Mouse keratinocytes express c98, a novel gene homologous to bcl-2, that is stimulated by insulin-like growth factor 1 and prevents dexamethasone-induced apoptosis

Hung-Yi Su a,*, Winston T.K. Cheng b, Shih-Chu Chen c, Chen-Tse Lin a, Yi-Yang Lien c, Hung-Jen Liu c, R. Stewart Gilmour d

a Graduate Institute of Biotechnology, National Pingtung University of Science and Technology, I, Hseuh-Fu, Road, Pingtung 91201, Taiwan, ROC
b Institute of Animal Science, National Taiwan University, Taipei, Taiwan, ROC
c Department of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan, ROC
d Liggins Institute, School of Medicine, University of Auckland, Auckland, New Zealand

Received 8 July 2003; received in revised form 5 November 2003; accepted 13 November 2003

Abstract

Many studies have been undertaken to investigate the mechanisms of skin differentiation. In particular, growth factors and hormones are believed to play important roles in skin proliferation, differentiation and survival. Insulin-like growth factor-1 (IGF-1) has been identified as a survival factor in many tissues including the skin, but the molecular mechanism of IGF-1 in epidermal differentiation is not completely understood. Neonatal mouse skin is useful for studying changes in gene expression, as the mitotic activity of skin cells changes shortly after birth. Using RNA differential display (DD), a 357-nt message that is specifically expressed in the epidermal keratinocytes of IGF-1-injected newborn mice but not in controls, has been identified. Confirmation of expression of this gene by ribonuclease protection assay (RPA) showed that its mRNA expression in the epidermal keratinocytes is induced by IGF-1. Using RNA ligase-mediated rapid amplification of 5′ cDNA ends (RLM-5′-RACE), we have successfully isolated a 3473-bp full-length gene, c98, that has 97% sequence homology to a bcl-2-like gene, bcl-w. The latter has been identified as a proto-oncogene in several murine myeloid cell lines. Amino acid sequence analysis of the c98 showed that it has 97% sequence identity to the bcl-w protein and possesses bcl-2 homology domains (BH) 1, 2 and 3. Immunoblotting data revealed similar increases of c98 protein expression to its mRNA expression in the keratinocytes of IGF-1-injected animals. Weak expression of other bcl-2 family member proteins, bax, bcl-2 and bcl-xL, were also found in the immunoblots. Additionally, IGF-1 was found to be able to protect epidermal keratinocytes from dexamethasone (DEX)-induced apoptosis, based on the findings that after the cells were treated with DEX, DNA laddering was present in the control mice but not in those injected with IGF-1. Further, using a photometric enzyme-linked immunoassay to quantitate keratinocyte death, we found that after addition of DEX, the amounts of cytoplasmic histone-associated DNA fragments were not significantly (P>0.05) different in IGF-1-treated cells compared with untreated control cells during the high mitotic stage of skin epidermis. To assess the role of c98 in these anti-apoptotic processes, we have generated a recombinant plasmid that contains an expression vector and c98 and transfected this plasmid into the keratinocytes from mice without IGF-1-treatment. Expression of the c98 protein was found to completely (P<0.05) block DEX-induced apoptosis after cell transfection. Taken together, our current data demonstrated that IGF-1 plays an anti-apoptotic role in the DEX-induced apoptosis in epidermal keratinocytes and this, at least in part, may be mediated through expression of c98.

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Keywords: RNA differential display; Rapid amplification of 5′ cDNA ends; Neonatal mouse skin

Abbreviations: IGF-1, insulin-like growth factor-1; DD, RNA differential display; DEX, dexamethasone; RPA, ribonuclease protection assay; RLM-5′-RACE, RNA ligase-mediated rapid amplification of 5′ cDNA ends; BH, bcl-2 homology domains; PBSA, phosphate buffered saline with albumin; RT-PCR, reverse transcription-polymerase chain reaction; PI 3-kinase, phosphatidylinositol 3-kinase

The nucleotide sequence data reported in this paper has been submitted to GenBank and has been assigned accession number AY170344.

* Corresponding author. Tel.: +886-8-7740511; fax: +886-8-7229796.
E-mail address: suhy@mail.npust.edu.tw (H.-Y. Su).
1. Introduction

The morphogenesis of skin epidermis is a dynamic process that requires a balance between proliferation and cell death for the maintenance of tissue homeostasis [1]. Epidermal keratinocytes undergo a series of temporal and spatial morphologic/biochemical changes that result in the production of terminally differentiated cells [1,2]. Terminal keratinocyte differentiation leads to the generation of anucleated nonviable cells that have been thought as a specialized form of apoptosis [3,4]. The skin epidermis therefore provides an excellent model for studying coordinate regulation of cell proliferation, differentiation and cell death [5].

Insulin-like growth factor-1 (IGF-1), a single chain polypeptide of 70 amino acids, is a member of the insulin-related proteins along with IGF-II, insulin and relaxin [6]. Unlike insulin, which regulates metabolic processes such as glucose transport, glycogen, and fat biosynthesis, IGF-1 functions mainly as a mitogenic factor and a regulator of differentiation [7]. This growth factor is mainly expressed in the liver, and mediates endocrine action of growth hormone (GH) with the assistance of binding proteins (BPs) in circulation [8,9]. The latter control IGF transport, efflux from vascular compartments and are associated with cell surface receptors [9]. In tissues, IGF-1 is produced by mesenchymal type cells and acts in a paracrine and autocrine fashion by binding to its specific receptor, type 1 IGF receptor (IGF-1R) [10]. This binding activates the receptor tyrosine kinase (RTK) that triggers the downstream responses and finally stimulates cell division [11].

IGF-1 has been identified as a potent stimulator of cellular proliferation and differentiation in the skin [12,13]. In human skin, DNA synthesis and keratinocyte growth have been reported to increase after the exogenous administration of IGF-1 to cultured epidermis [14]. Mice with disrupted IGF-1R have a thinner epidermis and fewer keratinocytes compared to their control littermates [15]. In addition, IGF-1R and BPs have been found in the dermis and the epidermis, although IGF-1 is only produced in the former [13,16–18]. It has therefore been speculated that the dermal production of IGF-1 may participate in epidermal cell proliferation in a paracrine fashion [13], the BPs and IGF-1R may play a role in transporting and binding IGF-1 from the dermis to epidermis and IGF-1 may act as a mitogen in the development of skin (reviewed in Ref. [19]).

Neonatal mice provide a useful model for investigating the development of skin as in mice, differentiation of skin cells starts shortly after birth and follows a temporal and spatial pattern [20]. Skin epidermis increases its thickness at day 2, reaches its maximum at day 3 after birth, and lasts for about 3 days [20]. In this paper, the techniques of differential display (DD) [21] have been used to investigate the molecular basis of IGF-1 in skin differentiation, by studying differences in gene expression in mouse epidermal keratinocytes during this period. These techniques have previously been used to investigate changes in gene expression in neonatal and prenatal mouse skin, during development of hair follicles [22,23]. It is reported here that a novel gene, c98, which is homologous to a bcl-2 gene, is differentially expressed in the epidermal keratinocytes between the IGF-1-treated and the control animals. The potential role of c98 in the effect of skin differentiation and apoptosis is outlined.

2. Materials and methods

2.1. Animals and IGF peptides

C57Bl mice were purchased from the National Laboratory Animal Breeding and Research Center of National Science Council in Taipei, Taiwan. A single male was mated to a female in individual cages with food and water supplied ad libitum. Fluorescent light was provided from 5:00 am to 5:00 pm each day. At birth of pups, the sire was removed from the cage. Offspring from the same age of mice were used for the experiments. Recombinant human IGF-1 obtained from GroPep Pty. Ltd., Adelaide, Australia was prepared for injection as described [24]. One hundred micrograms of IGF freeze-dried powder was dissolved in 100 μl of 0.01 M HCl and diluted in 900 μl of phosphate buffered saline with albumin (PBSA) prepared by autoclaving a mixture of 0.05 M K2HPO4 and 0.15 M NaCl (pH 7.4), and adding 0.1% (w/v) filter-sterilized bovine serum albumin. Twenty-five micrograms (0.25 ml) of the IGF peptide was given per injection. Injections were given intraperitoneally twice a day from birth (day 0) to 4 days after birth. The control group of mice received only PBSA with same treatment scheme as the above IGF-treated mice.

2.2. Tissue culture

Primary keratinocytes cultures were prepared from dorsal skin of neonatal mice as described previously [25]. Briefly, epidermis and dermis were separated with 0.25% trypsin [26]. Subsequently, single cell suspensions of epidermal cells were seeded into 25-cm² plastic culture flasks (Corning, Corning, NY) with keratinocyte basal medium (Sanko Junyaku, Tokyo, Japan) without fetal bovine serum at the density of 25 × 10⁶ cells per flask.

2.3. RNA isolation and mRNA differential display

In this study, the technique of DD [21] was used to investigate changes in gene expression in the epidermal keratinocytes on 3–5-day-old mice between IGF-1- and PBSA-treated mice. Total RNAs were isolated from freshly cultured primary keratinocytes using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD). These RNAs were
treated with DNase I and quantified by absorbance at 260 nm in a spectrophotometer. The integrity of the RNA was verified by ethidium bromide staining of ribosomal RNA on a 1% agarose gel. The RNA samples were run in duplicate in each experiment. Synthesis of the first strand cDNA was accomplished by reverse transcription (RT) using 300 U of MMLV reverse transcriptase (Life Technologies) and 2.5 µM of 3’-primer [27]. After heat inactivation of the reverse transcriptase at 95 °C for 5 min, 2 µl of the sample was added to 18 µl of polymerase chain reaction (PCR) mix containing a 5’-primer and AmpliTag DNA polymerase (Perkin Elmer, Foster City, CA). The preparation of PCR mix was based on a nonradioactive procedure described by Doss [28] except that the amount of AmpliTag DNA polymerase was doubled.

As DNase I-treated RNAs are sometimes contaminated with residual chromosomal DNA [29], a control RT-PCR lacking added reverse transcriptase was included. The RT-PCR products from each day were run on a sequencing gel (Model SA, Life Technologies). Six 3’-primers (5’-T12AT-3’, 5’-T12CA-3’, 5’-T12CT-3’, 5’-T12CC-3’, 5’-T12CG-3’, 5’-T12GA-3’) and five 5’-primers (5’-AGTAGCTGGA-3’, 5’-GCTCGCTAC-3’, 5’-ATGTGGGT GGT-3’, 5’-AGATGCTGGA-3’) were synthesized by Generet (Singapore) were used to accomplish 30 RT-PCRs. To measure the sizes of differentially expressed genes, the pGEM DNA markers obtained from Promega (Madison, WI) were included in each display gel. After electrophoresis to display the PCR products, the gels were stained with silver based on the procedures as described [30]. A control RNA, which contained pooled RNA of day-1 keratinocytes from IGF-1-treated mice, was included in each differential display. The cDNA patterns of this control RNA had already been consistently shown on several display gels. DNA bands that were present differentially on different treatment groups were isolated from the gel based on the methods as described [31]. The recovered DNAs from display gel were PCR-reamplified and cloned into a pCR II vector (Invitrogen, San Diego, CA), and the plasmid DNA was prepared as previously described [22]. The size of the PCR products was analyzed by agarose gel electrophoresis. Six independent clones per original DNA bands were sequenced, with the M13 forward and reverse primers using an automated sequencer by the MDBio, Inc., Taipei, Taiwan. The resulting sequences were tested for homology to known sequences in the GenBank databases by BLAST search [32].

2.4. RNA ligase-mediated rapid amplification of 5’ cDNA ends (RLM-5’-RACE)

Isolation of the full-length differentially expressed cDNA clone was accomplished by RLM-5’-RACE using the GeneRacer Kit (Version J, Invitrogen). In brief, total RNA extracted from epidermal keratinocytes of 3–5-day-old mice was treated with calf intestinal phosphatase (CIP) to remove 5’ phosphates, according to the manufacturer’s instruction. The dephosphorylated RNA was then treated with tobacco acid pyrophosphatases (TAP) to remove the 5’ cap structure from full-length mRNA. Five micrograms of the 5’ end of mRNA was ligated with 0.25 µg of GeneRacer RNA oligo using T4 RNA ligase. First-strand cDNA synthesis was performed by reverse-transcribing the ligated mRNA in the presence of the GeneRacer oligo dT (54-mer) primer (Invitrogen). Second-strand synthesis was performed with a 3’ reverse gene-specific primer, the sequences designed from the differentially expressed cDNA sequence and the GeneRacer 5’ Primer (homologous to the GeneRacer RNA oligo). This product was then amplified by PCR and the PCR product was cloned into a pCR II vector (Invitrogen). Plasmids from positive colonies were purified and sequenced by MDBio, Inc., Taiwan. The resulting sequences were tested for homology to known sequences in the GenBank databases by BLAST search [32].

2.5. Ribonuclease protection assay (RPA)

A nonradioactive RPA was performed to confirm the expression of differentially expressed cDNA, c98, in epidermal keratinocytes of IGF-1-treated mice from day 1 to day 5 of age using an Ambion RPA III kit (Ambion, Austin, TX) following the manufacturer’s recommended protocol and the method as described [33]. A parallel set of RPA done on 1–5-day-old keratinocytes from un.injected mice was also performed. The antisense RNA probe was prepared by transcription of a BamHI-linearized plasmid template of c98 with T7 RNA polymerase using the Sp6/T7 transcription kit (Boehringer Mannheim, Mannheim, Germany) in the presence of digoxigenine (DIG)-UTP (Roche Molecular Biochemicals, Mannheim, Germany). This antisense riboprobe was subsequently purified in a 1-ml Sephadex G-50 (Sigma Chemical Co., St. Louis, MO) spin column as previously described [22]. After hybridization with RNA from epidermal keratinocytes, the unhybridized single-stranded RNA was digested by RNases A and T1 (provided with the RPA III kit). Protected fragments were analyzed on a 6% polyacrylamide gel containing urea, transferred to a nylon membrane by electrophorosetting, and detected using a DIG Nucleic Acid Detection kit (Roche Molecular Biochemicals). Mouse actin DNA template used as a control was included in the RPA III kit, and the methods used for purification of the actin hybridization probe were the same as those for the c98.

2.6. DNA fragmentation and cell death detection

To determine apoptosis of epidermal keratinocytes, dexamethasone (DEX) was used as an anticancer drug for the induction of cell death. Cells (5 × 10⁶) from IGF-1- and
PBSA-injected groups were treated with DEX (prepared in absolute ethanol and diluted in PBS to $1 \times 10^{-5}$ M) (Sigma) for 12 h. In addition, cells from the two groups of animals were treated with only control vehicle (i.e. identical concentrations of ethanol and PBS) to determine if the effects on cell apoptosis were due to treatment of DEX. Genomic DNAs were isolated from the two treated groups and one control vehicle group according to the methods as described [34]. In brief, cells were rinsed with PBS, homogenized in 100-$\mu$l volume of lysis buffer [10 mM EDTA, 50 mM Tris (pH 8.0), 0.5% sodium lauryl sarcosine, and 0.5% mg/ml proteinase K], and then incubated at 50 °C overnight. RNase A (100 $\mu$g/ml) was mixed with DNA lysate and incubated at 37 °C for 1 h. DNAs were separated by electrophoresis on a 1% agarose gel in 1 x Tris–borate ethylenediaminotetraacetic acid (EDTA) (50 mM Tris base, 50 mM boric acid, and 1 mM disodium EDTA) at 100 V for 1.5 h and then stained with ethidium bromide (0.5 $\mu$g/ml).

For the detection of cell death, confluent keratinocytes from IGF-1- and PBSA-injected mice in 24-well plates were treated with DEX (1 $\times 10^{-5}$ M) or control vehicle in Dulbecco’s modified Eagle’s medium (DMEM). After 12 h of incubation, cell death was measured in a photometric enzyme-immunoassay for the quantitative in vitro determination of cytoplasmic histone-associated DNA fragments (mono- and oligo-nucleosomes) using the “Cell Death Detection ELISA PLUS.” of Roche Diagnostics (Mannheim, Germany), as described previously [35]. In brief, cells were pelleted at 300 x g, washed with PBS (pH7.2), and lysed with lysis buffer (provided with the kit). The lysates were then centrifuged at 300 x g for 5 min and 20 $\mu$l of the supernatant was added to a streptavidin-coated 96-well microtiter plate. After addition of 80 $\mu$l of a solution containing anti-histone-biotin and anti-DNA-peroxidase conjugates in incubation buffer, the samples were incubated at room temperature (RT) with shaking for 2 h. Subsequently, the wells were washed three times with incubation buffer. 100 $\mu$l of the substrate solution was added, and the samples were incubated at RT for 10 min in the dark. The absorbance was measured at 405 nm. The value of cells that were treated with control medium was set as 100% and the apoptosis rates of treated cells were calculated as a percentage of control.

2.7. Immunoblotting

For the generation of c98 antibodies, the cDNA from nt 522–591 was subcloned into NdeI/BamHI sites of the pET28a vector (pET system, Norvagen) and the plasmid was transformed into BL21(DE3) bacteria. The protein was induced by incubating the bacterial cells with 1 mM isopropyl-1-thio-β-D-galactopyranoside and 50 ml of bacterial suspension was treated with lysozyme. The resulting solution was subjected to sonication and freeze/thaw followed by centrifugation. The pellet was extracted with 6 M urea and 3% SDS and electrophoresed on a 16% polyacrylamide gel. The protein band of interest was excised and injected into mice for the production of c98 antibodies. Bax, bcl-2 and bcl-xL antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-β-actin was from Sigma. For immunoblotting analysis, cells from IGF-1-treated mice from day 3 to day 5 of age were lysed in the lysis buffer (50 mM Tris–Cl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40 and 150 mM NaCl) containing protease inhibitor (Sigma). Lysates were pelleted to remove the debris and protein concentrations measured using a modified Lowry assay (Protein Assay Kit, Bio-Rad). Fifty micrograms of protein per sample was electrophoresed on 12.5% SDS polyacrylamide gels (SDS-PAGE). After transfer of proteins to nitrocellulose, the filters were blocked for 1 h in the blotting solution [TBST (10 mM Tris, 150 mM NaCl and 0.05% Tween 20) and 5% nonfat dry milk] and incubated with primary antibodies diluted 1:500 in the blotting solution overnight at 4 °C. After five washes in the blotting solution, the filters were further incubated with secondary peroxidase-labelled antibodies diluted 1:500 in TBST and immunoreactive proteins were detected using the ECL kit (Amersham).

2.8. Cell transfection

The c98 was digested with SacI at the nt position of 27 and subcloned into the unique SacI site of the vector pcDNA3.1/GS (Invitrogen). The pcDNA3.1 vector was previously shown to be able to carry foreign genes and transfect primary keratinocytes [36] and thus was used in our experiments. Isolation of the plasmid DNA, c98/pcDNA3.1/Zeo(–), was performed using the S.N.A.P. MiniPrep Kit (Invitrogen) based on the manufacturer’s recommendation. Primary keratinocytes from PBSA-treated mice were transfected with c98/pcDNA3.1/Zeo(–) or pcDNA3.1/Zeo(–) using lipofectamine according to the manufacturer’s procedures (Invitrogen). Briefly, 5 x 10^4 confluent cells were plated in serum-free medium and transfected with 10 μg of plasmid DNA. Forty-eight hours later, cells were transferred to 100-mm plates and selected in 400 μg/ml of zeocin. Zeocin-resistant colonies were subcultured individually, treated with DEX or control vehicle, and cell death was measured as described in Section 2.6. Expression of c98 protein in the culture was confirmed by immunoblotting (data not shown).

3. Results

3.1. Identification and isolation of c98

In this paper, DD techniques were used to identify changes in gene expression of epidermal keratinocytes between IGF-1- and PBSA-injected mice at 3–5 days after birth. Our results showed that a particular gene, c98, was expressed in IGF-1- (lanes 3–8, Fig. 1) but not in PBSA-
injected animals (lanes 9–14, Fig. 1). The c98 DNA bands shown on day 5 (lanes 7–8) had higher intensity compared to day 3 (lanes 3–4) and day 4 (lanes 5–6). In addition, the c98 band was not present in the pooled RNA samples from day 1 (lane 1, Fig. 1). Repeated experiments showed a similar display pattern (data not shown). Apart from the c98 band, six bands that were also differentially expressed in the IGF-1-treated animals but not shown in repeated experiments (data not shown) and thus were excluded from further investigation. RNA samples free from DNA contamination were confirmed by no amplification of cDNA in the control where RT-PCR was performed in the absence of reverse transcriptase (lane 2, Fig. 1). Thus, cDNA bands shown on the display gel were fingerprints of mRNA. To determine nucleotide sequence of c98, the c98 band from a display gel was eluted, PCR-reamplified and cloned (Fig. 2). The c98 DNA was consistent in size (lane 2) with the bands in the display gel. After cloning into the pCRII vector (Invitrogen), the released c98 insert was slightly larger in size (lane 3) and this is due to the addition of vector sequences.

3.2. Nucleotide and amino-acid sequencing of c98

Both strands of c98 were sequenced and their sequence alignment showed 100% complementary between the two strands (data not shown). Nucleotide sequencing of c98 (nt 3111 to 3467, Fig. 3A) revealed that they were flanked by the primer set used (shown in italic and bold), although there is one nucleotide mismatch at the penultimate base between the 3' -primer and the c98 (lower case, Fig. 3A). The mismatch between primer and c98 suggests the primer may exhibit degeneracy during annealing in the RT step [29]. After performing RLM-5'S'-RACE, the full-length c98 sequence (Fig. 3A) was searched with BLAST and showed 97% identity to the nucleotides of a murine bcl-2-like mRNA in myeloid cell lines [37]. The latter was identified as an anti-apoptotic function similar to that

Fig. 1. Identification and isolation of c98 from epidermal keratinocytes of neonatal mice by DD. Total RNAs isolated from epidermal keratinocytes of IGF-1-(lanes 3–8) and PBSA-injected mice (lanes 9–14) at 3–5 days were run in duplicate, and reverse-transcribed with MMLV reverse transcriptase in the presence of a 3' -primer (5' -TTTTTTTTTTAT-3') and PCR-amplified with an additional 5' -primer (5' -AGTAGCTGGA-3'). A control RNA from epidermal keratinocytes of IGF-1-treated mice on day 1 was included in lane 1. Lane 2 represented RT-PCR results of the same RNA used in lane 1 except lacking added reverse transcriptase. The methods used for isolation of c98 bands were as described in Section 2.

Fig. 2. Cloning of c98. DNA of c98 from DD gel was cloned into the pCRII vector (Invitrogen). Ligated products were transformed into Escherichia coli and the plasmid DNA was subsequently purified as described in Section 2. Lane 1 represented a size marker of the lambda DNA with HindIII digest (Promega), lane 2 the PCR product of c98 DNA and lane 3 cloned c98 digested with EcoRI to release the inserted PCR product.
for bcl-2 in myeloid cells [37]. Amino acid sequence analysis of c98 revealed that it has 97% sequence homology from residues of 16–327 with the mouse bcl-w protein (Fig. 3B) [37], confirming the same identity found in the corresponding positions of their nucleotide sequences (Fig. 3A). In addition, c98 possesses bcl-2 homology domains (BH) 1, 2 and 3 that are found in other bcl-2 members (Fig. 3B) [37,38]. The derived protein sequence of c98 has 96% identity to that of the murine bcl-w protein.

3.3. Confirmation of c98 mRNA expression in the skin

To verify the expression of c98 mRNA in the epidermal keratinocytes of IGF-1 treated mice, a nonradioactive RPA was performed (Fig. 4). To extend the investigation on c98
expression before day 3 of mouse age, total RNAs extracted from epidermal keratinocytes at 1–2 days were also included in the assay. Hybridization of a DIG-labeled c98 antisense RNA probe with RNA of keratinocytes from each day resulted in the formation of a 357-bp RNA–RNA hybrid that was protected from subsequent RNase digestion (lanes 4–6, Fig. 4). A stronger expression of c98 was found on day 5 (lane 6, Fig. 4) compared to days 3 and 4 (lanes 4–5, Fig. 4), whilst c98 did not express at 1–2 days (lanes 2–3, Fig. 4). Results of RPA from untreated animals showed no signal during 1–5 days (data not shown). To verify if the RNAs were evenly loaded for all samples, they were hybridized with a 300-nt mouse h-actin probe (lane 8, Fig. 4). The actin mRNA was detected with similar intensity from day 1 to day 5 (lanes 10–14, Fig. 4). The absence of a signal from the negative control, yeast RNA, suggests a complete digestion of single-stranded unhybridized probes by RNases (lanes 1 and 9, Fig. 4). The RPA results confirmed the presence of c98 mRNA in the epidermal keratinocytes of IGF-1-treated mouse as previously shown with DD.

3.4. In vitro detection of anti-apoptotic activity in IGF-1-treated mice

To determine the anti-apoptotic activity in IGF-1-treated neonates, genomic DNAs isolated from epidermal keratinocytes of IGF-1- and PBSA-injected animals treated with DEX were examined for molecular integrity. Intact DNAs were visualized in IGF-1-injected animals (lanes 3–5, Fig. 5) but DNA laddering was seen in PBSA-injected animals (lane 6–8, Fig. 5), suggesting an anti-apoptotic functions for IGF-1-injected animals at 3–5 days. In addition, keratinocytes from 1–2-day-old neonates treated with IGF-1 were not protected from DEX (lanes 1–2, Fig. 5). Intact DNAs were observed in IGF-1- and PBSA-injected animals after treat-
3.5. Detection of anti-DEX-induced cell death in IGF-1-treated mice

To determine the anti-DEX-induced cell death in epidermal keratinocytes, we measured DNA fragmentation in cells from IGF-1- and PBSA-injected mice. Our results demonstrated that at 3–5 days, IGF-1 completely ($P > 0.05$) blocked DEX-induced cell death compared with untreated control cells (D3–D5/IGF-1 columns, Fig. 6A), while the cell death triggered by DEX in epidermal keratinocytes was observed ($P < 0.001$) in IGF-1-injected cells at 1–2 days (D1–D2/IGF-1 columns, Fig. 6A). The amount of cell death reduced by IGF-1 on day 3, 4 and 5 was 46%, 49% and 54%, respectively (D3-D5/IGF-1 and D3-D5/PBSA columns, Fig. 6A). In addition, the cell-death rate was unchanged ($P > 0.05$) in the IGF-1-compared with PBSA-injected animals at 1–2 days (D1–D2/IGF-1 and D1–D2/ PBSA columns, Fig. 6A).

3.6. Confirmation of anti-DEX-induced apoptosis by c98 in PBSA-treated mice

To determine if the anti-DEX-induced apoptosis by IGF-1 is due to c98, the keratinocytes from PBSA-treated mice were transfected with the c98(pcDNA3.1 plasmid. Our data demonstrated that c98(pcDNA3.1 was capable of preventing ($P>0.05$) DEX-induced apoptosis in the keratinocytes from PBSA-treated mice, whilst pcDNA3.1-transfected cells showed significantly ($P<0.001$) higher cell-death rate compared with control vehicle-treated groups (Fig. 6B). The reduced cell-death rate by c98 was 53% (Fig. 6B).

3.7. Detection of c98 protein expression in IGF-1-treated mice

To verify if the protein expression of c98 corresponds to its mRNA expression, immunoblotting was performed at 3–5-day-old keratinocytes from IGF-1-treated mice. Our
results showed that c98 protein was expressed at days 3–5 and the highest level was seen at day 5 (Fig. 7), confirming the similar mRNA increases shown with RPA. In addition, hybridization signals were present in the immunoblots after treatment with bcl-xL, bcl-2 and bax antibodies during this period (Fig. 7).

4. Discussion

To better understand the molecular control of IGF-1 in skin differentiation, DD was used to identify potential new genes that may be up- or down-regulated by IGF-1. IGF-1 injections were performed intraperitoneally from days 0–4 and DD was done during the period of increasing mitotic activity of skin epidermis from days 3–5 of mouse age [20]. Using 30 combinations of primer sets, one up-regulated gene, c98, was identified in the keratinocytes of IGF-injected animals (Fig. 1). Similar increases of c98 expression were also found in the keratinocytes that were directly injected with less IGF-1 (i.e. 0.25 mg/ml twice daily from days 0–4) during days 3–5 (Su, H.-Y., Lin, C.-T. and Gilmour, R.S., unpublished data), suggesting more pronounced effect of IGF-1 on the stimulation of c98 expression in vitro compared to in vivo. RPA results demonstrated up-regulation of c98 persisted at days 3–5 but no expression was seen during days 1–2 (Fig. 4), suggesting IGF-1-dependent differential expression of c98 only at the high mitotic stage of skin epidermis. Hybridization signals were not evident in the RPA of 1–5 day keratinocytes from uninjected mice (data not shown), demonstrating that expression of c98 was not due to natural accumulation of this gene during this period. Further, the highest expression was seen on day 5 compared to days 3 and 4 (Fig. 4), suggesting an accumulated expression of c98 from days 3–4 to day 5. Taken together, our data demonstrated that the expression of c98 in days 3–5 keratinocytes was not due to the natural ontogeny of this gene but is a consequence of a high mitotic index and that an external agent like IGF-1 is required.

Apoptosis refers to a series of characteristic morphological changes in cells, and these changes include cell shrinkage, dense chromatin condensation, cellular budding, fragmentation, rapid phagocytosis by nearby cells and DNA fragmentation into units of about 200 base pairs [39,40]. DEX-induced apoptosis has been reported in several myeloma cell lines and primary myeloma cultures [41,42]. We demonstrated here that DEX is able to induce apoptosis in primary keratinocyte cultures of mouse skin based on the finding that DNA fragmentation was detected in PBSA-injected animals after treatment of DEX (lanes 6–8, Fig. 5). The number of these DNA fragments was significantly ($P<0.001$) greater in the DEX-treated compared to untreated control mice (D1–D5/PBSA columns, Fig. 6A).

Apoptosis may play a major role in skin development and bcl-2 is believed to be involved in the developmental process. Evidence has shown that apoptosis is responsible for the homeostasis of skin, in particular, the terminal differentiation of the epidermal keratinocytes [40]. It is also reported that expression of bcl-2 is associated with the survival of differentiating keratinocytes [43,44]. The main signaling pathway for IGF-1R-mediated protection from apoptosis was suggested to be involved in the phosphorylation of the Bcl-2 family member proteins [45]. Bcl-2 family member proteins such as bcl-2, bcl-xL and bax contain conserved amino acid sequences of BH 1, 2, and 3 domains [37,46–49]. These domains are required for the formation of homodimers or heterodimers with other Bcl-2 family members [50], and BH1 and BH2 domains are required for the anti-apoptotic function of bcl-2 and for heterodimerization with the proapoptotic protein bax or bad [51]. The BH3 domain has been found in bax and bak and functions by heterodimerizing with bcl-2 and bcl-xL, respectively [38,52]. Activated IGF-1R has been reported to be able to increase the level of bcl-xL expression by increasing its protein level [53]. Recent studies have identified a linear survival cascade consisting of IGF-1, IGF-1R, phosphoinositide 3-OH kinase [PI(3)K], and the serine/threonine kinase Akt (also termed protein kinase B or PKB) [54]. Activated Akt phosphorylates bad and abolishes its apoptotic properties [55,56].

In this paper, we suggest that the anti-apoptotic function of IGF-1 to keratinocytes is mediated through the expression of c98 interacting with several Bcl-2 family member proteins, bcl-xL, bcl-2 and bax, as these four proteins were all found in the IGF-1-treated animals (Fig. 7). To support this hypothesis, we demonstrated that expression of c98 alone conferred resistance to DEX-induced apoptosis in PBSA-treated mice (Fig. 6B). We speculate that the anti-apoptotic function of c98 may be due to its BH 1-3 domains (Fig. 3B) and the formation of homodimers or heterodimers with other Bcl-2 family member proteins through these domains. Additionally, these anti-apoptotic effects of IGF-1 by c98 were more pronounced during the high mitotic stage of skin epidermis. For instance, during days 1-2 when c98 was absent (Fig. 4), the anti-apoptotic activity was not detected (Fig. 5; D1–D2/IGF-1 columns, Fig. 6A); whilst during days 3–5 when c98 was expressed (Fig. 4), the anti-apoptotic function of IGF-1 was completely ($P>0.05$) restored (Fig. 5; D3–D5/IGF-1 columns, Fig. 6A). The rate of anti-apoptosis may be correlated to the quantity of the c98 expression as suggested by the finding that, on day 5 when the greatest reduced amount of apoptosis was detected (Fig. 6A), the strongest expression of both c98 mRNA and protein was detected (Figs. 1, 4 and 7). Taken together, it is suggested that expression of c98 is directly affected by IGF-1 treatment during the high mitotic stage of skin epidermis. The effect of IGF-1 on protection of epidermal keratinocytes from apoptosis may be due to interaction of c98 with at least some Bcl-2 family member proteins downstream of IGF-1 signaling pathway. Further studies on in situ hybridization would be important to identify cellular locations of c98 with...
reference to the skin cells. It would be also important to investigate expression of other growth factors, GH, insulin and IGF-1R in the IGF-1-treated animals and determine whether they are involved in the induction of c98 for future studies.

In summary, our preliminary data revealed that epidermal keratinocytes of the newborn mice provide a useful tool for the study of differential gene expression, and IGF-1 may have an anti-apoptotic function against DEX-induced apoptosis in these cells through, at least in part, expression of c98.

Acknowledgements

This work was supported by National Science Council Grant NSC90-2311-B-020-001, Taiwan, ROC.

References


