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Serum factors regulate the expression of the proliferation-related genes α5 integrin and keratin 1, but not keratin 10, in HaCaT keratinocytes

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Abstract **In the highly coordinated programme of gene expression during keratinocyte proliferation and differentiation, α5 integrin and keratins 1 and 10 (K1/K10) may play important regulatory roles. We were interested in seeing whether, in continuously growing, immortalized HaCaT keratinocytes, similar to normal keratinocytes, the expression of α5 integrin and K1/K10 was related to cell proliferation and differentiation. After release from cell quiescence the expression of α5 integrin, both at the mRNA and protein levels, was upregulated in the cells. At the same time, K1/K10 mRNA and protein expression decreased dramatically, while the mRNA for D1 cyclin became detectable, and the cells became highly proliferative. These findings indicate that α5 integrin and K1/K10 are involved in the regulation of HaCaT proliferation and differentiation, as in normal keratinocytes. However, HaCaT cells are different from normal keratinocytes in their ability to lose K1/K10 expression. There is no evidence that the expression of K1/K10 can be reversed in normal keratinocytes. This ability of dedifferentiation might be a unique feature of HaCaT cells and may be a key component of their immortalized nature. We also found that serum factors regulate mRNA expression of α5 integrin and K1, but not of K10, in HaCaT cells. This information could be relevant to the understanding of normal epidermal differentiation.**

Keywords HaCaT keratinocytes · Proliferation · Differentiation · α5 integrin · Keratins 1 and 10

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Introduction

In the multilayered epidermis of normal human skin the keratinocytes undergo a continuous process of proliferation and differentiation. In this self-renewing process quiescent basal stem cells are triggered to produce suprabasal, transiently amplifying cells, which then exit the cell cycle and undergo terminal differentiation during their movement to the tissue surface. Proliferation and differentiation involve a highly coordinated programme of gene expression that includes both induction and suppression. While most stages of squamous differentiation are well characterized, including changes in gene expression, factors that regulate the induction and suppression of genes remain largely unknown. Integrins and keratins are two major groups of proteins that are closely related to keratinocyte proliferation and differentiation [1, 2, 13, 14, 26, 36]. Although it is unclear what mechanisms regulate the exit of keratinocytes from the cell cycle into senescence, both the functional downregulation of integrins and the expression of keratins 1 and 10 (K1/K10) seem to be involved in this fundamental step in the differentiation programme [1, 14, 19].

For a long time it was thought that keratins 1 and 10 (K1/K10) only have a structural role in cells, but more recent findings suggest that in addition to providing mechanical integrity to the cells in the context of a tissue, keratins also participate in signalling processes fundamental to cell physiology [28]. Ectopic expression of K10 has been shown to inhibit the proliferation of HaCaT keratinocytes [28], although in the in vivo epidermis, keratinocytes in the immediate suprabasal compartment, that already express K1/K10, can still proliferate (K1/K10$^+$ transiently amplifying keratinocytes) [4].

α5 integrin is poorly expressed in normal skin, but in highly proliferative skin such as fetal epidermis and wounded skin, and in psoriasis, its expression is upregulated [18, 21, 30, 35]. In all cases, α5 integrin is expressed only in undifferentiated keratinocytes, and it is lost from the cell surface as keratinocytes differentiate [1, 19, 26]. Fibronectin, through its main receptor α5β1 integrin, can

provide anchorage-independent direct cell cycle regulatory signals [34] by the direct modulation of cyclin-dependent kinase activity [33]. In ex vivo keratinocyte culture anti-α5 integrin monoclonal antibody inhibits the growth of keratinocytes already adhered on fibronectin [5].

HaCaT cells, although immortalized and genetically abnormal, are considered to be a good model for human keratinocytes [7, 31]. Therefore, they are commonly used in experiments examining effects of therapeutic drugs on keratinocyte physiology and studying keratinocyte biology [16, 23, 27, 28]. HaCaT cells form an almost normal epidermis with similar architecture to that formed by normal adult keratinocytes, including the formation of a basement membrane and keratinization, when transplanted on nude mice or cultured in organotypic culture with human dermal fibroblasts [8, 32]. However, the formation of normal tissue architecture is delayed in HaCaT transplants, indicating a reduced sensitivity to environmental signals compared to adult epidermal keratinocytes [8].

It is known that, in a similar manner to normal keratinocytes, HaCaT cells also express K1/K10 and α5 integrin [6, 31]. To explore the relationship between these proteins and cell proliferation in HaCaT cells, we forced the cells into quiescence by serum withdrawal and high density culturing, then released them into an almost synchronized, highly proliferative state by passaging into serum-containing medium, and looked at the expression of K1/K10 and α5 integrin at both the mRNA and protein levels in relation to cell proliferation. We found a close correlation between the transcriptional regulation of α5 integrin and K1/K10 keratins and cell proliferation in the HaCaT cells. Applying a different culture system, we were able to show that serum factors regulate the mRNA expression of α5 integrin and K1, but not of K10. Our results demonstrate that the application of different culture conditions for in vitro growing HaCaT cells results in useful model systems for investigating the mechanisms regulating keratinocyte proliferation and differentiation.

Materials and methods

Cell culture

Human HaCaT cells, kindly provided by Dr N.E. Fusenig (Heidelberg, Germany), were grown in 75-cm^2 cell culture flasks (Costar, Cambridge, Mass.) and maintained in high-glucose Dulbecco's modified Eagle's medium (high-glucose DMEM; Gibco, Eggstein, Germany), supplemented with 10% fetal bovine serum (FBS; Gibco), L-glutamine, penicillin/streptomycin and fungizone (Sigma, Budapest, Hungary) at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was changed every 2 days.

Synchronization procedure

HaCaT cells were synchronized by cultivation at high density in the absence of serum. After the cells had grown to 100% confluence in medium containing 10% FBS for 5 days, the medium was replaced by serum-free high-glucose DMEM. The cells were grown in serum-free medium for 1 week. The synchronized cells were trypsinized and were then seeded into 75-cm^2 culture flasks at a density of 5×10^3 cells/cm^2 in 10% FBS high-glucose DMEM. Cells were counted and their viability determined by trypan blue staining. Samples for propidium iodide (PI) staining, RT-PCR and Western blot analysis were taken from parallel cultures at different times after passaging the cells to the 10% FBS-containing high-glucose DMEM.

Immunocytochemistry

Serum-starved, confluent HaCaT cells were passaged into slide chambers (Nunc, Roskilde, Denmark) at a density of 4×10^4 cells/cm^2. The cells were grown in these chambers for different times and the medium was changed every 2 days. For immunocytochemical staining, the cells were fixed for 20 min at 4 °C in 2% paraformaldehyde (Sigma). They were then incubated overnight at 4 °C in a humid chamber with the primary antibodies. Mouse monoclonal antibodies specific for human α5 integrin at a dilution of 1:200 (clone SAM-1, Immunotech, Prague, Czech Republic) and mouse monoclonal antibodies specific for human K1 and K10 at a dilution of 1:100 (ICN, Budapest, Hungary) were used as primary antibodies. Mouse IgG1 was used for isotypic control staining. Immunostaining was performed using an avidin-biotin immunoperoxidase kit (Vectastain Elite Kit; Vector Laboratories, Burlingame, Calif.) with 3-amino-9-ethylcarbazol (Sigma) as the chromogen. The slides were counterstained with haematoxylin.

Immunoblotting

Total protein extracts from synchronized HaCaT cells at different times after the end of the synchronization process were prepared in a lysis buffer of 1.5% sodium dodecyl sulfate (SDS), 62.5 m*M* Tris-HCl, pH 6.8, 5 m*M* ethylenediamine tetraacetic acid, 5% 2-mercaptoethanol, 1 μg/ml antipain, 1 μg/ml chymostatin, and 1 μg/ml leupeptin (all from Sigma). Lysates were precleared by centrifugation and supernatants were stored at –20 °C. The constituent proteins of the keratinocyte extracts were separated by SDS/PAGE on a 9% separating gel and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, Calif.). In order to verify equivalence of the loadings of proteins in the wells, the gel and the nitrocellulose were stained with coomassie brilliant blue and ponceau S, respectively (Sigma). Membranes were blocked by incubation in Tris-buffered saline (150 m*M* NaCl, 25 m*M* Tris, pH 7.4) containing 0.05% Tween 20 (Sigma) and 3% nonfat dried milk (Fluka Chemie, Neu-Buchs, Switzerland) for 2 h at room temperature and then incubated overnight at 4 °C with the appropriate dilution of primary antibodies (mouse monoclonal anti-human α5 integrin (Transduction Laboratories, clone 1) diluted 1:250 and mouse monoclonal anti-human K1/K10 (ICN Biomedicals, clone AE2) diluted 1:400 in blocking buffer. Alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) was used as secondary antibody at a dilution of 1:2500 in the blocking buffer for 2 h at room temperature. Blots were developed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as substrate (BCIP/NBT, Sigma).

Propidium iodide DNA analysis

HaCaT cells were harvested by trypsinization and washed in PBS twice. The cell density was adjusted to 1.5×10^6 cells/ml, then 1 ml cell suspension was centrifuged at 1000 rpm for 10 min. The pellet was suspended in 70% cold (–20 °C) ethanol and fixed for at least 24 h at 4 °C. After fixation the cells were centrifuged at 3000 rpm and suspended in 1 ml PI staining buffer (50 μg/ml PI, and 100 U/ml RNase A; all from Sigma) and stained for 30 min at room temperature. The samples were analysed using Facscalibur (Becton Dickinson) and Modfit (Verity Software House, Topsham, Me.).

RT-PCR

Total RNA was isolated from 1×10^6 HaCaT cells using TRIzol reagent (Gibco) following the instructions of the manufacturer. First-strand cDNA was synthesized from 3 μg total RNA in 20 μl

final volume using a First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania). After reverse transcription, amplification was carried out by PCR using Taq DNA polymerase and dNTP Set of MBI Fermentas. A 10-µl aliquot of the reverse transcription solution was used as a template for α5 integrin-specific PCR, a 0.5-µl aliquot for K1- and K10-specific PCR, a 2-µl aliquot for cyclin D1-specific PCR and a 1-µl aliquot as a template for the β actin-specific PCR. Primers specific for human α5 integrin (5′-ATTATCAGAGCAAGAGCCGGATAGA-3′ and 5′-GGAGATGAGGGACTGTAAACCGA-3′), for human K1 keratin (5′-GGACATGGTGGAGGATTACCG-3′ and 5′-TGCTCTTCTGGGCTATATCCTCG-3′), for human K10 keratin (5′-GCAAAATCAAGGAGCGGTATGA-3′ and 5′-GAGCTGCACACAGTAGCGACC-3′) for human cyclin D1 (5′-AGGAGAACAAACAGATCA-3′ and 5′-TAGGACAGGAAGTTGTTG-3′) and for human β-actin (5′-AGAGATGGCCATGGCTGCTT-3′ and 5′-ATTTGCGGTGGACGATGGAG-3′) were included in the reactions at a final concentration of 0.66 pmol/µl.

The PCR reactions amplified a 358 bp product for α5 integrin, a 316 bp product for keratin 1, a 685 bp product for keratin 10, a 162 bp product for cyclin D1 and a 406 bp product for β-actin. The same PCR conditions were used for all PCR reactions: 94 °C 90 s, 60 °C 90 s, 72 °C 120 s. The number of cycles were as follows: 25 cycles for α5 integrin, 27 cycles for K1 and K10, 25 cycles for cyclin D1 and 25 cycles for β-actin. The concentration of $MgCl_2$ was 1.5 m*M* in all PCRs. The products were run on 2% agarose gel, stained with ethidium bromide, photographed and evaluated using a Kodak Edas 290 densitometer and Kodak 1D Digital Science software (Scientific Imaging Systems, New Haven, Ct.).

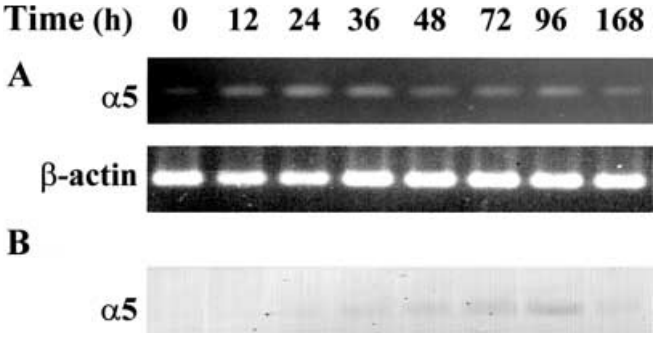

Fig. 1 A, B α5 integrin expression in HaCaT cells. HaCaT cells were arrested by serum deprivation and contact inhibition before passage to serum-containing culture medium. The expression of α5 integrin was analysed by RT-PCR (**A**) and by immunoblotting (**B**) at the times indicated after releasing the cells from serum starvation and contact inhibition

Results

Expression of α5 integrin in HaCaT cells is regulated by environmental conditions

In order to achieve homogeneity, we synchronized the HaCaT cells by cultivation at high density in the absence of serum for 1 week, thus avoiding the use of chemical treatments. We then forced the cells to re-enter the cell cycle by passaging them into 10% FBS-containing medium. The expression of α5 integrin, both at the mRNA and protein levels, was determined at 0, 12, 24, 36, 48, 72, 96 and 168 h following the end of the synchronization process, using RT-PCR, Western blot analysis and immunocytochemistry. The level of α5 integrin mRNA expression as determined by RT-PCR was very low at 0 h (Fig. 1A), but had risen by 12 h after release from quiescence and then gradually increased (24 h, 36 h) to a maximum at 48 h, remaining high until 96 h (Fig. 1A). This high-level expression coincided with intense proliferation of the cells. Before the cells became 100% confluent, the expression of α5 integrin mRNA started to decrease (168 h, Fig. 1A).

The level of α5 integrin protein showed very similar kinetics, but as expected, the increase in the α5 integrin mRNA preceded that of the protein. In synchronized serum-starved cells the level α5 integrin protein increased grad-

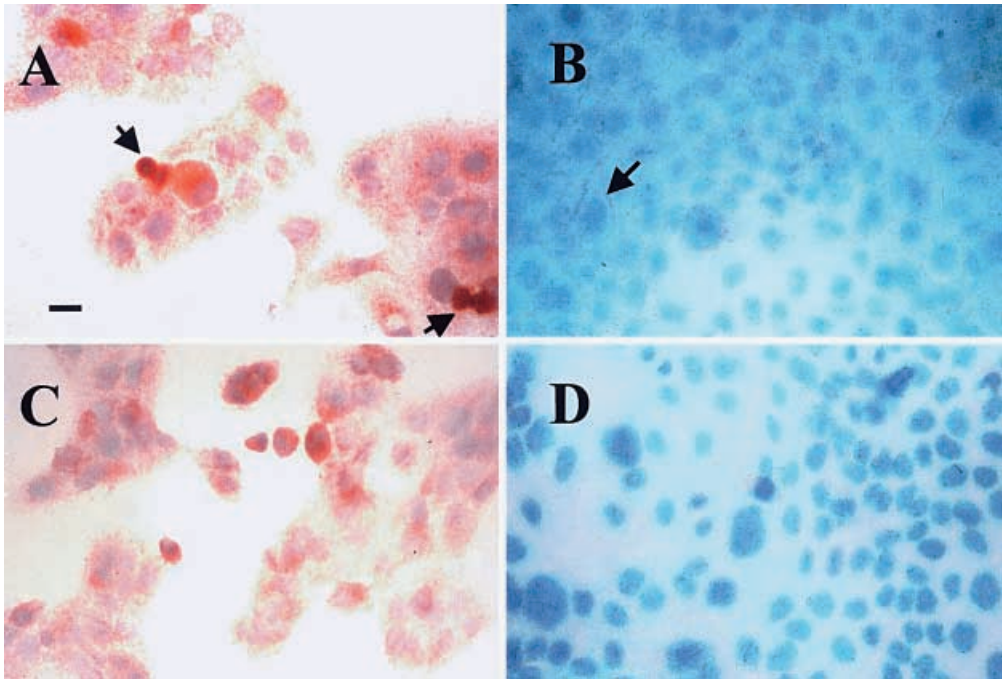

Fig. 2 A–D Immunocytochemical staining of HaCaT cells for α5 integrin and K1/K10 at different times after release from cell quiescence. Staining for α5 integrin is shown 36 h after serum addition and passage all cells in the culture (**A**). Mitotic cells in the culture (*arrows*) appear to have a stronger α5 integrin expression relative to the other cells (**A**). Later, when the cells reach higher density, the α5 integrin expression decreases (**B**). HaCaT cells stain strongly for K1 and K10 shortly after release from cell quiescence (**C**). Later, in the more confluent culture, the expression of K1 and K10 is decreased (*bar* 50 µm) (**D**)

ually from undetectable at 0 h to a maximum at 96 h, and had decreased considerably by 168 h (Fig 1B). Differences in the expression of α5 integrin among individual cells were also examined by immunocytochemistry. α5 integrin was similarly expressed in all cells (Fig. 2A) with the exception of mitotic cells, in which the expression appeared to be stronger (Fig. 2A, arrows). In the immunocytochemical analysis, as in the Western analysis, α5 integrin expression was highest before the cells started to become confluent after which, at higher cell densities, the expression faded (Fig. 2B). In the more confluent cultures when the protein level was low with an almost undetectable signal at 168 h in the Western blot analysis (Fig. 1B), a clear membrane localization of the protein was seen (Fig. 2B, arrow). This distinct membrane staining was not visible in highly proliferative HaCaT cells at the time of high protein synthesis (Fig. 2A).

HaCaT keratinocytes lose K1/K10 expression after release from synchronization

The expression of K1/K10 was investigated in the same system. Both K1 and K10 mRNAs were highly expressed in the synchronized, serum-depleted, and contact-inhibited HaCaT cells (Fig. 3A, 0 h). After release from quiescence the level of K1 and K10 mRNAs decreased dramatically. A very low level of K1 mRNA was still detectable at 12 h in culture, but K10 mRNA had become undetectable at 12 h. We were not able to detect K1 or K10 mRNAs after 12 h (Fig. 3A). Changes in the levels of K1 and K10 proteins were followed by Western analysis. The proteins were present in the cells in extremely large amounts in the synchronized serum-depleted cultures at 0 h but then started to decrease very slowly (12 h, 24 h, 36 h, 48 h, 72 h) and were still high even at 72 h after the end of synchronization, despite the apparent lack of new protein synthesis during this period (Fig. 3B). K1 protein had become undetectable by 96 h while K10 protein was still detectable even at 168 h (Fig. 3B), although its amount had decreased considerably.

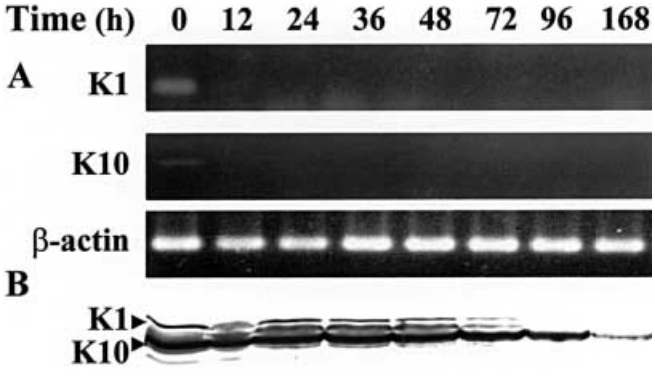

Fig. 3 A, B K1 and K10 expression in HaCaT cells. HaCaT cells were arrested by serum deprivation and contact inhibition, then stimulated to re-enter the cell cycle by passage to serum-containing medium. Changes in the expression of K1/K10 mRNAs were analysed by RT-PCR (**A**), K1 (67 kDa) and K10 (56.5 kDa) proteins were analysed by immunoblotting of whole-cell lysates (**B**) at the times indicated after release from cell quiescence. Amplified products of K1 (316 bp), K10 (685 bp) and the internal control β-actin gene (406 bp) were visualized by ethidium bromide staining on agarose gel

The expression of K1/K10 proteins was also examined by immunocytochemistry to make certain that the decrease in the levels detected by Western analysis was not due to differential expression by a subpopulation of cells in the culture. Individual cells did not show differences in K1/K10 expression and the expression kinetics observed by immunocytochemistry were very similar to those found in the Western analysis. The level of K1/K10 proteins decreased dramatically from an initial high level (Fig. 2C) to a very low level as the culture moved toward another confluent state (Fig. 2D). In contrast to α5 integrin, the expression pattern of K1/K10 always appeared cytoplasmic. It has been previously reported that K1/K10 expression is induced in HaCaT cells when the cells reach a sufficiently high density in the culture [31]. In our culture system, the high cell density culture is the one evaluated at time 0, at which point the expression of both K1 and K10 was very high in the cells. At the other end of our observation period (168 h), the cells were not yet confluent, only forming a monolayer and still with gaps between colonies (Fig. 2B, D).

The suppression of K1 and K10 genes and the disappearance of the proteins from the cells could not have been a result of a selection within the HaCaT cell population of a less-differentiated phenotype, because K1/K10 was expressed in all the cells at the time of seeding (0 h), and at no time during the culture process did we observe high numbers of dead cells in the culture. At seeding (0 h), cell viability was 98% and the culture supernatant did not contain unattached cells at the times of medium change. The above-described kinetics of K1/K10 mRNA and protein expressions in HaCaT cells indicate that both keratins are very long-lived proteins. In fact there is no evidence in normal keratinocytes that K1/K10 once synthesized can disappear from the cells. The ability to degrade K1/K10 may be characteristic of HaCaT cells and could be a key ability in their immortality.

α5 integrin mRNA and protein expression increase while K1 and K10 mRNAs and protein decrease dramatically with proliferation in HaCaT cells

D1 cyclin mRNA expression and DNA staining with PI were used to follow the proliferation state of synchronized HaCaT cells after release from contact inhibition and serum starvation. Cyclin D1 is an early G_1 phase marker [3]. After 1 week of serum starvation the cells had undergone almost complete cell cycle withdrawal. At 0 h both the cyclin D1 mRNA (Fig. 4) and the number of $S/G_2/M$ phase cells (Fig. 5A) were very low in culture, and they both remained low during the first 24 h after release from quiescence. A gradual increase in cyclin D1 message was de-

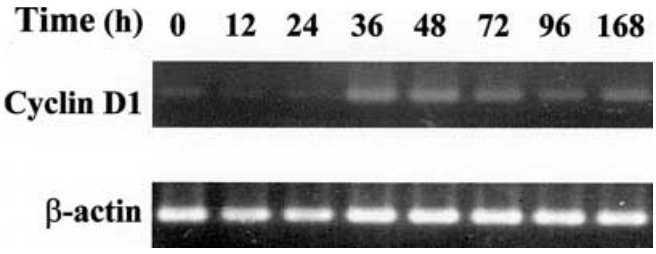

Fig. 4 Expression of cyclin D1 mRNA in HaCaT cells after release from cell quiescence. RT-PCR analysis was used to detect the expression of cyclin D1 mRNA at the times indicated after release from cell quiescence. Amplified products of cyclin D1 (162 bp) and the internal control β-actin gene (406 bp) were visualized by ethidium bromide staining on agarose gel

tected between 24 and 72 h (Fig. 4), closely followed by increasing numbers of cells entering S phase (Fig. 5A), indicating a high rate of proliferation by day 3. K1/K10 mRNAs decreased to undetectable levels between 24 and 48 h (Fig. 5B). Although the cells still contained high levels of K1/K10 proteins, there was a substantial relative decrease in both keratins by day 3 (24.3% for K1 and 46.2% for K10; Fig. 5C). A dramatic increase in α5 integrin mRNA preceded the high proliferation of the cells at 24 h (Fig. 5B), followed by a gradual increase in α5 integrin protein expression (Fig. 5C). The increase in the amount of α5 integrin protein paralleled the increase in the number of $S/G_2/M$ phase cells (Fig. 5A, C), indicating a close connection between α5 integrin protein expression and cell proliferation.

Serum factors regulate the expression of K1 and α5 integrin genes but not the K10 gene in HaCaT cells

To determine whether the dramatic downregulation of K1/K10 gene expression and the significant upregulation of α5 integrin mRNA expression were driven by serum factors or contact inhibition, RT-PCR analysis was used. The effect of serum deprivation was examined on freshly seeded HaCaT cells to exclude the effects of contact inhibition on the expression of these genes. These cells were not starved and synchronized prior to seeding. Seeding was done in the presence of serum and the cells were kept in the serum-supplemented medium for 12 h before serum withdrawal. Only very low levels of K1 and K10 expression could be detected in the cells 12 h after seeding (Fig. 6, 12 h). The cells showed a dramatic increase in K1 mRNA expression 24 h after serum withdrawal (Fig. 6, 36 h), suggesting a strong suppression of the K1 gene by serum factors, followed by a gradual decrease between 48 and 72 h (Fig. 6, 60 and 84 h), indicating that other regulatory mechanisms also existed. Surprisingly, the K10 mRNA level remained unchanged upon serum withdrawal (Fig. 6), indicating that the expression of the keratin 10 gene is not regulated by serum factors. A relatively strong α5 integrin expression was found at 12 h in culture in the presence of serum

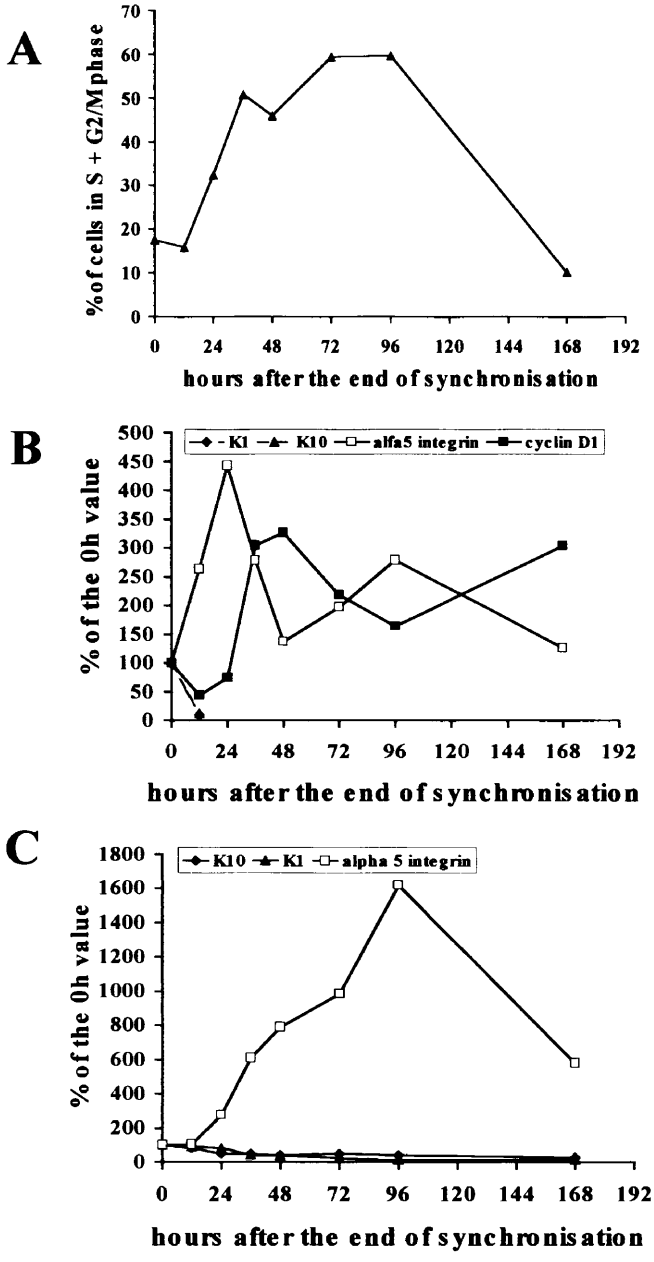

Fig. 5 A–C Comparison of cell proliferation and the expression of α5 integrin, K1, and K10 in HaCaT cells. The percentages of $S/G_2/M$ phase cells were determined by PI staining and DNA analysis using flow cytometry and Modfit cell cycle analysis software (**A**). The expression levels of cyclin D1, α5 integrin, K1 and K10 mRNAs were defined by RT-PCR (**B**). The relative intensities of the bands measured by densitometric analysis were normalized to the intensities of the bands of β-actin. The values are expressed as percentages of the 0-h values (**B**). Changes in the amounts of α5 integrin, K1 and K10 proteins during the experiments were determined by immunoblotting and densitometry (**C**). Values are expressed as percentages of the 0-h values

(Fig. 6, 12 h). After removal of the serum from the culture medium, the α5 integrin expression decreased to an almost undetectable level (Fig. 6, 36 and 60 h), indicating that serum factors regulate the α5 integrin gene expression. The expression of α5 integrin mRNA reappeared in

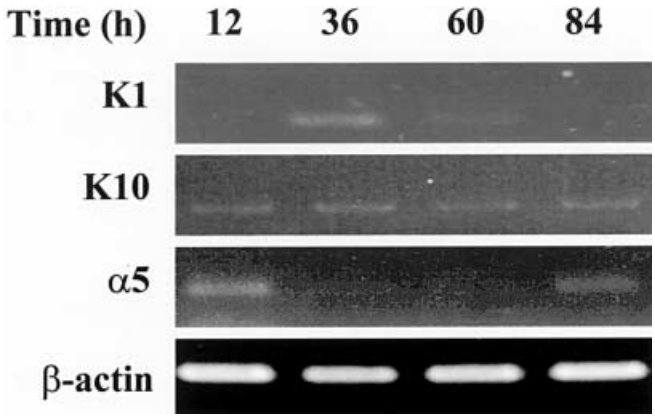

Fig. 6 Effect of serum withdrawal on the expression of α5 integrin and K1/K10 mRNAs in HaCaT cells. HaCaT cells were seeded and grown in serum-containing medium for 12 h, then the serum was removed from the medium. Cells were analysed by RT-PCR for the expression of α5 integrin, K1 and K10 24, 48 and 72 h after serum withdrawal (36, 60 and 84 h after seeding). The amplified products of α5 integrin (358 bp), K1 (316 bp), K10 (685 bp) and the internal control β-actin (406 bp) were visualized by ethidium bromide staining on agarose gel

the cells 72 h after serum withdrawal (Fig. 6, 84 h), indicating that other regulatory mechanisms also existed.

Discussion

Upon serum starvation and high-density culturing, quiescent HaCaT cells expressed K1/K10 proteins, as well as mRNAs at high levels while α5 integrin expression was suppressed. In contrast, after release from cell quiescence, highly proliferating HaCaT cells expressed α5 integrin at a high level. These highly proliferating cells still contained large amounts of the differentiation-associated markers, K1/K10 proteins, but there was no K1/K10 mRNA present in the cells, indicating a lack of new protein synthesis.

Analysis of the expression of α5 integrin and K1/K10, and the proliferation state of the synchronized HaCaT cells revealed a close correlation between cell proliferation and the expression of α5 integrin at both the mRNA and protein levels. The α5 integrin mRNA expression preceded and the α5 integrin protein expression coincided with intense proliferation of the cells, defined by the expression of cyclin D1, an early G_1 marker, and PI DNA analysis, suggesting that α5 integrin may play a role in the promotion of HaCaT proliferation. This finding is not surprising, since increased expression of α5 integrin on keratinocytes in the in vivo epidermis is associated with hyperproliferation of the cells [18, 29]. The in vivo significance of α5 integrin expression and function in the epidermis have been demonstrated by forced suprabasal expression of α5 integrin in transgenic mice that resulted in a phenotype reminiscent of the hyperproliferative skin disease, psoriasis [9].

In contrast to α5 integrin, the downregulation of K1/K10 at the mRNA level seems to be a prerequisite for proliferation of HaCaT cells, indicating an inhibitory role for these keratins in proliferation. Although at the time of intense proliferation, K1/K10 proteins were still present in the cells, new proteins could not be synthesized, since no message was present for these keratins. It is possible that continuous synthesis of keratins is required to block cell proliferation. The newly synthesized keratins may differ from the already existing keratins in various functions, such as inhibition of proliferation. Changes in keratin function may be due to posttranslational modifications that are known to occur during differentiation of keratinocytes [14]. Together with actin microfilaments and microtubules, keratin filaments make up the cytoskeleton of epithelial cells. Although these proteins are thought to be involved in maintaining the mechanical integrity of epithelial cells, their function in the complex differentiation process is still unclear. It has been shown that ectopic expression of K10 inhibits the proliferation of HaCaT cells in culture [28].

Our results also indicate that the expression of the K1 gene is tightly regulated by serum factors and the K1 and K10 genes are regulated differentially. Although it has been previously reported that the expression of K1 and K10 could be regulated independently, the background of this differential regulation was not clarified [31]. Based on the results of our experiments, we conclude that the expression of K1 is regulated by serum factors, but other regulatory mechanisms also exist. On the other hand, serum factors seem to have no direct regulatory effect on K10 gene expression.

The suppressive effect of serum on the expression of the K1 gene could be due to retinoic acid contained in the FBS. Retinoic acid has been shown to suppress the expression of K1 and K10 genes as well as the differentiation process in keratinocytes [12, 15]. Another possibility is that cytokines provided by the FBS are responsible for the suppression of K1 in HaCaT cells. In cultured keratinocytes, epidermal growth factor stimulates proliferation and inhibits the expression of K1 through the epidermal growth factor receptor (EGFR) [25]. It is known that TGF-β can influence biochemical markers of keratinization and inhibit K1, K10 and filaggrin synthesis [10, 24]. Another factor that may be responsible for the suppression of K1 is interleukin 6 (IL-6). Increased levels of IL-6 have been detected in the plasma of patients with active psoriasis and IL-6 has been shown to induce proliferation of keratinocytes [17].

The expression of α5 integrin is linked to an undifferentiated, intensely proliferating state of HaCaT keratinocytes and α5 integrin is expressed in an opposite way to K1/K10 and in a similar manner to its expression in normal human keratinocytes in which the expression coincides with a highly proliferative, undifferentiated state of the cells [26]. We found a strong expression of α5 integrin in HaCaT cells growing in the presence of serum that decreased dramatically after the removal of the serum from the culture medium, indicating that serum factors play a role in the regulation of α5 integrin gene expression. This finding may be relevant to the situation of wound healing,

where keratinocytes are exposed to serum factors, that could be responsible for the induction of α5 integrin expression on keratinocytes in the wound. One important factor present in the serum that is known to regulate α5 integrin expression in various cells is fibronectin [11, 20, 22].

HaCaT keratinocytes under serum-free contact-inhibited conditions showing strong expression of K1/K10 and suppressed expression of α5 integrin resemble suprabasal nonproliferating keratinocytes of normal human epidermis in vivo. It is possible that serum factors, that are able to diffuse through the basement membrane from the dermis into the epidermis, regulate the expression of K1 in the in vivo epidermis. As keratinocytes move away from the basement membrane, the concentration of these membrane-diffusible serum factors gradually decreases and the cells start to express K1. However, in normal keratinocytes there is no evidence that the expression of K1 and K10 can be reversed. The ability of HaCaT cells to dedifferentiate seems to be a unique feature of these cells and may be related to their immortal nature.

Our data support the findings of previous investigations indicating similarities between HaCaT cells and normal keratinocytes and suggest that HaCaT cells are excellent candidates for studying external regulators of proliferation and differentiation and the underlying molecular mechanisms [31]. Our findings also emphasize the importance of the in vitro conditions under which the cells are cultured. HaCaT keratinocytes under different culture conditions represent different types of keratinocytes. In serum-starved contact-inhibited culture, HaCaT cells resemble suprabasal nonproliferating differentiated (K1/K10$^+$) keratinocytes of normal epidermis, while the highly proliferative HaCaT cells (α5 integrin$^+$, K1/K10$^+$), after release from contact inhibition and addition of serum, resemble the activated (α5 integrin$^+$) differentiated (K1/K10$^+$) transiently amplifying keratinocytes.

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