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Identification and Interpretation of Epidermal Growth Factor and c-*erb*B-2 Overexpression

Barry A. Gusterson

Overexpression of normal cellular genes may be one mechanism by which malignant cells can acquire a selective growth advantage. The epidermal growth factor receptor and the c-*erb*B-2 protein are members of the *erb*B family and are good examples of genes that appear to act through this mechanism. Molecular and biochemical analyses of these two proteins also illustrate how studies of growth factors, growth factor receptors and oncogenic retroviruses may lead to new approaches to diagnosis and treatment. In particular, overexpression of these growth factor receptors has identified clinical subgroups that may respond differently to chemotherapy and provides the opportunity for antibody targeted therapy. Overexpression of these proteins can be identified using immunocytochemistry on both histological sections and fine-needle aspirates, thus enabling these parameters to be assessed preoperatively and to be monitored during therapy.
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INTRODUCTION

OVER 40 genes have now been described that appear to have a role in the malignant process. These genes often encode for proteins that are important components of the growth regulatory pathways in the normal cell [1]. In many cases these oncogenes are members of 'families' encoding proteins with similar structures and possibly similar functions [2].

Growth regulatory mechanisms usually involve the binding of a growth factor to a specific receptor on the cell surface, which then through an intracellular biochemical cascade leads to cell division. As different cells respond to different growth stimuli it is necessary for them to have cell type associated receptors. As the final response of the cell is similar, (i.e. cell division), it is not surprising that receptors have evolved with similar structures and often very close sequence homology in their amino acid composition. Where this homology is overt they are grouped into the same family often before their function is known. Thus newly identified proteins often have an assumed function based on our knowledge of the physiological role of other well characterised family members.

The epidermal growth factor receptor (EGFR) and the c-*erb*B-2 genes are examples of one of these families. They both encode transmembrane proteins that are putative growth factor receptors although they may have other functions in normal tissues [3–5]. Both of these receptors are overexpressed in certain tumour types. It is presumed that overexpression in some way increases the 'sensitivity' of the tumour cell to the normal levels of the growth factor that binds to the receptor. Alternatively increased levels of the receptor may produce a constitutively activated receptor cascade in the absence of the growth factor. It is proposed that both of these mechanisms would result in selectively triggering the proliferation of the tumour cell with a consequent selective growth advantage for the tumour population. It is not known *in vivo* which, if any, of these hypotheses is correct. However, antibodies to both the EGFR and the c-*erb*B-2 protein have been shown to inhibit tumour growth indicating that these molecules are important components of the growth response in these model systems. At

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least two peptide growth factors EGF and tumour derived growth factor alpha (TGFα) will activate the EGFR [4] and a possible ligand for the c-*erb*B-2 receptor has been recently identified [6]. Thus at the present time these two members of the *erb*B family, together with a recently identified third member, (c-*erb*B-3) [7] provide future potential targets for cancer therapy.

ISOLATION AND IDENTIFICATION OF EGFR AND c-*erb*B-2

In order to define a growth factor receptor it is necessary to identify the growth factor that activates it and to demonstrate a biological response. It is, however, important to realise that these receptors and their ligands are usually defined using *in vitro* assays and in many parts of the body the growth factors appear to have diverse effects, depending on the target cell type and the local tissue environment. Thus EGF and TGFα can induce osteolysis and have both growth stimulatory and growth inhibitory effects.

EGFR

Many tumours that express the EGFR can also be shown to produce TGFα, thus raising the attractive hypothesis that they may have an autocrine feed back loop as found in other systems [8]. This is supported by *in vitro* studies that have demonstrated that such cells will grow in tissue culture in the absence of serum.

The EGFR has been isolated by its ability to bind EGF. This was carried out using a squamous carcinoma cell line (A431), that has in excess of a million receptors per cell. This same cell line was used as an immunogen to raise antibodies to the receptor. Although the first antibody to work on tissue sections was produced over 10 years ago, immunohistochemical studies are still limited owing to the difficulty of producing a reagent that will work consistently on paraffin embedded tissue. This has resulted in numerous reports of small series on tumours in which it is difficult to assess the clinical significance of the results.

Two methods have been used to assess the levels of EGF receptor in tissues. The more commonly used has been indirect immunocytochemistry using antibodies directed to the receptor. This method has the advantage that it is possible to identify the cell type expressing the tumour and can thus distinguish between staining of the tumour and the normal stroma. The disadvantage is that the method depends on the staining protocol used and many of the antibodies currently available are not specific for the receptor. One of the best reagents is still that originally produced by Waterfield and Ozanne [9] as it reacts with the protein core of the receptor and not with the blood group carbohydrate epitopes that characterise many other reagents. The other method is to make a membrane preparation of the tissues and to use the growth factor's ability to bind to the receptor to quantify the number of receptors. This method provides quantitative results, but has the disadvantage that it is not possible to assess the effect of or the level of stromal contamination.

Using both methods, however, it can be demonstrated that some tumours notably, squamous cell carcinomas of the head and neck, squamous carcinomas of the lung, bladder carcinomas, breast carcinomas and gliomas have overexpression of the receptor. This is often associated with increased numbers of copies of the EGFR receptor gene. For a more detailed account of the structural and functional relationships of EGF and TGFs the reader is referred to a recent review [4]. The EGFR has been the subject of an excellent review by Harris and Neal [10].

In terms of clinical application it must be said that the EGFR has been a disappointing area. With the exception of papers, notably from Harris and his colleagues, there have been few reports on the prognostic value of measuring receptor levels [11]. This may be largely due to the fact that the levels of the receptor tend to correlate with high tumour grade and thus many groups may have thought it not worth pursuing this marker as an independent variable. When antibodies become available that work consistently on paraffin embedded material it will be possible to rapidly screen large tumour data sets, but at the present time few groups are using EGFR measurements routinely. In the case of breast carcinomas, expression of the EGFR is associated with a poor prognosis [11]. More recently it has been shown that the prognostic significance of EGFR measurements are stronger if combined with measurements of the c-*erb*B-2 protein [12].

In summary, measurements of EGRF are not yet a routine in the assessment of any tumours, but it is to be predicted that in the case of gliomas and breast cancers in particular, they may turn out to be of value as independent prognostic variables. The final outcome will, however, rely on data from much larger series than are currently available. In terms of therapy, there is still the hope that some of the antibodies may either alone or coupled to toxins be of therapeutic value, but that is currently a long way off. In view of the high levels of EGFR expression in normal tissues it seems unlikely that such an approach would ever be successful as a systemic treatment, although there is some evidence that radiolabelled anti-EGFR antibodies can be used in the treatment in brain gliomas resistant to conventional forms of treatment [13].

C-erbB-2

It is now known that many of the acutely transforming retroviruses have sequences within their genome which have been acquired from host cells and through mutational events have converted protooncogenes into dominantly acting viral oncogenes [14–16]. One group of these viral oncogenes is characterised by the first member of the family to be identified, v-*erb*B, which induces erythroblastosis and sarcomas in chickens. This gene, like many others subsequently identified, originated from a normal cellular gene or protooncogene, which in this case is the epidermal growth factor receptor gene (c-*erb*B-2) [17–20]. Another member of the *erb*B family, c-*erb*B-2 was identified by the classical method employed to isolate oncogenes, namely the NIH-3T3 fibroblast transfection assay [21, 22]. More recently a third member of the familiy (c-*erb*B-3) has been identified using reduced stringency hybridisation of v-*erb*B to normal human genomic DNA [7]. Elevated levels of mRNA for the product of this gene in some human breast cancer cell lines indicated that it may play a role in some human malignancies [23].

Based on its amino acid sequence the protein encoded by the c-*erb*B-2 gene (p185) appears to be a member of a large group of growth factor receptors [24]. There is also some provisional data suggesting that a *ras* transformed 3T3 cell line may produce a putative ligand [25]. Lippman's group at the Lombardi Cancer Centre has recently reported the isolation of a putative ligand for the receptor. It is possible to generate a mitogenic signal in a chimeric receptor in response to EGF [26, 27] which also supports the role of c-*erb*B-2 as a novel growth factor receptor. Taken together these data would suggest that the c-*erb*B-2

product may produce transformation by an effect on cell proliferation. There are tissues in which the c-*erb*B-2 gene product appears to be expressed, where it is unlikely to have a relationship to local growth control.

Studies of c-*erb*B-2 expression have been carried out by analyses of the mRNA, or by estimations of protein using western blotting or immunocytochemistry. In the majority of the publications in this area the antibodies used have been characterised against extracts of cell lines with the assumption that the reactivity seen in tissues is a true reflection of the distribution of the protein. Another major potential problem relating to many of the immunohistochemical studies relates to the controls employed. In the absence of a demonstration of the extracted protein from the tissue in question, the most that can be claimed for these studies is c-*erb*B-2 like immunoreactivity. It should also be remembered that even demonstration of the mRNA may not necessarily be equated with protein as appropriate RNA may not be translated.

In a detailed immunocytochemical study of the adult human gut, Cohen *et al.* [28] has demonstrated prominent immunoreactive c-*erb*B-2 in the stomach, small intestine, colon, pancreas, salivary gland and liver. It should be noted that the staining in the small intestine was absent [29] or weak [28] in the crypts and strong in the villi, raising doubts about the role of this protein as a growth factor receptor in this site.

With the availability of antibodies directed against the p185 protein that appear to be able to inhibit tumour cell growth both *in vitro* [30] and *in vivo* [31, 32] the way is open to use these reagents to examine effects on other normal tissues that express the protein. Antibodies against different epitopes on the external domain will be particularly useful in investigations of ligand binding and the analyses of functional domains [33]. In this way, the physiological and pathological role of this protein may become more apparent. Although the case for a role for the p185 protein in normal growth control is not entirely convincing in the tumour situation the evidence is more compelling.

A range of tumour cell lines derived from glandular tissues overexpress c-*erb*B-2 and this usually, but not always, correlates with gene amplification [23]. Subsequently, it was also shown that overexpression of c-*erb*B-2 in NIH 3T3 cells was transforming in a similar way to presence of the mutant gene, suggesting that a dosage effect of the normal gene product could produce the same end result as the mutation [34]. The numerous publications in the literature on c-*erb*B-2 in human tumours as reviewed by Gullick and Venter [35] have clearly demonstrated that gene amplification and overexpression is a common finding in human tumours. In most reports gene amplification directly correlates with overexpression of the product [36]. Amplification has now been demonstrated in a broad range of glandular tumours including breast, ovary [37], stomach [28, 38, 39], salivary [40], renal [41], colonic carcinomas [42, 43] and adenocarcinomas of the lung [43]. The highest incidence of amplification and overexpression appears in breast and ovary [37]. There have been few studies of benign lesions but in our own report of proliferative breast disease we found no evidence of immunocytochemical positivity, even in cases demonstrating atypia [5].

Clinical interest in this cellular gene in breast cancer was initiated by the work of Slamon *et al.* [44] which suggested that amplification of the gene was a bad prognostic indicator in lymph node-positive patients. Amplification is associated with overexpression of this gene, thus enabling the localisation of overexpressing tumours by demonstration of the product by immunocytochemistry [5, 45, 46]. Fundamental to the studies in this area is the question of whether in the analyses of tumours the different methods of assessing the c-*erb*B-2 gene and its products are comparable. Only Slamon *et al.* [37] have measured amplification in relation to RNA, protein and immunocytochemistry and there was generally good correlation between all four methods. It can be concluded that for practical purposes the results of different methods are broadly comparable. Many studies, including our own, have now recorded a high incidence of c-*erb*B-2 overexpression in breast cancer and some have reported a correlation with nodal status [44, 47–49]. Both benign and malignant breast disease have now been studied using immunocytochemistry [5, 37, 50–54] and these data indicate that c-*erb*B-2 is a good discriminant of cytologically malignant cells in positive tumours [5, 51]. Combining the results of many studies up to 27% of all invasive breast carcinomas show gene amplification [37]. In intraduct carcinomas [5, 55, 56] and Paget's disease [5, 57], the level of overexpression is much higher with figures of up to 100% in some subtypes [55].

We have recently shown that there is a significantly increased incidence of overexpression in ductal carcinoma *in situ* (DCIS) compared with invasive carcinomas [5, 55]. Another recent study confirmed this high incidence of c-*erb*B-2 overexpression in ductal carcinoma (DCIS), with an 83% incidence in comedo and 29% incidence in non-comedo DCIS [56].

c-*erb*B-2 overexpression has naturally been of interest to the clinicians through its potential role as a novel growth regulator, as overexpression of a receptor may give the tumour a selective growth advantage and this may be a poor prognostic factor. Some of the earlier studies, including our own, showed a trend towards a relationship with decreased disease-free survival and overall survival [51, 58], but did not reach statistical significance. Subsequent papers have clearly demonstrated a strong correlation with both disease-free survival [37, 52, 59, 60] and overall survival [37, 44, 53, 59–61]. Recent data from an NSABP study also demonstrated a 5-fold increase in mortality rate ($P = 0.00001$) for immunocytochemically positive cases [52]. When nuclear grade and c-*erb*B-2 overexpression were combined they were predictive, regardless of lymph node status [52]. We would support their suggestion that a combination of c-*erb*B-2 overexpression and cytological features may be a good way to identify patients at increased risk.

More recently it has been shown that in lymph node negative disease that c-*erb*B-2 is a poor prognostic indicator [62]. This has been confirmed in a large prospective randomised trial of over 1500 cases. In this study it was also shown that c-*erb*B-2 overexpression correlated with unresponsiveness to chemotherapy [63].

FUTURE PROSPECTIVES

In the next few years there will be moves by many groups to exploit the present findings and to translate them into antibody-targeted therapy, radioimmunolocalisation of micrometastases and possible analyses of circulating c-*erb*B-2 proteins to detect early recurrence. None of these approaches are new, but the overexpression of a membrane associated molecule such as c-*erb*B-2 in a defined group of tumours makes this a more favourable prospect than in any other solid epithelial tumour to date. When considering prognosis it would appear that c-*erb*B-2 overexpression does identify patients with a reduced survival group, but unfortunately these patients appear not to benefit from some forms of chemotherapy. The significance of the EGFR will have to await more extensive evaluation.

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Residual DNA Damage: What is Left Over and How Does This Determine Cell Fate?

Trevor J. McMillan

Radiation-induced DNA damage may remain unrepaired for a number of reasons: it may be too severe, it may be in inaccessible parts of the genome, it may be induced at critical points in the cell cycle or it may be converted into large DNA deletions. This residual damage is likely to be responsible for cell death either by physical restriction of replication or transcription or by metabolic disruption due to loss of function of critical genes. Although residual damage is important, cells may differ in their ability to tolerate it, which may be a factor that determines the relative radiosensitivity of a given cell population.
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INTRODUCTION

IF FULLY efficient, the repair of DNA damage after insult by chemical and physical agents would result in a cell having no remaining damage. With drugs this can sometimes be achieved if the cells are kept in a strictly non-proliferative state, such that within the sensitivity of the appropriate assays, damage is totally removed and cell kill is zero. With ionising radiation the situation is different since there always appears to be a degree of residual damage no matter what the radiation quality, dose, dose-rate or culture conditions.

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At the level of cell survival this is demonstrated by experiments on plateau-phase cells irradiated at low dose-rate in which a terminal slope to the survival curve is reached as the dose rate is lowered (Fig. 1). Because of problems with proliferation such studies are rare but in the one study where this has been done thoroughly [1] this pattern was produced exactly, indicating that there is an amount of nonrecoverable damage produced following treatment with ionising radiation. At the level of naked DNA isolated form irradiated cells the demonstration of residual damage (usually strand breaks) is hampered by the lack of sensitivity of the techniques available. However, rarely has complete rejoining of strand breaks been observed. This is especially true for DNA double strand breaks (dsb) which are believed to be the most significant lesions in terms of cell death.

It is likely that this level of residual damage is important in the determination of the degree of killing by radiation treatment.