EGF mutant receptor vIII as a molecular target in cancer therapy

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Introduction

In the year 2000, an estimated 1220 100 new cases of invasive cancer will be diagnosed in the United States, and about 552 200 people are expected to die of it (Greenlee et al. 2000). Treatment of cancer has conventionally consisted of surgery, radiation and chemotherapy. Despite improved cancer therapy involving early detection, the surgical removal of solid tumor masses, and the use of radiotherapy, cytotoxic agents, or both, truly effective therapeutic approaches are still lacking and thus urgently needed. Extensive analyses have defined tumor-associated and, less frequently, tumor-specific, surface antigens displayed on the malignant cell surface. Targeting cells selectively through these surface antigens by molecular probes is inherently different from surgery, radiation, and chemotherapy and has been considered as a favored modality for cancer therapy. A considerable effort has been made over the past decade to develop new immunotherapeutic strategies against cancer; recently, such strategies have shown promise (Waksal 1999, Yang et al. 1999, Kreitman et al. 2000). Targeted therapy can be accomplished by using tumor-specific monoclonal antibodies (Mabs) alone or Mabs armed with radionuclides, prodrugs, or toxins, which selectively kill tumor cells while not destroying normal cells.

Many growth factors and their receptors play important roles in modulating cell division, proliferation and differentiation, and the possibility of disrupting these processes has led to the development of novel therapeutic agents for cancer treatment. Therefore, growth factor receptors are attractive candidates for targeted therapy, as they are often overexpressed on the surface of cancer cells. Among these, the type I epidermal growth factor (EGF)-related family of tyrosine kinase growth factor receptors are expressed in a broad spectrum of tumor types, which classifies them as one of the most frequently implicated cell-surface markers for human cancers. This family consists of four members: EGF receptor (EGFR), ErbB-2, ErbB-3 and ErbB-4 (Gullick 1998). EGFR was the first cell-surface glycoprotein identified to be amplified and rearranged in glioblastoma multiforme (GBM) and to act oncogenically to stimulate the growth and spread of cancer cells. Overexpression of ErbB-2 and ErbB-3 also has been found commonly in solid human tumors.

The involvement of increased and/or aberrant EGFR activity in human cancer is well documented (Libermann et al. 1985, Wong et al. 1987, Ekstrand et al. 1991), and it was originally thought that EGFR amplification promoted tumor development exclusively by increasing ligand-activated signaling through wild-type EGFR (EGFRwt). However, it is now known that many GBMs exhibiting EGFR amplification have EGFR mutations (Frederick et al. 2000). Several types of EGFR gene mutations have been reported in GBM, and in other tumors, and in nearly all cases the alterations have been reported in tumors with EGFR amplification (Libermann et al. 1985, Wong et al. 1992, Frederick et al. 2000). A mutant EGFR (EGFR class III variant, or so-called EGFRvIII) that lacks 267 amino acids from its extracellular domain, resulting in the formation of a new, tumor-specific target, is commonly found in GBM (Wikstrand et al. 1998a), and has been reported also in breast, ovarian, prostate and lung carcinomas (Garcia de Palazzo et al. 1993, Moscatello et al. 1995, Wikstrand et al. 1995). Antibodies with specific activity against this receptor have been developed that are internalized into the cell after receptor binding. This review will summarize current research in cancer therapy on the tumor-specific marker EGFRvIII as an attractive target for immunotherapeutic regimens, primarily in GBM, but also potentially in breast, ovarian, lung and prostatic carcinomas.

EGFR in human cancer development

Overexpression of receptors has been implicated as an important factor in the proliferation of malignancies and has also been identified as a marker of poor prognosis. For example, up to 90% of high-grade astrocytic gliomas express EGFR, which is associated with EGFR gene amplification in 40–50% of GBM (Libermann et al. 1985, McLendon et al. 2000). EGFR has been implicated in supporting the oncogenesis and progression of human solid tumors, which
makes it an attractive target for anti-cancer treatment, as summarized extensively elsewhere (Wikstrand et al. 1998a). Candidate tumors for EGFR targeting therapy include head and neck squamous cell cancer and cervical, renal cell, lung, prostate, bladder, colorectal, pancreatic, and breast cancer, in addition to melanoma, glioblastoma, and meningioma.

EGFR is encoded by the c-erbB1 proto-oncogene and has a molecular mass of 170 kDa. It is a transmembrane glycoprotein with a cysteine-rich extracellular region, an intracellular domain containing an uninterrupted tyrosine kinase site, and multiple autophosphorylation sites clustered at the C-terminal tail. The extracellular portion has been subdivided into four domains: domains I and III, which have 37% sequence identity, are cysteine-poor and conformationally contain the site for ligand (EGF and transforming growing factor [TGF]α) binding. Cysteine-rich domains II and IV contain N-linked glycosylation sites and disulfide bonds, which determine the tertiary conformation of the external domain of the molecule. In many human cell lines, TGFα expression has a strong correlation with EGFR overexpression, and therefore TGFα was considered to act in an autocrine manner, stimulating proliferation of the cells in which it is produced via activation of EGFR. Binding of a stimulatory ligand to the EGFR extracellular domain results in receptor dimerization and initiation of intracellular signal transduction, the first step of which is activation of the tyrosine kinase. The earliest consequence of kinase activation is the phosphorylation of its own tyrosine residues (autophosphorylation). This is followed by association with activation of signal transducers leading to mitogenesis (Carpenter & Cohen 1990).

Ligand-induced activation of the c-erbB receptors encourages receptor dimerization, which in turn initiates a signaling cascade via the Ras/mitogen-activated protein kinase pathway, resulting in transcriptional activation. Not only do receptors of the same type produce dimers (homodimerization), but different members of the type I kinase family can interact (heterodimerization); for example, experimental evidence demonstrating interactions between EGFR and c-erbB-2 has been found in ovarian cancer (Marth et al. 1992).

In gliomas, gene amplification is the predominant underlying mechanism of EGFR protein overexpression, and estimates of the frequency of EGFR gene amplification by genetic analyses in human tumors of astrocytic origin range from 37 to 58% (Wikstrand et al. 1998a). Amplification is primarily associated with GBM and not with astrocytomas of grades II/III or pilocytic astrocytomas (Agosti et al. 1992, Ekstrand et al. 1992, Schiegel et al. 1994). Up to 90% of high-grade astrocytic gliomas express the EGFR antigen, a hyperexpression associated with EGFR gene amplification in more than 50% of glioblastomas (McLendon et al. 2000). Protein overexpression without gene amplification, however, has been reported in 12–38% of GBMs (Chaffanet et al. 1992, Tsug et al. 1997), suggesting that multiple systems of deregulation in EGFR expression may exist at translational and post-translational levels.

**Anti-EGFR Mabs**

Mabs reactive with EGFR that are most promising for therapeutic applications are the chimerized/human Mabs MC-C225 and E7.6.3. As reviewed by Waksal (1999), the administration of the chimerized (human IgG1) version of anti-EGFRwt Mab 225 (IMC-C225; cetuximab) in Phase I trials of patients with recurrent squamous cell carcinoma of the head and neck (SCCHN) has yielded promising results. Virtually 100% of SCCHN biopsy tissues express high amounts of EGFRwt homogeneously throughout the tumor and, in preliminary trials, the combination of cetuximab with either chemotherapy (cisplatin) or radiation resulted in an overall response rate of 91.6% (15 complete, six partial, and one minor response). The mode of action is presumed to be competition with a ligand for EGFR, resulting in internalization, but absence of downstream signaling, and eventual apoptosis. Even more promising is the recent report of the fully human (IgG1) Mab E7.6.3, derived from transgenic mice producing human Ig (Yang et al. 1999). Like cetuximab, E7.6.3 completely blocks the binding of a ligand to EGFR, which abolishes cell proliferation, EGFR tyrosine phosphorylation, and other downstream effects. Most importantly, in preclinical therapy of A431 xenografts in athymic mice, administration of E7.6.3 not only prevented the formation of tumors, but also induced eradication of established tumors with a total dose of 0.6 µg in 65% of the mice, with no tumor recurrence being observed for more than 8 months after cessation of treatment. This result was obtained in the absence of any concomitant chemotherapy, which expands its potential applicability to human disease. E7.6.3 is expected to exhibit minimal immunogenicity and a longer half-life as compared with mouse or mouse-derivatized Mabs, thus allowing repeated antibody treatments in patients (Yang et al. 1999).

**EGFR mutations and gene amplification**

Overexpression of EGFR in human malignancy has been the subject of extensive study, in which it has become increasingly apparent that alterations in EGFR may be as important as amplification with respect to the oncogenic effects; furthermore, such alterations have been demonstrated to correlate with a poor prognosis (Libermann et al. 1984, Di Fiore et al. 1987, Velu et al. 1987, Neal et al. 1990). Recent studies have confirmed that approximately 37–58% of GBMs amplify EGFR, with accompanying gene rearrangement (reviewed in Wikstrand et al. 1998a). Several types of EGFR gene mutations have been reported in GBM, and in nearly all cases the alterations have been reported in
tumors with EGFR amplification (Frederick et al. 2000). It was initially suggested that EGFR amplification promoted tumor growth only by increasing mitogen-activated, growth-stimulatory signaling by EGFRwt. However, it is now known that many GBMs with EGFR amplification have EGFR mutations (Ekstrand et al. 1992, Wong et al. 1992); consequently, different types of EGFR existing in individual tumors play their corresponding roles in contributing to the highly proliferative nature of GBM. This leads to simultaneous expression of both wild-type and mutant receptors (Libermann et al. 1985, Wong et al. 1987, Humphrey et al. 1988, Bigner et al. 1990, Wikstrand et al. 1997, McLendon et al. 2000).

As summarized recently (Bigner et al. 1998), several genomic variants have been detected in multiple biopsies. EGFR mutant receptors in malignancies arise from gene rearrangements with internal deletions (Sugawa et al. 1990, Humphrey et al. 1991). Most mutants contain a deletion of specific exons encoding part of the extracellular domain of the EGFR molecule. Tumor progression may be promoted by constitutive receptor activation (ligand-independent), impaired receptor downregulation, activation of alternative signaling cascades, abrogation of apoptotic mechanisms, and some other mechanisms (Nishikawa et al. 1994, Nagane et al. 1996, Chu et al. 1997, Huang et al. 1997). As shown in Table 1 and Fig. 1, EGFRvI with an extensive N-terminal truncation is similar to the viral erbB-1 gene product that induces malignant transformation and potent constitutive receptor activation (Wong et al. 1992). The EGFRvII mutant has an in-frame deletion of 83 amino acids outside the ligand site on domain IV of the extracellular region (exons 14 and 15) (Humphrey et al. 1991). Class IV and V mutants carry deletions in the intracellular domain (Fig. 1), in which Class IV receptors truncate at amino acid 958 and Class V receptors lack amino acids 959–1030 (Frederick et al. 2000). Class VI and VII mutants are Class IV and V coexisting with Class III, which carries defined extracellular domain deletions (Batra et al. 1994). Class III mutants (EGFRvIII) are the most frequently detected genomic variant; in GBMs that exhibit EGFR gene amplification, more than 50% express the class III deletion (Bigner et al. 1990, Humphrey et al. 1990, Sugawa et al. 1990). The 145-kDa EGFRvIII mutant receptor contains an in-frame deletion of exons 2–7 from the extracellular region. It appears that each of these EGFR deletion mutants arises from loss of specific exons from the genes that encode them. For instance, EGFRvIII may generate from recombination between introns 1 and 7 (Sugawa et al. 1990). The EGFRvIII transcript encodes a protein lacking the cysteine-rich domain II and a portion of domain I. Double-mutant EGFRvIII with an additional extracellular region deletion (∆12–13) has been reported (Callaghan et al. 1993); these deletions correspond to a loss of exons 2–7 and exons 12–13 respectively. The size of EGFRvIII/∆12–13 is 125 kDa and, in addition to loss of domain II, it also has lost a portion of domain III. Because of the loss of exons 2–7, both EGFRvIII and EGFRvIII/∆12–13 contain a unique amino acid residue not present in EGFRwt (Table 1). The joining of exons 1 and 8 in these two mutants creates a novel epitope with a glycine residue at the junction of the two exons, as published previously (Humphrey et al. 1991). EGFRvIII/∆12–13 has a second novel residue, histidine, at the junction of exons 11 and 14.

In addition to deletion of EGFR exons, several mutants with tandem duplication of portions of either the extracellular or the intracellular regions of EGFR have been reported in glioma cell lines and biopsy specimens (Fenstermaker & Ciesielski 2000). Two EGFR mutants, EGFR.TDM/18–25 and EGFR.TDM/18–26, contain tandem duplications of

### Table 1: Comparison of EGF wild-type and mutant receptors.

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Ligand dependence</th>
<th>Δ or TD</th>
<th>Protein size (kDa)</th>
<th>Predicted key inter-exon boundaries†</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFRwt</td>
<td>+</td>
<td>None</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td>EGFRvI</td>
<td>–</td>
<td>N-terminal truncation</td>
<td>145</td>
<td></td>
</tr>
<tr>
<td>EGFRvII</td>
<td>+</td>
<td>∆ exons 14–15</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>EGFRvIII</td>
<td>–</td>
<td>∆ exons 2–7</td>
<td>145</td>
<td>KK[G]NY (exons 1–8)</td>
</tr>
<tr>
<td>EGFRvIV</td>
<td>+</td>
<td>∆ exons 25–27</td>
<td>167</td>
<td></td>
</tr>
<tr>
<td>EGFRvV</td>
<td>+</td>
<td>C-terminal truncation</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>EGFR.TDM/2–7</td>
<td>+</td>
<td>TD exons 2–7</td>
<td>180</td>
<td>CP[L]CQ (exons 7–2)</td>
</tr>
<tr>
<td>EGFR.TDM/18–25</td>
<td>–</td>
<td>TD exons 18–25</td>
<td>185</td>
<td>SSL-LVE (exons 25–18) SSL-SAT (exons 25–26)</td>
</tr>
<tr>
<td>EGFR.TDM/18–26</td>
<td>–</td>
<td>TD exons 18–26</td>
<td>190</td>
<td>RNG-LVE (exons 26–18) RNG-LQS (exons 26–27)</td>
</tr>
</tbody>
</table>

Δ = deletion; TD = tandem duplication.

†Predicted amino acid sequences of mutant molecules at key exon junctions are shown: Bracketed amino acids are unique to the mutants (Wong et al. 1992, Wikstrand et al. 1998a, Ciesielski & Fenstermaker 2000; Fenstermaker & Ciesielski 2000; Frederick et al. 2000).
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**Figure 1** Schematic protein structure of EGFRwt and mutant forms. NH₂, amino terminus; COOH, carboxyl terminus; TM, transmembrane segment; TK, tyrosine kinase domain; amino acid residue numbers lie below the structure.

exons 18–25 and exons 18–26 respectively, which encode the tyrosine kinase domain and a portion of the calcium internalization domain of the molecule (Ciesielski & Fenstermaker 2000, Frederick et al. 2000). There is also a distinct class of EGFR mutants with tandem duplication of exons 2–7 in the extracellular region from some gliomas (Fenstermaker & Ciesielski 2000). The 180-kDa tandem duplication mutant (EGFR.TDM/2–7) contains a unique residue, leucine, as a result of the joining of the 3'-end of exon 7 with the 5'-end of exon 2 (Fenstermaker & Ciesielski 2000). The TDM transcript encodes an additional cysteine-rich domain II and a partial duplication of domain I. The molecular processes leading to the development of deletion and tandem duplication mutations in EGFR may be fundamentally related.

Recently, Frederick et al. (2000) reported the diversity and frequency of EGFR mutations by sequencing cDNA products representing the entire EGFR coding region and found that 50% of GBMs examined had EGFR amplification, in which all cases were identified to have coding sequence alterations (Frederick et al. 2000); among the EGFR mutants, 67% exhibited the most common mutation – lack of the coding sequence for amino acids 6–273, also known as EGFRvIII. These data are consistent with EGFR mutations being exclusively and frequently associated with EGFR amplification, and the existence of multiple EGFR mutations within individual tumors suggests that GBMs with EGFR amplification have the capacity to produce a variety of functionally distinct EGFRs (Frederick et al. 2000).

**EGFRvIII**

The type III EGF deletion-mutant receptor (EGFRvIII) is the most common mutation and was first identified in primary human glioblastoma tumors; EGFR gene amplification is correlated with the structural rearrangement of the gene. The EGFRvIII gene has an in-frame deletion of 801 base pairs, corresponding to exons 2–7 in the mRNA, resulting in the deletion of amino acids 6–273 in the extracellular domain and the generation of a glycine at the fusion point (Wikstrand et al. 1998a). The new tumor-specific epitope is situated near the amino terminus of the receptor extracellular domain.
Immunohistochemistry, flow cytometry and confocal microscopy have been used to demonstrate that monoclonal antibodies specific for this epitope decorate the surface of cells (Wikstrand et al. 1997; Fig. 2). This truncated mutant receptor has a molecular mass of 145 kDa compared with that of 170 kDa for EGFRwt. Mutant receptor EGFRvIII has been found in brain neoplasms, where it is present in 60–70% of GBM and gliosarcoma, but at a low frequency in anaplastic astrocytomas (Bigner et al. 1990, Yamazaki et al. 1990). Immunohistochemical evaluation of high-grade gliomas reveals a predominant membrane and cytoplasmic staining pattern for EGFRvIII as detected by specific Mabs; the antigen distribution is relatively homogeneous, with frequent increased density at cell–cell contacts (Fig. 3; Wikstrand et al. 1997, 1998b). It is also present in squamous cell, adenosquamous cell and undifferentiated non-small-cell lung cancer (Garcia de Palazzo et al. 1993), intraductal, infiltrating ductal breast cancer, and ovarian carcinoma (Moscatello et al. 1995, Wikstrand et al. 1995).

A cascade of ligand–receptor interactions involving ligand binding, autophosphorylation, receptor dimerization and signaling by EGFRvIII and normal EGFR has been extensively reviewed by Wikstrand et al. (1998a). A number of functional differences between EGFRvIII and EGFRwt have been characterized. There is no evidence for regulation of EGFRvIII by EGF or TGFα; EGFRvIII is constitutively activated. Introduction of EGFRvIII into the U87MG human glioma cell line or murine NR6 cells resulted in cell-surface expression of a truncated receptor having a ligand-independent, weak but constitutively active and unattenuated kinase and enhanced tumorigenicity in nude mice (Nishikawa et al. 1994, Batra et al. 1995), which was mediated by both an increase in proliferation and a decrease in apoptosis of tumor cells. In contrast, overexpression of EGFRwt did not confer a similar growth advantage (Nagane et al. 1996, Huang et al. 1997).

The molecular mechanism by which the EGFRvIII transfectants acquire transforming activity is not yet clear. First, EGFRvIII has been found constitutively associated with signaling adapter proteins Shc and Grb2, which are involved in the recruitment of Ras to activated receptors, even if dimerization does not occur (Moscatello et al. 1996, Prigent et al. 1996, Chu et al. 1997). Studies by Prigent et al. (1996) and Feldkamp et al. (1999) demonstrated that constitutively active EGFRvIII enhanced the growth of glioblastoma cells through increased activity of Ras-GTP. However, Moscatello et al. (1998) demonstrated that high levels of phosphatidylinositol (PI) 3-kinase activity are constitutively present in EGFRvIII-positive cells; these levels were dependent upon the kinase activity of the receptor. Therefore, PI 3-kinase played an essential role in EGFRvIII transformation. Similarly, the c-Jun N-terminal kinase (JNK)
pathway was found to be constitutively active with high levels in EGFRvIII-positive cells that were not present in cells overexpressing EGFRwt; this implicated constitutive activation of the JNK pathway in transformation by EGFRvIII (Antonyak et al. 1998).

Contrary to earlier published results, a recent study showed that there is no difference between the dimeric EGFRvIII and the ligand-stimulated EGFRwt with respect to kinase activity as determined by the extent of autophosphorylation (Fernandes et al. 2000). The authors proposed that the high kinase activity of EGFRvIII is due to self-dimerization. The patterns of phosphorylation of both the EGFRwt and EGFRvIII receptors are similar, and the receptor–receptor self-association is highly dependent on a conformation induced by N-linked core glycosylation (Fernandes et al. 2000).

Nishikawa et al. (1994) have demonstrated the tumorigenic potential and shorter latency of U87MG.ΔEGFR cells in both subcutaneous and intracerebral implantations. A continuation of this study, using EGFRvIII autophosphorylation site point mutations (Huang et al. 1997), established that mutation of any of the autophosphorylation sites, singly or in combination, dramatically reduced the enhanced tumorigenic activity conferred by the mutant receptor. Nagane et al. (1998) have shown that EGFRvIII-mediated transformation is accomplished by enhancing cell growth and reducing apoptosis by upregulation of Bcl-X. This upregulation suppressed cisplatin-induced apoptosis in U87MG.ΔEGFR cells as compared with EGFRwt-expressing U87MG cells, presumably through suppression of caspase-3-like proteases.

Treatment of EGFRvIII-positive cell lines with the PI 3-kinase inhibitors wortmannin or LY294002 decreased JNK activity and induced the loss of transformed cell properties (anchorage-independent growth, growth in low-serum medium), which suggests that the transforming activity of EGFRvIII involves the constitutive activation of both PI 3-kinase and JNK activity (Moscatello et al. 1998, Antonyak et al. 1998). Therefore, the growth advantage enjoyed by EGFRvIII cells both in vitro and in vivo is dependent upon the constitutive activity of the tyrosine kinase portion of the receptor and the activation of the JNK pathway, presumably through PI 3-kinase; these associations provide targetable processes for therapeutic intervention.

Cell lines transfected with EGFRvIII are more resistant to paclitaxel-mediated cytotoxicity, and tubulin polymerization induced by paclitaxel is suppressed as compared with that in cells expressing EGFRwt (Montgomery et al. 2000). Differential expression of β-tubulin isotypes has been proposed to modulate paclitaxel
resistance, and EGFRvIII-expressing cells demonstrated increases in class IV β-tubulin mRNA; inhibition of EGFRvIII kinase activity was also found to decrease expression of class IV of β-tubulin by 50% and to partially reverse resistance to paclitaxel (Montgomery et al. 2000). Recently, an interleukin (IL)-3-dependent murine hematopoietic cell line (32D cells) transfected with EGFRvIII was reported to abrogate the IL3-dependent pathway in the absence of ligands (Tang et al. 2000). EGFRvIII expression significantly enhanced tumorigenicity of MCF7 cells in athymic nude mice, which indicated that EGFRvIII may play a pivotal role in human breast cancer progression (Tang et al. 2000).

Anti-EGFRvIII Mabs

Because of the truly tumor-specific nature of EGFRvIII, our group has developed both polyclonal and monoclonal antibodies directed against this mutant form of EGFR (Humphrey et al. 1990, Wikstrand et al. 1995). Several murine Mabs specific to EGFRvIII with high-affinity constants (0.13–2.5 × 10^9 M^−1) have been reported (Wikstrand et al. 1997). These EGFRvIII-specific Mabs have been used to assess both the quantitative and qualitative expression of EGFRvIII in disaggregated cells and tissue samples derived from human tumor biopsies.

The development of Mabs and single-fragment chain constructs specific for the mutant EGFRvIII, including L8A4, Y10, P14, X32, MR1, MR1-1 and 14E1, has been well described (Reist et al. 1995, Wikstrand et al. 1995, 1997, Kuan et al. 1999, 2000, Schmidt et al. 1999, Beers et al. 2000). As EGFRvIII does not bind EGF or TGFα, such ligand-induced pathways are irrelevant; far more significant in terms of targeting approaches, however, is the fate of an anti-EGFRvIII Mab after binding to EGFRvIII. As defined by indirect immunofluorescence and radiolabeled EGFRvIII-specific Mab assays, EGFRvIII-bound receptor-specific Mabs are internalized by HC2 20d2 cells to intracellular vesicles within 5 min (Reist et al. 1995). In a separate quantitative assay for Mab internalization, conventionally radioiodinated Mabs L8A4 and Y10 were rapidly lost from the surface of HC2 20d2 cells; the percentage of trichloroacetic acid-soluble cell culture supernatant counts representing catabolized Mab after cellular processing was shown to be predominant, indicating that the Mab, and probably the Mab-EGFRvIII complex, were rapidly catabolized after internalization.

The production of a human IgG1/mouse chimeric anti-EGFRvIII Mab has been described (Reist et al. 1997a); the binding characteristics of the chimeric Mab have been shown to be similar to those of the murine parent, but comparative tissue distribution studies of the chimeric construct indicate that it is superior to the murine Mab as a targeting agent in vivo. Of great potential is the generation of MR1, and its affinity-matured derivative MR1-1 (Kuan et al. 1999, 2000). MR1 was isolated from a single-chain antibody variable domain (scFv) phage display library developed from the spleen of a mouse immunized with a synthetic peptide representing the EGFRvIII fusion junction and purified EGFRvIII (Lorimer et al. 1996); the immunotoxin formed with this scFv and domains II and III of Pseudomonas exotoxin A exhibited a binding affinity (Kd) of 2.2 × 10^8 M for the EGFRvIII peptide. Combinatorial variation of CDR3 of V_H and V_L followed by phage display was then used to select affinity-matured mutants of the parental scFv (Beers et al. 2000). MR1-1, which differs from the parental MR1 by three amino acid residues (one in V_H CDR3 [F92W] and two in V_L CDR3 [S98P, T99Y]), has a 15-fold greater K_d (1.5 × 10^9 M) for the extracellular domain of EGFRvIII than does MR1 (Kuan et al. 2000).

Current approaches to EGFRvIII-expressing tumor cells for cancer therapy

As summarized above, clinical trials of both murine and chimerized Mabs reactive with EGFRwt have been initiated; despite the lack of apparent systemic toxicity via normal EGFRwt distribution, targeting of a truly tumor-specific marker is still preferable. A variety of approaches, including passive and active approaches to immunotherapy directed to EGFRvIII, are currently being evaluated in experimental model systems.

Targeting with naked Mabs

As demonstrated by Goldstein et al. (1995) and Yang et al. (1999), passive administration of chimerized or fully human EGFRwt-specific Mabs can repress the growth of EGFRwt-positive xenografts and, in the case of E7.6.3, eradicate established tumors without accompanying chemotherapy. To determine if similar effects could be elicited for EGFRvIII, we investigated a panel of anti-EGFRvIII-specific Mabs (Wikstrand et al. 1995). Most effective in in vitro assays was the murine IgG2a Mab Y10, which recognizes both the human EGFRvIII and a murine homologue of this mutation (Sampson et al. 1996). We demonstrated that incubation of unarmed Y10 with EGFRvIII-expressing cells inhibited DNA synthesis and cellular proliferation, which led to cell death. In addition, Y10 was found to mediate cell death of EGFRvIII-positive cells in the presence of complement, as well as with both murine and human cells bearing Fc receptors (Sampson et al. 2000). As a result of these encouraging in vitro data, mice with EGFRvIII-expressing tumors were treated with Mab Y10. In initial experiments intraperitoneal injections of Mab Y10 led to long-term survival in all mice bearing subcutaneous EGFRvIII-expressing tumors. In addition, this
treatment produced a long-lasting and potent anti-tumor immune response to subsequent rechallenge of tumor subcutaneously. Similar therapy with intraperitoneal Mab administration in animals with intracerebral tumors failed to increase survival significantly, presumably because large molecules, such as Mabs, fail to gain access to tumors within the brain because of the blood–brain barrier and related factors such as tumor interstitial pressure. However, a single, direct intratumoral injection of Mab Y10 into mice with invariably lethal EGFRvIII-positive brain tumors produced an average 286% increase in survival, with more than 25% of the animals being cured. These data indicate that the unarmered, IgG₂, EGFRvIII-specific Mab Y10 may be a significant immunotherapeutic agent without further manipulation.

**Targeting with internalizing EGFR–Mab complex**

As previously described for the EGFRwt–C225 Mab complex (Goldstein *et al.* 1995, Waksal 1999), the EGFRvIII–Mab complexes are also rapidly internalized (Reist *et al.* 1995). This property has afforded the opportunity to produce potent toxin–Mab constructs for efficient target cell killing and also has necessitated the development of unique labeling strategies to enhance intracellular retention of isotypically labeled Mabs, fragments and constructs for radioimmunotherapy.

**Immunotoxin**

The Food and Drug Administration recently approved the Mabs rituximab and Herceptin (Genentech, South San Francisco, CA, USA), which are effective and well tolerated, but 50% or more of patients do not respond and are in need of alternative therapy. Because EGFRwt–Mab and EGFRvIII–Mab complexes have been shown to internalize rapidly (Goldstein *et al.* 1995, Reist *et al.* 1995), and a single internalized toxin molecule has the capacity to mediate cell death, the prospect of using specific immunotoxins is very attractive. In patients with superficial bladder carcinoma, the anti-EGFR recombinant toxin TP40 – composed of TGFα (binds to the EGFR) and truncated Pseudomonas exotoxin A – resulted in pathologic improvement in carcinoma in situ when instilled into the bladder (Goldberg *et al.* 1995). EGF itself was fused to DAB₃₈₉ (a diphtheria toxin derivative) for systemic therapy of EGFRwt-positive carcinomas and has resulted in a response in lung cancer (Foss *et al.* 1998). A Phase I/II study is currently being conducted in patients with non-small-cell lung cancer.

Using the scFv 14E1, which recognizes both EGFRwt and EGFRvIII, Schmidt *et al.* (1998, 1999) created an immunotoxin with a truncated form of Pseudomonas exotoxin A (scFv14E1–ETA). ScFv14E1–ETA displayed a cell-killing activity of EGFRvIII-positive cells that was up to 100-fold greater than that of EGFRwt-positive cells; as no difference in binding affinity for the two molecules could be demonstrated, it was postulated that events downstream of the immunotoxin binding were responsible for the enhanced kill. In addition, systemic treatment of mice with the toxin resulted in complete suppression of EGFRvIII-positive metastasis formation.

After the initial demonstration of the cytotoxic efficacy of immunotoxins constructed by conjugating a modified version of Pseudomonas exotoxin A (PE) to the anti-EGFRvIII-specific Mabs L8A4, H10 and Y10 (Lorimer *et al.* 1995), the immunotoxin MR1(scFv)PE38 was prepared (Lorimer *et al.* 1996); MR1 immunotoxin is an extremely potent and specific killing agent of EGFRvIII-positive cells. The *in vivo* therapeutic efficacy of MR1 immunotoxin was evaluated in a dose–response study in an athymic rat model of neoplastic meningitis established with U87MG ΔEGFR cells; three tested doses of MR1 immunotoxin were delivered via an indwelling subarachnoid catheter every other day. As compared with sham treatment with saline or irrelevant Mab, MR1 immunotoxin increased the median survival of tumor-bearing rats from 430 to 657% and produced 6/8, 4/7, or 4/7 long-term survivors, the survival rate being dependent upon MR1 immunotoxin dose (Archer *et al.* 1999a). These results further establish the feasibility of using EGFRvIII as a specific immunotherapeutic target, and the safety and efficacy of immunotoxins directed to EGFRvIII; toxins generated with the affinity-enhanced MR1-1 scFv are currently undergoing evaluation. As dimerization is not required during internalization of Mab-EGFRvIII complexes, EGFRvIII–toxin-mediated treatments may be effective even for targeting cells with relatively low receptor density.

**Radiolabeled antibody**

We have extensively reviewed the development of labeling strategies for anti-EGFRvIII Mabs designed to maximize stability and minimize lysosomal degradation and dehalogenation (Wikstrand *et al.* 1999a). The method of Mab labeling used had a major influence on the selectivity and specificity of Mab distribution *in vivo* (Reist *et al.* 1995, 1997b). *N*-Succinimidyl 5-iodo-3-pyridine carboxylate (SIPC), which binds to the lysine ε-amino groups on Mabs, carries a positive charge on the nitrogen atom of its pyridine ring at lysosomal pH, and has been shown to remain trapped inside lysosomes because of this charge. As compared with Iodogen (Pierce Chemical Co.), SIPC increased intracellular retention of delivered radioactivity up to 65% *in vitro* assays. Paired-SIPC labeled experiments showed that tumor localization indices were at least 10 by 72 h, a degree of specificity three to four times greater than that obtained with labeling by tyramine cellobiose (Reist *et al.* 1997b). In addition, the tumor-to-tissue ratios for liver, spleen, and kidneys were three times greater for SIPC-labeled Mab at
the later time points, indicating faster clearance from organs known to be involved in Mab processing.

Because lysosomes are known to retain positively charged molecules, a polycationic peptide composed of D-amino acids (D-Lys-D-Arg-D-Tyr-D-Arg-D-Arg; D-KRYRR) was designed to enhance cellular retention. In vitro assays with the U87MG.ΔEGFR cell line indicated that internalized activity and total cell-associated activity for the 125I-labeled D-KRYRR-L8A4 conjugate were up to four and five times greater respectively, than that for L8A4 labeled with 125I using Iodogen. Paired-label comparisons in athymic mice with s.c. U87MG.ΔEGFR xenografts demonstrated up to fivefold greater tumor uptake for Mab labeled with D-KRYRR. Another paired-label study, directly comparing L8A4 labeled with radioiodinated D-KRYRR and L-KRYRR, confirmed the role of D-amino acids in enhancing tumor uptake. These results suggest that D-KRYRR is a promising reagent for the radioiodination of internalizing Mabs, such as the anti-EGFRvIII Mab, L8A4 (Foulon et al. 2000). In biodistribution studies using athymic mice bearing s.c. U87MG.ΔEGFR tumor xenografts, animals received intratumoral or intravenous infusions of paired-label [131I]SIPC-MR1(scFv) and [131I]SIPC-anti-Tac(scFv) as a control. When given by the intratumoral route, MR1(scFv) retained a high tumor uptake of 85% and 16% of the injected dose per gram at 1 h and 24 h after administration respectively. Specific/control scFv tumor uptake ratios of more than 20:1 at 24 h demonstrated specific localization of MR1(scFv) (Kuan et al. 1999). In the same biodistribution tumor model, paired-label intratumoral infusions of 131I-MR1-1 scFv and 125I-MR1 scFv resulted in a significantly greater tumor localization by MR1-1 than by MR1, presumably as a result of its higher affinity. This was reflected by an up to 244±77% increase in tumor uptake for MR1-1 compared with that for MR1. This enhanced tumor retention combined with the rapid clearance from blood and high tumor-to-normal-tissue ratios of scFv suggests that the engineered scFv MR1-1 will significantly impact glioma targeting and immunotherapy (Kuan et al. 2000).

Other cytoidal agents

In addition to the antibody-mediated therapeutic approaches described here, targeting of EGFRvIII-expressing cells is being pursued by a variety of other agents, including inhibitors of EGFR kinase and other downstream enzymes. An increase in activated Ras-GTP in EGFRvIII-positive cells was demonstrated by Prigent et al. (1996) and confirmed by Feldkamp et al. (1999). These authors also reported that the farnesyl transferase inhibitor L-744,832, which exerts its effect through inhibition of the Ras signaling pathway, induces greater levels of cytostasis and cytotoxicity in EGFRvIII cells than in EGFRwt cells (Feldkamp et al. 1999). Proposed mechanisms include the induction of apoptosis and cell cycle arrest at both the G1/M and G2/S phase boundaries. A similar preferential effect on inhibition of cell proliferation by the EGFRwt kinase inhibitor tyrophostin AG1478 on EGFRvIII-expressing cells compared with EGFRwt-expressing cells has been reported by Han et al. (1996), and the presumed mechanism of action is as yet undefined, but selective inhibition of autophosphorylation of EGFRvIII as compared with EGFRwt was suggested. O’Rourke et al. (1998) have shown the p185neu ectodomain-derived mutant (carboxyl terminal deletion mutant) forms heterodimers with EGFRvIII proteins and reduces the phosphotyrosine content and kinase activity of EGFRvIII monomers. The authors reported that receptor-based inhibitory strategies exploit the thermodynamic preference for erbB ectodomains to heterodimerize, thereby creating erbB receptor assemblies that are defective in signaling and do not internalize. Pharmaceutic agents that mimic the p185neu ectodomain may therefore have important therapeutic applications in advanced human malignancies expressing erbB receptors, such as EGFRvIII.

Active immunization against EGFRvIII

The ability to induce humoral reactivity to EGFRvIII in various mammalian species by immunization either with a 14mer peptide representing the unique EGFRvIII sequence (Pep-3) or with complete extracellular domain as expressed on EGFRvIII-positive cells (Hills et al. 1995, Wikstrand et al. 1993, 1995) suggested that the induction of CD8+ cytotoxic T cells was possible. Various ways of presenting the unique EGFRvIII epitope in vaccine format are currently being investigated.

Immunogenic peptide

The first demonstration of humoral and CD8+ T lymphocyte-mediated responses induced in mice by immunization with the EGFRvIII epitope 14 mer peptide Pep-3 (LEEKKGNYVVTDHC) coupled to keyhole limpet hemocyanin (KLH) plus complete Freund’s adjuvant was reported by Moscatello et al. (1997). The authors claimed a protective effect in 75% of vaccinated mice (12/16) to challenge tumor cells, and only 81% (13/16) of the control vaccinated mice developed tumors, the results are difficult to interpret. To clarify the effect of vaccination with peptide in preventing EGFRvIII tumor take, we created a murine homologue of the EGFRvIII gene and used this to transfect the B16 melanoma cell line to create a tumor–host syngeneic system in C57Bl/6 mice (Sampson et al. 1996). Animals vaccinated with Pep-3 alone or Pep-3 conjugated to KLH had a statistically significant decrease in the rate of subcutaneous tumor growth and tumor take that was dependent on natural killer (NK) cells and CD8+ cytotoxic T lymphocytes, according to the authors (Sampson et al. 1999). In addition,

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CD8+ T cells derived from mice vaccinated with this specific peptide were able to mediate lysis of tumor cells expressing this antigen in vitro. Significant antibody titers were also demonstrable in the serum isolated from these animals and were predominantly of the IgG1 isotype. Subsequent experiments have demonstrated that passive transfer of serum from previously vaccinated animals also produces an anti-tumor effect and cure in some tumor-bearing animals (Sampson et al. 1999). These data indicate that vaccination with this tumor-specific peptide produces a diverse and potent anti-tumor immune response that is specific and long lasting.

**Conformationally intact immunogenic proteins**

The induction of humoral and cellular immunity by conformationally intact EGFRvIII as expressed on the cell membrane was first investigated by Ashley et al. (1997a), by vaccination of C57Bl/6 mice with the allogeneic pre-B cell line 300.19 alone, or transfected to express EGFRvIII. Demonstration of CD8+ T lymphocytes cytotoxic for H-2a EGFRvIII-positive syngeneic B16–F10 melanoma cells or allogeneic murine astrocytoma 560 cells, but not for H-2a-mismatched EGFRvIII-positive NR6M cells, was reported only in mice receiving 300.19–EGFRvIII. Significant NK activity was also reported. Only mice vaccinated with 300.19–EGFRvIII cells exhibited longer latency after challenge with B16–F10 tumor, with 50% long-term survival. This protective effect was dependent upon CD8+ and CD4+ cells, but not NK cells, as established by depletion experiments. Whether the antigen-presenting properties of the 300.19 cell line are required for this effect can now be investigated by direct immunization with the biologically active, apparently conformationally appropriate extracellular domain of the EGFRvIII recently produced by Kuan et al. (2000).

An alternative form for presentation of the EGFRvIII epitope is by anti-idiotype immunization (Wikstrand et al. 1999b). A Mab specific for antigen (Ab1) can be used as immunogen to elicit Ab2, which binds to Ab1. A subset of Ab2 Mabs capable of specifically inhibiting the binding of Ab1 to antigen (Ab2β) have an antigen-binding site that conformationally mimics the original antigen epitope and can subsequently induce the formation of a tertiary response (both Ab3 and T-cell 3) to Ab2β. The subset of Ab3 Mabs that recognizes the binding site of Ab2β is designated Ab1’; and the Ab1’ Mabs differ from Ab1 in that, having been induced by the conformational Ab2β binding site, they have binding sites that are antigen-specific, but conformationally novel. Important for vaccine production is the fact that Ab2β immunization can induce both humoral and cellular immunity, which antigen immunization, especially with lipids and carbohydrates, frequently does not (Bowen & Bona 1991). We recently reported the production of rat anti-Ab1 Mab Y10 anti-idiotype antibodies (Wikstrand et al. 1999a,b); only one of six specific Ab2β Mabs (Mab 2C7) was capable of inducing protection from challenge with syngeneic B16–F10 EGFRvIII-positive cells after an intensive regimen of Mab 2C7–KLH + CFA vaccination; six of 20 mice so immunized did not develop tumor, and regression of emerging tumors was seen in an additional three mice. The remaining 11 mice developed tumor with a latency and growth rate indistinguishable from control (PBS) and other Ab2β-immunized mice. The use of anti-idiotype vaccines for sensitization to EGFRvIII in patients may be a valuable adjunct to other active immunotherapy procedures, especially if the Pep-3-induced response in humans should prove to be primarily humoral. The ability of anti-idiotype Mab 2C7 to induce anti-EGFRvIII in non-human primates is being investigated to determine its potential clinical utility.

**Dendritic cell immunization**

Another format for the presentation of EGFRvIII antigen is via the use of antigen-presenting dendritic cells (DCs) capable of initiating potent primary antitumor immune responses in vivo (Fernandez et al. 1998). As summarized by Wikstrand et al. (1999a), peptide-pulsed autologous DCs have been demonstrated to induce detectable cell-mediated immunity in Phase I trials in melanoma, prostate carcinoma and renal cell cancer patients. In preliminary studies, we have demonstrated that DC-based vaccines utilizing B16–F10 cell extracts or total RNA induce specific cytotoxic T lymphocytes and prolonged survival in mice bearing intracerebral B16–F10 tumors induced either before or after administration of DC vaccine (Ashley et al. 1997b). We have demonstrated the capacity to generate DC from the peripheral blood of glioma patients (Archer et al. 1999b), thereby establishing this as a feasible approach for Phase I study once an optimal pulsing antigen form is determined in vitro. An intriguing combination of techniques would be to combine the DC and anti-idiotype approaches: Ab1’-pulsed DC could be used to maximize the induction of cell-mediated immunity; this approach has been successful for the production of anti-viral antigen antibodies and memory cells in mice (Brait et al. 1991).

**Summary and conclusions**

The major factors that influence antibody-targeted therapy for cancer treatment, including glioma therapy, are specificity, affinity, tumor penetration, toxicity and immunogenicity. Above all, the search for a truly tumor-specific surface antigen to be targeted by antibodies, either unarmed or armed with radioisotopes or toxins for immunotherapy, is important. Optimal antigens for targeting should exhibit high (>1 × 105 sites/cell) expression on the surface of tumor cells, with little or no expression on normal
cells. EGFRvIII is an excellent example of a normal molecule that has undergone genetic rearrangement to provide a truly tumor-specific target. This unique tumor antigen is internalizable after binding with specific Mabs or antibody fragments. Immunotargeting approaches that have been shown to be efficacious with acceptable toxicity in animals include the use of: (1) immunotoxins, (2) radiolabeled Mabs and fragments against EGFRvIII, (3) an ‘unarmed antibody,’ and (4) vaccine approaches.

The armed antibody or antibody fragment approaches found to be effective have been with both radiolabeled anti-EGFRvIII Mabs and immunotoxins. New radiolabeling methods have been developed to prevent dehalogenation of internalized anti-EGFRvIII Mabs. These methods include targeting of an anti-EGFRvIII Mab in tumor xenografts with radiolabeled N-succinimidyl 5-iodo-3–pyridinecarboxylate (Reist et al. 1997a) and radiodiination via α-amino acid peptide (Foulon et al. 2000). An affinity-matured, single-fragment chain–Pseudomonas exotoxin conjugate has been prepared, and this immunotoxin is now being produced under GMP conditions, and a clinical trial in patients with glioma, with administration by intracranial microdiffusion, will be initiated shortly.

The unarmed antibody approach described by Sampson et al. (2000) with Mab Y10 against EGFRvIII is very similar to the successful use of Herceptin (Genentech). Unarmed antibody is effective against both subcutaneous and intracranial tumors. The mechanism is most probably both a direct anti-proliferative effect with the induction of apoptosis and an indirect effect through the mobilization of antibody-mediated immune effector functions, such as complement and antibody-dependent cell-mediated cytotoxicity.

Vaccine approaches both to prevent tumor take or recurrence and to treat actively growing tumors have been successful in animals. The approaches being developed for Phase I clinical trial include intradermal administration of KLH-conjugated Pep-3 and pulsing of human DCs with Pep-3 and other EGFRvIII antigenic formats, followed by re-administration of the pulsed DCs.

Initial trials will be carried out in patients with malignant glioma who show high levels of expression of EGFRvIII. After those initial clinical trials have been completed, similar Phase I trials will be carried out in patients with other major cancers that may have a sufficient level of EGFRvIII to contemplate using it as a therapeutic target. These include prostate, breast, ovarian and non-small-cell lung carcinoma.

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