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The *BCL6* Gene Is Predominantly Expressed in Keratinocytes at Their Terminal Differentiation Stage

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We analyzed the expression of the *BCL6* gene in mouse tissues by *in situ* hybridization. The expression was strong in the upper layer but undetectable in the basal layer of epidermis from adult mice. When human keratinocytes were cultured with a high concentration of calcium ion, these cells stopped their proliferation and differentiated to their terminal stage. In these keratinocytes, *BCL6* expression was induced after stimulation and progressively up-regulated. The kinetics was very similar to that of a cyclin dependent kinase inhibitor $p21^{Sdi1/Cip1/WAF1}$ in these cells. These results suggest that BCL6 plays a role in keratinocytes at terminal differentiation stage.

The nonrandom chromosomal translocations are often found in hematologic malignancies (1). The *BCL6* gene was isolated from the translocation breakpoint involving 3q27 of diffuse large B-cell lymphomas (2-4). Since the *BCL6* gene encodes a protein containing *Krüppel*-type zinc finger motifs (4-6) and BTB/POZ domain (7), BCL6 can bind to a specific DNA sequence (8) and may function as a transcriptional repressor (9,10). However, a physiological function of this protein is not known. We have recently cloned the murine homologue of *BCL6* (11). This gene is ubiquitously expressed in adult tissues, predominantly in skeletal muscle. We further analyzed the expression in developing tissues by *in situ* hybridization. Here we show that *BCL6* expression was strongly detected in embryonic epidermis. Furthermore, *BCL6* was progressively induced in human keratinocytes after the terminal differentiation was induced. We discuss a correlation between *BCL6* expression in keratinocytes and their terminal differentiation.

MATERIALS AND METHODS

Animals. (C57BL/6xDBA/2) F1 female mice were purchased from Japan SLC Co. Ltd. (Hamamatsu, Japan).

In situ hybridization. Frozen sections fixed with 4% paraformaldehyde in PBS were incubated with proteinase K solution at the concentration of 10 μg/ml in 10 mM Tris-HCl pH 8.0 and 1 mM EDTA at 37°C for 10 min. The sections were hybridized with 1 μg/ml of a riboprobe at 55°C for 16 hours. After hybridization, RNase A (20 μg/ml) treatment was carried out at 37°C for 30 min. Positive signals were detected by the Nucleic Acid Detection Kit (Boehringer Mannheim, Mannheim, Germany). The riboprobes were synthesized on a 0.65 kbp *Apa*I fragment of the murine *BCL6* cDNA by SP6 or T7 RNA polymerase using the digoxigenin (DIG) RNA labeling kit (Boehringer Mannheim).

Cell culture. Primary human keratinocytes purchased from the Cell Systems (Kirkland, WA) were cultured as recommended by the manufacturer. Terminal differentiation was induced in these cells by changing the medium to modified MCDB 153 containing 1.5 mM calcium chloride without bovine pituitary extracts. These cells were also cultured with phorbol myristate acetate (PMA) (Sigma Chemical Co., St. Louis, MO) (5 ng/ml) or transforming growth factor-β (TGF-β) (Sigma) (5 ng/ml).

Northern blot analysis. Northern blot analysis has been described previously (11). A 0.7 kbp *Xma*III/*Sph*I fragment of the human *BCL6* cDNA and a 1.0 kbp *Kpn*I fragment of the human *p21* cDNA kindly provided by Dr. A. Noda

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(Meiji Institute of Health Science, Odawara, Japan) were used as probes. These fragments were subcloned into pGEM vectors and labeled by DIG using polymerase chain reaction with T7 and SP6 primers.

Western blot analysis. Western blot analysis has been described previously (12). The cultured keratinocytes were lysed with NP40 lysis buffer (150 mM NaCl, 1.0% NP40, 50 mM Tris pH8.0) with protainase inhibitors on ice for 30 min. Amounts of protein were estimated by the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA). Total proteins (75 μg) were fractionated on a 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham International plc., Buckinghamshire, England) using electroblot (Bio-Rad). The filter was incubated with rabbit anti-BCL6 antiserum followed by horseradish peroxidase conjugated donkey anti-rabbit immunoglobulin (Amersham) for 1 hour at each step. The antibodies detection reaction was performed using the ECL chemiluminescence detection system (Amersham).

Gel retardation assay. Gel retardation assay has been described previously (8). Double-stranded oligonucleotide containing the BCL6 recognition sequence (5′-TATGCTTTCTAGGAATGTCG) was labeled with DIG using the DIG Oligonucleotide 3′-End Labeling Kit (Boehringer Mannheim).

RESULTS

BCL6 mRNA Is Detected in Epidermis

We analyzed expression of the *BCL6* gene in sections of mouse embryos between embryonic day 11.5 (E11.5) and E16.5 by *in situ* hybridization. The expression was strongly detected in developing epidermis from an embryo at E15.5 (Fig. 1). At this embryonal age the structure of epidermis is immature. We therefore further analyzed the expression in epidermis from adult mice. The expression was strong in spinous and granulous layer but undetectable in basal layer (Fig. 2), indicating that *BCL6* is up-regulated in keratinocytes after comittment of differentiation.

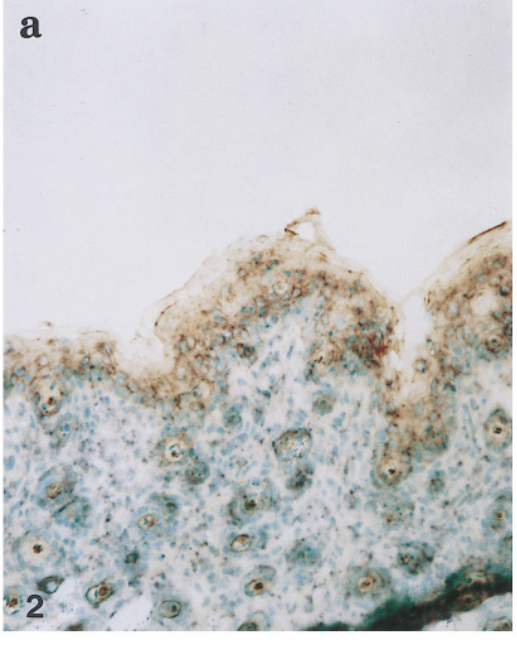
BCL6 Is Induced in Human Keratinocytes at the Terminal Differentiation Stage

The process of epidermal development can be mimicked by the *in vitro* human keratinocyte culture. Supplement of calcium ion to growing keratinocytes triggers a terminal differentiation program including growth arrest and induction of differentiation markers (13). We measured *BCL6* mRNA in the keratinocytes stimulated with calcium ion by Northern blot analysis (Fig. 3a). The expression was undetectable in growing keratinocytes (Day 0) but detectable in the cells within one day after stimulation and was progressively up-regulated thereafter. Recently it is reported that a cyclin-dependent kinase inhibitor $p21^{Sdi1/Cip1/WAF1}$ is induced in keratinocytes after the same stimulation (14). Therefore, we rehybridized the same filter with the *p21* probe. *p21* was also induced in the cells after stimulation, and kinetics of the expression was very similar to that of *BCL6*. But the level of *p21* reached to plateau about 1 day earlier than that of *BCL6*.

Induction of BCL6 in the keratinocytes was further examined at protein level. Total cell lysates from the cultured keratinocytes were immunoblotted with anti-BCL6 antibody (Fig. 3b). The protein was not detected in the growing keratinocytes (Day 0) but was detectable within two days after stimulation. Since BCL6 protein can bind to double-stranded DNA with the specific sequence (8), we analyzed binding activity of the proteins from the cultured keratinocytes by gel retardation assay (Fig. 3c). This assay might detect some non-specific binding since a small amount of the retardation band was detected in the protein from growing keratinocytes (Day 0). Amounts of the band progressively increased within four days after stimulation. This band was completely inhibited by the addition 200-fold excess of the cold competitor in the binding reaction. These results suggest that active form of BCL6 protein is induced in keratinocytes at terminal differentiation stage.

The BCL6 Expression in Keratinocytes Correlates with Their Terminal Differentiation

Other stimulations like PMA (15) and culture confluence (16) are also known to induce the keratinocyte differentiation. When keratinocytes were cultured at confluent state or stimulated with PMA for 4 days, *BCL6* expression was induced like the calcium stimulation (Fig. 4). *p21* was also up-regulated in these keratinocytes. However, TGF-β stimulation did not signifi-



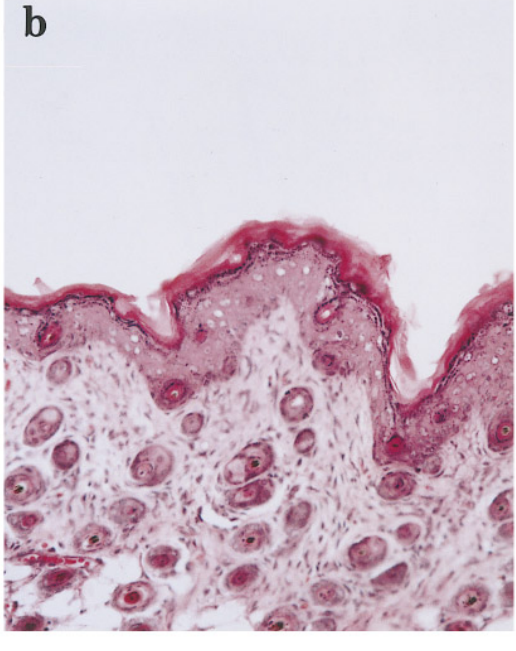


FIG. 1. *In situ* hybridization of *BCL6* expression in epidermis from a mouse embryo at E15.5. *BCL6* mRNA was detected by the *in situ* hybrydization with the *BCL6* anti-sense riboprobe (a) but not with the sense riboprobe (b). Original magnification (a and b) ×300.

FIG. 2. *In situ* hybridization of *BCL6* expression in adult mouse epidermis. (a) *BCL6* mRNA was detected in spinous and granulous layer by the *in situ* hybridization with the anti-sense riboprobe. (b) Serial sections of the epidermis were stained with hematoxylin-eosin dye to identify the layers. Original magnification, (a and b) ×300.

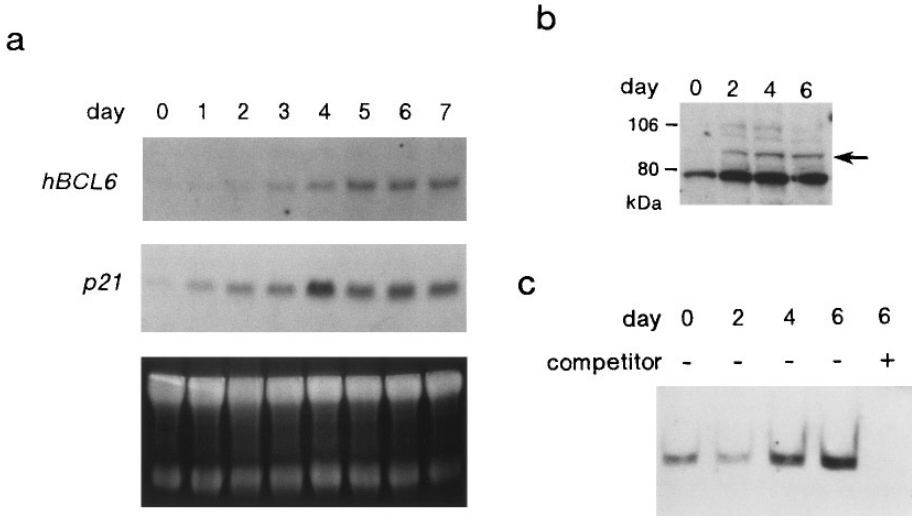


FIG. 3. *BCL6* expression in keratinocytes after induction of terminal differentiation by calcium ion. Human keratinocytes were cultured with 1.5 mM calcium chloride. Total RNAs and proteins were isolated from the cells. (a) Expression of *BCL6* and *p21* mRNA was analyzed by Northern blot. The ethidium bromide-stained gel, prior to transfer, is shown below for normalization of RNA loading. (b) BCL6 protein was detected by Western blot with anti-BCL6 antibody. The arrow indicates BCL6 specific bands. (c) DNA binding activity of proteins from the cells was analyzed by gel retardation assay.

cantly induce *BCL6* expression. Although stimulation of keratinocytes with TGF-β can inhibit their cell growth, it cannot trigger the differentiation (14). These results suggest that the *BCL6* expression in keratinocytes correlates with their terminal differentiation.

DISCUSSION

In this study, we showed that *BCL6* expression in keratinocytes closely correlated with their terminal differentiation. The expression is also reported in skeletal muscle from human and

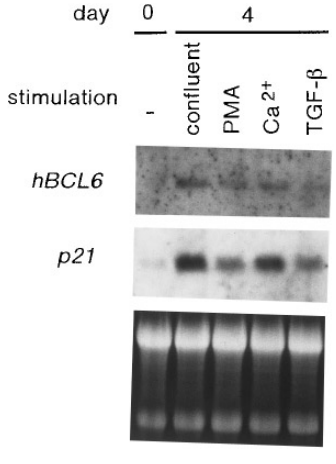


FIG. 4. *BCL6* expression in keratinocytes after induction of terminal differentiation by various inducers. Human keratinocytes were cultured at confluent condition or with PMA, calcium chloride, or TGF-β for 4 days. Total RNAs were isolated from the cells. Expression of *BCL6* and *p21* mRNA was analyzed by Northern blot. The ethidium bromide-stained gel, prior to transfer, is shown below for normalization of RNA loading.

mouse (6,11). Adult skeletal muscle is known to be a terminally differentiated tissue, and *BCL6* is up-regulated in myoblasts after induction of differentiation (Miki, *et al.* in preparation). Furthermore, the expression was detected in terminally differentiated spermatids from adult mouse testis (data not shown). The expression was undetectable in proliferating spermatogonia but was strong in spermatids just before releasing to the lumen of the seminiferous tube. Therefore, the expression in cells at terminal differentiation stage is not a unique phenomenon to keratinocytes.

The *BCL6* expression was induced in keratinocytes within one day after stimulation and increased progressively at terminal differentiation stage (Fig. 3). This kinetics was very similar to that of *p21*$^{Sdi1/Cip1/WAF1.}$ Within one day after stimulation, differentiation markers such as transglutaminase 1 have been already expressed (17), and DNA synthesis has been almost totally inhibited in the keratinocytes (13). The cyclin-dependent kinase inhibitor p21 may function as an inducible growth inhibitor of the keratinocytes. However, BCL6 may not play a role in the expression of *p21* as a transcription factor, since the kinetics of *BCL6* expression seemed to be about one day slower than that of *p21* (Fig. 3a) and recent reports demonstrated transcriptional repressor activity of BCL6 (9,10). This kinetics also suggests that BCL6 plays a role not in commitment of differentiation but in maintenace and/or function of the terminally differentiated cells.

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