Expression of Epidermal Growth Factor in Transgenic Mice Causes Growth Retardation*

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The epidermal growth factor (EGF) family of peptides signals through the erbB family of receptor tyrosine kinases and plays important roles in development and tumorigenesis. Both EGF and transforming growth factor (TGF)-α only bind to erbB1 and activate it. The precursor of EGF is distinct from that of TGF-α in having eight additional EGF-like repeats. We have recently shown that the EGF precursor without these repeats is biologically active and leads to hypospermatogenesis in transgenic mice. Here we present evidence that the growth of transgenic mice widely expressing this engineered EGF precursor is also stunted. These mice were consistently born at half the normal weight and reached almost 80% of normal weight at adulthood. The mechanism involved a reduction of serum insulin-like growth factor-binding protein-3. Chondrocyte development in the growth plate was affected, and osteoblasts accumulated in the endosteme and periosteum. Besides these novel findings on the in vivo effects of EGF on bone development, we observed no sign of tumor formation in our transgenic animals. In contrast to previous reports on TGF-α transgenic mice, we show that the biological functions of EGF and TGF-α are clearly distinct.

Epidermal growth factor (EGF) was initially identified from mouse submaxillary gland extract as a stimulator of eyelid opening and incisor eruption when injected into newborn mice and rats (1). Mature human EGF is composed of 53 amino acids but is derived from a much larger transmembrane precursor of 1207 amino acids (2). It belongs to the EGF family of peptides that signals through the erbB receptors, with EGF receptor being the prototype (3). EGF is released from its precursor by a specific arginine estero-peptidase that, in many cells, appears to be limiting (4). However, processing occurs in granular convoluted tubules of the submandibular gland, and EGF is released mainly into saliva (5).

Transforming growth factor (TGF)-α binds to the EGF receptor with an affinity similar to that of EGF, and the two share many biological effects. TGF-α is a 50-amino acid polypeptide derived from a 160-amino acid membrane-bound precursor. It was initially isolated as one of the transforming peptides from sarcoma virus-transformed fibroblasts (6). EGF, TGF-α, and amphiregulin only bind and activate EGF receptors (also called erbB1 and HER1) (7), and they are referred to as group one of the EGF family. In recent years, information on the EGF family and erbB receptor family has expanded rapidly. In in vitro studies on cells expressing multiple erbB family members, signal specificity was shown to be controlled by ligand specificity as well as receptor homo- and heterodimerization (8).

Important information on the in vivo functions of erbB signaling has been gained from transgenic mice that overexpress the ligand as well as loss of function mutants (9). Mice deficient in one or all three of EGF, TGF-α, and amphiregulin revealed their distinct role in mammary gland development (10). Mice without TGF-α or with a mutant EGF receptor showed an identical phenotype of affected hair and eyelid development (11–13). In addition to these defects, mice with a null mutation in EGF receptor died at peri-implantation, midgestation, or shortly after birth, depending on their genetic background (14–16). In cancer tissues, overexpression of EGF receptor, TGF-α, and amphiregulin, but not EGF, is frequently found (7). In agreement with this observation, transgenic mice overexpressing TGF-α showed epithelial hyperplasia of several organs, pancreatic metaplasia, and breast carcinoma (17, 18). Amphiregulin was found to be a preneoplastic tumor marker in transgenic models of mammary tumors, including transgenic mice of TGF-α and erbB2 (19). Expression of amphiregulin in basal keratinocytes induced a psoriasis-like phenotype in transgenic mice (20). To provide further information on the physiological and pathological roles of EGF and to distinguish its in vivo effects from those of other EGF receptor ligands, we have generated transgenic mice widely expressing a shortened human EGF precursor (hEGF). The eight EGF-like repeats were deleted, leaving the active EGF domain in the transmembrane form. This would release the effect of EGF-like repeats, if any, on the exposure of the EGF domain and allows direct comparison of its effects with TGF-α. Our previous study has shown that hEGF, like the full-length precursor, is biologically active in transforming NIH3T3 (21).

Various in vitro studies have shown that EGF reduces synthesis of insulin-like growth factor (IGF) and IGF-binding protein-3 (22, 23). In vivo, IGF action is influenced by the IGF-binding proteins (IGFBPs). Six IGFBPs have been found that differ in their influence on IGF activity. Besides increasing the half-life of IGFs in circulation, IGFBPs can potentiate activities of IGFs on cell proliferation. In addition, IGF-independent regulatory mechanisms of IGFBPs have been described. IGF-independent growth inhibition by IGFBP-3 is believed to occur through IGFBP-3-specific cell surface association proteins or receptors and involves nuclear translocation (24).

*This work was supported by a grant from the Hong Kong Research Grants Council (to S.-Y. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: EGF, epidermal growth factor; TGF, transforming growth factor; hEGF, shortened human EGF precursor; IGFBP, insulin-like growth factor-binding protein; IGF, insulin-like growth factor; PCR, polymerase chain reaction.
have been developed over the past few years (25). The overexpression of IGFBP-3 under the control of a ubiquitous promoter resulted in selective organomegaly (26). Recent data indicate that low levels of IGFBP-3 are associated with stunted growth and an increased risk of at least several types of carcinoma that are common in economically developed countries (24, 27). Additional studies are required to determine the clinical relevance of these findings.

To elucidate the role of EGF in vivo, we have recently overexpressed hEGF in transgenic mice. The two major phenotypes were infertility and stunted growth (28). Here we investigated the possible mechanisms leading to the growth problem and the relationship between EGF and IGFBP-3.

**EXPERIMENTAL PROCEDURES**

*Generation of Transgenic Mice—*The procedures for microinjection have been described previously (29). The DNA construct consisted of EGF with the \(\beta\)-actin promoter to give widespread expression in transgenic animals. The eight EGF-like repeats in the extracellular domain of hEGF were removed as described previously (21). Transgenic mice were characterized by Southern analysis, immunoblotting, and immunohistochemistry of hEGF (28, 30).

*Radioimmunoassay of Serum IGFBP-3—*Blood was collected by cardiac puncture immediately after the animal was sacrificed by cervical dislocation. The blood samples were allowed to clot for 15 min on ice, and then serum was collected by centrifugation. Aliquots were stored at \(-20^\circ C\). Serum IGFBP-3 was measured using undiluted serum with the immunoradiometric assay kit from Diagnostic Systems Laboratories, Inc. Controls were age- and strain-matched normal mice including nontransgenic littermates. The statistical difference between the transgenic and control groups was analyzed using the Mann-Whitney test.

*Histology of Long Bone in Postnatal Mice—*The hind limb was dissected away from tendon and muscle and then fixed in 4% paraformaldehyde overnight at 4 \(^\circ C\). Bone was decalcified using a procedure described in Ref. 31 modified as follows: bone was washed four times with water for 15 min each and then immersed in 20% EDTA in water and kept at 4 \(^\circ C\). EDTA solution was changed every second day during the first week and every third day during the second week or a longer period. Finally, bone was washed for a total of at least 6 h with five changes of water before embedding in fibrowax (Gurr, BDH). Sections were cut at 6-\(\mu\)m thickness.

*Immunohistochemistry—*Antigen detection was based on the streptavidin-biotinylated peroxidase system (Dako). The procedures have been described in detail previously (32). To detect human but not mouse EGF protein, the polyclonal antibody Ab-3 (Calbiochem) was used at a dilution of 1:1000 for bone sections. Sections from nontransgenic mice were used as negative controls. Endogenous EGF expression was identified using a polyclonal anti-mouse EGF antibody (Serotec) at a dilution of 1:500 and 1:1000. To confirm the specificity of signals obtained with anti-mouse EGF antibody, the diluted antibody was preincubated with 10 \(\mu\)m murine natural EGF (Life Technologies, Inc.) overnight at 4 \(^\circ C\) before use.

*Reverse Transcription-PCR—*Expression of EGF was studied in a chondrocyte cell line, MCT, which was derived from mouse rib primary chondrocytes immortalized with temperature-sensitive \(\beta\)-actin antigen. At a nonpermissive temperature of 37 \(^\circ C\), these cells stop growing and acquire characteristics of hypertrophic chondrocytes (33). Total RNA was extracted, DNase-digested, quantified by absorbance at 260 nm, reverse-transcribed with oligo(dT), and PCR-amplified as described previously (34). EGF was amplified with primers 5'-GAGAATTCCGCCTGCACCAAC and 5'-TCTGCTGGATTACATTAAAGCACTG and 5'-GCTGCTGGATTACATTAAAGCACTG and 5'-TCTGCTGGATTACATTAAAGCACTG. After 30 cycles of 94 \(^\circ C\) for 30 s, 57 \(^\circ C\) for 30 s, and 72 \(^\circ C\) for 1 min, half of the product was electrophoresed. cDNA samples from mouse adult kidney and embryos at day 17.5 were used as positive controls. Amplification with primers for hypoxanthine phosphoribosyl transferase (bpt), 5'-CCTGCTGGATTACATTAAAGCACTG and 5'-GCTAAGGGCCATATC-CAACACAAAC, served as a PCR control. After 30 cycles (94 \(^\circ C\) for 30 s, 55 \(^\circ C\) for 30 s, and 72 \(^\circ C\) for 1 min), one-eighth of the product was electrophoresed.

*RESULTS AND DISCUSSION* We have recently reported the generation of EGF transgenic mice. They all expressed human EGF protein at high levels in various organs, and their fertility problem has been reported previously (28). Growth rate and body weight were compared with those of nontransgenic littermates. All transgenic animals were born at only half the weight of their normal littermates. They caught up by day 20 and reached 78% of the weight of nontransgenic littermates at adulthood (Fig. 1A). The transgenic mice appeared to be proportionate dwarfs. In the current study, we focused on investigating the mechanism leading to stunted growth.

**EGF Reduced Serum IGFBP-3—**IGF-I is known to be a mediator of growth hormone action in pubertal growth (35). It also acts from gestation day 13.5 onward in prenatal mice in a growth hormone-independent manner, whereas IGF-II controls growth earlier in gestation (36). In humans, IGF-I, but not IGF-II, has also been shown to be involved in the control of fetal size during the later months of intrauterine life (37). The molar concentration of serum IGFBP-3 roughly equals the sum of the IGFI and IGF-II molar concentrations (38). We speculated that EGF exerted its effect on growth through the IGF system, and we measured the concentration of serum IGFBP-3. Serum IGF-I could not be reliably quantified with the system we have been using for measuring human IGF-I. The mean IGFBP-3 level of transgenic mice (182.5 ± 94.4 ng/ml; \(n\) = 4 founders; 2–9 months old) was significantly lower than that of wild type littermates (94.4 ng/ml; \(n\) = 4) at various time points after birth. Serum IGFBP-3 levels of the four above-mentioned founders as determined by radioimmunoassay. The wild type value was obtained from 12 mice including their 4 littermates. Values shown are the mean ± 1 S.D.

The blood samples were allowed to clot for 15 min on ice, and then serum was collected by centrifugation. Aliquots were stored at \(-20^\circ C\). Serum IGFBP-3 was measured using undiluted serum with the immunoradiometric assay kit from Diagnostic Systems Laboratories, Inc. Controls were age- and strain-matched normal mice including nontransgenic littermates. The statistical difference between the transgenic and control groups was analyzed using the Mann-Whitney test.
IGFBP-3 in liver and kidney. Transgenic mice overexpressing different IGFBPs have been very useful for addressing the specific functions of IGFBPs (25). Overexpression of IGFBP-3 resulted in selective organomegaly that differed from the major sites of transgene expression (26). We believe that in our transgenic mice, reduced serum IGFBP-3 is the result of EGF overexpression rather than a secondary effect of growth retardation. In a recent study (40), EGF administered for 7 days to young adult rats was shown to significantly lower IGFBP-3 levels to 44% of control values without affecting the body weight, whereas circulating IGFBP-1 and -2 levels were unaffected. It has also been shown by Frystyk et al. (41) that injection of EGF for 4 weeks into adult rats decreased serum IGF-I and IGFBP-3. The authors discussed that most in vitro studies, including those on hepatocytes, reported an increase in IGFBPs. In both situations, EGF reduced IGFBP-3.

**In vitro,** reduced IGFBP-3 would increase free IGF-I. In *vivo,* reduced IGFBP-3 would decrease circulating IGF-I because most IGF-I is bound to IGFBP-3 (41). In transgenic mice overexpressing interleukin-6, growth impairment was also correlated with reduced IGF-I (42). In IGF-I null mutants, the mice were smaller from embryonic day 12.5 (36). In our case, EGF also acted prenatally because we noticed that all transgenic mice identified at weaning were small from the day of birth. Our data are in agreement with the hypothesis that EGF affects the production/secretion of IGFBP-3, hence decreasing the availability of IGFs and resulting in slower growth before and after birth.

**Abnormal Proliferation of Osteoblasts**—To gain further insights into the effects of EGF overexpression on bone development, we investigated the histology of long bones of transgenic mice. In wild type mice, osteoblasts were found as an even lining along the bone cortex both on the outer surface (periosteum) and inner surface along the marrow cavity (endosteum). In transgenic mice, hEGF immunostaining was found in both the periosteum (Fig. 2A) and the endosteum (Fig. 2B). In addition, abnormal accumulation of osteoblasts in the periosteum and/or endosteum was found in some areas (Fig. 2D). This imbalance in bone remodeling, however, did not result in thickening of the cortical bone. In contrast, we found that the thickness of the cortical bone in transgenic mice was reduced compared with that of normal mice (data not shown). It has been shown that in cultured fetal rat long bone, EGF stimulated thymidine incorporation at a low concentration, whereas it stimulated bone resorption at a higher concentration (43). The long bone has also been shown to harbor EGF receptors in osteoblast-like cells (44). Our data raised the possibility that EGF overexpression increased osteoblast proliferation in *vivo.*

*Endogenous EGF Is Expressed Mainly in Hypertrophic Chon-
Unlike normal mice at 6 months of age (Fig. 3A), the growth plate of our transgenic animals still contained columns of chondrocytes consisting of a considerable number of prehypertrophic chondrocytes (Fig. 3B). However, the signal of hEGF immunostaining in the growth plate of our transgenic animals was too weak to be detected. Ideally, the growth plate of younger transgenic animals should be studied. To gain insight into the normal role of EGF in bone development, we studied endogenous EGF expression in the growth plate of fetal (day 14.5–17.5), 2-day-old, 2-week-old, and 4-week-old mice. EGF was strongly expressed in some proliferating and all hypertrophic chondrocytes at all stages studied (Fig. 4, A and B). The specificity of immunostaining was shown by the fact that it could be blocked by preabsorbing the antibody with 10 mM EGF. Similar results were obtained by Tajima et al. (45), who reported staining in resting, proliferating, and hypertrophic zones of the adult mouse femur epiphyseal plate. We further substantiated our findings by studying the expression of EGF in a mouse chondrocyte cell line, MCT. At a nonpermissive temperature of 37 °C, the cells stop growing and express molecular markers of hypertrophic chondrocytes such as type X collagen and osteopontin (33). By reverse transcription-PCR, we found EGF expression only when MCT cells differentiated to hypertrophic chondrocytes at 37 °C (Fig. 4C). Although TGF-α expression has been reported in a number of cell lines, to our knowledge, cell lines expressing EGF are rare. We suggest a specific role for EGF in the last stages of chondrocyte differentiation. The MCT cell line will allow us to study the regulation of EGF production and its role in chondrogenesis.

Comparison with TGF-α Transgenic Mice: A Role for EGF in Tumorigenesis?—Because both EGF and TGF-α, as well as
their precursors, activate EGF receptors (46–48), we compared the phenotype of our mice with that reported for transgenic mice overexpressing TGF-α or the TGF-α precursor (17, 18). Transgenic mice overexpressing TGF-α weighed approximately 10% less than the control mice (49). None of the neoplastic changes reported in liver, coagulation gland, and pancreas of TGF-α mice was observed in our mice at a gross or histological level, despite the expression of hEGF in these organs as detected by immunohistochemistry and/or Western blotting. Indeed, we observed patch necrosis in the liver of all of our transgenic animals (Fig. 5). This was in sharp contrast to liver enlargement and increased proliferation in TGF-α mice (49).

These data suggested an important functional difference between EGF and TGF-α. Of the four features originally observed when EGF was injected into newborn animals, accelerated eyelid opening and incisor eruption were most striking. In addition, abnormal skin structure and stunted growth occurred at high doses of EGF (1). In our transgenic mice, only growth retardation was remarkable and would be attributed to the decrease of IGFBP-3. Because other phenotypes encountered in the previous study were not observed in our transgenic mice, the mechanism of action of EGF on eyelid opening and incisor eruption might be different from that on growth. To our knowledge, this is also the first report on the in vivo effects of EGF on chondrocyte and osteoblast proliferation. During bone development, EGF may play a role in chondrocyte hypertrophy. We also provide in vivo evidence that EGF overexpression did not lead to tumorigenesis in our transgenic animals. Additional studies to reveal the distinct biological effects of EGF and TGF-α in vivo are under way in our laboratory. We are also generating transgenic mice expressing EGF in a tissue-specific manner to distinguish the systemic versus paracrine effects of EGF.

Acknowledgments—We thank Dr. Véronique Lefebvre for the gift of MCT, our colleagues at Hong Kong University (Dr. Danny Chan, Victor Leung, Keith Leung, Kingston Mak, Davy Lee, and Anthony Chan) for enthusiastic help, and Prof. Louis Low for help with radioimmunoassay. We thank Dr. Kuma Kaluarachchi for expert advice on bone analysis. We are also grateful to Drs. M. Setou, Y. Okada, and S. Takeda (University of Tokyo, Tokyo, Japan) for constructive comments on the manuscript.

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FIG. 5. Liver necrosis in transgenic mice. Hematoxylin and eosin staining of wild type (A) and transgenic (B) liver is shown. Immunostaining of hEGF in wild type (C) and transgenic (D) liver is shown. Positive staining (brown) was detected in sinusoid cells of transgenic mice. Scale bars, 25 μm.
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J. Biol. Chem. 2000, 275:38693-38698.
doi: 10.1074/jbc.M004189200 originally published online September 22, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M004189200

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