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Upregulation of endogenous heparin-binding EGF-like growth factor and its role as a survival factor in skeletal myotubes

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Abstract To investigate the role of heparin-binding EGF-like growth factor (HB-EGF) in skeletal muscle, we studied its function in skeletal myotubes in vitro using mouse C2C12 cells. Expression levels of membrane-anchored HB-EGF (proHB-EGF) protein were increased specifically during their differentiation among epidermal growth factor receptor (EGFR) ligands. Production levels of EGFR on the cell surface were constant. Tyrosine phosphorylation of EGFR, however, was constitutively increased during differentiation. Quenching of endogenous HB-EGF significantly rendered myotubes sensitive to apoptotic cell death induced by hypoxic stress, suggesting that proHB-EGF in the skeletal muscle is specifically upregulated to function as a survival factor.

Key words: Heparin-binding EGF-like growth factor; Epidermal growth factor receptor; Cell survival; Skeletal muscle; C2C12; Hypoxia

1. Introduction

Skeletal muscle cells provide a typical example of terminal differentiation [1]. Their proliferating precursor cells, myoblasts, express at least one muscle regulatory gene of the MyoD family [2] and can be introduced into the muscle differentiation lineage by the withdrawal of growth factors in vitro. Growth factor-starved myoblasts irreversibly exit from the cell cycle, becoming terminally differentiated myotubes which are multinucleated fused cells [1], and do not proliferate further. However, they simultaneously produce both growth factors and their receptors, indicating that some autocrine mechanism may exist. Heparin-binding EGF-like growth factor (HB-EGF) transcript is highly expressed in skeletal muscle [3] and is driven by MyoD [4]. Since epidermal growth factor receptor (EGFR), which binds to HB-EGF, is expressed in myotubes, possible interactions of HB-EGF and EGFR and their physiological function may play a critical role in the formation or the functional maintenance of terminally differentiated myotubes.

HB-EGF was originally identified as a mitogenic protein with 76–87 amino acid residues for vascular smooth muscle cells [5,6]. HB-EGF, like other members of the EGF family, is initially synthesized as a membrane-anchored form (proHB-EGF) that can be processed to release the soluble form (sHB-EGF). ProHB-EGF tethered to the cell surface also stimulates adjacent cell growth through cell-cell contact, termed a juxtacrine manner [7]. In addition to the growth factor activity, proHB-EGF also functions as a diphtheria toxin receptor [8,9] and exists in muscle cells [3]. These data are consistent with the fact that the skeletal and cardiac muscles are the most sensitive organs to diphtheria toxin [10]. The present study reports an investigation of the physiological role of HB-EGF, particularly its membrane-anchored form in the skeletal muscle cells.

2. Materials and methods

2.1. Materials

Sulfo-NHS-biotin was purchased from Pierce (Rockford, IL, USA). An enhanced chemiluminescence (ECL) kit was purchased from Amersham (Buckinghamshire, UK). Human HB-EGF neutralizing antibodies #197 and #H1 were described previously [11,12]. Tyrphostin AG1478 was purchased from Calbiochem (La Jolla, CA, USA). Phosphotyrosine antibody PY-20 was from Transduction Laboratory (Lexington, KY, USA). An antisense phosphorothiolate oligonucleotide, complementary to the 1–27 nucleotide sequence of the open reading frame of HB-EGF cDNA, and the corresponding sense phosphorothiolate oligonucleotide were prepared by Griner Japan (Tokyo, Japan). [^{125}I]EGF was purchased from ICN (Costa Mesa, CA, USA).

2.2. Cell culture

A mouse muscle myoblast C2C12 cell line was obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FCS) and, unless otherwise indicated, the cell culture media contained penicillin (100 U/ml) and streptomycin (100 μg/ml). In order to differentiate C2C12 myoblasts into myotubes, the medium was changed to a differentiation medium (DMEM supplemented with 2% horse serum).

2.3. Biotinylation and immunoprecipitation of proHB-EGF

In order to detect proHB-EGF, cell surface biotinylation, immunoprecipitation and Western blotting were carried out as described previously [13].

2.4. Growth factor assay

To measure the mitogenic activity of HB-EGF, an EP170.7 cell assay was carried out as described previously [7].

2.5. Detection of tyrosine-phosphorylated EGF receptor

Tyrosine-phosphorylated EGF receptor was identified using phosphotyrosine antibody PY-20. Subconfluent undifferentiated and differentiated C2C12 cells in 10 cm dishes were lysed with a lysis buffer and immunoprecipitated with PY-20 as described previously [6]. Precipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred electrically to a nitrocellulose membrane and probed with PY-20, followed by horse-

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Abbreviations: EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; proHB-EGF, membrane-anchored heparin-binding EGF-like growth factor; sHB-EGF, soluble heparin-binding EGF-like growth factor; EGFR, epidermal growth factor receptor

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radish peroxidase (HRP)-conjugated goat anti-mouse IgG (Amersham). The membranes were then treated with detection reagents for ECL Western blotting for 1 min (Amersham) at room temperature.

2.6. Northern blot analyses

Total RNA from cultured C2C12 cells was prepared by the acid guanidinium thiocyanate/phenol/chloroform method [14]. Eighteen μg of total RNA/lane was fractionated on a 1.0% formaldehyde-agarose gel and transferred onto a Zeta probe (Bio-Rad) membrane by capillary action as described previously [15].

2.7. Released lactate dehydrogenase (LDH) measurements

Released LDH was measured using a MTX'LDH' kit (Kyokuto Pharmaceutical Industrial, Tokyo, Japan). C2C12 myotubes, cultured in a 96-well plate, were incubated for 16 h under hypoxic conditions using an AnaeroPack (Mitsubishi Gas Chemical, Tokyo, Japan). Twenty-five μl of each cultured medium was transferred to another 96-well plate. Twenty-five μl of coloring solution (0.74 mg/ml 2-*p*-iodophenyl-3-*p*-nitrophenyl tetrazolium chloride, 50 mg/ml lithium lactate) was then added to each well. The plates were incubated at 37°C for 5 min and the reaction was stopped by addition of 50 μl of 1 M HCl and the absorbance at 560 nm was measured. The amount of released LDH was calculated according to the following formula: % absorbance = ((T−PC)/(NC−PC))×100 (T, absorbance of total LDH from cells; PC, absorbance of the released LDH from cells cultured under normal conditions; NC, absorbance of the released LDH from cells cultured for 24 h under hypoxic conditions).

2.8. [^{125}I]EGF binding

[^{125}I]EGF binding was carried out according to the method described previously [5,6]. Briefly, the C2C12 cells, plated in 24-well plates, were washed twice with ice-cold phosphate-buffered saline (PBS) containing 1 mg/ml bovine serum albumin (BSA-PBS). Increasing amounts of [^{125}I]EGF (3.93×10^8 cpm/μg) were added and the cells were incubated for 1 h at 4°C. The cells were washed three times with ice-cold BSA-PBS and lysed with 0.2 N NaOH. Cell-associated radioactivity was measured on a γ-counter (Beckman). Non-specific binding of [^{125}I]EGF was assessed in the presence of a 60-fold excess of cold EGF. Specific binding was determined by subtracting the non-specific binding. The non-specific binding was less than 10% of the total binding.

2.9. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the C2C12 cell line as described above. Five μg of total RNA was used for cDNA synthesis in a reaction mixture containing 75 mM KCl, 50 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 10 mM DTT, 0.5 μg of oligo(dT) primer (Gibco BRL, Gaithersburg, MD, USA), 0.25 mM dNTPs, 6 U of Rous-associated virus 2 reverse transcriptase (Takara, Osaka, Japan). After incubation for 60 min at 37°C, the cDNA obtained was subjected to 35 cycles of PCR [16] (94°C, 1 min; 55°C, 2 min; 72°C, 3 min) using the following specific primers for EGF: 5′-CACTGAGCAACTCCACTCAG-3′ and 5′-CACATCCCCAAGAGGAGCAG-3′, TGF-α: 5′-ATGGTCCCCGCGACCGGACA-3′ and 5′-TTCAGACCACTGTCTCAGAG-3′, betacellulin: 5′-GGTCTTGCAATTCTCCACTG-3′ and 5′-GCTTGTGATAACTTTATAAC-3′, amphiregulin: 5′-GGTGGAACCAATGAGAACTC-3′ and 5′-CATCCGAAAGCTCCACTTCC-3′, epiregulin: 5′-CGCAAGCTGCACCGAGAAAG-3′ and 5′-CTTGTCCGTAACTTGATGGC-3′ and the PCR products were amplified. The PCR products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide prior to visualization using ultraviolet light.

2.10. Detection of apoptotic cell death

Apoptotically dead cells were detected using an in situ detection kit (Takara) according to the manufacturer's protocol. Differentiated C2C12 cells were cultured in a two well chamber slide and incubated for 8 h under hypoxic conditions. Cells were fixed with freshly prepared 4% paraformaldehyde in PBS, pH 7.4, for 30 min at room temperature, and washed three times with PBS. The fixed cells were incubated with a permeation buffer for 5 min on ice and washed three times with PBS. The cells were then incubated with a labelling buffer for 90 min, washed with PBS and viewed on an inverted fluorescence microscope.

3. Results

3.1. HB-EGF mRNA and protein expression levels in C2C12 cells

To analyze the physiological roles of HB-EGF in the myotube, we examined HB-EGF expression in pre- and post-differentiated mouse myoblast C2C12 cells in vitro. Northern blot analyses were carried out to examine the expression level of HB-EGF mRNA in C2C12 cells. A 2.5 kb transcript of HB-EGF was detected in both pre- and post-differentiated C2C12 cells and the HB-EGF mRNA expression level was

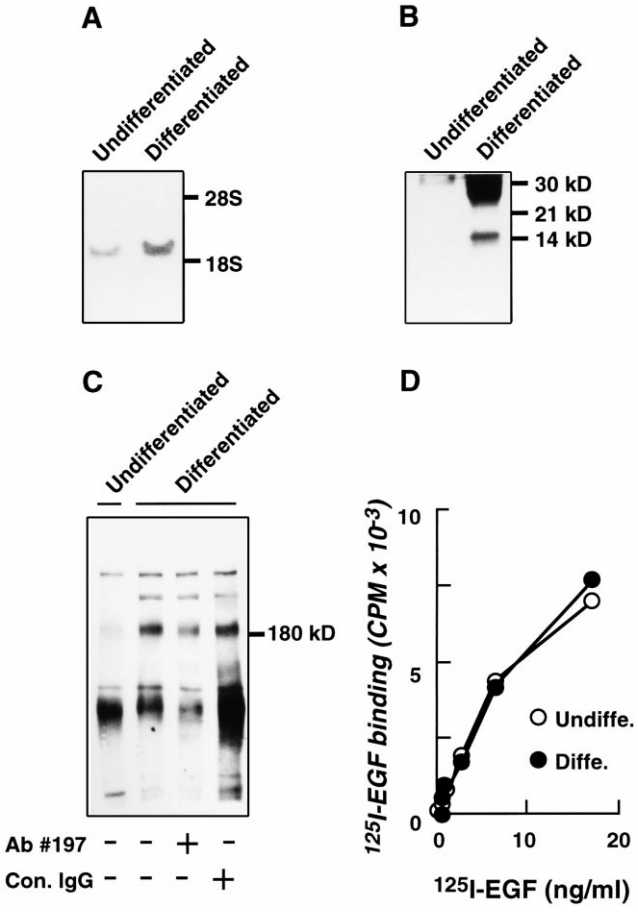

Fig. 1. Expression of HB-EGF and EGFR in C2C12 cells. (A) Northern blotting analyses of HB-EGF mRNA. Total RNA (18 μg), prepared from undifferentiated and differentiated C2C12 cells, was electrophoresed in a 1% agarose, immobilized to Hybond N^+ (Amersham) and hybridized with a ^{32}P-labelled HB-EGF cDNA probe. (B) Immunoprecipitation of biotinylated proHB-EGF. Undifferentiated and differentiated C2C12 cells were biotinylated and immunoprecipitated by HB-EGF antibody #H1. The precipitated proHB-EGF was then subjected to SDS-PAGE, Western blotting and avidin-HRP detection. ProHB-EGF protein is detected as heterogeneous bands of 14–29 kDa [13]. (C) Western blot analysis of proteins with phosphorylated tyrosines. Differentiated and undifferentiated C2C12 cells were lysed with 1% Triton X-100 solution and immunoprecipitated with phosphotyrosine antibody PY-20. The precipitated proteins were subjected to SDS-PAGE, followed by Western blotting with PY-20. Differentiated C2C12 cells treated with HB-EGF neutralizing antibody #197 or normal goat IgG at a final concentration of 100 ng/ml were also subjected to immunoprecipitation and Western blotting by phosphotyrosine antibody PY-20. (D) [^{125}I]EGF binding to differentiated and undifferentiated C2C12 cells. [^{125}I]EGF specific binding was estimated in the presence or absence of a 60-fold excess of cold EGF.

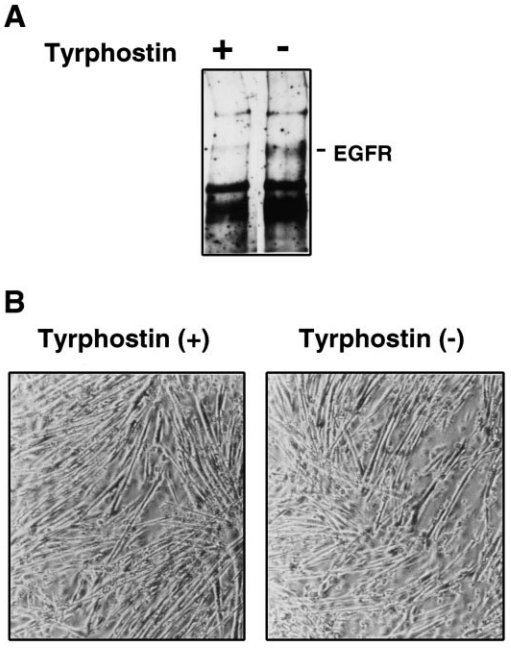

Fig. 2. Effect of anti-tyrosine phosphorylation of EGFR reagent tyrphostin AG1478 on C2C12 cell differentiations. (A) Blocking of EGFR tyrosine phosphorylation by tyrphostin AG1478. Undifferentiated C2C12 cells were pre-incubated for 8 h with and without 3 μM tyrphostin. Cells were lysed and immunoprecipitated with phosphotyrosine antibody PY-20. The precipitates were then subjected to a 6% SDS-PAGE, followed by Western blotting with PY-20. (B) Phase-contrast photographs of differentiated C2C12 cells in the presence and absence of tyrphostin AG1478. Subconfluent C2C12 cells were cultured in DMEM supplemented with 20% FCS and 10 nM tyrphostin AG1478 for 12 h. Cells were exposed to differentiation medium containing 10 nM tyrphostin AG1478 for 4 days.

elevated by approximately two-fold during differentiation (Fig. 1A). To investigate the upregulation of the protein production during the differentiation, qualitative analyses were carried out to estimate processed HB-EGF (sHB-EGF) and proHB-EGF. sHB-EGF activities in the conditioned media of 48 h cultured pre- and post-differentiated C2C12 cells were not detected by the growth factor assay using EP170.7 cells (data not shown). The proHB-EGF protein was detected by combination analyses of cell surface biotinylation and immunoprecipitation and was identified as heterogeneous bands of 14–29 kDa as reported previously (Fig. 1B) [13]. The level of HB-EGF production, mainly proHB-EGF, was upregulated more than five-fold during differentiation. We further examined whether upregulated proHB-EGF is capable of activating

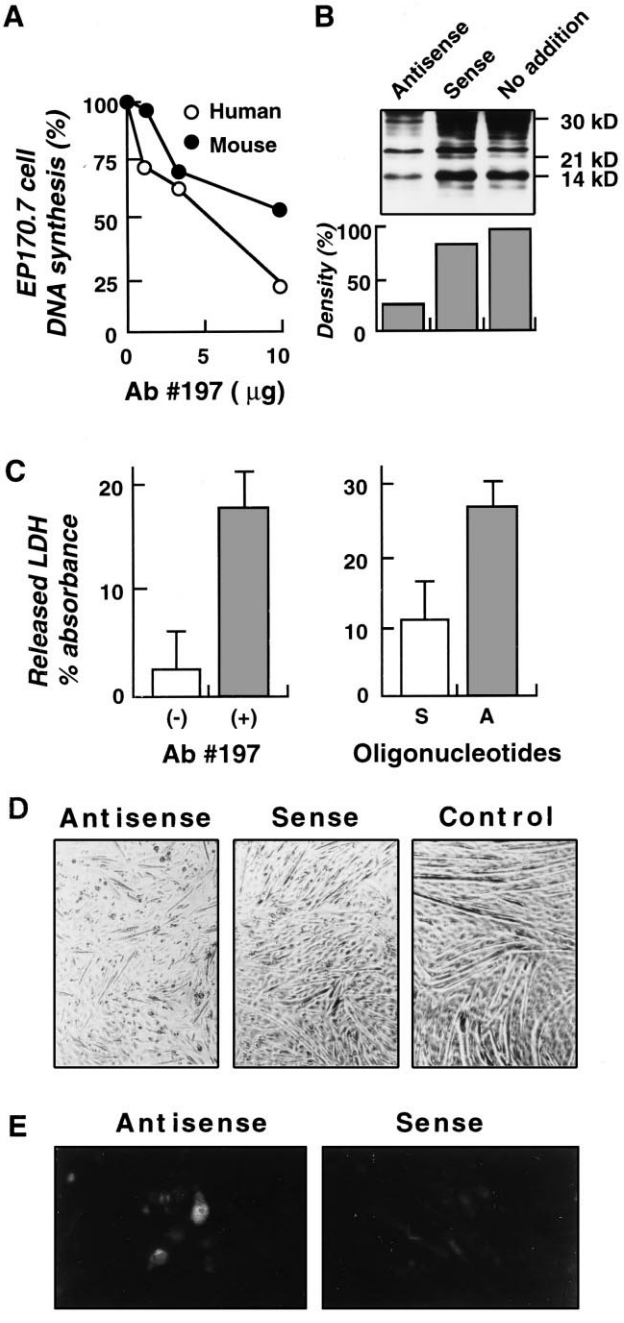

Fig. 3. Effects of anti-HB-EGF reagents on differentiated C2C12 cells under hypoxic conditions. (A) Neutralization of mouse HB-EGF by anti-human HB-EGF neutralizing antibody #197. Approximately 10 ng of mouse (closed circle) and human HB-EGFs (open circle) were pre-incubated with increasing amounts of anti-human HB-EGF neutralizing antibody #197 and tested for an EP170.7 cell assay. (B) Blocking of HB-EGF production by antisense oligonucleotide. Differentiated C2C12 cells treated with 10 μM of antisense or sense oligonucleotide were biotinylated and immunoprecipitated by HB-EGF antibody #H1. (C) Measurements of released LDH from differentiated C2C12 cells treated with and without anti-HB-EGF reagents. Differentiated C2C12 cells were cultured for 8 h in the presence of 10 μg/ml HB-EGF neutralizing antibody #197 or 10 μM HB-EGF antisense oligonucleotide under hypoxic conditions using AnaeroPack. (D) Phase-contrast photographs of differentiated C2C12 cells treated with oligonucleotides. Myotube disruption induced by hypoxia in the presence of antisense oligonucleotide (left), but not the sense oligonucleotide (center) of HB-EGF. Differentiated C2C12 cells were cultured for 16 h in the presence of HB-EGF antisense or sense oligonucleotide. After transferring cells from hypoxic to normal, cells were further cultured under normal conditions for 24 h. Representative phase-contrast photographs of cells are shown. As a control, the same volume (10 μl/2 ml) of H_2O was added into the culture medium (right). (E) Apoptotic cell death of differentiated C2C12 cells. Differentiated C2C12 cells treated with antisense (left) or sense (right) oligonucleotide of HB-EGF were cultured under hypoxic conditions. Cells were fixed, permeated and labelled with fluorescenced dUTP. Cells were observed by fluorescence microscopy.

endogenous EGFR. Since anti-mouse EGFR antibody for immunoprecipitation is not yet available, immunoprecipitation and Western blotting with phosphotyrosine antibody PY-20 and specific blocking of EGFR tyrosine kinase activity by tyrphostin AG1478 were employed to identify tyrosine-phosphorylated EGFR. A tyrosine-phosphorylated 180 kDa band was apparent in post-differentiated C2C12 cells, whereas it was only faint in pre-differentiated C2C12 cells (Fig. 1C). Human HB-EGF neutralizing antibody #197 moderately blocked tyrosine phosphorylation of the 180 kDa band (Fig. 1C), suggesting that the upregulated proHB-EGF activated the EGF receptor tyrosine kinase. Furthermore, [^{125}I]EGF binding on pre- and post-differentiated C2C12 cells was carried out to estimate the upregulation of EGFR during differentiation. [^{125}I]EGF binding on both types of cells was observed in a dose-dependent manner (Fig. 1D), suggesting that EGFR production was not affected during differentiation. These results indicate that proHB-EGF production and EGFR activation are elevated during differentiation of C2C12 cells.

3.2. EGFR signaling was not involved in differentiation of C2C12 cells

Since HB-EGF production and activation of EGFR occur during differentiation of C2C12 cells, involvement of EGFR signaling in their differentiation was investigated. C2C12 cells were cultured with differentiation medium in the presence or absence of 10 nM tyrphostin, which specifically blocks EGFR autophosphorylation. The addition of tyrphostin effectively blocked the tyrosine phosphorylation of a 180 kDa band (Fig. 2A). However, it had no effect on myotube formation in C2C12 cells, as shown in Fig. 2B. Collectively, these data suggest that HB-EGF-EGFR signaling in C2C12 cells is not involved in their differentiation, despite the fact that its signaling pathway is enhanced (Fig. 2).

3.3. Blocking of HB-EGF or EGFR significantly enhanced sensitivity to apoptosis induced by hypoxic stress

To investigate the role of proHB-EGF produced by differentiated C2C12 cells, HB-EGF blocking reagents, HB-EGF neutralizing antibody #197 and antisense oligonucleotide were used. Firstly, HB-EGF neutralizing antibody #197, raised against human HB-EGF, partially inhibited mouse HB-EGF activity (approximately 50% inhibition), while it inhibited more than 80% of human HB-EGF activity (Fig. 3A). Secondly, the antisense oligonucleotide reduced the production level of proHB-EGF protein by approximately 30%, as compared to that of sense oligonucleotide-treated cells (Fig. 3B). C2C12 cells were treated with both reagents under normal and hypoxic conditions. Measurements of the LDH released from cells revealed that the amount of released LDH from cells treated with both reagents was considerably increased as compared to that of their control cells under hypoxic conditions (Fig. 3C). After transferring cells from hypoxic to normal conditions, myotubes were disrupted in the presence of the antisense oligonucleotide, whereas they were well maintained in the presence of sense oligonucleotide or without oligonucleotides (Fig. 3D). In spite of myotube disruption, undifferentiated myocytes showed no differences in their growth among the three conditions. On the other hand, myotube disruption was not induced by antisense oligonucleotide treatment under the normal conditions (data not shown). These HB-EGF blocking experiments facilitated apoptotic cell death caused by hypoxic stress (Fig. 3E).

Altogether, this suggests that proHB-EGF-EGFR signaling is involved in the protection of apoptotic cell death of myotubes induced by hypoxic stress.

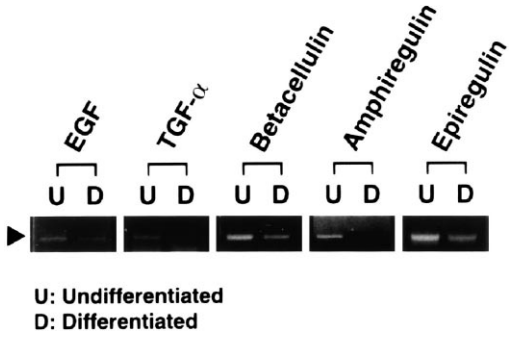

Fig. 4. Expression of the EGF family members during differentiation of C2C12 cells. Thirty-five cycles of PCR were carried out using cDNAs prepared from differentiated and undifferentiated C2C12 cells and specific primers for EGF, TGF-α, betacellulin, amphiregulin and epiregulin. PCR products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide. An arrowhead shows appropriate PCR products.

3.4. Expression of other members of the EGF family in pre- and post-differentiated C2C12 cells

Thus far, six members, including HB-EGF, have been identified as a ligand for EGFR. To investigate whether other members of the EGF family are capable of playing a role as an EGFR ligand in pre- and post-differentiated C2C12 cells, RT-PCR analyses were carried out to detect their mRNAs. As shown in Fig. 4, transcripts of EGF, TGF-α, betacellulin, amphiregulin and epiregulin were detected in pre-differentiated C2C12 cells. The mRNA expression of TGF-α and amphiregulin was not detected in the post-differentiated C2C12 cells, whereas betacellulin, amphiregulin and epiregulin transcripts were detected. However, none of their transcriptional levels was elevated during the differentiation of C2C12 cells. These results suggest that the specific elevation of HB-EGF mRNA occurs during differentiation of C2C12 cells into myotubes.

4. Discussion

Although HB-EGF has also been shown to be abundantly expressed in adult skeletal muscle tissues [3], the functional role of the expressed HB-EGF in skeletal muscle tissues has not yet been elucidated. It has been reported that EGFR signaling was not involved in myocyte differentiation [17]. It is well known that differentiated myotubes do not grow any more. Then, we focused on the role of HB-EGF in the survival of myotubes.

Treatment of C2C12 cells with differentiation medium upregulated HB-EGF mRNA expression and proHB-EGF protein levels as shown previously [4]. Activation of EGFR that binds to HB-EGF was also observed, along with the upregulation of HB-EGF unless EGFR was upregulated. Further analyses revealed that active proHB-EGF was produced and not effectively processed to sHB-EGF. This suggests that a juxtacrine mechanism of HB-EGF might be physiologically important rather than a paracrine one in myotubes. These

findings strongly suggest that the proHB-EGF-EGFR signaling may well be involved in some biological events of myotubes. This view is also supported by the facts that none of the EGFR ligands, except for HB-EGF, was upregulated during differentiation.

To investigate the possible roles of HB-EGF in myotubes, we examined whether or not the survival of the differentiated C2C12 cells was blocked by HB-EGF neutralizing antibody or antisense oligonucleotide. These experiments would be expected to function effectively because of the dominant expression of HB-EGF among EGFR ligands in skeletal myotubes, although the blocking efficiencies of both treatments were estimated to be approximately 50–70%. These treatments to quench HB-EGF, however, apparently enhanced the release of LDH, as a result of cell death of the differentiated C2C12 cells under hypoxic conditions. The number of surviving myotubes was also significantly different after treatments with and without the quenching reagents under hypoxic conditions. These findings strongly support the hypothesis that proHB-EGF expressed on myotube cell surfaces is able to function to protect cells from some influences of environmental changes, such as hypoxia, often referred to as a survival factor. The molecular mechanism underlying this phenomenon remains to be elucidated. The idea that proHB-EGF also functions as a survival factor of cardiac myotubes might be supported by the fact that an early and greatly enhanced response of HB-EGF gene expression was induced by hypertrophic stimuli in neonatal and adult rat cardiac myocytes [18]. Recent studies relating to the molecular mechanisms of the cell division cycle have suggested that the upregulation of cyclin-dependent kinase inhibitors, such as $p21^{CIP1/WAF1}$, could be involved in the terminal differentiation states and might play an important role in maintaining the quiescent state [19]. Furthermore, it has been shown that proHB-EGF, but not sHB-EGF, can induce the upregulation of $p21^{CIP1/WAF1}$, resulting in the acquisition of cell survival potency in hepatoma cells in our laboratory [15]. Based on these findings, $p21^{CIP1/WAF1}$ regulation by HB-EGF in myotubes was examined after treatments with anti-HB-EGF reagents. However, direct regulation of $p21^{CIP1/WAF1}$ by proHB-EGF was not observed in this case (Horikawa et al., unpublished observation). ProHB-EGF might regulate other molecules involved in the cell cycle or redox regulations.

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