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EGF receptor in neoplasia and metastasis

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Summary

EGFR is a member of the tyrosine kinase family of cell surface receptors with a wide range of expression throughout development and in a variety of different cell types. The receptor can transmit signals to cells: i) upon interaction with ligands such as EGF, TGFα, amphiregulin or heparin binding EGF, ii) upon truncation or mutation of extracellular and/or intracellular domains, iii) upon amplification of a basal receptor activity (in the absence of ligand) through cooperation with other cellular signaling pathways or nuclear events (e.g. expression of v-erbA). The activated EGFR can exert pleiotropic functions on cells, depending on their tissue origin and state of differentiation. Under certain conditions it can also contribute to neoplasia and development of metastases. Such conditions can exist upon aberrant receptor/ligand expression and activation (e.g. in the wrong cell; at the wrong time; in the wrong amounts). Aberrant signalling can also occur through constitutive EGFR activation. Oncogenic potential of EGFR has been demonstrated in a wide range of experimental animals. EGFR is also implicated in human cancer, where it may contribute both to the initiation (glioblastoma) and progression (epithelial tumors) of the disease. EGFR may influence key steps in the processes of tumor invasion and dissemination. Involvement of EGFR in tumor spread may indicate a potential use of this receptor as a target for antimetastatic therapy.

1. Introduction

The seed and soil hypothesis formulated by Steven Paget more than 100 years ago to explain the organotropism of cancer metastases appears still as a valid hypothesis which can be examined at the cellular and even molecular level. Signals from the soil, the microenvironment, are transmitted via cell-surface receptors into normal or neoplastic cells which then react according to their state of differentiation and development in a specific way. Members of the receptor tyrosine kinase family are frequently implicated in experimental models of neoplasia as well as in human cancer. One of the best studied receptor signaling systems from this family is the EGF-receptor (EGFR). The receptor can be stimulated upon autocrine or paracrine interaction with corresponding ligands such as EGF and TGF-α. Abnormal receptor signaling can occur with truncated forms of receptors or receptor over-expression which is observed in some forms of neoplastic development. Receptor stimulation by itself or in combination with other signals can have a variety of biological consequences.

Expression and activity of EGFR have been linked with a number of human neoplastic diseases or pre-neoplastic stages. We shall discuss influences of the EGFR on cell growth and differentiation, cell-cell interactions, cell matrix adhesion, cell motility, ECM-degradation, invasion and metastasis. From these observations it becomes clear that this receptor system can influence a variety of cellular functions of importance for malignant growth and metastasis and could therefore also play an important role in various forms of human cancer and its progression towards metastasis.

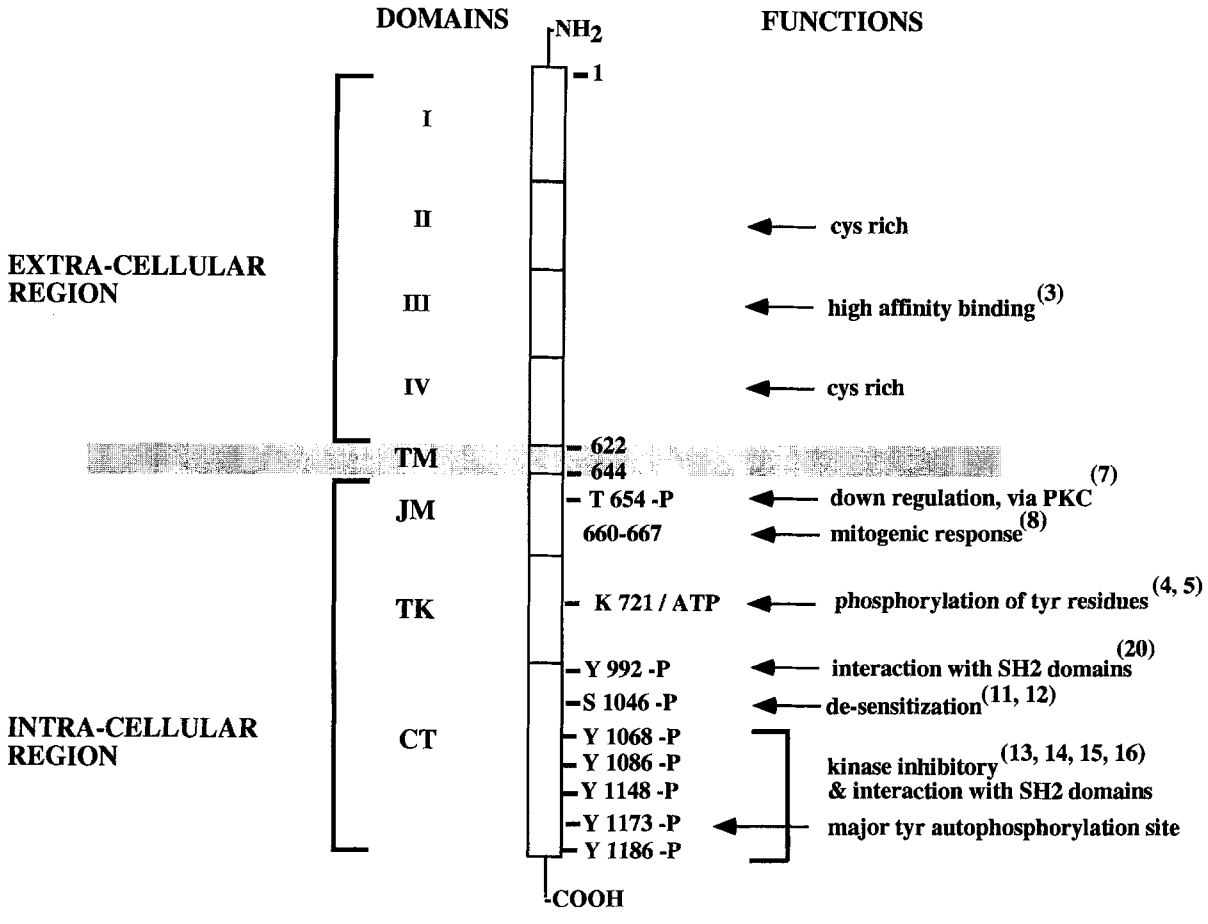

Fig. 1. A schematic diagram representing EGFR with its distinct domains and the functions attributed to them (on the right). Relevant literature are cited accordingly. TM: transmembrane domain, JM: juxta-membrane domain, TK: tyrosine-kinase domain, CT: carboxy-terminal domain.

2. The EGFR: a subclass I tyrosine kinase receptor

2.1. structure and function

A model of the EGFR domain structure is drawn in Fig. 1. The mature human EGFR (HER1) is a single polypeptide chain of 1186 amino acids, M_r170,000 daltons, containing approximately 40,000 daltons of N-linked oligosaccharide and in some cell types mannose phosphate [for review see: 1]. The receptor traverses the plasma membrane with a single hydrophobic anchor sequence. The extracellular amino terminal end can be divided into four domains, with the third domain being responsible for high affinity binding to EGF and probably also other specific ligands of the receptor. The intracellular carboxy-terminal sequences encode tyrosine kinase and carboxy-terminal regulatory functions. The structural organisation of the EGFR is commonly shared by at least four other monomeric growth factor receptors, HER2/neu, HER3, HER4 [2] and Xmrk, which together with EGFR/HER1 comprise the family of subclass I tyrosine kinase receptors (for review see: 3).

The binding by EGFR of EGF was reported to have a 1:1 stoichiometry but two possible affinity states, with the majority of the cell surface expressed receptors exhibiting the lower affinity state. Binding of ligand has been proposed to drive the dimerization or oligomerization of receptors. This process promotes the interaction between kinase domains leading to their activation [for review see:

3]. Binding of ATP to a lysine residue at position 721 within the EGFR kinase domain is the key event required to initiate tyrosine kinase activity of the receptor. All known functions of the EGFR, excluding ligand binding, appear to depend on the tyrosine kinase activity [4, 5].

At least two cytoplasmic regions, the juxtamembrane and the carboxy terminal domains, regulate the affinity for ligand as well as the activity and specificity of the protein tyrosine kinase function [6, 7; for review see 8]. The major regulatory sites are targets for phosphorylation by PKC or cross phosphorylation by EGFR. Phosphorylation of C-terminal tyrosine residues is also important for the physical interaction of EGFR with other cellular proteins that carry compatible so called src homology 2 (SH2) domains (see Fig. 1, and below). Truncation of the carboxy terminal domain has led to constitutive EGFR activity [17, 18]. However, surprisingly receptor activation does not always seem correlate with receptor phosphorylation [19].

The SH2 domain is a common feature of many nonreceptor kinases which act in the signal cascade downstream of activated growth factor receptors. This is the major structural feature responsible for interaction of PLC-γ, PI3-kinase, and ras-GAP with the activated EGFR. Raf, a serine/threonine kinase which also associates with EGFR does not contain an SH2 domain. Recruitment of these molecules to the cell surface, their phosphorylation and/or conformational modulation through interaction with EGFR have been proposed to lead to their activation and secondary signal transduction. The affinity of EGFR for these molecules is variable, being high for PLC-γ but particularly low for PI3-kinase. Therefore, for some molecules and in some cell types successful interactions may dependent on the level of EGFR expression. The complexities of such interactions have been used as a basis to explain the cooperation of different receptors in the activation of growth as well as transformation associated changes [for reviews see: 21–23].

2.2. Naturally occuring truncations

At least two EGFR transcripts of 10.5 and 5.8 kb and occasionally a smaller 2.6 kb transcript have been reported in RNA from a variety of human cell lines [6, 24]. These transcripts correspond to mRNAs of 10, 5 and 2 kb in rat cell lines and tissues [19, 25] and, 12 and 9 kb in normal chicken embryo [26]. The two larger EGFR mRNAs have been generally assumed to differ in size because of different lengths of poly-adenylated tails. However the smaller transcript (2–2.6 kb) is truncated, and hybridises only to probes corresponding to the extracellular domain of EGFR. This truncated receptor has been detected in the A431 human vulva carcinoma cell line [6] and other human squamous carcinoma cell lines [27] as well as in the WS1 diploid human fibroblast line (Khazaie, unpublished). A corresponding truncated EGFR was detected in normal rat liver and in the MTLn3 rat mammary adenocarcinoma cell line [19, 25].

The truncated EGFR transcript is by virtue of its expression in nomal rat liver and in diploid human fibroblasts, most likely a natural product arising from differential splicing of the EGFR gene transcript. At least in the case of A431 cells the variant EGFR was shown to contain sequences from a novel gene fused at the C-terminal end [6] and to be secreted extracellularly [27]. The truncated EGFR can form *in vitro* a heterodimer with the intact receptor and inhibit both basal and EGF-dependent kinase activity [28], however expression of a genetically engineered soluble extracellular EGFR domain had little if any effect on the growth and phenotype of EGF stimulated NIH-3T3 cells [29]. Although the physiological function of the protein is as yet unknown, it is tempting to speculate on the possibility of a potential regulatory interaction at the cell surface with the complete EGFR.

2.3. Ligands

The first known specific ligand for the EGFR was epidermal growth factor (EGF)/urogastrone, which seemed to have an epidermal proliferative function and antagonistic action on gastric acid secretion. EGF is expressed as a 1200 amino acid residue glycosylated transmembrane precursor or a 53 amino acid secreted product [30–32]. The secreted EGF is

usually considered to be a processed form of the transmembrane precursor. Several distinct peptides with specific EGFR binding properties are now known to exist. These include TGF-α, the pox virus growth factors, amphiregulin, and heparin binding EGF [33, 34; for reviews see: 35–37]. Common features of all these ligands are a cysteine rich region spanning a length of approximately 50–60 amino acid residues, and occurrence in two forms: as shorter secretory peptides, and as large membrane bound glycosylated putative precursors, which for EGF and TGF-α are also biologically active [38, 39].

In adult tissues, EGF has been primarily localized to differentiated cells rather than to stem cells. In epidermis and various glandular tissues, both EGFR and EGF were expressed in differentiating cells [40]. Concentrations of TGF-α in the kidney are increased upon injury affecting a number of renal responses [for reviews see: 41, 42]. The kidneys are considered to be the major source of urinary EGF, while the prostate is the major source of EGF in the seminal fluid [43, 44]. Likewise, the mammary tissue is the source of relatively high concentrations of EGF in milk [45]. The major sources of EGF in the CNS are macrophages, glial cells and neurons as well as uptake from the peripheral blood [for review see: 46, 47].

EGF-like peptide sequences are present in a variety of cell surface and extracellular proteins [45]. The potential function of these peptides as EGFR agonists is of interest, particularly in view of the reports on mitogenic activities of extracellular matrix proteins [48, 49].

3. Role in normal development

It is now established that the EGFR is expressed throughout development and in a variety of undifferentiated as well as differentiated cells [for review see: 50]. EGFR and TGF-α are expressed in the preimplantation conceptus and may play a role in blastocoel expansion, embryo-uterine signalling, and the implantation process [51–54]. Among the functions attributed to EGFR activity are the proliferation and development of specific epithelial territories in the embryo, including branch point morphogenesis and maturation of early embryonic lung tissue, skin development, and promoting survival of early progenitor cells of the cleft palate [55, 56; for review see: 57].

EGFR exhibits a broad expression throughout the brain tissue, primarily in the early post-natal astrocytes and purkinje cells and in the adult neurones of the cerebral cortex, where it may be important in promoting terminal differentiation [58, 59] and determining the viability of neurones [60; for reviews see: 45]. Purified and cultured astrocytes but not oligodendrocytes respond mitogenically to EGF, in agreement with the higher levels of expression of EGFR and EGF in astrocytic cells of glial origin [61, 62]. In the hypothalamus, EGFR mediates the release of luteinizing hormone releasing hormone (LHRH) [63].

An interplay of the actions of EGFR and estrogen receptor has been proposed to be required for the differentiation of normal mammary epithelial cells as well as the induction of uterine and vaginal growth [64, 65]. EGFR expression is high in the cap-cell layer of the terminal end buds [66], a proliferating cell population [67] which is presumed to be the stem cell population of both the luminal and myoepithelial cells of the mammary ducts [68]. The cap cell layer is devoid of estrogen receptors which instead are abundant in the surrounding stromal cells [66]. It has been proposed that estrogen may regulate the growth of cap-cells through a paracrine mechanism by stimulating the production of a peptide factor for which EGF or TGF-α are prime candidates [69]. In ovariectomized mice, the exogenous delivery of either EGF or TGF-α was sufficient to restore the pattern of normal ductal growth in the involuted mammary gland. In normal mice distinctly different patterns of immunolocalisation were observed for EGF (inner layers of terminal end buds and in ductal cells of mammary epithelium) and TGF-α (epithelial cap cell layer of the advancing terminal end bud and in stromal fibroblasts at the base of the terminal end bud) suggesting that each polypeptide plays a different role in normal mammary gland morphogenesis [70].

4. Role in malignant development

Expression and activity of EGFR have been linked with a number of pre-malignant or malignant diseases. These include skin hyperplasia, erythroblastosis, and fibrosarcoma in animals; and, in humans, notably benign hyperplasia of the skin, mammary carcinoma, glioblastoma, and hepatic carcinoma. In some instances truncations of the EGFR may be necessary to allow for its function as a dominant oncogene. In others, over-expression may be needed to amplify a tumor promoting signal. However, it is also apparent that in some instances truncations or overexpression of EGFR are not necessary. Overall, the combination of activation of EGFR, through autocrine or paracrine loops, and accumulation of appropriate genetic alterations may lead to neoplasia and metastasis.

4.1. The oncogenic potential of EGFR in experimental systems

The nature of events subverted by EGFR activity may vary depending on the type of cancer studied. This conclusion is most evident when comparing the contribution to different neoplasias of, A: aberant expression, B: paracrine activation, C: truncation of EGFR, or D: receptor activation in the context of complementing nuclear events.

4.1.1. Truncations and aberrant expression in experimental neoplasia

Initial interest in a transforming potential for the EGFR came from the realisation of the sequence homology between the cloned human receptor and the chicken v-erbB oncogene [71]. The v-*erb*B oncogenes are retrovirally transduced and truncated form of the "chicken EGFR". Expression of v-e rbB by the transforming retroviruses AEV-ES4 and AEV-H, led to erythroleukemia and fibrosarcoma in infected chicks, as well as to the transformation of bone marrow erythroblasts and chicken embryo fibroblasts (CEFs) in culture [for reviews see: 72, 73].

A direct comparison of the transforming functions of v-erB and human EGFR was performed by simply using the AEV-ES4 retrovirus to express a complete normal EGFR cDNA instead of v-erB [17]. Surprisingly, the complete human EGFR promoted the EGF dependent outgrowth of erythroblasts from *in vitro* infected bone marrow cultures. Infection of young chicks with retroviral vectors that co-expressed v-erbA, an altered form of the thyroid hormone receptor gene [74, 75], and EGFR led to acute erythroleukemia. The major consequence of truncations affecting the extracellular amino terminal end (removal of the ligand binding domain) was constitutive activation of the receptor. Truncations of the intracellular carboxy terminal end had much wider consequences affecting not only receptor activity but also the biological function of EGFR kinase.

Truncation of 32 carboxy terminal amino acids removing the last two tyrosine autophosphorylation sites (see Fig. 1) conferred additional erythropoietin receptor properties to human EGFR as assayed in primary chicken erythroblasts. Removal of a further 94 amino acids deleting more sites of tyrosine autophosphorylation inactivated erythroid transformation without diminishing fibroblast transformation (assayed *in vitro*) by human EGFR. Thus, expression of the complete EGFR was sufficient to transform (promote aberrant growth of) immature erythroblasts, while truncations of the EGFR changed the function of EGFR in a lineage specific manner.

In contrast to the apparent absence of EGFR in mature hematopoietic cells, recent observations indicate that this receptor is normally expressed in immature/progenitor hematopoietic cells [76, 77]. Other reports suggest that the EGFR signal transduction pathway may function in transformed hematopoietic cell lines [78–82]. Together these observations suggest that EGFR may be a naturally occuring growth/survival factor for immature hematopoietic cells, which in part explains how inappropriate expression of this receptor may lead to manifestations of leukemia, the expansion of immature hematopoietic cells.

EGFR is abundantly and universally expressed in mature fibroblasts. Ligand induced activation and/or overexpression of the EGFR led to a number of changes in the *in vitro* behaviour of primary chicken fibroblasts [17] as well as in established mu-

rine or rat fibroblast cell lines [83, 84] which are associated with neoplastic transformation, but did not promote invasive growth of fibroblasts in the chicken embryo [85] or induce sarcoma in transgenic mice [86; Thomas von Rüden, pers. comm.]. These observations confirm earlier reports on the non-sarcomagenic character of v-erbB isolates with a complete C-terminus. Truncations of at least 23 carboxy terminal amino acids of the chicken c-erbB seemed to be required for inducing sarcoma [87–89].

On the other hand, signals transmitted by the complete EGFR may cooperate with otherwise non-sarcomagenic nuclear oncogenes and lead to tumorigenic growth of fibroblasts [85]. This observation may in part explain the apparently contradictory reports on the tumorigenic properties of EGF or TGF-α expressing established rodent cell lines [90, 91], suggesting that cooperative nuclear events [92] might have been overlooked in these cell lines.

It is now understood that changes in the intracellular region of the EGFR not only induce ligand independent activation of the EGFR but also change substrate recognition by EGFR kinase. Removal of the carboxy terminal tail of the EGFR significantly broadened the spectrum of cellular substrates for EGF dependent tyrosine phosphorylation [93]. Similar truncations markedly impaired EGF dependent increase of inositol phosphate formation in NIH3T3 cells [20] and the EGF dependent activation of phospholipase A2 in CHO cells expressing ectopic human EGFR [94]. Even a single amino acid substitution, threonine for arginine at position 662 in the juxtamembrane domain, was sufficient to change both the pattern of intracellular proteins phosphorylated and mitogenic behavior of different transfected established cell lines in response to EGF [95]. Therefore the v-erbB oncogenes as well as truncated or mutated forms of the EGFR may be more than constitutively activated EGFR molecules, and can be expected to have distinct and novel properties.

4.1.2. Aberrant activation in TGF-α transgenic mice

Paracrine, autocrine, and more recently adhesion activation of EGFR have been the focus of attention for developmental biologists and tumor biologists, attempting to explain controlled as well as self propagating mechanisms for growth, development, and neoplasia.

Experiments with transgenic mice suggest that an autocrine mechanism involving the EGFR could be expected to play a role in the initiation and/or progression of mammary and hepatocellular carcinoma as well as pancreatic hyperplasia [96, 86]. Transgenic mice expressing TGF-α were reported to develop hepatic carcinoma and abnormal breast tissue. Mammary carcinomas were observed in the post lactating gland. Evidently none of these neoplias required amplification or truncation of the EGFR, but rather depended on the paracrine activation of the endogenous receptor (and perhaps complementing nuclear and/or environmental events).

Transgenic mice over-expressing TGF-α specifically in the stratified squamous epithelia, developed thicker epidermis and stunted hair growth as well as benign papillomas in regions of mechanical irritation or wounding. Areas of the skin that were subjected to mild irritation displayed localized leukocytic infiltration and granular layer loss, characteristic of psoriasis in humans [97]. These observations are in agreement with those made on human cells, where an interplay of EGFR autocrine activity and IGF-I receptor activity is believed to promote the appearance of psoriatic lesions [98, 99], and in other instances promote skin carcinogenesis [100–102]. Interestingly, murine epidermal cells may be equally responsive to EGF as to other classical chemical tumor promoters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) for neoplastic transformation [103–105].

4.2. Involvement of EGFR in human tumors

In the following sections we shall survey the evidence concerning the involvement of EGFR in human mammary carcinoma, which so far is the most intensively investigated human cancer in connection with EGFR. Other epithelial tumors will not be discussed in detail but only refered to. Glioblastoma, a non-epithelial tumor and the only human can-

cer where truncation and over-expression of EGFR may play an important role, is briefly reviewed. The mechanism by which EGFR may contribute to malignant transformation or progression of epithelial cells is not limited to mitogenic stimulation but is likely to involve a variety of cellular responses that have been associated with cellular migration and invasiveness. These will be discussed in the context of mechanisms of tumor progression and metastasis.

4.2.1. EGFR and mammary carcinoma

In human breast carcinoma a strong inverse correlation between the expression of estrogen receptor (ER) and EGFR [106–109; also see review: 110] as well as between EGFR and ER plus progesterone receptor (PR) [111–118] has been established. In human breast carcinomas the percentage of EGFR positive tumors reported in the literature varies from 22% to 67% [117]. Differences in assay methodology, tumor biopsy sample selection and cut-off level seem to offer plausible explanations for this variation. In addition, endogenous TGFα may lead to the occupation of EGFR ligand binding domain and receptor downregrulation resulting in underestimations of receptor number when analysed by receptor binding [119]. EGFR expression, particularly in ER negative patients [107] has been a marker of morphological and functional de-differentiation related to a poor prognosis [106, 109, 111, 113, 115, 117].

Expression of EGFR in ER negative/EGFR positive tumors was reported to be heterogeneous, supporting the existence of subsets of tumor cells with differential aggressive potentials [118, 119]. Furthermore it was demonstrated that expression of the EGFR in breast tumor metastases is frequently elevated compared to the primary tumor [106, 109], which suggests involvement of EGFR in the metastatic process.

Recent reports indicate that cross-talk between EGFR and ER or PR in the mammary gland may have important consequences on the regulation of normal and aberrant growth. Elevated and sometimes estrogen inducible expression of TGF-α has been reported in both human and experimental mammary cancers as well as mammary tumor cell lines [for review see: 120, 121]. In cultured human breast tumor cell lines expressing constitutively either ER and EGFR [122] or ER plus a transfected EGFR [123] a heterospecific receptor modulation could be identified. Thus the simultaneous induction of ER and EGFR signal transduction in these cells was not tolerated. Prolonged cultivation of EGFR plus ER expressing cells with EGF resulted in loss of estrogen-dependent proliferation, despite the presence of high amounts of ER [123]. Furthermore, prolonged cultivation of these cells with tamoxifen resulted in anti-hormone resistant subclones expressing EGFR but lacking ER and PR [122, 123]. It remains to be established if this observed unresponsiveness or down-regulation of ER in EGFR expressing cultured mammary tumors during antiestrogen treatment could contribute to the failure of endocrine treatment in the clinic.

4.2.2. Involvement of EGFR in other epithelial malignancies

A number of other epithelial malignancies have (through experimental observation and or clinical correlations) been linked with EGFR function. The most convincing of these are hepatic carcinoma, where an interaction of TGF-α/TGF-β signal transduction pathways may play a key role [124–126] and prostatic hyperplasia/cancer [for reviews see: 127, 128]. Other epithelial carcinomas associated with EGFR activity include renal carcinoma [129, 130], bladder cancer [131], epithelial malignancies derived from human oral tissue [132], laryngeal cancer [133], oesophageal tumors [134–136], stomach cancer [137], colon carcinoma [138, 139], ovarian adenocarcinomas [140], and lung cancer [141, 142].

4.2.3. Truncations of EGFR in glioblastoma

Glioblastoma is the only human cancer so far causatively linked to expression of truncated EGFR. Amplification and rearrangement of the EGFR locus are also common features, and therefore presumably constitutive over-activity of the receptor is involved [143–146]. However the extent to which mutations and truncations other than those affecting the extracellular domain, may contribute to the onset of glioblastoma is not sufficiently documented. Characteristic rearrangements of the EGFR gene in glioblastoma give rise to novel antigenic

epitopes. An antibody made to the rearranged sequences of the EGFR in glioblastoma multiforme, the most malignant of human primary brain tumors, was shown to react with rearranged EGFRs in several patients with the same particular EGFR deletion mutation, demonstrating the potential use of common antibodies for diagnosis and treatment [147].

5. EGFR and mechanisms of tumor progression and metastasis

Metastatic spread of tumors is a consequence of a series of events in which growth factors could be involved. Sequentially, tumor cells must proliferate, loose their anchorage dependence on the extracellular matrix (ECM) and their contacts with neighboring cells, pass through the vessel wall, enter the blood stream, seed the target organ and form a new colony [148–150]. During all these processes, tumor cells are submitted to a variety of environmental controls including growth factors from the host or from the tumor itself, as well as various substrates in contact with cells. EGF and TGFα are well known for their growth stimulating effects in a wide variety of systems. Accumulating evidence is presented that these growth factors have pleiotropic effects on cell motility, chemotaxis, secretion and differentiation which in some cases correlate with metastatic potential.

5.1. Effects of EGF on tumor growth

Besides the well known stimulatory effect, activation of EGFR can inhibit growth of cells in tissue culture depending on the cell type, the number of receptors and the assay conditions. This may explain occasional discrepancies in correlating the response of tumor cells to EGF *in vitro* with their response to EGF after transplantation into host animals.

5.1.1. Differential effects of EGF on growth of cultured cells

It has been shown that the effect of EGF on cell proliferation is dependent on the quantity of occupied EGFR and that occupation of additional EGFR in excess led to decreased growth stimulation and even to an inhibition of cells grown in monolayer tissue culture [151, 152]. However, the growth of human epidermoid A431 cells expressing high numbers of EGFR (2×10^6/cells) was inhibited by EGF in monolayer culture, while it was stimulated under 3-dimensional culture conditions [153]. Furthermore, under tissue culture conditions the degree of cell-cell contacts may determine if EGF and TGFα induce either mitogenic or inhibitory signals as demonstrated with a human renal adenocarcinoma cell line [154].

5.1.2. Xenotransplants of EGFR expressing tumors in immune deficient mice

A clear correlation between tumor growth and EGFR expression could be demonstrated in tumor xenografts. A relationship between a high number of EGFR and the tumorigenic potential in nude mice has been described for the human A431 cells [155], human mammary MDA 468 cells [156] and a feline mammary carcinoma [152]. Ozawa [134] showed that growth rates of A431 human epidermal xenografts were markedly enhanced by EGF supplied by implanted osmotic pumps. Surgical removal of the submaxillary glands, a major source of EGF in male mice, decreased tumor growth rates in animals bearing transplanted mammary cancer [157] or human squamous cell carcinoma [158]. This inhibitory effect was reversed by supplying exogenous EGF. Implants of a human EGFR-expressing melanoma line in scid mice metastasized spontaneously to multiple distant sites. Resection of the primary tumor followed by the application of an EGFR specific MAb resulted in suppressed growth of established micrometastases [159]. Furthermore, an EGFR specific MAb inhibited growth of human epidermoid cells when transplanted subcutaneously, intravenously or into the peritoneum of nude mice [160]. Moreover, the antitumor effect persisted when animals were treated with the $F(ab)'_2$ fragment of the antibody suggesting that the antitumor effect was not due to immune mechanisms.

5.2. The effects of EGF on metastasis in experimental systems

5.2.1. Syngeneic animal models in which metastatic capability correlates with growth factor responsiveness

Tumor cells have been shown to demonstrate *in vitro* proliferative responses to defined growth factors, such as platelet-derived growth factor, insulin-like growth factor, EGF and others. The ability to proliferate when stimulated with growth factors correlates in some systems with the metastatic capability of the malignant cells as was shown in the mouse colon adenocarcinoma 26 for IGF-1 [161] and in the 13762NF rat mammary adenocarcinoma system for transferrin [162] and EGF [163].

5.2.2. Syngeneic animal models in which metastatic capacity is enhanced by EGF

In the rat rhabdomysarcoma (RMS) 9–4/0 system, treatment of cultured tumor cells with 20 ng/ml EGF for 48 hours enhanced the lung colonising potential of i.v. injected tumor cells significantly [164]. In addition, tumor growth in the mediastinal lymphatic tissue was observed in rats receiving EGF-treated cells. Furthermore, treatment of rats with EGF following ablation of the primary tumor resulted in a dramatic increase in the median number of spontaneous lung metastases, and high incidence of axillary lymph node and extrapulmonary (mediastinal) metastases as compared to saline treated animals. While this study clearly demonstrates that EGF can enhance the metastatic potential of EGFR expressing tumors, it does not allow discrimination between the effects of EGF on the tumor cells or those on the host.

In order to elucidate this question, closely related tumor cell clones with different metastatic potential and different levels of EGFR expression have to be used. Introduction of the gene for EGFR into the receptor negative clone should then confer metastatic capability to this cell clone. In order to perform these studies we have chosen clones MTC and MTLn3 from the 13762 NF rat mammary adenocarcinoma and introduced the gene for the human EGFR into low metastatic clone MTC. Our initial observations suggest that in this model system expression of EGFR may be a determining factor for metastasis of the tumor cells from the mammary fat pad to their target organ, the lungs.

5.3. Effects of EGF on specific steps of the metastatic cascade

While definitive experimental proof is still lacking that EGFR is involved in spontaneous metastasis, there are some indications that EGF can enhance the ability of cells to succeed in some steps of the metastatic cascade. Recent reviews have discussed in detail the basic mechanisms of tumor cell adhesion, invasion and motility [165, 166]. Here we will concentrate on examples where stimulation of EGFR increased the potency of cells to succeed in these important steps of the metastatic cascade.

5.3.1. Influence of EGF on the integrin receptor family, their ligands and adhesion to ECM

Early work by Briles and Kornfeld [167] had indicated a correlation between tumor cell adherence to extracellular matrix proteins (ECM) *in vitro* and increased lung colonising potential of intravenously injected tumor cells. Subsequently the importance of cellular adhesion in lung colonising potential has been well documented by using closely related tumor cell clones of defined adhesive and metastatic properties [for reviews see: 149, 150].

Recently the integrins were identified as the major family of receptors by which cells attach to ECM. Accumulating data indicate that EGFR may directly influence the expression, organisation and function of the integrins. It has been known for a long time that EGF induces rapid changes in the interaction of tumor cells with their own ECM or defined matrices. For example, in the case of human epidermoid carcinoma A431 cells it was observed that within minutes of exposure to this factor, the cells undergo rapid morphological changes resulting in retraction of the cells from the tissue culture substrata as they become significantly more rounded [168–170] and exhibit membrane ruffling, and filopodia [171, 172]. When A431 cells where plated onto collagen, addition of EGF did not interfere with attachment but modulated spreading of cells [169].

This effect was inhibited by cytochalasin B, a compound which interferes with the actin-associated microfilament system, indicating dissociation of linked integrin-cytoskeleton interaction. Our group recently described that EGF increased within 5 minutes adhesion of highly metastatic rat mammary adenocarcinoma cell clone MTLn3 to fibronectin. Treatment of closely related mammary adenocarcinoma cell lines with EGF enhanced lung colonising potential only when the cells expressed EGFR (Lichtner *et al.*, in preparation).

Recently evidence has been provided that EGFR activation can influence the interaction of cells with defined matrix components, by modulating the subunit organisation of the integrins. For example, enterocyte sheet migration was stimulated by EGF on laminin but not on collagen I or fibronectin, indicating modulation of a specific integrin subunit [173]. Indeed, integrin α1 subunit pools were decreased after EGF treatment on collagen-I but increased on laminin. The EGF induced changes in immunreactivity required protein synthesis since they were inhibited by cylcoheximide. This is in line with a report [174], that in quiescent Swiss 3T3 cells EGF induced rapid increase in vinculin and β1-integrin mRNA levels. Elucidation of the mechanism by which EGF affects integrin organisation and pool size awaits better understanding of the mechanisms which modulate integrins. In this respect it is of considerable interest, that the integrin β subunit contains in its intracellular domain a tyrosine residue whose neighboring sequences show high degree of homology with the tyrosine autophosphorylation site of the EGFR [175]. Perhaps critical interaction of integrins are influenced by EGFR mediated phosphorylation of these residues. However, EGFR mediated phosphorylation of integrins does not seem to happen in human KB cells [176], and may be dependent on the cell type investigated.

Several integrin subunits are phosphorylated upon binding to ECM, suggesting that in addition to providing adhesive interactions with immobilised ECM proteins, integrins also modulate transmission of intracellular signals [177–180]. Moreover transient tyrosine phosphorylation of protein(s) of 130–150 kd has been observed in KB carcinoma cells following cross linking of α3β1 integrins [176]. In mouse fibroblasts adhesion and spreading on fibronectin led to rapid tyrosine phosphorylation of a protein of similar size, termed focal adhesion kinase and suggested to be a component in the putative integrin signalling pathway [181, 182]. It has been postulated that integrins may even share some of the intracellular signal transduction pathways of tyrosine kinase receptors [for reviews see: 183, 184].

Integrins have been recognised not only as systems to provide adhesive strength by interaction with immobilised ECM proteins, but also as systems which aid the cell in recognising and responding to environmental signals. EGFR activity has been reported to induce the production and secretion of matrix proteins in several cell lines. Increased secretion of fibronectin was reported for normal rat liver cells [185], and of fibronectin and laminin for the human breast cancer cell line PMC42 [186]. In the latter study EGF induced increased production of matrix proteins might have been causative for the increased adherence of cultured PMC42 cell organoids. The modulation of integrin function as well as production/secretion of ECM proteins by EGFR could have significant biological consequences, making the EGFR system perhaps a key regulator of the cellular response to the microenvironment.

5.3.2. Influence of EGFR on cell-cell contact and cytoskeleton

Recent reports have opened the possibility that EGFR activity may also directly influence cell-cell contact, another critical parameter known to define epithelial invasiveness [188]. Both EGFR and E-cadherins were shown to co-localise in the basolateral membrane of A431 cells [189]. Changes in the level of expression of E-cadherin or tyrosine phosphorylation of the associated protein β-catenin, in MDCK epithelial cell line were shown to lead to rapid loss of cell-cell contact, acquisition of fibroblastoid morphology and invasive phenotype [190, 191; for reviews see; 192, 193]. Direct phosphorylation of β-catenin by EGFR or contact of EGFR with transmembrane TGF-α or EGF on neighboring cells are possible mechanisms that may relate to the role of EGFR in the acquisition of invasive properties.

We had reported recently that EGFRs localise preferentially in the cell-cell contact areas of A431 cells and that negative control mechanisms preventing EGFR activation may be exerted by adjacent cells [194]. However, in detergent-permeabilized cells the cytoskeleton-associated EGFRs were fully active. It is of interest that in the highly metastatic mammary adenocarcinoma clone MTLn3, cytoskeleton-associated EGFRs are highly susceptible to phosphorylation in permeabilized cells, while in intact cells mitogenic stimulation occurs without detectable receptor phosphorylation [19].

A number of recent studies indicate that EGFR, is itself in part associated with the cytoskeleton [194–196]. Furthermore, a number of cytoskeletal components have been shown to be phosphorylated *in vivo* and *in vitro* by EGFR kinase, such as fodrin, spectrin, tubulin and microtubulin associated protein 2, ezrin and lipocortin 1 [196, 197, 199]. Cytoskeletal associated EGFRs may have specialised functions since they are mainly of the high affinity class [196, 197, 200].

It has been proposed that activation of PI3-kinase, which occurs through association with tyrosine kinase receptors, may directly influence actin filament reorganisation. However, due to the low affinity of EGFR for PI3-kinase, overexpression of this receptor may be needed to allow for these events [for review see: 22].

5.3.3. Effects of EGF on cell motility

Another parameter often associated with epithelial invasiveness is motility [for reviews see: 192, 193, 201–203]. Cell motility requires several distinct steps that must occur in a coordinated fashion for cellular translocation to occur. Following the establishment of adhesion to the underlying substratum, the cell must be able to form protrusions, establishing new adhesions and be able to break older adhesions for translocation to occur [for review see: 165].

In order to clearly demonstrate an effect of EGF on cell motility it had to be separated from its effect on cellular growth. This has been demonstrated in several cell lines grown in tissue culture, such as rat intestinal epithelium cells [204], the human embryonal carcinoma cell line Tera-2 [205], and keratinocytes [206] [for review see: 202]. Similarly, the human epidermoid carcinoma KB cells [207] or human glioma line K-343 M6a [172] showed relatively high motility and grew dispersely as single cells when cultured with EGF, while in the absence of EGF cells grew in clusters. The migration and spread of closely related human glioma cells from spheroids on a plastic substratum was increased by EGF [208].

5.3.4. Effects of EGF on the production/release of ECM degradative enzymes and on invasion

Many different types of ECM degradative enzymes have been implicated in invasion by metastatic cells, such as metalloproteinases, aminopeptidases, serine proteinases, cysteine proteinases and aspartic proteinases [for reviews see: 209, 210]. EGF has been shown, among other growth factors and cytokines, to modulate the level of cell-secreted serine proteinases and metalloproteinases. In human squamous cell carcinoma, EGF influences plasminogen activator-mediated proteolysis of ECM [211]. In lung and colon carcinoma EGF induced the synthesis of urokinase type plasminogen activator activity (uPA) [212, 213] and in normal fibroblasts of collagenase respectively [214]. In mouse mammary adenocarcinoma cell lines EGF dependent secretion of proteinases was correlated with the metastatic properties of the cell lines [215]. Expression of transfected TGFα in a rat bladder carcinoma cell line resulted in highly mobile cells which produced a gelatinolytic activity not normally synthesized by untransfected or control neo transfected cells [216]. In RL 95-2 human endometrial adenosquamous carcinoma cells, EGF stimulated an increase in uPA [217].

In three cell lines with similar numbers of EGFR established from one patient with maxillary tumor, only one line responded to EGF with increased invasiveness into fibrin gels [218]. Subsequent analysis revealed that in this particular EGF-responsive cell line the production of the proteinase inhibitors PAI-1 and TIMP was increased while the production of type IV collagenase and membrane bound PA were unaltered.

6. Conclusions

1. Expression of the EGFR, in contrast to initial expectations, is not restricted to a specific cellular lineage but has been observed in a variety of different cell types throughout development.
2. Biological functions of EGFR are equally variable, ranging from providing signals for survival or terminal differentiation to inducing mitogenic response, cell motility, and invasion.
3. EGFR activity has been shown to initiate or contribute to progression of neoplasia in a wide range of experimental systems (cultured cells, retrovirally infected birds, rodent tumor model systems, and transgenic mice).
4. In human malignancies, truncations or mutations of EGFR are rare events with exception of glioblastoma. This contrasts with the frequent deletions observed in the transduced EGFR genes of avian oncogenic retroviruses.
5. In various human epithelial malignancies expression of EGFR is associated with tumor progression. In such carcinomas, paracrine or autocrine activation of EGFR seems to be a common means of promoting growth and/or dissemination.
6. In human mammary tumors, an inverse correlation between expression of EGFR and ER indicates involvement of EGFR in tumor progression. Interactions of EGFR and ER were also implicated in TGFα transgenic animals, leading to development of mammary carcinoma.
7. EGF may support metastatic capacity of tumor cells by enhancing their ability to succeed in specific steps of the metastatic cascade such as invasion, lodgement, extravasation, cell locomotion and growth in distant organs. The pleiotropic effects of EGFR on cellular cytoskeletal reorganisation, adhesion, motility, expression and activation of proteases may be in many circumstances a key to the success of cancer as a lethal disease.
8. Future research should further elucidate the signaling pathways that are affected by EGFR and that can lead to transformation and/or tumor progression. Clarification is also needed with regard to the role of other tyrosine kinase receptors and of specific domains in these processes.

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