Distribution of mRNA for human epiregulin, a differentially expressed member of the epidermal growth factor family

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We have recently identified epiregulin as a new growth regulator and a member of the epidermal growth factor (EGF) family. Epiregulin has certain characteristics that are different from those of the classical members of the EGF family, EGF and transforming growth factor α, including mitogenic responses on several normal cells and binding to EGF receptors on epidermoid carcinoma A431 cells. In the present study we cloned and identified the expression of human epiregulin transcript. The human epiregulin gene encoded a 163-residue putative transmembrane precursor containing an EGF-like domain in the internal segment, and the structural organization was similar to that of other members of the EGF family that bind to EGF receptors. Northern blot analysis showed the expression of human epiregulin to be mainly on peripheral blood macrophages and the placenta in normal tissues, and was highest on epithelial tumour cell lines in various types of tumour cell lines. The expression profile was quite different from that of other members of the EGF family in normal and tumour cells. Recombinant expression in mammalian cells also showed that human epiregulin was secreted as a soluble form of approx. 5 kDa that is biologically active on the basis of the stimulation of DNA synthesis. Our findings suggest that epiregulin is involved in certain physiological processes such as maintenance or development of normal cell growth, and the progression of carcinomas.

INTRODUCTION

Several cellular processes, such as cell survival, proliferation, differentiation, adhesion, migration and axon guidance, are controlled by a variety of peptide growth factors. These factors bind to specific membrane receptors to trigger a cascade of intracellular signals, resulting in specific events for target cells. These molecules, together with their receptors, exhibit powerful signalling effects; when activated by mutation or overexpression they become potent oncogenes and cause drastic cellular transformation [1,2]. Epidermal growth factor (EGF) is one of these growth factors and was the first to be identified [3]. In recent years, several new members of the mammalian EGF family, which share six conserved cysteine residues at similar spacings, have been identified on the basis of their biological activities on different target cells. At present, EGF-related growth factors can be divided into two subgroups on the basis of their direct binding to specific receptors and the genes' structural organization [3–13]. Members of the first group, which includes EGF, transforming growth factor α (TGF-α) [4], heparin-binding EGF-like growth factor (HB-EGF) [5], betacellulin [6] and amphiregulin [7] (also known as schwannoma-derived growth factor [8] or keratinocyte autocrine growth factor [9]), bind directly to a classic EGF receptor (EGFR) [3]. Members of the other group, including neu differentiation factor [10], also known as heregulin [11], glial growth factor [12] or acetylcholine receptor-inducing activity [13], bind directly to the recently cloned EGF receptor family, HER-3/erbB-3 [14,15] and HER-4/erbB-4 [16]. Results of recent studies showed that unlike EGF, TGF-α and HB-EGF, betacellulin mediates the signal directly through EGFR and HER-4/erbB-4 [17,18]. Although the binding and biological activities of the groups of growth factors are similar, the potencies of the factors are different. TGF-α and EGFR are ubiquitously expressed in normal tissues, whereas homogeneous null mutant mice for the TGF-α gene showed relatively minor phenotype alterations, including wavy hair and curly whiskers due to a disorganization of the hair follicle structure [19,20]. These results suggest that other family members can replace the function of these factors. In contrast, recent studies with mice homozygous for a disrupted EGFR gene showed that mutation of these molecules results in peri-implantation, mid-gestation and postnatal mortality depending on the genetic background [21–23]. Although it is well known that EGFR plays an essential role in morphogenesis/organogenesis, the physiological significance of the newly identified ligands for EGFR has not so far been elucidated.

We have recently purified the newest member of the EGF family, epiregulin, as a tumour growth-inhibitory factor inducing morphological changes in human epidermoid carcinoma HeLa cells. Epiregulin was obtained from a conditioned medium of mouse fibroblast-derived highly tumorigenic cell line NIH 3T3/cloned T7 [24]. Purified mouse epiregulin contains 46 amino acid residues with 24–50 % sequence identity with other members of the EGF family and exhibits low affinity for EGFR on human epidermoid carcinoma A431 cells compared with EGF and TGF-α.

In the present study we report the cloning and expression of human epiregulin. Our results show that, although other members of the EGF family are ubiquitously expressed in normal tissues, the level of expression of epiregulin was extremely low but clearly detected in the macrophages and placenta, and was high in carcinoma cells. This is the first study that reports the tissue and cellular distribution of human epiregulin and represents the initial step in our understanding of the physiological role of epiregulin in the regulation of normal and tumour cells.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, EGF receptor; HB-EGF, heparin-binding EGF-like growth factor; MEM, Eagle's minimal essential medium; MTN blot, multiple tissue Northern blot; TGF-α, transforming growth factor α.

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The nucleotide sequence reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number D30783.

MATERIALS AND METHODS

Cells and materials

HeLa, A431, A549, ACHN, Colo201, HCT-15, KB, NRC-12, WI38-VA13, HT-1080, Hos, A-172, CCF-STTG1, IMR-32, U-937, K-562, THP-1, HL-60 and Balb/3T3 clone A31 cells were purchased from Dainihon Pharmaceutical (Osaka, Japan). TR-24, TR-13 and TM-4 cells were tumour cells maintained in our laboratory. They were established from patients by members of our group, Dr. T. Ohnishi and Dr. T. Hashimoto (Jikei University School of Medicine, Tokyo, Japan).

Isolation of genomic and cDNA clones

Human placenta genomic DNA (Clontech Laboratories, Palo Alto, CA, U.S.A.) digested with *Hae*III was subjected to electrophoresis on 0.8 % agarose gel. A gel fraction of approx. 1.2 kb was excised; extracted DNA was ligated with *Not*I/*Eco*RI adaptor (Pharmacia LKB Biotechnology, Uppsala, Sweden), and inserted into λgt10 (Stratagene, La Jolla, CA, U.S.A.) as a vector. The library was screened with a 138 bp probe corresponding to 46-residue mouse mature epiregulin [25], which was amplified by PCR and labelled with [α-^{32}P]dCTP with a multiprime labelling kit (Amersham, Little Chalfont, Bucks., U.K.). Filter hybridization was performed at 37 °C for 16 h in a hybridization buffer containing 30 % formamide, 5 × SSC (1 × SSC, 0.15 M NaCl/0.015 M sodium citrate), 5 × Denhardt's solution, 0.5 % SDS, 100 μg/ml of sonicated heat-denatured salmon sperm DNA, and a ^{32}P-labelled probe. The blots were washed once in 2 × SSC containing 0.1 % SDS for 30 min at room temperature, then twice in 0.1 × SSC containing 0.5 % SDS for 30 min at 37 °C; Kodak X-Omat AR films were then exposed to the filters at −70 °C with intensifying screens. Twenty-four positive clones were isolated from approx. 3.0×10^5 phages and the insert was subcloned into the *Eco*RI site of pUC18 (htg-geno1.2) for DNA sequencing.

To construct the cDNA library, poly(A)$^+$ RNA of HCT-15 cells was prepared with a Quick prep mRNA purification kit (Pharmacia) and oligo(dT)-primed cDNA was synthesized with a cDNA synthesis kit (Pharmacia) in accordance with the instructions provided by the manufacturer. The cDNA library was constructed in λgt10. Library screening was performed with a 154 bp *Pst*I fragment of htg-geno1.2 under conditions of high stringency. Hybridization was performed with the above hybridization buffer containing 50 % formamide at 42 °C, after which the filters were washed at 50 °C. The positive clone was subcloned into the *Sma*I site of pUC18 (hTG-4.3) for DNA sequencing. We also amplified the 5′- and 3′-terminal portions of the cDNA with the rapid amplification of cDNA ends (RACE) system (Gibco BRL). The 5′-terminal portion was synthesized with a complementary primer corresponding to the C-terminal segment of putative mature epiregulin (nt 469–490 in Figure 2, 5′-TAAAAAGAAGTGTTCACATCGG-3′), and an oligo(dC) anchor sequence was added to the 5′-end of cDNA by terminal deoxynucleotidyltransferase, as the PCR template. PCR was performed with AmpliTaq DNA polymerase in a DNA thermocycler (Model 480; Perkin–Elmer Instruments, Norwalk, CT, U.S.A.) for 35 cycles at 94 °C for 1 min, 40 °C for 2 min, and 72 °C for 3 min with primers (nt 445–425, 5′-CCTGCAGT-AGTTTTGACTCAT-3′) and a commercially supplied anchor primer (5′-CUACUACUACUAGGCCACGCGTCGACTAG-TACGGGIIGGGIIGGGIIG-3′). The 3′-terminal portion was synthesized with a commercially supplied adaptor primer [5′-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3′]. The PCR was performed under the conditions described above, with primers (nt 4338–4359, 5′-GAAGAGCCATTTTGGTAAACG-3′) and a commercially supplied universal amplification primer [5′-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3′]. The resultant products were subcloned into the *Sma*I site of pUC18 for DNA sequencing. Amplified 5′ and 3′ fragments contained 136 bp and 240 bp overlapping regions with hTG-4.3 respectively.

DNA sequencing

The nucleotide sequence was determined by the dideoxynucleotide chain terminator method [26] with the Sequenase DNA-sequencing kit (United States Biochemical Co., Cleveland, OH, U.S.A.). The longest cDNA insert, hTG-4.3, was digested with several restriction enzymes. The fragments were subcloned into pUC, followed by a partial determination of the nucleotide sequence. The complete nucleotide sequence was determined by the primer walking method. Both strands of the cDNA were completely sequenced at least twice to confirm the sequence.

Northern blot analysis

Human multiple tissue Northern (MTN) blot I, MTN blot II and a cancer cell line were purchased from Clontech Laboratories (Palo Alto, CA, U.S.A.). Poly(A)$^+$ RNA species from tumour cell lines were isolated with a Quick prep mRNA purification kit (Pharmacia). Poly(A)$^+$ RNA species were separated through 1 % (w/v) agarose gel containing formaldehyde and transferred to nylon membranes (Hybond N; Amersham). Northern blot hybridization was performed with a PCR-amplified 550 bp fragment of human epiregulin cDNA (nt 149–698) as a probe, which contained the entire open reading frame, human TGF-α or β-actin cDNAs (Clontech), under conditions of high stringency, as recommended by the manufacturers of the MTN blots. Probes were labelled with [α-^{32}P]dCTP by using a multiprime labelling kit. Human TGF-α cDNA of 524 bp as a probe, which contained the entire open reading frame, was amplified by PCR with 5′-primer (5′-AAGAGCTCTGCCCGCCCGCCCGTAA-3′) and 3′-primer (5′-AAGGTACCAAACTCCTCCTCTGGGCT-3′), digested with *Sac*I and *Pst*I, and subcloned into pUC18 (the underlined bases indicate *Sac*I and *Pst*I respectively).

Separation of human peripheral blood cells

Heparinized peripheral blood samples were withdrawn from healthy donors who had signed a consent form. The peripheral blood cells were separated into mononuclear and polymorphonuclear leucocytes by Monopoly resolving medium (Dainihon Pharmaceutical Co., Osaka, Japan) gradient method, or separated into mononuclear leucocytes by the Ficoll-paque gradient method. A macrophage-enriched fraction was prepared as follows. Approximately 2.0×10^7 mononuclear leucocytes, suspended in 10 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) foetal bovine serum, were plated in a 90 mm diameter dish coated with human serum. After 2 h the plates were washed vigorously three times with PBS. Mouse peritoneal macrophages were isolated similarly. The tightly adherent cells were cultured with staphylococcal clumping factor (Sigma, St. Louis, MO, U.S.A.) for 30–60 min at 37 °C, fixed with methanol and stained with Giemsa solution (Merck). Phagocytic cells constituted more than 95 % of the macrophage fraction. Macrophage depletion was performed with KAC-2 (Japan Antibody Institute, Takasaki, Japan) containing silica suspension, in accordance with the instructions provided by the manufacturer. Briefly, heparinized peripheral blood was incu-

bated with 0.1 vol. of KAC-2 suspension for 30 min at 37 °C. The two phases were separated by Ficoll-paque and the upper phase was collected.

Recombinant expression in Verots S-3 cells

We constructed the blunt-ended 817 bp *Kpn*I–*Hin*dIII fragment (nt 53–869) of mouse epiregulin cDNA [25] and the PCR-amplified 550 bp fragment (nt 149–698) of human cDNA into a blunt-ended *Pst*I–*Kpn*I site of a plasmid vector pcDL-SRα-296 [27]. One day before transfection, 10^6 Verots S-3 cells (RIKEN Cell Bank, Tukuba, Japan) were plated into a 90 mm diameter dish containing Eagle's minimal essential medium (MEM; Nissui, Tokyo, Japan) supplemented with 5% (v/v) foetal bovine serum at 37 °C. The cells were transfected with 5 μg of plasmid DNA by the lipofection method with DOTAP (Boehringer Mannheim, Germany) at 33 °C for 6 h in MEM. This was followed by replacement of the medium with MEM supplemented with 5% foetal bovine serum. After 72 h the medium was replaced again with MEM or methionine-free DMEM, supplemented with 100 μCi of [^{35}S]methionine and [^{35}S]cysteine (Amersham). The cells were cultured at 33 °C for approx. 48 h. The supernatants of these cells were immunoprecipitated with anti-epiregulin polyclonal antibodies as described below.

Cellular proliferation assay

Balb/3T3 clone A31 cells were plated into 96-well plates at a density of 2×10^4 cells per well in 0.1 ml of DMEM supplemented with 5% (v/v) calf serum. After 48 h the medium was replaced with DMEM supplemented with 0.1% BSA, and cells were incubated with the conditioned media for 20 h. [^{3}H]Thymidine (Amersham) was added to 2.5 μCi/ml during the final 4 h of incubation. The cells were harvested by trypsin treatment, and radioactivity was counted with the Betaplate system (Pharmacia). In these assays, two independent experiments on triplicate plates were performed.

Preparation of anti-epiregulin antibodies and immunoprecipitation

Anti-human epiregulin polyclonal rabbit antibodies were prepared as described previously [24] by immunizing recombinant human epiregulin (residues 63–108 in Figure 2) produced by *Bacillus brebis* (K. Nakazawa, T. Komurasaki, M. Takahashi, T. Ohmuki, D. Uchida, H. Toyoda and S. Morimoto, unpublished work). The antibodies did not recognize human EGF or TGF-α. The conditioned media were incubated with 2.5 μg/ml of normal rabbit IgG for 60 min in the absence or presence of 1 μg/ml of recombinant human epiregulin, and then with 20 μl of Protein G–Sepharose (50%) (Pharmacia) for approx. 15 h at 4 °C. Similarly the supernatants were incubated with 2.5 μg/ml of rabbit anti-(human epiregulin) IgG and Protein G–Sepharose. After incubation, the pellets were washed five times with 20 mM Tris/HCl, pH 7.4, containing 0.5% Triton X-100 and 0.15 M NaCl. The precipitates were suspended in 20 μl of SDS/PAGE sample buffer. The supernatants were resolved by SDS/PAGE [10–25% (w/v) gel] under reducing conditions. Finally, the film was exposed to the fixed gel for 16–63 h and proteins were detected with a FUJIX Bio-imaging Analyser BAS2000 (Fuji Film Co., Tokyo, Japan).

RESULTS

Molecular cloning of human epiregulin

Epiregulin transcript-related signal was not detected in several normal human tissues by using mouse epiregulin cDNA encoding the mature form under conditions of low stringency. Accordingly, we attempted to isolate human genomic DNA fragments for

Figure 1 Nucleotide sequence of the segment containing the exon coding for the first 33 residues of human epiregulin

Boxes indicate the consensus sequence for the acceptor and donor sites of intervening sequences [28]. The lower numbers at the right indicate the amino acid positions cited in Figure 2.

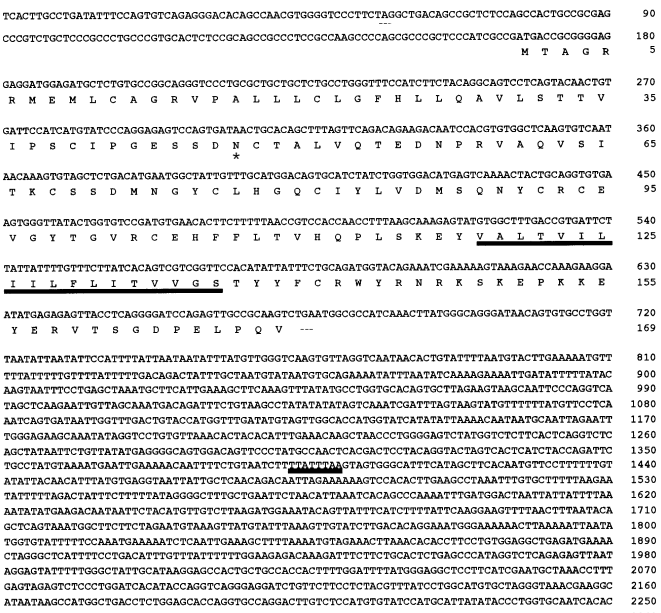


Figure 2 Nucleotide sequence and deduced amino acid sequence of human epiregulin cDNA

The bold lines underneath amino acids represent the strong hydrophobic stretch based on the algorithm of Kyte and Doolittle [60]. The destabilizing signals (TTATTTA/TA/T) for cytokine mRNA and polyadenylation signal (AATAAA) are underscored with single and double lines respectively. The potential N-glycosylation site is denoted by an asterisk. The thin line indicates the region homologous to the *Alu*I family. Numbers at the right indicate the position of nucleotide (above) and amino acid (below).

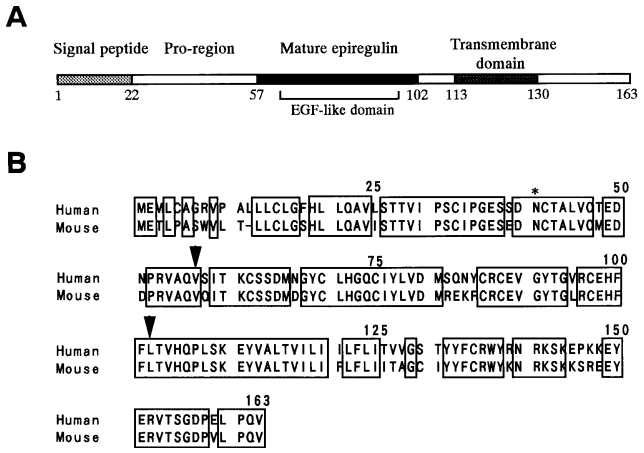
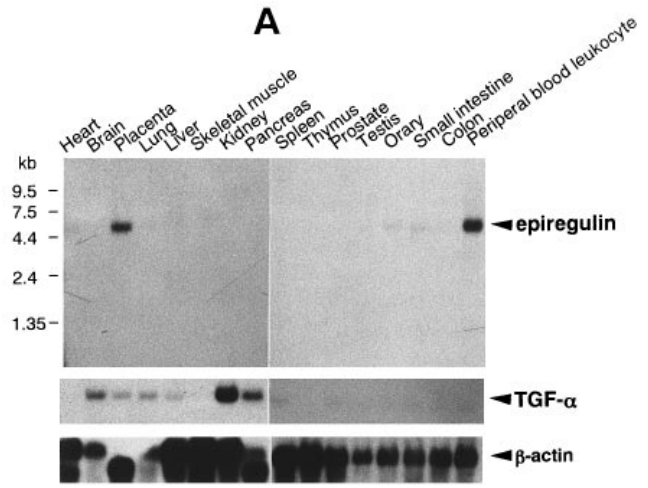


Figure 3 Amino acid sequence of the human epiregulin precursor

(**A**) Schematic diagram of the structural organization of epiregulin precursor. Signal peptide was deduced from the criteria of von Heijne [61,62]. (**B**) Protein sequence comparison between human and mouse epiregulin precursors deduced from cDNA. A potential N-glycosylation site is denoted by an asterisk. Arrows indicate N- and C-termini of a purified preparation of mouse epiregulin.



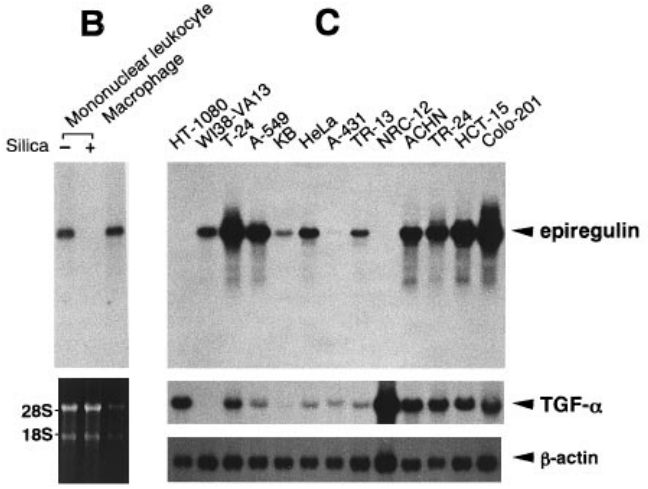


Figure 4 Northern-blot analysis of epiregulin expression in normal human tissues and tumour cell lines

(**A**) Normal tissues; (**B**) mononuclear leucocytes; (**C**) tumour cell lines. Nylon membranes filters, to which 2 μg and 10 μg of poly(A)$^+$ RNA species were transferred in (**A**) and (**C**) respectively, were probed with human epiregulin and reprobed with human TGF-α cDNA. Filters probed with epiregulin or TGF-α cDNA were exposed for 120 or 40 h in (**A**) respectively, and for 40 h in (**C**), at -80 °C. Human MTN blot I and II filters were used in (**A**). The signal for epiregulin in the heart was also detectable in another MTN blot I filter and commercially supplied poly(A)$^+$ RNA. (**B**) Mononuclear leucocytes in peripheral blood cells were separated by the Ficoll-paque gradient method, and macrophages were isolated by the panning method with a dish coated with human serum. Depletion of macrophages from mononuclear leucocytes was achieved by incubation with silica suspension. Lanes of macrophages, mononuclear leucocytes ($-$silica) and macrophage-depleted leucocytes ($+$silica) were loaded with 5, 30 and 30 μg of total RNA respectively. RNA species were stained with ethidium bromide (lower panel) and then hybridized with epiregulin cDNA (upper panel).

epiregulin. Among the human genomic DNA fragments digested by several restriction enzymes, approx. 1.2 kb of completely digested *Hae*III fragments was constructed in the λgt10 phage, and the library was screened with the above probe. An isolated clone containing an insert of 1239 bp was found to code the first 33 residues of mature human epiregulin on the basis of similarity to mouse epiregulin (Figure 1). The codon for the 33rd residue was followed by a stop codon, suggesting that the GT sequence at that position marks the donor site of the intervening sequence [28]. No other region was found to code human epiregulin in this fragment. To identify the source of the human epiregulin mRNA, we screened several human tumour cell lines by Northern hybridization with a 154 bp *Pst*I fragment containing a single exon as a probe. A hybridized signal was detected in a colon carcinoma cell line, HCT-15. The screening of approx. 6×10^6 recombinant phages from the HCT-15 cDNA library, with the above probe and conditions of high stringency, yielded a single positive clone. The nucleotide sequence covered nt 310–4576. Because the length of epiregulin transcript was estimated to be approx. 4.8 kb as described below, the sequence of the ends of the cDNA was determined with a RACE–PCR system.

Nucleotide sequence and deduced amino acid sequence analysis of human epiregulin

Figure 2 shows the complete nucleotide sequence of the 4627 bp cDNA and the deduced amino acid sequence. An in-frame stop codon was located 109 bp upstream from the first ATG. Although the first to third ATGs did not conform to the optimal consensus sequence for eukaryotic mRNA translational initiation sites [29], it was assumed that the second ATG was the translation initiator owing to the similarity of the amino acid sequence to that in the mouse homologue. The cDNA encoded a single open reading frame for a protein of 163 residues. The termination codon was followed by a 3951 bp 3′-untranslated region containing two destabilizing signals (TTATTTA/TA/T) for cytokines and proto-oncogene mRNA species [30,31], and five polyadenylation signals (AATAAA) were present. Sequences similar to the *Alu*I repeat were located at approximately nt 2650–2950. The identity of the amino acid sequence between human and mouse epiregulin precursor was 85% (Figure 3B). In addition, a highly hydrophobic stretch was observed at residues 113–132, which was considered to be a putative transmembrane domain. A single potential N-linked glycosylation site was found in the N-terminal pro-region of the epiregulin precursor as well as TGF-α. A

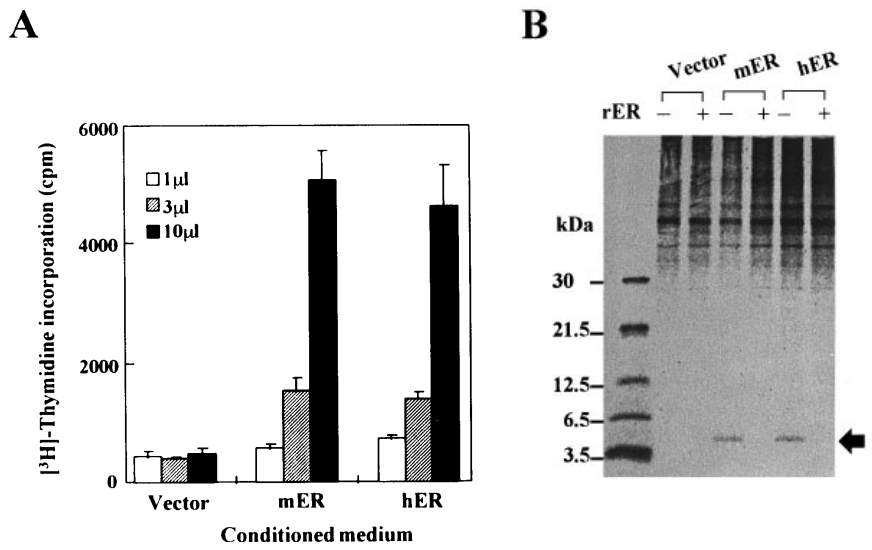


Figure 5 Expression of recombinant epiregulin in Verots S-3 cells

(**A**) Mitogenic activities on Balb 3T3/clone A31 cells of conditioned media from transfected Verots S-3 cells. Balb 3T3/clone A31 cells were cultured with indicated amounts of conditioned media from Verots S-3 cells transfected with a pcDL-SRα-296 alone (Vector), or constructs containing precursors of mouse (mER) or human epiregulin (hER) cDNA. The DNA synthesis was determined by measuring the incorporation of [^{3}H]thymidine. (**B**) Immunoprecipitation with anti-epiregulin antibodies of conditioned media from transfected Verots S-3 cells. ^{35}S-labelled conditioned media (4 ml) from transfected cells with a pcDL-SRα-296 alone (Vector), or constructs encoding precursors of mouse (mER) or human epiregulin (hER) cDNA were immunoprecipitated with anti-epiregulin antibodies in the presence (+) or absence (−) of an excess of recombinant human epiregulin (rER). The arrow indicates an immunospecific band for epiregulin.

relatively basic region, rich in arginine and lysine residues, was detected in a putative cytoplasmic domain at residues 140–148, as well as other members of the EGF family. The exact function of this region is not known at present.

Expression of epiregulin mRNA in normal tissues and tumour cell lines

Northern-blot analysis showed an epiregulin transcript of approx. 4.8 kb expressed predominantly in the human placenta and peripheral blood leucocytes and to a lesser degree in the heart of normal adults (Figure 4A). In addition, very weak signals, requiring a long exposure time, were observed in samples from the bone marrow, ovary, small intestine and colon. Very faint bands were also detected in samples from the lung and liver, depending on the particular mRNA preparation. The expression level of epiregulin in the placenta and peripheral blood leucocytes was markedly lower than that of TGF-α in the kidney and pancreas (Figure 4A). These results indicate that the expression of epiregulin in normal adult tissues examined in the present study was extremely low compared with that of other members of the EGF family. In the next series of experiments, human peripheral blood cells were freshly fractionated as described in the Materials and methods section. Epiregulin transcript was detected in macrophages from mononuclear leucocytes (Figure 4B). Incubation of peripheral blood cells with a silica suspension to deplete macrophages decreased the expression of epiregulin in mononuclear leucocytes. Epiregulin transcript was also detected in mouse peritoneal macrophages (results not shown).

Several human tumour cell lines were examined for the expression of epiregulin (Figure 4C). High levels of expression were detected in carcinomas of the bladder (T-24), lung (A-549), kidney (ACHN and TR-24) and colon (Colo201 and HCT-15). Furthermore, the expression was also detected in epidermoid carcinomas (HeLa and KB, but not A-431), carcinomas of the kidney (TR-13, but not NRC-12), and in transformed fibroblasts (WI38-VA13), but not in fibrosarcomas (HT-1080). In contrast, no expression was present in osteogenic carcinoma (Hos), glioblastoma (A172), astrocytoma (CCF-STTG1), neuroblastoma (IMR-32) or melanoma (G361 and TR-4) (results not shown). In haematopoietic cell lines the expression was detected in histiocytic monocyte-like lymphoma (U-937), but the level was very low in chronic myelogenous leukaemia (K562). No expression was present in monocytic leukaemia (THP-1), myelocytic leukaemia (HL-60), Burkitt's lymphoma (B-cell line) (Raji) or acute lymphoblastic leukaemia (T-cell line) (Molt-4) cells (results not shown). The pattern of epiregulin expression was not identical with that of TGF-α. For example, the expression of TGF-α was observed in HT-1080, and highly in NRC-12 cells, whereas no expression of epiregulin was detected in the same cell lines. In contrast, a signal for epiregulin but not TGF-α was detected in WI38-VA13 and KB cells.

Recombinant expression of epiregulin in mammalian cells

The precursor regions of human and mouse epiregulin cDNA were transiently expressed in monkey fibroblasts (Verots-S3) under the control of a strong eukaryotic promoter, SRα. The conditioned media from mouse and human epiregulin cDNA transfectants stimulated DNA synthesis in mouse fibroblast Balb 3T3/clone A31 cells in a dose-dependent manner, in marked contrast with that from transfectant with vector alone (Figure 5A). In the next step, the conditioned media were immunoprecipitated with anti-epiregulin polyclonal antibodies and subjected to SDS/PAGE (Figure 5B). The results identified a single radiolabelled band of approx. 5 kDa in the supernatants of mouse and human cDNA transfectants. The band was immunospecific for epiregulin because it diminished when immunoprecipitation was performed in the presence of an excess of epiregulin. No other immunospecific band was observed with a

molecular mass larger than 5 kDa. These findings indicate that human epiregulin is secreted from normal cells in a biologically active mature form of 5 kDa.

DISCUSSION

We cloned the complete human epiregulin cDNA from colon carcinoma HCT-15 cells and a partial genomic DNA fragment containing an exon from human placenta. The structural features of human epiregulin precursor, including the signal peptide, pro-region and EGF-like motif, followed by a spacer region, putative transmembrane domain and a putative cytoplasmic tail, were characteristic of other members of the EGF family that bind to EGFR. In addition, the structure of epiregulin was most similar to that of TGF-α among the EGF family members on the basis of the length of each segment and the location of a single potential N-glycosylation site in the pro-region. Furthermore, a single exon encoded the first 33 residues of putative mature epiregulin, which contains only the first and second disulphide bonds of the EGF-like motif. The genes for EGF family members, including TGF-α [32], amphiregulin [33] and HB-EGF [34], are divided into six exons, and the EGF gene [35] has 24 exons. The exon organization of the EGF-like motif of these members is conserved. Interestingly, the sequence encoding between the second and third disulphide bonds of the EGF-like motif was identically interrupted by an intron in epiregulin and other EGF members. Thus the exon/intron organization of epiregulin might be similar to those of other members. On the basis of these structural and biochemical characteristics, epiregulin is considered to be the sixth member of the EGF family present in the human genome that interacts with EGFR.

Our results showed that human epiregulin was predominantly expressed in peripheral blood macrophages and the placenta. Although it was also expressed in other tissues of normal adults, the level of expression was markedly lower than that of TGF-α. This represents a unique profile compared with other members of the EGF family. Members of the EGF family are also abundantly detected in a variety of normal adult tissues. For example, EGF is predominantly expressed in the submaxillary glands, distal tubules of the kidney and mammary glands [36], whereas amphiregulin is expressed to a large extent in the ovaries and placenta [33], HB-EGF in the heart, lung, brain and skeletal muscles [37], and betacellulin in the liver and kidney [38]. Initial studies failed to identify the source of expression of TGF-α in normal adult cells, partly owing to the low expression and cell type specificity [39]. The expression of TGF-α was detected in the kidneys, pancreas, brain, placenta, lungs and liver after exposure for a short time. In contrast, the expression of epiregulin was not clearly evident under similar conditions and the use of a similar blot filter. We have reported recently that epiregulin mRNA is not detected in several normal tissues of the adult mouse by Northern-blot analysis with the MTN blot [25]. However, performing Northern-blot analysis with freshly isolated mRNA allowed the detection of weak signals of mouse epiregulin transcript, particularly after long exposure. These signals were detected predominantly in the lungs, smooth muscles and heart. Thus the expression level of epiregulin seems to be extremely low in normal adult tissues examined in the present study.

Macrophages produce a number of polypeptide mediators, including platelet-derived growth factor, TGFs, fibroblast growth factor, interferon α and interleukins, in response to inflammation and wound repair [40–44]. Epiregulin transcript was also detected in macrophages. In contrast, expression of TGF-α was not clearly observed in the human peripheral blood lymphocytes fraction when experiments similar to those on epiregulin were performed with 20 μg of total RNA, indicating that the expression of epiregulin was markedly higher than that of TGF-α in resting cells. Previous studies showed that HB-EGF, heparin-binding macrophage-derived growth factor, is found in pig wound fluid within the first few days of injury [45]. A large body of evidence indicates that EGF and TGF-α promote wound healing *in vivo* [46–50]. Thus, on the basis of the known function of other EGFs, epiregulin is potentially involved in wound healing as a non-heparin-binding macrophage-derived growth factor. Furthermore, the relatively restricted distribution of epiregulin suggests that it might have a functional significance in macrophages. In addition, epiregulin transcript was detected in the human placenta. We have recently shown that the transcript is detected in 7-day-old mouse embryo, diminishing thereafter to very low or undetectable levels [25]. Considered together, epiregulin is likely to play a role in morphogenesis/organogenesis during the early stages of development.

Our results also showed that epiregulin was expressed in 9 out of 11 human carcinoma cell lines, and was particularly over-expressed in colon carcinomas. These studies also showed that epiregulin was not expressed in three brain-derived tumour cell lines, two melanomas and four out of six haematopoietic cell lines. It should be noted that EGF and/or TGF-α, as well as EGFR, are also co-expressed in a number of human tumours [51–56]. Their overexpression is thought to be involved in the pathogenesis of these malignancies as well as in their poor prognosis. For example, approx. 40–50 % of oestrogen-receptor-negative breast tumours overexpress EGFR and are associated with early relapse and poor survival [57]. Furthermore, approx. 80–90 % of colorectal carcinomas express EGFR [58,59]. Preliminary results from our laboratory have shown that the expression of epiregulin is extremely low in mouse normal fibroblasts, Balb 3T3/clone A31 cells and NIH 3T3 cells, whereas it is overexpressed in transformed fibroblasts L-929 and NIH 3T3/clone T7 that acquired tumorigenicity in athymic mice (T. Komurasaki, H. Toyoda, D. Uchida and K. Hanada, unpublished work). These experiments also demonstrated a much weaker anchorage-independent growth of rat kidney NRK 49F cells by epiregulin compared with EGF in the presence of TGF-β. Considered together, our findings suggest that epiregulin is probably involved in the progression of carcinomas of certain phenotype.

Finally, we are currently investigating the ability of ^{125}I-labelled epiregulin to bind to four ErbB-family receptors and following tyrosine phosphorylation by using breast carcinoma cell lines that express different subsets of receptors. Our preliminary results have shown that the binding of ^{125}I-labelled epiregulin to breast carcinoma SK-BR-3 cells that express EGFR, ErbB-2 and ErbB-3 is completely inhibited by pretreatment with anti-EGFR antibody that binds to the extracellular domain of EGFR. Activation of tyrosine phosphorylation of proteins by epiregulin was also inhibited completely in the presence of anti-EGFR antibody in SK-BR-3 cells (T. Komurasaki, H. Toyoda, D. Uchida and S. Morimoto, unpublished work). These results suggest that the signal of epiregulin is mediated through EGFR. Studies designed to examine receptors of epiregulin will be conducted in our laboratory in the near future.

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REFERENCES

1 Aaronson, S. A. (1991) Science **254**, 1146–1152
2 Cross, M. and Dexter, M. (1991) Cell **64**, 221–280

3 Carpenter, G. and Cohen, S. (1990) J. Biol. Chem. **265**, 7709–7712
4 Marquardt, H., Hunkapiller, M. W., Hood, L. E. and Todaro, G. J. (1984) Science **223**, 1079–10824
5 Higashiyama, S., Abraham, J. A., Miler, J., Fiddes, J. C. and Klagsbrun, M. (1991) Science **251**, 936–939
6 Sing, Y., Christofori, G., Hanahan, D., Ono, Y., Sasada, R., Igarashi, K. and Folkman, J. (1993) Science **259**, 1604–1607
7 Shoyab, M., Plowman, G. D., McDonald, V. L., Bradley, J. B. and Todaro, G. J. (1989) Science **243**, 1074–10765
8 Kimura, H., Fischer, W. H. and Schubert, D. (1990) Nature (London) **384**, 257–260
9 Cook, P. W., Mattox, P. A., Keeble, W. W., Pittelkow, M. R., Plowman, G. D., Shyoab, M., Adelman, J. P. and Shipley, G. D. (1991) Mol. Cell. Biol. **11**, 2547–2557
10 Wen, D., Peles, E., Copples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Sibiger, S. M., Levy, R. B. et al. (1992) Cell **69**, 559–572
11 Homes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D. et al. (1992) Science **256**, 1205–1210
12 Marchionni, M. A., Goodearl, A. D. J., Chen, M. S., Bermingham-McDonough, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K. et al. (1993) Nature (London) **362**, 312–318
13 Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S. and Fiscchbach, G. D. (1993) Cell **72**, 801–805
14 Kraus, M. H., Issing, W., Miki, T., Popescu, N. C. and Aronson, S. A. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 9193–9197
15 Plowman, G. J., Whitney, G. S., Neubauer, M. G., Green, J. M., McDonald, V. L., Todaro, G. J. and Shoyab, M. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 4905–4909
16 Plowman, G. J., Culouscou, J.-M., Whitney, G. S., Green, J. M., Carlton, G. W., Neubauer, M. G. and Shoyab, M. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 1746–1750
17 Riese, II, D. J., Bermingham, Y., van Raij, T. M., Buckley, S., Plowman, G. J. and Stern, D. F. (1996) Oncogene **12**, 345–353
18 Beerli, R. R. and Hynes, N. E. (1996) J. Biol. Chem. **271**, 6071–6076
19 Luetteke, N., Qui, T. H., Peiffer, R. L., Oliver, P., Smithies, O. and Lee, D. C. (1993) Cell **73**, 263–278
20 Mann, G. B., Fowler, K. J., Gabriel, A., Nice, E. C., Williams, R. E. and Dunn, A. R. (1993) Cell **73**, 249–261
21 Thresdgill, D. W., Dlugosz, A. A., Hansen, L. A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R. C. et al. (1995) Science **269**, 230–234
22 Sibiia, M. and Wanger, E. F. (1995) Science **269**, 234–238
23 Miettinen, P. J., Berger, J. E., Meneses, J., Phung, Y., Pedersen, R. A., Werb, Z. and Derynck, R. (1995) Nature (London) **376**, 337–341
24 Toyoda, H., Komurasaki, T., Uchida, D., Takayama, Y., Isobe, T., Okuyama, T. and Hanada, K. (1995) J. Biol. Chem. **270**, 7495—7500
25 Toyoda, H., Komurasaki, T., Ikeda, Y., Yoshimoto, M. and Morimoto, S. (1995) FEBS Lett. **377**, 403–407
26 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
27 Takebe, Y. M., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M. and Arai, N. (1988) Mol. Cell. Biol. **8**, 466–472
28 Cech, T. R. (1983) Cell **34**, 713–716
29 Kozak, M. (1984) Nucleic Acids Res. **12**, 857–872
30 Lagnado, C. A., Brown, C. Y. and Goodall, G. J. (1994) Mol. Cell. Biol. **14**, 7984–7995
31 Zubiaga, A. M., Belasco, J. G. and Greenberg, M. E. (1995) Mol. Cell. Biol. **15**, 2219–2230
32 Brissenden, J. E., Derynck, R. and Francke, U. (1985) Cancer Res. **45**, 5593–5597
33 Plowman, G. D., Green, J. M., McDonald, V. L., Neubauer, M. G., Disteche, C. M., Todaro, G. J. and Shoyab, M. (1990) Mol. Cell. Biol. **10**, 1969–1981
34 Fen, Z., Dhadly, M. S., Yoshizumi, M., Hilkert, R. J., Quertermous, T., Eddy, R. L., Shows, T. B. and Lee, M. E. (1993) Biochemistry **32**, 7932–7938
35 Brissenden, J. E., Ullrich, A. and Francke, U. (1984) Nature (London) **310**, 781–784
36 Crawfold, R. J., Penschow, J. D., Niall, H. D. and Coghlan, J. P. (1985) Nature (London) **313**, 228–231
37 Abraham, J. A., Damm, D., Bajardi, A., Miler, J., Klagsbrun, M., Alan, R. and Ezekowitz, B. (1993) Biochem. Biophys. Res. Commun. **190**, 125–133
38 Sasada, R., Ono, Y., Taniyama, Y., Shing, Y., Folkman, J. and Igarashi, K. (1993) Biochem. Biophys. Res. Commun. **190**, 1173–1179
39 Derynck, R. (1992) Adv. Cancer Res. **58**, 27–52
40 Ross, R. (1987) Annu. Rev. Med. **38**, 71–79
41 Sporn, M. and Robert, A. (1988) Nature (London) **332**, 217–219
42 Folkman, J. and Klagsbrun, M. (1987) Science **235**, 442–447
43 Oppenheim, J. J. and Neta, R. (1994) FASEB J. **8**, 158–162
44 Madtes, D. K., Raines, E. W., Sakariassen, K. S., Assoian, R. K., Sporn, M. B., Bell, G. I. and Ross, R. (1988) Cell **53**, 285–293
45 Marikovsky, M., Breuing, K., Liu, P. Y., Eriksson, E., Higashiyama, S., Farber, P., Abraham, J. and Klagsbrun, M. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 3889–3893
46 Brown, G., Curtsinger, L., Brightwell, J., Ackerman, D., Tobin, G., Polk, H., George-Nascimento, C., Valenzuela, P. and Schultz, G. (1986) J. Exp. Med. **163**, 1319–1324
47 Schultz, G., White, M., Mitchell, R., Brown, G., Lynch, J., Twardzik, D. and Todaro, G. (1987) Science **235**, 350–352
48 Lynch, S., Nixon, J., Colvin, R. and Antoniades, H. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 7696–7700
49 Brown, G., Nanney, L., Griffen, J., Cramer, B., Yancey, J., Curtsinger, L., Holtzin, L., Schultz, G., Jurkiewicz, M. and Lynch, B. (1989) New Engl. J. Med. **321**, 76–79
50 Musoe, T., Pierce, G., Morishima, C. and Deuel, T. (1991) J. Clin. Invest. **87**, 694–703
51 Smith, J. J., Derynck, R. and Korc, M. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 837–841
52 Nister, M., Libermann, T. A., Betshholtz, C., Pettersson, M., Claesson-Welsh, L., Heldin, C.-H., Schlessinger, J. and Westermark, B. (1988) Cancer Res. **48**, 3910–3918
53 Mydlo, J. H., Michaeli, J., Cardo, C. C., Goldenberg, A. S., Heston, W. D. W. and Fair, W. R. (1989) Cancer Res. **49**, 3407–3414
54 Markowitz, S. D., Molkentin, K., Gerbic, C., Jackson, J., Stellato, T. and Willson, J. K. (1990) J. Clin. Invest. **86**, 356–362
55 Tateishi, M., Ishida, T., Mistudomi, T., Kaneko, S. and Sugimachi, K. (1990) Cancer Res. **48**, 137–141
56 Stromberg, K., Collins, IV, T. J., Gordon, A. W., Jackson, C. L. and Johnson, G. R. (1992) Cancer Res. **52**, 341–347
57 Harris, A. L., Nicholson, S., Sainsbury, J. R. C., Farndon, J. and Wright, C. (1989) J. Steroid Biochem. **34**, 123–131
58 Yasui, W., Sumiyoshi, H., Hata, J., Kameda, T., Ochiai, A., Ito, H. and Tahara, E. (1988) Cancer Res. **48**, 137–141
59 Steele, R. J. C., Kelly, P., Ellul, B. and Eremin, O. (1990) Br. J. Cancer **61**, 345–326
60 Kyte, J. and Doolittle, R. F. (1982) J. Mol. Biol. **157**, 105–132
61 von Heijne, G. (1983) Eur. J. Biochem. **13**, 17–21
62 von Heijne, G. (1985) J. Mol. Biol. **184**, 99–105

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