). Moreover, recent reports have identified transcription factors that may participate in the various stages of epidermal differentiation. One factor, basonuclin, has been cloned and hypothesised to play a role in the early events of basal cell transition to the differentiating suprabasal cell compartment (18). Two other factors, Skn-1a and Skn-1i have been cloned and are able to transactivate the promoter of the squamous-specific gene keratin K10 (19). It has been difficult to discriminate between factors specifically affecting proliferation or differentiation in squamous differentiating keratinocytes since the two phases may only be separated by a few hours (9). In contrast, the regulation of these two phases in senescing keratinocytes are separated by several days. This lag between the downregulation of proliferation markers and the induction of differentiation markers in senescing keratinocytes coupled with the proposed specificity of some transcription factors for different steps in the differentiation pathway may indicate that a cascade of transcriptional events is required during keratinocyte senescence and possibly during squamous differentiation. The senescent keratinocyte model reported herein may provide a useful model for the study of these transcriptional effectors due to the relatively large time interval between downregulation of proliferation markers and induction of differentiation markers.

Both cdc2 and E2F-1 expression were downregulated at the onset of replicative senescence. The downregulation of cdc2 in two distinct epithelial cell types in the present study taken with the elevated levels of cdc2 expression previously reported in spontaneously immortalised human keratinocytes (8) and the downregulation of cdc2 in senescing fibroblasts (4,5) suggests that cdc2 downregulation may be common to all senescing cells. Cell cycle-dependent expression of E2F-1 was recently reported (20,21) and coupled with our previous report of the downregulation of E2F-1 mRNA in differentiating keratinocytes (9) it is apparent that E2F-1 may also be a marker of replicative senescence. In addition, other proliferation-associated genes such as cyclins A and B are also downregulated during senescence (5). It is unlikely that all these genes are causally involved in senescence. Indeed, transfection of cdc2 into senescent fibroblasts fails to reverse the senescent phenotype (5). The above evidence indicate that it is more likely that senescence may be the product of a single or very few transcriptional event(s) initiating the downregulation of many genes such as E2F-1, cdc2 or the cyclins that are

associated with proliferation. This is supported by the ability of a single chromosome to induce senescence (3) and the mapping of at least four complementation groups to the control of senescence (2).

A model of growth control in cycling cells has been proposed in which E2F is an essential regulator of cell proliferation (12,22,23). Many effectors of proliferation such as the tumour suppressor genes Rb or p107 as well as the DNA tumour viruses SV40, papillomavirus 16 and 18 as well as adenovirus A5 appear to operate via interactions with E2F (24). In cycling cells, hypophosphorylated Rb binds E2F presumably disabling its ability to transactivate S phase specific genes (22,24). Since the phosphorylation state of Rb can dictate E2F transcriptional activity, the regulation of Rb phosphorylation will be of importance in proliferation control. Cdc2 has been shown to phosphorylate Rb and p53 (22) thus alleviating growth suppression. Since both cdc2 and E2F-1 mRNA's are downregulated during NHEK and MEC senescence it is possible that the regulation of growth arrest during senescence differs to that controlling the cell cycle. As outlined above, cell cycle traverse requires activation and deactivation of cell cycle controllers. In contrast, the irreversible growth arrest accompanying senescence may be due to the termination of synthesis of cell cycle controllers.

In summary, the present study shows that senescence of keratinocytes involves both activating (differentiation markers) and repressing (proliferation-associated markers) activities separated by a large time interval. This program shares many similarities with the changes in gene expression that occur during keratinocyte differentiation and suggests a link between the signalling pathway leading to senescence and the pathway(s) inducing squamous differentiation.

ACKNOWLEDGMENTS: The authors appreciate the many thoughtful and constructive comments made by Dr. Kevin Mills. We are grateful to Dr's Lawrence Ostrowski and Russell D. Owen for their criticisms of this manuscript prior to submission.

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