# PART I

# The Regulation of Cancer

# 1

# The Type 1 Growth Factor Receptor Family, Their Ligands and Their Role in Human Cancers

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## INTRODUCTION

Peptide and polypeptide growth factors, unlike classical hormones, are produced in a variety of tissues throughout the body. These bind to their cell surface receptors and thereby initiate a cascade of intracellular events culminating in either a positive or a negative growth signal. Growth factors can act by an autocrine, juxtacrine paracrine or endocrine process (Figure 1.1). Autocrine action is due to the secretion by a cell of growth factors for which it possesses receptors. Juxtacrine stimulation occurs when one cell possessing cell surface bound growth factors interacts with an adjacent cell possessing receptors. Paracrine action is defined as the release of soluble factors by cells that diffuse to and act upon adjacent or closely located cells. In the final case of endocrine stimulation, growth factors may act on distant sites very much like a classical hormone; for instance, epidermal growth factor (EGF) produced in mice in the submandibular gland has been shown by sialadenectomy to stimulate spermatogenesis.

### General Characteristics of the EGF Ligand Family

The EGF family of growth factors are among the most studied of this class of molecules (Prigent and Lemoine, 1992). Several of these, in addition to displaying a role in normal development and wound repair, have been implicated in malignant transformation of cells (Aaronson, 1991). All the members of the EGF family of ligands, whether they bind to the EGF receptor or another receptor, have six cysteine residues that occur with a conserved spacing. Table 1.1 shows

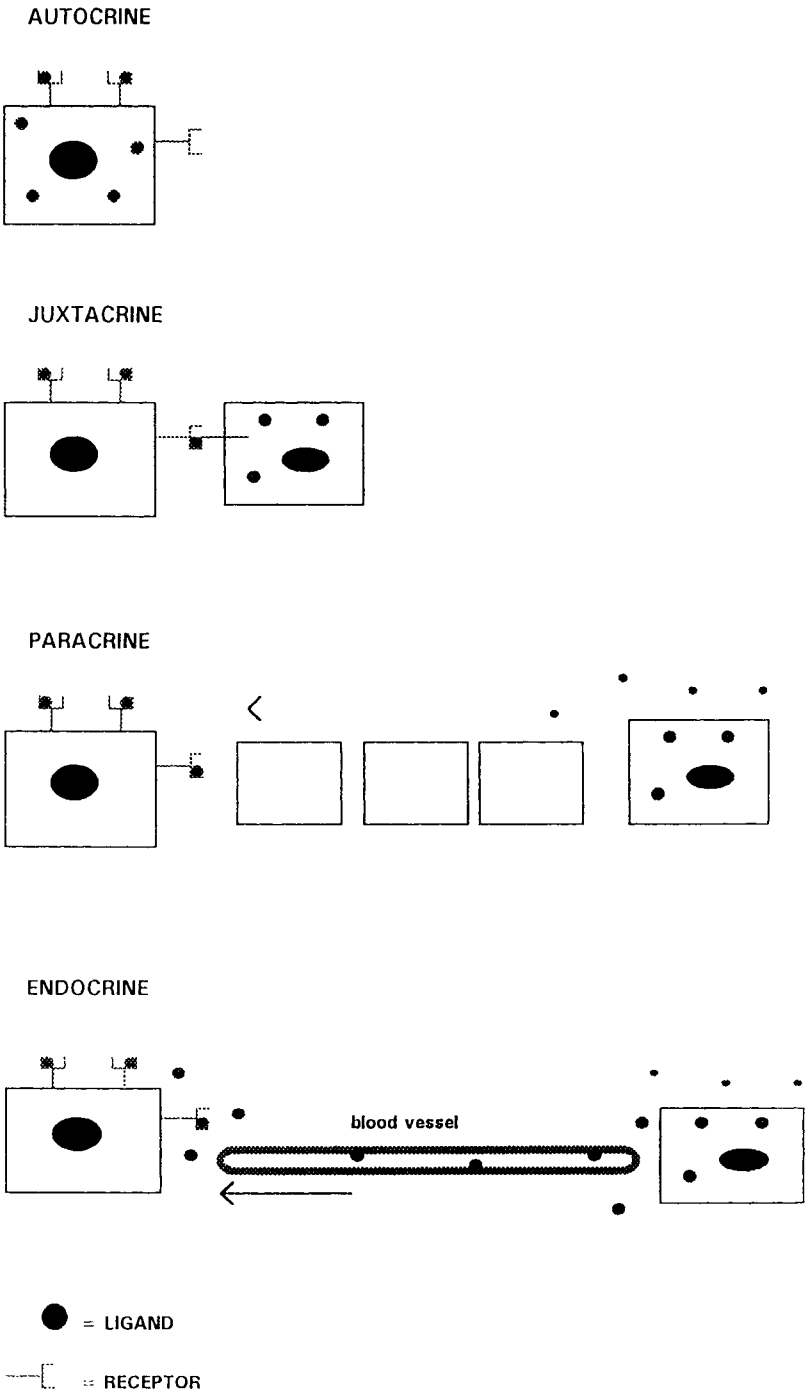


**Figure 1.1** Mechanisms of action of growth factors.

**Table 1.1.** Currently Known Members of the EGF Ligand Family

| Ligands | Receptors |
|---|---|
| EGF, TGF-$\alpha$, amphiregulin (AR), heparin binding EGF, betacellulin, viral growth factors | EGFR |
| ? | c-*erb*B2 |
| Heregulin/NDF/GGF/ARIA | c-*erb*B3 |
| Heregulin/NDF/GGF/ARIA | c-*erb*B4 |
| Cripto | ? |

the currently known members of the family with their receptors. We will now review briefly the properties of the ligands and receptors identified thus far in this family. Several reviews have also been published on specific members of the family.

## EGF

The 53 amino acid form of human EGF is derived by proteolytic cleavage from a 1,217 amino acid precursor. Thirty-seven of the 53 amino acids are homologous with mouse EGF and 14 of the 16 amino acids that differ could have resulted from single base pair changes. In fact, most members of this family have been found to be fairly well conserved in amino acid sequence during evolution. The EGF gene has been localized to chromosome 4q2.5, which transcribes an mRNA of 4.75 kb. In the adult human, EGF is produced by the submandibular salivary gland, gastric epithelium, Brunner's glands in the duodenum, pancreas, kidney, sweat glands (especially the apocrine glands), breast, thyroid gland, pituitary gland and the nervous system. EGF expression is also induced in areas of gastric, pancreatic or intestinal epithelium surrounding sites of chronic inflammation, necrosis or ulceration (Browne, 1991). In addition to existing in a secreted form, the EGF protein precursor is membrane bound, occurs widely and appears to be functional by a juxtacrine mechanism.

EGF induces its effect on cells through the EGF receptor, which undergoes tyrosine autophosphorylation and phosphorylates other proteins such as phospholipase C-II on specific tyrosine residues, which in turn leads to their activation and relocation to the cell membrane. The phosphorylated enzyme then activates the inositol phosphate pathway, leading to stimulation of protein kinase C. In addition, other pathways are also stimulated. The nature of the second messenger systems utilized by individual type 1 growth factor recep-

tors is currently a topic of much research. It is clear that not all the receptors activate the same pathways providing a mechanism for their specificity of action. Later events following receptor activation include increased protein synthesis, inhibition of protein catabolism and increased synthesis of the c-*fos* and the c-*myc* gene products. EGF by itself is often not effective in inducing cell division but requires the presence of other factors such as insulin-like growth factor type 1 (IGF-1) or platelet-derived growth factor (PDGF), suggesting that more than one intracellular signalling pathway needs to be activated to stimulate mitogenesis.

Addition of EGF to cells in culture leads to down-regulation of expression of the receptor protein; however, somewhat paradoxically, EGF has been shown to stimulate EGF receptor synthesis by inducing increased mRNA transcription. EGF can induce proliferative responses in cancer cells. Increased EGF levels have, for instance, been reported in high-grade brain tumours (glioblastoma multiforme), which also tend to have a high incidence of gene amplification of EGFR receptor, suggesting the possibility of an autocrine loop in these tumours. However, the incidence of increased expression of EGF in human cancers is much less frequent and reliably documented than some other ligands of this family.

### Transforming Growth Factor Alpha (TGF-$\alpha$)

TGF-$\alpha$ shares structural similarity with EGF in both its precursor (consisting of 160 amino acids) and mature forms (50 amino acids) (Derynck, 1992). TGF-$\alpha$ binds to the EGFR with high affinity and generally produces the same effects on the target cells as EGF itself. Some reports have suggested, however, that TGF-$\alpha$ is half as potent as EGF at the same concentration. In addition, the two ligands display different abilities to bind to the EGF receptor at high and low pH values, although it is not clear if this is physiologically significant. TGF-$\alpha$ differs in its distribution in that it seems to be one of the main ligands for the EGF receptor during foetal development when the levels of EGF are either very low or absent. In adults, it is expressed very widely and its distribution includes keratinocytes, bronchus, intestine, renal tubule, pituitary and decidua. Most notably, however, its production is enhanced in some transformed cells. The proliferation of squamous cell carcinoma cell lines has been found to be sustained by a TGF-$\alpha$ autocrine pathway with the growth factor produced constitutively while its receptors are simultaneously overexpressed. Similar pathways have been demonstrated in high-grade brain tumours and breast carcinomas.

Apart from its direct effect on tumour cells, it has been reported that TGF-$\alpha$ as well as EGF can also promote bone resorption leading to hypercalcemia of malignancy, although TGF-$\alpha$ appeared to be ten times as potent as EGF. Another important property of this class of ligands first demonstrated with TGF-$\alpha$ is that they can induce angiogenesis, suggesting a second function in tumourigenesis.

One of the most compelling pieces of evidence for the transforming capabilities of TGF-$\alpha$ is the demonstration that transgenic mice expressing TGF-$\alpha$ in breast cells develop adenocarcinomas in the postlactational mammary gland. Expression in other transgenic mouse strains leads to cancers developing in the liver and pancreas.

### Amphiregulin

Amphiregulin is the third member of the family to be identified. The gene for amphiregulin is located on the long arm of chromosome 4 (4q13-4q21), which is transcribed into a 1.4-kb mRNA. The mRNA is translated to yield a precursor with 252 amino acids that is then cleaved to yield a mature peptide of either 78 or 84 amino acids. The mature peptide has structural homology with EGF (43% sequence identity) and the other EGF-like ligands, conserving all six cysteine residues. High levels of mRNA have been detected in human ovary and placenta; intermediate levels were seen in pancreas, colon, lung, breast, cardiac muscle, spleen, kidney and testis; it was absent or at very low levels in adrenal, parathyroid, thymus, prostate, epidermis, duodenum, brain and liver. The distribution of the amphiregulin protein has not, however, yet been studied in detail.

Amphiregulin binds to EGF receptor with a reportedly lower affinity than EGF and apparently has some different biological properties. It also inhibits the growth of tumour cell lines derived from different sites (A431cells, breast tumour cell lines HTB 132 and 26, the ovarian adenoma cell line HTB 75 and the neuroblastoma cell line HTB 10). It was, however, found to stimulate growth of several fibroblast cell lines, the pituitary tumour cell line CRL7386 and the ovarian carcinoma cell line HTB 77. Amphiregulin did not have any significant effect on the growth of other tumour cell lines such as the breast tumour cell line MCF-7, the lung carcinoma cell line A 549, the squamous carcinoma of the larynx Hep 2 and the colon carcinoma cell line H3347. One possible factor explaining these early observations is that amphiregulin can inhibit cell growth at low concentrations but will stimulate division at higher levels. In ovarian tumour cell lines (3/6) and surface epithelial cell lines (2/3), it inhib-

ited growth at picomolar concentrations but stimulated their growth at nanomolar levels.

Amphiregulin has a unique pattern of expression in human colon, being expressed in the cytoplasm and nucleus of terminally differentiated, nonproliferative surface columnar and secretory epithelial cells of the mucosa but not in the proliferative epithelial cells of the crypts. In a series of colonic tumours, 50% showed moderate levels of amphiregulin expression, with a greater proportion (71%) of positivity being observed in well-differentiated tumours than in poorly differentiated tumours (18%). A colon carcinoma cell line, GEO, was found to secrete amphiregulin and co-express EGFR, thereby completing the autocrine loop.

In breast cancer cell lines, amphiregulin was found at high levels in ER positive cell lines, MCF-7, ZR75-1 and T47D and was absent or expressed at low levels in SKBR 3, MDA MB 231, MDA MB 468 and Hs 578T. Amphiregulin mRNA was found to be increased after oestradiol treatment of oestrogen-responsive MCF-7 cells. Amphiregulin protein detected by immunostaining was predominantly cytoplasmic, but nuclear and occasional nucleolar staining was also observed in some of the cells.

Similar immunocytochemistry of ovarian cancer cells revealed the protein to be localized to the nucleus of all the cells. However, in the carcinoma cell lines, the nuclear staining was concentrated in the nucleolus but was diffuse in the nucleus of the normal surface epithelial cells.

Keratinocyte autocrine factor (KAF), secreted by human keratinocytes, has been shown to be structurally, immunologically and biologically identical to amphiregulin.

### Heparin Binding EGF Like Growth Factor

Heparin binding EGF like growth factor was purified from the conditioned medium of a human histiocytic cell line. The protein is encoded by a 2.5-kb mRNA and appears to be synthesized as a membrane-bound precursor of 208 amino acids that is then cleaved to release a mature protein of about 75 amino acids. The growth factor has one of the putative nuclear targeting sequences found in amphiregulin. Heparin binding EGF like growth factor binds to smooth muscle cells with a greater affinity than EGF and has been found to be mitogenic to keratinocytes. The growth factor has been postulated to play a role in wound healing. Recently the gene structure and chromosomal location (chromosomes) of the human heparin binding EGF like growth factor have been reported.

### Betacellulin

Betacellulin is another recent addition to the EGF family of ligands. It is a 32-kDa glycoprotein first identified in the conditioned medium of cell lines derived from mouse pancreatic $\beta$ cell tumours. Betacellulin binds to and stimulates tyrosine phosphorylation of the EGF receptor. It has been shown to be a potent mitogen for vascular smooth muscle cells and retinal pigment epithelial cells, suggesting a role in the vascular complications of diabetes.

### Viral Growth Factors

Pox viruses including vaccinia virus, Shope fibroma virus, myxoma virus and molluscum contagiosum synthesize and release EGF-like peptides from cells they infect. Vaccinia virus growth factor has been purified and found to have structural homology with EGF. Vaccinia virus growth factor binds to EGF receptor and activates its kinase and stimulates mitogenesis. However, the exact role of these factors in the pathogenicity of the viruses is unknown, as it has been reported that vaccinia virus can infect cells without EGF receptor as efficiently as cells with high levels of them. Other reports, however, have contradicted this, suggesting that very high levels of EGF ($>10^{-8}$ M) or antibodies to the EGF receptor can inhibit infection of cells, perhaps by inhibiting viral particle binding.

### Heregulin/Neu Differentiation Factor (NDF)

Heregulin is the human homologue of the murine Neu differentiation factor (NDF). The gene for heregulin is located on the short arm of chromosome 8 (8p12-p21). The mature protein is expressed in at least ten forms due to differential splicing; however, each form contains a region structurally similar to the other members of the EGF family. The factors do not bind to EGF receptors nor c-*erb*B2 but bind to c-*erb*B4 and c-*erb*B3. Heregulin/NDF related factors derived from alternate gene splicing during transcription have been identified as acetylcholine receptor inducing factor (ARIA) and glial growth factor (GGF). The expression pattern of NDF mRNA has been examined in adult rat tissues. The highest levels of NDF were detected in the spinal cord, followed by lung, brain, ovary and stomach. Low levels were seen in kidney, skin and heart but expression was undetectable in the liver, spleen and placenta.

Heregulin/NDF has been shown to induce tyrosine phosphorylation of c-*erb*B4/HER4 in a series of human tumour cell lines derived from breast, colon and neuronal tissue but not in ovarian tumour cells

lacking the receptor. Heterodimerization of c-*erb*B4 and c-*erb*B2 has been demonstrated, following stimulation with heregulin, in cells expressing both the receptors. Heregulin was found to be growth stimulatory for mammary tumour cell lines at low concentrations but inhibitory at higher levels. NDF, however, was shown to induce differentiation of mammary tumour cells to milk-producing nondividing cells. The exact interactions between the heregulin/NDF isoforms and individual members of the type 1 receptor family are currently a rapidly evolving area. Clarification of this will help in interpreting the biological activity of this complex ligand famiy.

### Cripto

Cripto is a human gene encoding a protein similar in sequence to EGF, and in particular to EGF's six conserved cysteine residues. It differs substantially in not possessing an A loop structure and in not being synthesized in a membrane-bound form. No receptors have yet been identified for cripto. Expression of cripto in certain cell types can promote their growth in soft agar and leads mouse NOG8 mammary epithelial cells to form tumours in nude mice. In this, however, it resembles TGF-$\alpha$ which in cells with low or moderate levels of EGF receptors produces the same behaviour.

Several authors have now examined cripto mRNA and protein expression in different tumour types. Cripto has been reported to be expressed in 79% of colon tumours with almost equal incidence between well-differentiated and poorly differentiated tumours. In one study 60% of adenomas and 12% of the noninvolved normal colon samples adjacent to tumours were positive but none of the nine normal colon samples showed expression. This suggests that cripto could be evaluated as a potential marker for colonic tumourigenesis. Seventy-five percent of a series of breast tumours were found to overexpress cripto protein as determined by immunohistochemistry. The staining was predominantly in the cytoplasm, with some specimens showing membrane staining. The stroma and the adjacent uninvolved breast epithelium were negative. Cripto was found to be expressed in all the seven breast cancer cell lines studied and oestradiol failed to modify the expression of cripto in MCF-7 cells. Overexpression of cripto has also been reported in gastric carcinomas.

## EGF RECEPTOR FAMILY

Growth factor receptors with tyrosine kinase activity have been classified into nine different families based on the structure of the extracellular domain, their kinase domain and the nature of their ligand.