The epidermal growth factor system of ligands and receptors in cancer

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Introduction

Multicellular organisms have evolved systems to allow information to be exchanged between cells in tissues. One example of these utilises proteins or peptides which are synthesised and released under stringent controls from one cell which then diffuse through the interstitial space until they encounter another cell which possesses cell surface proteins which bind this "first" messenger. These latter proteins, termed receptors, alter their activity and convey information across the plasma cell membrane to the cytoplasm, the "second" message, to elicit responses which include inducing the cell to exit a quiescent state and to enter the cell replicative cycle or to induce the cell to transit this cycle more rapidly. Other responses may also occur such as alterations in the cell cytoskeleton which may affect motility or the release of further signals such as those inducing neovascularisation. Typically in normal tissues these act during development or are evoked in processes such as wound healing.

Mutations in genes involved directly or indirectly with these systems can activate this program of events [1], recapitulating the wound healing response. However, due to the fixation of the mutations this process cannot be switched off, hence the remark that "cancer is a wound that never heals". It is no surprise therefore that aberrant activation of signalling pathways is inevitably associated with processes such as the secretion of proteolytic enzymes, which digest basement membranes, and the promotion of revascularisation, which are both essential to the spread and growth of solid tumours. In fact few mutations have been identified in genes which participate in these "secondary" events, presumably as they are necessary but insufficient in themselves to act as drivers of metastatic cancer [2].

One subset of growth factors and their receptors quite frequently involved in instigating and maintaining cell transformation are the receptor tyrosine kinases (RTKs). There are 58 RTK genes present in the human genome which can be subdivided into several families based on their sequence similarity, the similarity of their ligands and their known physical and functional interactions [3]. Some of these receptor types have barely been studied at all, but one family, partially because of its prominence in tumour development, is now known in some detail and has suggested paradigms which may (or may not) be instructive in the study of other less regarded receptors. This is the epidermal growth factor receptor (EGFR) family which consists of four receptors and (at least) eleven growth factors [4].

The EGF family of ligands

EGF was discovered by Cohen and Levi-Montalcini in the late 1950s as a side fraction in the purification of nerve growth factor from extracts of bovine hypothalami which was able to promote epithelial growth [5,6]. Its importance historically is not least as an example of a purifiable activity which reproducibly activated cell division after many years of often fruitless attempts to purify ephemeral activities called chalones thought to limit cell growth [7].

Of course the first ligand represented only a very small part of the whole system as we now know it. The full family of ligands described to date consist of eleven genes, however, since the EGF sequence motif and associated three dimensional fold is the second most common in the human genome (after the immunoglobulin sequence) of which there are three thousand examples, it is not impossible that further ligands may exist (http://pfam.sanger.ac.uk/clan?id=EGF). The eleven accepted ligand genes may be subdivided into those which are unspliced (EGF, transforming growth factor-α (TGF), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC), epigen and epiregulin) and those which are spliced (neuregulins 1–4) and based on their pattern of receptor recognition (EGF, TGF-α and AR bind the EGFR only; HB-EGF, BTC and epiregulin bind EGFR and HER4; the neuregulins bind HER3 and/or HER4:

epigen seems to bind EGFR, HER3 and HER4 but with low affinity) [8].

Seven EGFR binding ligands are produced as a single gene product which encodes a transmembrane sequence as well as an EGF motif whereas the four neuregulin genes are subject to extensive splicing to produce multiple protein products [9]. These variants fall into three classes, those with a transmembrane domain, those with a nuclear localisation sequence and those that have neither and apparently accumulate in the cytoplasm. While the transmembrane neuregulin variants follow much the same fate as the EGFR ligands which traffic to the cell surface and are released by regulated proteolytic cleavage [10] the function of the intranuclear [11,12] and cytoplasmic [13] variants is still enigmatic.

The EGF family of receptors

There are four genes in the human genome which encode the related members of the EGF receptor family, EGFR, HER2, HER3 and HER4. The primary product of each gene encodes a long single polypeptide chain which contains a centrally located transmembrane sequence. The proteins are inserted in the cell membrane as they are synthesised and are conveyed via various vesicles from the endoplasmic reticulum via the Golgi apparatus (where they are extensively glycosylated on their extracellular sequence), until they reach the plasma cell membrane. All four receptors are internalised and degraded in the normal basal state but the EGFR can be internalised and degraded in lysosomes at a greater rate in response to ligand binding [14]. Whether the other receptors are internalised but then recycled to the plasma cell membrane is still an area of active research. This latter property is important as when cells are treated with antibodies such as cetuximab or trastuzumab they may affect the proportion of receptor recycled versus that destined for degradation [15].

The three dimensional structure of the four receptors has been determined in various conformational states [16,17]. This has revealed how the ligands bind to the extracellular part of the receptors (except HER2 which does not bind a ligand) and stabilise the protein conformation in a state in which two receptors can interact. Homodimers and heterodimers can form but the exact nature of the possible combinations and their relative preferences are not as yet fully established (in fact there are conflicting data, probably because some experiments have used soluble extracellular domains and others truncated or intact membrane associated molecules) [18]. In the cytoplasmic domain of each receptor there is a sequence which encodes an enzyme, a tyrosine kinase (TK) which catalyses the transfer of the terminal phosphate group of adenosine triphosphate (ATP) onto the para-hydroxyl group of selected tyrosine residues. On dimerisation the intracellular kinase domains re-orientate asymmetrically and one TK domain catalyses the phosphorylation of several tyrosines in the c-terminal region of the other molecule [19,20]. The exception to this is HER3 which appears to lack appreciable TK activity and acts as a passive substrate for phosphorylation by a different member of the family [21]. HER2 differs from the other receptors in not being able to bind a ligand and by being fixed in the active conformation. In this case the receptor seems to be poised to interact with any other family member it encounters which is also in the active conformation.

Alternative products of the HER2 and HER4 gene have been reported. In the case of HER4, due to alternative mRNA splicing, four full length receptor types are translated which differ at their extracellular juxtamembrane region and in their c-terminal region. The former give two alternative sequences which either permit or do not permit proteolytic cleavage to release a soluble extracellular domain [22]. The membrane associated truncated product is then subject to further cleavage to release a soluble cytoplasmic domain which is catalytically active, interacts with cytoplasmic proteins and translocates via nuclear pores to the nucleoplasm where it is thought to alter gene transcription [23]. The uncleaved variant is less well studied but apparently functions like the other full length products at the cell membrane [24]. The other two alternative splice variants concern the use or not of an exon which encodes an intracellular sequence which contains a site for phosphorylation on tyrosine which may couple with the phosphatidylinositol 3 (PI3)-kinase enzyme to affect intracellular signal transduction [25]. HER2 may be translated as a full length product or as several shorter products which encode soluble cytoplasmic domains which have active kinases. The latter are proposed to be influential in cell transformation [26].

The chemical modification of a tyrosine to a phosphotyrosine provides a charged structure to which other protein domains may bind. The system appears to work by increasing the affinity of interaction when the tyrosine is in the phosphorylated state (primarily by electrostatic interactions). The system is reversible in that phosphorylated tyrosines can be dephosphorylated by enzymes called phosphatases. The regulation of the reversible activity is less well understood than is

the phosphorylation process. Two domains, called SH2 (Src homology 2) and PTB (Phosphotyrosine-binding domain), are present either as adaptor proteins which form bridges between the phosphorylated receptor and a different protein or they may be an intrinsic part of a protein possessing an additional function [27]. Both domains not only require the phosphorylated tyrosine residue but also linear sequence motifs around the site allowing them to specifically engage with one or more positions selectively within the receptors cytoplasmic domain. Proteins containing or interacting with SH2/PTB domains encode various activities which propagate signals, often via cascades of other proteins, to various sites inside the cell. One rather well characterised pathway involving an enzyme called mitogen-activated protein kinase (MAPK) ultimately terminates inside the cell nucleus where it alters gene expression. These pathways induce the known responses the family can produce which include increases in cell division rates, motility, secretion or survival. Different combinations of receptor activities and strengths of activity (partially regulated by the number or fraction of active receptors) affect the balance of the outcomes. Thus the system is not simply an on/off switch but more resembles a calculating device but one in which its components can be present in larger or smaller numbers (a system sometimes termed a "soft machine") [28,29]. A phenomenon often also observed using appropriate techniques is receptor clustering where multiple receptors appear to form aggregates of up to 1000 molecules [30]. These may contain more than one receptor type but their internal organisation (if any) is not known. Clearly this "higher order" process may allow further information exchange between receptors and second messengers making the calculating device more complex, allowing the integration of more input information and more varied responses.

The EGF family of ligands and receptors in cancer

Hendler and Ozanne published the first report of receptor over-expression (a term used here to denote elevated receptor numbers relative to the normal levels found in that differentiated cell type) in a series of human lung cancer biopsies [31]. Despite the publication since then of many papers containing data on over-expression the literature is very incomplete and in some cases contradictory. A summary that states that the HER2 receptor is over-expressed in between 20% and 90% of cases of ovarian cancer (as an illustrative example I have seen in the literature) does not contribute to the precision of our knowledge. This inconsistency can have multiple causes such as variations in the method of measurement, whether mRNA or protein is measured, the definition of over-expression employed or whether tissue microdisection has been done before molecular analysis. Also, it is disappointing that studies of the complete family of ligands and receptors in particular tumour types are very rare, not least as we are aware now of the highly interactive nature of the system (and by inference the incompleteness of measuring one or only a few of its components). Despite this rather depressing picture it is now generally accepted that over-expression of one or more receptors and/or ligands is a feature of the majority of human carcinomas (there is less known about sarcomas where over-expression of this family appears to be rarer; and over-expression is very rarely reported in blood cell-derived tumours, probably as the family are not normally expressed in this lineage).

Over-expression of a substance involved in regulating cell growth is potentially one of the factors in cell transformation but much experimental work needs to be done to prove this concept. This information has been obtained in various models such as transgenic mice, in manipulating human cancer cells in culture and as xenografts and now in humans treated with drugs which target these systems. However, a caveat is that since each receptor and ligand clearly does not operate alone they may have a different influence depending on the composition and activity of the other parts of the system and the sensitivity of the cell to their actions [32]. Models in which multiple receptor types and amounts and availability of the eleven ligands (and their splice variants) are so much more complex that their full realisation remains a task for the future. This investment should however be worthwhile not only to understand the system more fully but also to predict the activity of various drugs whose effects on human disease are to a large degree currently unpredictable.

The mechanisms leading to over-expression involve elevated transcription and/or gene amplification. The alternative mechanisms are characteristic of particular tumour types (glioblastomas and head and neck cancer for instance display frequent amplification of the EGFR gene and 20% of breast cancers show amplification of the HER2 gene). One or two reports have suggested instances of amplification of the HER3 or HER4 gene but, where studied, these seem to be exceptions and whether these genuinely occur and at what frequency are not definitively established except to say that, if they do occur, it is rare.

The receptor genes may be activated by mutations. These have been reported in the EGFR [33], HER2 [34,35] and HER4 [36] genes and their effects studied in various model systems. They appear to activate the receptors kinase or have more subtle effects [36] which, none the less, lead to over activity of the system. Both over-expression and mutation are targets for anti-cancer drugs as described below.

Over-expression of the ligands is less easy to define as for reasons unknown they are not subject to gene amplification (again, reports in the literature are very rare but if this does indeed occur it is very uncommon). Understanding why amplification does not occur may in fact help us to understand the role of the ligands better. Over-expression without gene amplification has however been frequently reported but as most of the ligands are in fact released from cells by proteolysis (not secreted) the cell associated component may not represent the functional component [23, 37]. Again, very few studies seek to measure more than one or a few ligands and even less literature is available to help one to understand how the system as a whole acts in normal or malignant tissues. Here, experiments in Drosophila which have a smaller number of ligands (Spitz, Vein, Gurken and Kerren) and better models for tissue patterning may be instructive [38].

An emerging class of mutations which do affect ligand genes is the rearrangement of the neuregulin genes in breast and possibly other types of cancer. NRG1 is rearranged in 6% of human breast cancers making it the most frequent gene rearrangement in cancer due to the common occurrence of the disease [39]. How this affects the system is still largely a matter of speculation but it might cause the protein to be routed through the secretory pathway where it would evade the regulatory proteolytic controls normally imposed on its release [9]. The NRG3 gene is also apparently rearranged in breast cancer but the frequency of this event is not yet established (Dr P.A. Edwards, University of Cambridge). It may be that this mechanism is more common than thought at present but comprehensive studies are needed to demonstrate or refute this.

In summary, over-expression or over-activity of components of the EGFR system is the hallmark of common epithelial cancers. While genetic models in organisms such as flies have revealed much of the logic of the system, human disease and in particular cancer has shown the many ways in which it can malfunction to produce over-activity. The sum of these observations has strongly encouraged pre-clinical and clinical studies of drugs directed to parts of the system to assess their efficacy in repressing the growth of cancers.

The EGFR system of ligands and receptors as targets for cancer treatment

One of the attractive features of these molecules as targets is our understanding of how they normally function (unlike many other perhaps equally important oncogenes whose activity is only partially understood). Inhibitors or antagonists are the leitmotif of pharmacology and as such are well adapted to clinical development programmes. Indeed, a search of the PubMed database of scientific publications with the term “receptor tyrosine kinase inhibitors” found 18,208 papers and 2807 reviews on the subject. A factor against the system is its complexity, particularly in the case of the extensive ligand family. However, the principal predicted limitation in drug development was that all kinases act by binding ATP and that inhibitors acting at this binding site would be non-specific. There have been justified periods of optimism and pessimism in this subject and it is clear that many of the first generation of inhibitors have many off-target effects on other RTKs, on other kinases and ATPases, as well as the inevitable unpredictable chemical activities (including those of metabolic breakdown products). Indeed, one of the first drugs designed to be a specific inhibitor of the EGFR (whose development preceded the discovery of the HER3 and HER4 receptors) binds to and inhibits at least 37 other kinases in addition to its intended target [40]. Later drugs, probably by design, have a much more specific profile such as lapatinib which appears to inhibit only EGFR and HER2 (and to a lesser degree HER4) [41].

In parallel with the development of these cell membrane permeable small molecules, antibodies have been isolated and engineered which bind to the extracellular domain of the receptors (11,310 papers and 1613 reviews found in response to a PubMed search with “receptor tyrosine kinase antibodies”). In this case they are generally highly specific but have other drawbacks such as they cannot be taken orally (they would be digested like any proteins in food).

While there are several examples of attempts to make successful small molecule or antibody drugs targeted at the receptors in the family there are so far very few which seek to exploit the ligands. Two types of approaches might be envisaged: first, conversion of a growth factor by mutation into an antagonist [42] and second, making a neutralising antibody which by passive transfer (injection into the patient’s bloodstream) would prevent it competitively binding to its receptor. The discovery of the ligands has spanned at least 40 years and may not have reached its finale and as more is learned, (particularly of the

neuregulin family) more complexity has been revealed. However, an effective drug to a growth factor from a related family of molecules is in common use. Bevacizumab is a monoclonal antibody that binds to and competitively prevents the interaction of vascular endothelial growth factor (VEGF) with its receptor which shows useful clinical anti-cancer activity. However, making antibodies which would bind to many or all of the EGFR family of ligands would be a difficult challenge perversely due to their specific binding properties. This concept is not beyond the bounds of possibility, however, as exemplified by the conversion of trastuzumab to bind not only its primary target, the HER2 receptor, but to recognise also the structurally unrelated VEGF molecule [43]. Finally, with the fairly recent elucidation of the structure of a ligand bound to a receptor it was hoped that this might allow the design of genuine growth factor antagonists by selective structural modification. This has not yet been reported but remains an active issue.

One factor inhibiting the production of a range of effective antibody/receptor binding drugs is our limited understanding of how trastuzumab functions. At present, it may best be said that there are competing hypotheses including receptor downregulation, inhibition of recycling and antibody-mediated complement fixation [44]. Perversely, several reports have suggested that the antibody actually acutely activates the cytoplasmic kinase activity (similar reports also apply to cetuximab binding to the EGFR). It is true that some cells over-expressing the EGFR (for instance the A431 cell line) are killed by ligand-mediated activation of the over-expressed receptor and that treatment of cancers with the ligand has been mooted, but safety considerations seem to have limited enthusiasm for this strategy.

Drug resistance, both primary and induced, looms large in attempts to target receptors as it does with most chemotherapy. Reasonably, many investigators have hypothesised that if the target receptor is at low levels on tumour cells they are unlikely to respond to its inhibition. In the case of trastuzumab and HER2 this is clearly true as patients with less than 3+ (the highest quartile) of protein expression and/or evidence of gene amplification do not respond. In the case of cetuximab this is not so clear and responses have been reported in patients even apparently lacking EGFR (the target) expression. This seems improbable unless the activity is off-target and is more likely due to the expression level being too low to detect by the method employed. Some cell lines (MCF-7 is an example) have very low levels of EGFR but respond robustly to addition of an EGFR ligand. In this case it may be that the level of expression of the receptor is only one factor and that its level of activity also needs to be considered as well as the cells sensitivity to its effects, all of which are quite challenging to measure. Again, this returns to the issue of understanding the activity of the four receptor/eleven ligand system as a whole, at least as the first level of abstraction. More practically useful information on drug responsiveness has emerged in the use of cetuximab in the treatment of colorectal cancer where failure to respond has been associated with the presence of a mutationally activated ras or mutations occurring at various levels in the PI3kinase/PTEN pathway. This may be a useful paradigm in other systems too.

Conclusion

While there have been some notable successes in the development and introduction of small molecule or antibody-based drugs directed to the EGFR system in cancer, there have been many disappointments too. In chronic myeloid leukaemia and gastrointestinal tumours, imatinib stands as an example to be emulated. However, current thinking tends to favour the concept that these cancers are more dependent on the Bcr/Abl fusion kinase or the Kit/PDGF receptor pathways than adenocarcinomas being dependent on over-activity of (usually) one component of the EGFR pathway. Yarden has suggested that the EGFR system is selected in evolution to accommodate mutational events and this is presumably how it has evolved its considerable complexity [45]. In the same sense it retains adaptive power to dampen the effects of alterations to individual parts of its mechanism (achieved in the clinic through drugs rather than through random evolutional events over eons). This appealing hypothesis leads inexorably to the view that only by understanding the system more fully (and there clearly remains much to learn: the neuregulins are an evident example) will a greater evidence base be obtained to learn how to effectively interfere with one or more of its components to achieve clinically useful effects. Hopefully, the next 50 years of research on this system will be as interesting and lead to as many, if not more, practically useful products as has the first.

Conflict of interest statement

None declared.

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