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Review

The class III variant of the epidermal growth factor receptor (EGFRvIII): characterization and utilization as an immunotherapeutic target

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Any immunotherapeutic approach to cancer cell eradication is based upon the specific recognition of neoplastic cells and the sparing of surrounding normal tissue; perhaps nowhere is this distinction more important than within the central nervous system, due to the diffuse infiltrative nature of primary glial tumor cell growth. Whether ultimate effect moieties are immunoglobulins, fragments and/or their constructs with drugs, toxins, radionuclides, or immune cells, the specificity of effector : cell surface marker is crucial. This review describes the identification, immunologic characterization, and biologic behavior of a transmembrane tumor-specific altered growth factor receptor molecule which may well serve as a mediator of multiple immunotherapeutic approaches: the class III variant of the epidermal growth factor receptor, EGFRvIII.

Keywords: tumor-specific antigen; immunotherapy; growth factor receptors; internalization; glioblastoma

Introduction

Despite molecular biological advances in understanding human cancers, translation into therapy has been less forthcoming; targeting of neoplastic cells still requires that tumor-specific markers, preferably those on the cell surface, be identified. In addition to the capacity to localize putative effectors, a tumor-cell distinctive molecule which mediates biologic functions central to cell growth advantage, metabolism, adhesiveness/motility, or drug resistance, would have great therapeutic targeting potential. As growth factors and their receptors have a central role in regulating developmental and neoplastic processes, the investigation of the expression and action of various growth factor receptors in human neoplasia has been extensive. Following the identification of the epidermal growth factor receptor (EGFR) as the cellular homologue of v-*erb*B, several investigators have described the overexpression of EGFR at both the genotypic and phenotypic levels in many human tumors (breast, Arteaga *et al*, 1994; ovarian, Gullick *et al*, 1986; Owens *et al*, 1992; esophageal, Hollstein *et al*, 1988; non-small cell lung carcinomas, Garcia de Palazzo, 1993; Rusch *et al*, 1993), but most notably in human gliomas (Agosti *et al*, 1992; Bigner *et al*, 1990b; Humphrey *et al*, 1988; Libermann *et al*, 1985; Schlegel *et al*, 1994; Schwechheimer *et al*, 1995; Torp *et al*, 1991). As the normal EGFR is ubiquitous in normal human tissue, most notably liver, its value as a cytotoxic or cytostatic targeting agent is compromised. The observation of *EGFR* gene rearrangement as a frequent accompaniment to *EGFR* gene amplification, most notably in gliomas, has led to the identification of tumor-associated EGFR variant forms which provide the tumor target specifically desired (Batra *et al*, 1994).

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Description of EGFR and deletion variant families

EGFR and gliomas

Gene amplification, related to increasing grade of glioma malignancy, has been found to occur in approximately 50% of all glioblastoma multiforme (GBM) cases (Bigner *et al*, 1987; Wikstrand *et al*,

1998). Although amplification of N-*myc* and *gli* (2–4% overall) has been reported by different groups (Fuller *et al*, 1990; Kinzler *et al*, 1987; Wong *et al*, 1987), amplification of these genes and c-*myc* or K-*ras* are considered sporadic as compared to the amplification of c-*erbβ1*, or the epidermal growth factor receptor (EGFR) gene. The *EGFR* gene, 110 kb in size, 26 exons in organization, is localized to chromosome arm 7p11-13 (Merlino *et al*, 1985). Beginning with the initial description of *EGFR* gene amplification by Libermann *et al* (1985), subsequent studies have confirmed that approximately 37–58% of GBMs, but only isolated anaplastic astrocytomas, amplify the *EGFR* gene (Agosti *et al*, 1992; Ekstrand *et al*, 1992; Schlegel *et al*, 1994a,b; Torp *et al*, 1991; Wong *et al*, 1987). *EGFR* gene amplification is associated with high levels of EGFR mRNA or protein (ibid; Humphrey *et al*, 1988; 1990; Yamazaki *et al*, 1990; Sugawa *et al*, 1990; Ekstrand *et al*, 1994; Nishikawa *et al*, 1994), and in most cases, gene amplification is accompanied by gene rearrangement, as detected by the loss or altered size of bands in Southern blots. The rearrangements of the *EGFR* most commonly seen in gliomas are usually extensive deletions in the coding sequence of the gene.

As summarized recently (Bigner *et al*, 1998), essentially seven genomic variants have been detected to date in multiple biopsies: class I mutants, which essentially lack the extracellular domain of the encoded protein, resembling the v-*erb*B gene product; class II mutants, which contain an in-frame deletion of 83 aa in the extracellular domain outside the ligand site; class IV and V mutants, which carry deletions in the cytoplasmic domain, and class VI and VII mutants, (class IV and V respectively), coexisting with one of the defined extracellular domain deletions. Class III mutants are the most frequently detected genomic variant. As reported by several groups (Bigner *et al*, 1990b; Ekstrand *et al*, 1991, 1992; Humphrey *et al*, 1990; Schlegel *et al*, 1994a; Schwechheimer *et al*, 1995; Yamazaki *et al*, 1990), of the 40–50% of GBM which exhibit *EGFR* gene amplification, >50% express the class III deletion, with or without an additional intracytoplasmic region deletion.

Characterization of EGFRvIII

Class III mutants contain a deletion of exons 2–7 of the gene, resulting in an in-frame deletion of 801 base pairs of the coding sequence and the generation of a novel glycine residue at the fusion junction (Humphrey *et al*, 1988; Yamazaki *et al*, 1990; Figure 1a). This genomic deletion removes NH_2-terminal amino acid residues six through 273 from the extracellular domain of the intact wild type EGFR (170 kD), resulting in EGFRvIII molecule with an M_r of approximately 145 kD that has a unique primary sequence represented by an inserted glycine residue at position 6 between amino acid residues 5 and 274.

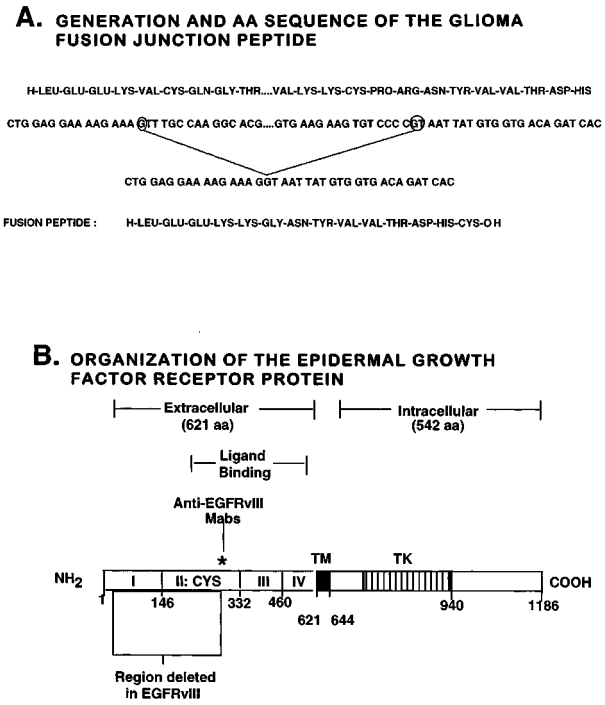

Figure 1 The EGFRvIII molecule: generation and organization. (**A**) Generation and amino acid sequence of the glioma fusion junction peptide. An 801 base pair in-frame EGFR gene deletion (upper row) results in the fusion of normally distant EGFR gene and protein sequences (lower rows). A glycine residue is created at the fusion point. (**B**) Schematic diagram of the wild type EGFR: NH_2, amino terminus; COOH, carboxy terminus; TM: transmembrane segment; TK: tyrosine kinase domain; aa residue numbers lie below the structure. (Compiled from Batra *et al*, 1994; Ekstrand *et al*, 1992; Humphrey *et al*, 1988).

This unique primary sequence and probable conformational alteration suggested the potential of an extracellularly located immunogenic moiety which might induce the production of antibodies specific for EGFRvIII. Following the demonstration by Gullick *et al* (1985) that high titer polyvalent and monoclonal antibodies (Mabs) specific for EGFR were produced following the immunization of rabbits and mice with short synthetic peptides derived from hydrophilic sequences of the proteins, we initiated the production of polyvalent serum to EGFRvIII by immunization with a 14 mer peptide (Pep-3) corresponding to amino terminus residues 1–5, the fusion junction glycine, and residues 274–280, with a terminal cysteine for KLH coupling. Rabbits and goats produced high titer specific activity for EGFRvIII following immunization with peptide alone (Humphrey *et al*, 1990; Wikstrand *et al*, 1993). The production of murine Mabs with similar affinity and specificity, however, required both presentation with the synthetic peptide-KLH complex and intact EGFRvIII as

expressed on cell surfaces (Wikstrand *et al*, 1995). A panel of specific anti-EGFRvIII Mabs has been assembled: L8A4, H10, P14 and X32, all of the IgG_1 isotype, and Y10, of the IgG_{2a} isotype (Wiksrand *et al*, 1997). These Mabs identify the EGFRvIII on the cell surface with relatively high affinity (K_A range, $0.13-2.5\times10^9$ M^{-1}) by live cell Scatchard analysis; specificity was determined with EGFR and EGFRvIII expressing cells and cell lysates by radioimmunoassay, ELISA, Western Blot, analytical flow cytometry, autophosphorylation and immunohistochemistry (Wikstrand *et al*, 1995, 1997). Recently, we have expanded this anti-EGFRvIII panel through the addition of a human IgG_2/mouse chimeric L8A4 molecule (Reist *et al*, 1997b). Of great potential is the generation of MR1, which was isolated from a single chain antibody variable domain (scFv) phage display library developed from the spleen of a mouse immunized with Pep-3 and purified EGFRvIII (Lorimer *et al*, 1996); the immunotoxin formed with this scFv and domains II and II of Pseudomonas exotoxin A exhibited a K_d or 11 nM for cell surface EGFRvIII. The activity of this and related constructs will be discussed below.

Population distribution and cellular localization of the EGFRvIII

The generation first, of Pep-3 affinity-purified polyvalent sera, and subsequently, specific Mabs, enabled us to analyze the normal and neoplastic human tissue distribution of EGFRvIII. As summarized previously (Garcia de Palazzo *et al*, 1993; Wikstrand *et al*, 1995), no normal tissues examined, including 2–5 cases per tissue of colon, kidney, liver, ovary, endometrium, placenta, testes, lung, peripheral nerve, lymph node, bone marrow, normal breast, skin, cerebellum or cerebral cortex, were found to express EGFRvIII. Although four cases of human spleen appeared positive in B cell regions, subsequent Western blot analysis of splenic lysates, and FACs analysis of dispersed splenic and bone marrow samples (total of two spleens and nine bone marrow samples) failed to reveal binding of the tested Mabs L8 and Y10. Analysis of frozen tumor tissue sections with Mabs L8A4 and Y10 revealed EGFRvIII protein positivity in 27% (3/11) infiltrating ductal and intraductal breast carcinoma cases, and an overall incidence of 52% (16/31) in gliomas (1/7 anaplastic astrocytomas, 2/3 gliosarcomas and 13/21 GBM; Wikstrand *et al*, 1995). In a subsequent series, Wikstrand *et al* (1997) reported that 50% of glioma biopsies (1/2 anaplastic astrocytomas, 7/12 GBM and 2/6 oligodendrogliomas) expressed EGFRvIII as detected by Mab L8A4 in either frozen tissue or microwave antigen-retrieved, formalin-fixed tissue. As shown in Figure 2, Mab L8A4 staining of frozen tissue from GBM TB 789 exhibits strong staining outlining both the tumor cell membranes and cytoplasm, with sparing of the nuclei; blood vessels were also entirely negative. Similar analysis with affinity-purified polyvalent rabbit-anti-EGFRvIII serum revealed that 16% (5/32) non-small cell lung carcinomas expressed EGFRvIII (Garcia de Palazzo *et al*, 1993). Using a similarly prepared and purified polyvalent rabbit anti-EGFRvIII serum in Western blot analyses of tumor tissue lysates, Moscatello *et al* (1995) reported that 57% (26/46) high grade and 86% (6/7) low grade glioma, 66% (4/6) pediatric gliomas, 86% (6/7) medulloblastomas, 78% (21/27) breast carcinomas and 73% (24/32) ovarian carcinomas expressed EGFRvIII. The high percentage figures reported here, however, include tumors which expressed only a 100 kD band also detected by the anti-EGFRvIII probe; although the authors postulate that this may be an intracellularly expressed, non-glycosylated form, the relatively lower incidence of the verifiable 140 kD form (e.g. 20.6% in ovarian tumors) is the more supportable observation.

At the cellular level, examination of 19 biopsy samples of human gliomas by indirect analytical and quantitative flow cytometry with the anti-EGFRvIII specific Mab L8A4 revealed that minimum estimates of the proportion of positive tumor cells in the 9/19 EGFRvIII-positive tumors ranged from 37–86%; in 4/5 cases in which quantitation of the EGFRvIII density/cell was performed, values of $2.7-6.8\times10^5$ EGFRvIII receptors/cell were obtained, levels consistent with successful *in vivo* immunotargeting (Wikstrand *et al*, 1997). This study also examined the predominant cell surface localization of EGFRvIII by a series of confocal microscopy experiments using indirect immunofluorescence with Mab L8A4 *versus* various cell populations, either endogenously expressing, or transfected to express, EGFRvIII. Following treatment with either formalin alone (membrane staining), formalin+Saponin (membrane and cytoplasmic staining) or formalin+Triton-X-100 (intranuclear staining), both EGFRvIII-transfected cells and biopsy-derived cells exhibited pronounced cell surface membrane staining, with small but detectable amounts in a predominantly perinuclear array

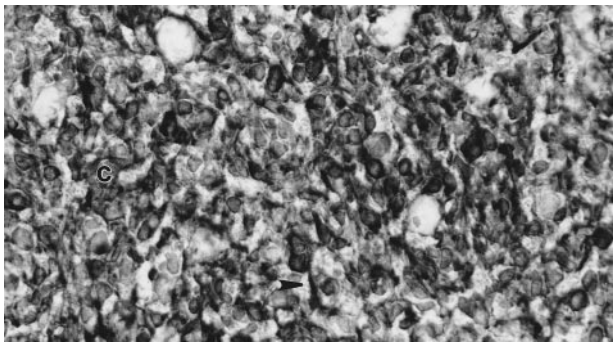

Figure 2 Immunohistochemical analysis of frozen tissue from human glioma case TB 789. Mab L8A4, 5 μg/ml; 400×. Note membrane (→) and cytoplasmic (**c**) staining.

in the cytoplasm. No cell population exhibited intranuclear staining for EGFRvIII. This pattern of predominant cell membrane expression and punctate perinuclear accumulation, and partition of approximately 75–86% in the membrane and the remainder in the cytoplasm, is quite similar to that described for wild type EGFR in this (Wikstrand *et al*, 1997) and other studies (Suarez-Quian and Byers, 1993; Herbst *et al*, 1994) and in the analysis of the EGFRvIII-transfected human glioma cell line U87MG.ΔEGFR as reported by Huang *et al* (1997).

Behavior of the EGFRvIII

Ligand binding, autophosphorylation, receptor dimerization and signaling

The ligand binding domain (domain 3), is presumably intact in EGFRvIII (Woltjer *et al*, 1992); however, the measurement of EGF or TGF-α binding capacity by EGFRvIII in intact cells is complicated by the usual coexpression of wild type EGFR (Moscatello *et al*, 1996; Schlegel *et al*, 1994a; Wong *et al*, 1992). True measurement of EGFRvIII ligand binding then, has only been possible in purposefully transfected cell lines lacking wild type EGFR (Batra *et al*, 1995; Moscatello *et al*, 1996). Batra *et al* (1995) have reported the transfection of the non-EGFR-expressing NIH3T3 cell line NR6 with cDNA for either EGFR (NR6W) or EGFRvIII (NR6M), resulting in stable cell lines expressing solely the 170 kD EGFR and 145 kD EGFRvIII proteins, respectively. Similarly, Moscatello *et al* (1996) reported the transfection of the same NIH3T3 cell line, to produce the CO12 20 c2 (EGFR expressing) and HC2 20 d2 (EGFRvIII expressing) cell lines. Both of these studies performed Scatchard analysis with ^{125}I-EGF; while HC2 20 d2 failed to bind EGF in both studies, the NR6M cell line was found to bind quite low (3–4 times over background), but consistent levels of EGF (Batra *et al*, 1995). The human glioma cell line U87MG, which expressed approximately 2×10^5 EGFR/cell (Wikstrand *et al*, 1997) was transfected with a constructed EGFRvIII cDNA yielding the cell line U87MG.ΔEGFR (Nishikawa *et al*, 1994), which expressed approximately 4×10^5 EGFRvIII/cell (Wikstrand *et al*, 1997). In Mab-mediated studies of the distribution and localization of EGFRvIII in U87MG.ΔEGFR following exposure to EGF, the authors observed that EGF had no effect upon the distribution of the EGFRvIII, as it did upon the EGFR receptor in EGFR-supertransfected cells or parent, EGFR-expressing U87MG cells, suggesting that binding of EGF to the EGFRvIII did not occur (Huang *et al*, 1997). The consensus, then, is that the class III deletion of exons 2–7 virtually abolishes all ligand binding by the EGFRvIII.

As summarized by several authors (Chu *et al*, 1997; Huang *et al*, 1997; Moscatello *et al*, 1996) following the binding of ligand to the intact, wild type EGFR, receptor dimerization, kinase activation, and autophosphorylation occur, which trigger signal transduction, receptor internalization and down-regulation. Receptor tyrosine phosphorylation regulates a series of interactions with intracellular signaling proteins, particularly SH2-domain-containing proteins such as the Shc adapter protein, STAT transcription factors and phospholipase C_γ, which are then themselves phosphorylated (Chu *et al*, 1997; Moscatello *et al*, 1996). A series of transcriptional factor activation occurs through the involvement of mitogen-activated protein kinase (MAPK) or the Jak-STAT system (Carter and Kung, 1994). Concomitantly, EGFR autophosphorylation results in a conformational change triggering internalization and lysosomal targeting, resulting in receptor down-regulation (Cadena *et al*, 1994). As receptors lacking internalization sequences have been associated with cell transformation (Chen *et al*, 1989), it has been postulated that constitutive receptor activation and/or impairment of down-regulating systems at any level can result in unregulated growth stimulation and oncogenesis (Chu *et al*, 1997).

As reported by different groups, the EGFRvIII as expressed in the cell lines NR6M, DH-EΔ801P, HC2 20 d2 and U87MG.ΔEGFR (Chu *et al*, 1997; Hills *et al*, 1995; Moscatello *et al*, 1996; Nishikawa *et al*, 1994; Prigent *et al*, 1996; Huang *et al*, 1997) is constitutively phosphorylated. In the NR6M study, which included denositometric analysis, the baseline phosphorylation of EGFRvIII, independent of TGF-α stimulation, was approximately 10 times lower than that of TGF-α stimulated EGFR in control NR6W (wild type EGFR-expressing cells; Chu *et al*, 1997). Although the results presented with U87MG and U87MG.ΔEGFR were not quantitated, the authors demonstrated that less than or equal amounts of phosphorylation are evident constitutively in U87MG. ΔEGFR cells as compared to wild type EGFR-expressing U87MG cells; as they claim a 4–5-fold excess of EGFRvIII protein as compared to EGFR in this analysis, it is supportable that the amount of the phosphorylation/protein is 4–5-fold less than that of stimulated wild type EGFR (Nishikawa *et al*, 1994). Further support of this observation is provided by the comparison of DH-EΔ801P (EGFRvIII-expressing NIH3T3 transfected cell line) as reported by Hills *et al* (1995) who concluded that 'an equivalent phosphotyrosine signal [by DH-EΔ801P cells] is, however, observed only when the truncated receptor protein is considerably overloaded with respect to that of the full-length EGFR'.

Although Moscatello *et al* (1996) reported that the EGFRvIII of HC 20 d2 cells, when treated with the cross-linker EDAC (1-ethyl-3-[3-(dimethylamino)-propyl] carbodiimide), exhibited readily detectable ligand-independent dimerization which was not increased by exposure to EGF, Chu *et al* (1997) were

unable to repeat this observation with EDAC or the crosslinker BS^3 (bis[sulphosuccinimidyl]subtrate), which differs from EDAC in terms of amino acid targets, chemical mechanism, spacer distance and geometry. Under no set of conditions (different cross-linkers, presence of ligands, assay temperature of 37°C), could these authors demonstrate dimerization of EGFRvIII, whereas EGFR dimerization was readily apparent. Huang *et al* (1997) reported a very small ($<5\%$) extent of dimerization of EGFRvIII in U87MG.ΔEGFR cells which was unaffected by the addition of ligand; as these cells also express approximately 2×10^5 EGFR per cell (Wikstrand *et al*, 1997), the authors concluded that EGFRvIII exhibits neither strong homo- nor heterodimerization.

Although not dimerized, EGFRvIII is constitutively active in its association with intermediate signaling molecules such as Shc and Grb2 (Chu *et al*, 1997; Moscatello *et al*, 1996). These studies also demonstrated that MAPK or PLC-γ tyrosine phosphorylation did not occur in EGFRvIII-expressing cells, suggesting that expression of the mutant receptor results not simply in a constitutively activated receptor, but in down-regulation of the ras-MAP kinase pathway, and alteration of the spectrum of signaling cascades utilized. Prigent *et al* (1996) also reported that U87MG.ΔEGFR cells exhibited phosphorylated Shc proteins and associated Grb2. As some of the endogenous wild type EGFR-expressing, EGFRvIII variants with mutated phosphorylation sites examined in this study required EGF to express phosphorylated Shc, the interactions in these dual wild type and variant-expressing cells are not clear. In support of this observation, however, Chu *et al* (1997) demonstrated in the EGFR-non-expressing, EGFRvIII-expressing cell line NR6M, that Shc immunoprecipitated from these cells has a higher phosphotyrosine content than Shc immunoprecipitated from ligand-stimulated EGFR-expressing NR6W cells. The study of Prigent *et al* (1996) also demonstrated an increase in activated Ras-GTP in U87MG.ΔEGFr cells as compared to U87MG cells, and as anit-Ras antibodies inhibited DNA synthesis in the former cells, concluded that the enhanced growth of U87MG.ΔEGFR cells is dependent upon Ras activity. As significant levels of Ras activation have not been detected in studies of cells expressing only EGFRvIII (Chu *et al*, 1997; Moscatello *et al*, 1996), the role of Ras in downstream signaling by EGFRvIII remains unresolved. More problematic are the conflicting reports of MAP kinase activity. Perhaps consistent with its involvement in the Ras pathway and its central role in transformation by ligand-stimulated wild type EGFR, MAP kinase was not found to be activated in NR6M cells (Chu *et al*, 1997). Although Montgomery *et al* (1995) initially reported comparatively low levels of MAP kinase activation by EGFRvIII in HC2 20 d2 cells, a subsequent paper by this same group (Moscatello *et al*, 1996) concluded that while some MAP kinase may be apparently active, there was no definite evidence for enhanced activation of either GTP-bound Ras of MAP kinase; in fact selective down-regulation of this pathway was suggested.

Transforming potential

Early studies *in vitro* of the effects of transfection of NIH3T3 cells with EGFRvIII cDNA demonstrated transformation and *in vitro* growth advantage in focus-forming assays in the absence of ligand, as opposed to wild type transfected cells (Yamazaki *et al*, 1990). Similarly, Batra *et al* (1995) reported a shorter population doubling time for EGFRvIII cDNA transfected NR6M cells at both high and low serum concentrations as compared to wild type expressing (NR6W) or control untransfected cells (NR6). In addition, this study investigated the classic parameter of transformation, anchorage-independent growth in soft agar; at a dose of 2.5×10^3 cells and low (2%) serum concentration, NR6M demonstrated an approximately fourfold increase in clonigenic potential over NR6W; NR6 cells formed no colonies. Similar results with the HC 20 d2 cell line were reported by Moscatello *et al* (1996).

Such purposefully transfected cell lines have been shown to be tumorigenic in athymic mice without exogenous EGF or TGF-α (Batra *et al*, 1995; Hills *et al*, 1995; Moscatello *et al*, 1996). For the transfected cell line NR6M, doses of 10^4 cells were sufficient for tumor induction, a dose 3 $\log_{10}$ lower than that required by NR6W cells (Batra *et al*, 1995). Nishikawa *et al* (1994) also demonstrated the tumorigenic potential and shorter latency of U87MG.ΔEGFR cells in both subcutaneous (s.c.) and intracerebral (i.c.) 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. Parallel experiments demonstrated that ligand-dependent EGFR transfected U87MG cells did not show enhanced tumorigenic activity in either sc or ic models, suggesting that the tumorigenic effect of EGFRvIII is ligand-independent and mediated through phosphorylation of its carboxyl-terminal tyrosine residues (Huang *et al*, 1997; Chu *et al*, 1997).

Internalization and processing

It has been well established that processing of the EGF-EGFR complex is characterized by endocytosis via clathrin-coated pits, and that internalization via this mechanism leads to delivery to lysosomes and subsequent degradation of EGFR (Willingham *et al*, 1981; Willingham and Pastan, 1982; Beguinot *et al*,

1984). As the EGFRvIII does not bind EGF or TGF-α, 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. Reist *et al* (1995) demonstrated that after binding to EGFRvIII, receptor-specific Mabs were internalized as defined by indirect immunofluorescence and radiolabeled EGFRvIII-specific Mab assays. HC 20 d2 cells were reacted with the anti-EGFRvIII Mab L8A4, followed by rhodamine-conjugated anti-murine IgG in the presence or absence of the permeabilizing agent saponin. Fluorescence patterns shown in Figure 3a represent both cell surface and internal staining with Mab. Cells in Figure 3b were subjected to blocking of mouse IgG determinants with Fab preparations of goat anti-mouse Ig, thus blocking surface bound L8A4; cells were then treated with rhodamine-conjugated anti-murine IgG in the presence of saponin to reveal only internalized L8A4. As shown by the arrows in Figure 3b, isolated intracellular vesicles containing antibody are evident within 5 min.

In a separate quantitative assay for Mab internalization, conventionally radioiodinated Mabs L8A4 and Y10 were rapidly lost from the surface of HC 20 d2 cells; as shown in Table 1, which presents the data for L8A4; this loss was accompanied by an increase in counts in the cell culture supernatant. The percentage of cell culture supernatant counts representing catabolized Mab following cellular processing (TCA soluble), was shown to be the majority of the supernatant counts, suggesting that the Mab, and probably the Mab-EGFRvIII complex, was rapidly catabolized after internalization. The high degree of Mab degradation in these studies suggests that the internalized Mab-EGFRvIII complex also may be routed to the lysosomal pathway (Reist *et al*, 1995).

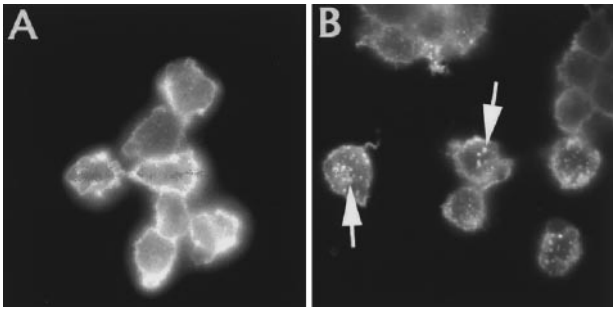

Figure 3 Immunofluorescence microscopy to detect anti-EGFRvIII Mab internalization. (**A**) Cells were incubated with Mab L8A4 at 4°C and then warmed to 37°C for 5 min. Cells in (**B**) were subjected to blocking of surface-associated IgG prior to staining with anti-murine IgG. Note Mab localized to vesicles in the perinuclear Golgi region (→). 315×.

Table 1 Cellular internalization and processing of anti-EGFRvIII Mab L8A4[a]

	Percentage of radioactive counts		
Time (h)	*Cell surface*	*Intracellular*	*Culture supernatant*
1	60±2	33±3	7±2
2	56±0	30±1	14±0
4	44±10	35±10	21±2
8	40±2	18±2	42±2
20	15±3	14±1	71±3

[a]Internalization by EGFRvIII expressing cells of Mab L8A4 labeled with ^{125}I using Iodogen.

Targeting of the EGFRvIII

Classical antibody targeting

The development of Mabs specific for the mutant EGFRvIII has been well described (Hills *et al*, 1995; Wikstrand *et al*, 1995, 1997; Reist *et al*, 1995, 1997b). These Mabs have been demonstrated to be specific for cells expressing EGFRvIII, but unreactive with cells expressing the wild type EGFR. *In vivo* targeting studies performed in nude mice bearing EGFRvIII positive tumor xenografts have indicated that the Mabs localize selectively and specifically to tumor tissue. Athymic mice bearing HC 20 d2 xenografts were injected with 2.5 μg of either L8A4 or H10 radiolabeled via the tyramine cellobiose (TCB) methods; as shown in Figure 4, the percent injected dose per gram (%ID/g) of the EGFRvIII specific Mabs in tumor peaked at 48 h, with maximum uptake of approximately 30% ID/g tumor; levels in excess of 15% ID/g were maintained through 7 days. Paired-label studies using L8A4, H10 and Mab P3X63Ag8, a nonspecific IgG_1 isotype matched control, demonstrated the specificity of tumor uptake; localization indices for both

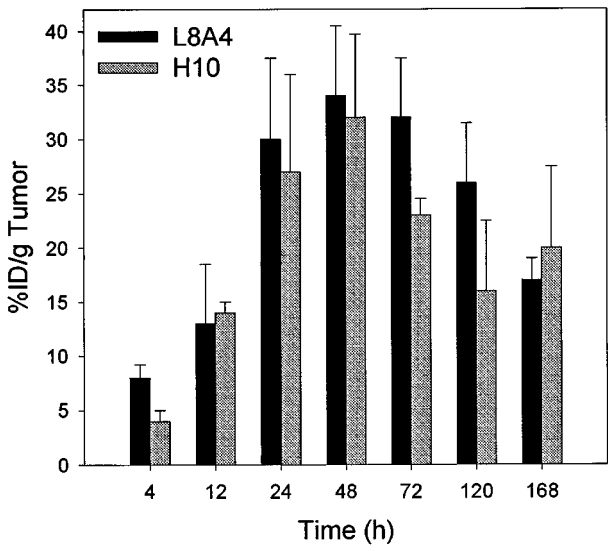

Figure 4 The percent injected dose per gram of EGFRvIII expressing tumor xenografts from mice given injections of ^{131}I-TCB-labeled anti-EGFRvIII Mab L8A4 or H10 (Reist *et al*, 1995).

L8A4 and H10 of 3–12 and good tumor-to-normal tissue ratios were obtained. The method of Mab labeling used had a major influence on the selectivity and specificity of Mab distribution *in vivo* (Reist *et al*, 1995, 1997a). Although the residualizing label TCB did enhance cellular retention of radiolabel as opposed to conventional lodogen labeling (Reist *et al*, 1995), cross-linking of TCB labeled Mab as well as non-specific uptake by the liver has been observed (ibid). Therefore, a second strategy involving the use of a novel radioiodination method which involves the reaction of N-succinimidyl 5-iodo-3 pyridine carboxylate (SIPC) with lysine ε-amino groups on Mabs has been investigated (Reist *et al*, 1996). SIPC carries a positive charge on the nitrogen atom of its pyridine ring at lysosomal pH and has been shown to remain trapped inside lysosomes due to this charge. As compared to Iodogen, or the non-charged N-succinimidyl 3-iodobenzoate (SIB) agent, SIPC increased intracellular retention of delivered radioactivity up to 65% in *in vitro* assays. In *in vivo* experiments in mice bearing EGFRvIII positive xenografts, comparison of SIPC- and TCB-mediated labeling of L8A4 revealed similar %ID/g in tumor for both methods over time (peak of 33–37%); in addition, paired-SIPC labeled experiments showed that tumor localization indices were $\geqslant$10 by 72 h, a degree of specificity 3–4 times higher than that obtained with labeling by TCB. In addition, the tumor-to-tissue ratios for liver, spleen, and kidneys were three times higher for SIPC-labeled Mab at the later time points, indicating faster clearance from organs known to be involved in Mab processing. Recently, the production of a human/mouse chimeric anti-EGFRvIII Mab was described (Reist *et al*, 1997b); the binding characteristics of the chimeric Mab have been shown to be similar to that of the murine parent, but comparative tissue distribution studies of the chimeric construct indicate that it may be a more superior targeting agent than the murine Mab *in vivo*.

As the EGFRvIII-Mab complex has been shown to internalize rapidly, the prospect of immunotargeting with an anti-EGFRvIII Mab-immunotoxin complex was investigated (Lorimer *et al*, 1995). Immunotoxins were constructed by conjugating a modified version of *Pseudomonas* endotoxin A (PE) to anti-EGFRvIII specific Mabs L8A4, H10 and Y10; these constructs were tested *in vitro* on NR6M cells, which express, on average, $5-8\times10^5$ EGFRvIII/cell (Wikstrand *et al*, 1997). All three immunotoxins specifically targeted EGFRvIII, as shown by inhibition of cytotoxicity by respective free antibody; 50% inhibition of protein synthesis occurred in a 15–50 pM range. Little or no cytotoxicity to cells expressing EGFR was detected (Lorimer *et al*, 1995). While the construction of these immunotoxins demonstrated that a specific immunoconjugate could be produced, chemical conjugation of PE often results in a heterogenous product. In addition, the large size of the intact Ig-PE construct may not be optimal for solid tumor penetration. Recently, following the immunization of mice with the specific EGFRvIII epitope and selection by phage display technology, we reported the isolation of an anti-EGFRvIII sFv which is extremely stable with a K_d of 22 nM (Lorimer *et al*, 1996). This sFv has been prepared with recombinant DNA technology into a *Pseudomonas* exotoxin construct with domains II and III of *Pseudomonas* exotoxin A. The resultant recombinant immunotoxin, designated MR1scFvPE38KDEL, (MR-1 immunotoxin) is an extremely potent killing agent with a 50% inhibitory concentration of 1–10 ng/ml, respectively, for NR6M and U87MG.ΔEGFR cells. Specificity was established by the lack of detected cytotoxin activity at 1000 ng/ml on untransfected U87MG cells, and the inhibition of toxicity by a 10-fold excess of MR1scFv. The *in vivo* therapeutic efficacy of MR-1 immunotoxin was evaluated in a dose response study in an athymic rat model of neoplastic meningitis established with U87MG.ΔEGFR cells; three doses of MR-1 immunotoxin were delivered via an indwelling subarachnoid catheter every other day. MR-1 immunotoxin increased the median survival of tumor-bearing rats from 105–300%, and produced 2/10, 3/10 or 5/11 long-term survivors, dependent upon MR-1 immunotoxin dose (Archer *et al*, 1997). A repeat of this study has produced 420–1240% increases in median survival, with 5/9 long term survivors (Archer *et al*, manuscript in preparation). These results further establish the feasability of EGFRvIII as a specific immunotherapeutic target, and the safety and efficacy of immunotoxins directed to EGFRvIII; as dimerization is not required for internalization of Mab-EGFRvIII complexes, even target cells with relatively low receptor density may be susceptible to EGFRvIII-mediated therapies.

Cell-mediated immune responses to the EGFRvIII

The demonstration of a humoral response to the 14 mer synthetic peptide representing the EGFRvIII-unique amino acid sequence suggested the possible induction of cytotoxic T lymphocytes (CTLs) which are $CD8^+$ with or without the association of MHC class I molecules (Restifo and Wunderlich, 1995; Huang *et al*, 1994). Ashley *et al* (1997) evaluated the immunogenic capacity of EGFRvIII for cell-mediated responses using transfected allogeneic cells; C57BL/6J mice were vaccinated with the allogeneic pre-B cell line 300.19 which has been transfected to express EGFRvIII, or with untransfected 300.19 cells. Splenic $CD8^+$ T lymphocytes cytotoxic for the H-2^b EGFRvIII positive, B16-F10 melanoma cells or EGFRvIII positive 560 astrocytoma cells were

induced; these effector cells were not cytotoxic for H-2^q mismatched NR6M cells. Significant NK activity was also detected. Similarly immunized mice were challenged intracranially with EGFRvIII+ or −B16-F10 cells; only mice vaccinated with 300.19 EGFRvIII+cells exhibited longer latency following challenge with B16-F10 EGFRvIII+tumors, with 50% long term survival. All mice challenged with B16-F10 EGFRvIII-cells died. Depletion of defined lymphocyte subsets in immunized animals 4 days before tumor challenge demonstrated the requirements for both $CD8^+$ and $CD4^+$ T cells but not NK cells in producing this protective effect. Similarly, Moscatello *et al* (1997) reported induction of both humoral reactivity and a $CD8^+$ T lymphocyte-mediated response in NIH Swiss mice vaccinated with EGFRvIII peptide-KLH in complete Freund's adjuvant; only 4/16 (25%) of mice so vaccinated developed HC 20 D2 tumors when challenged with a dose of HC 20 d2 cells which induced tumors in 13/16 (81%) control vaccinated mice. As the latter study was performed in a non-syngeneic host-tumor cell system, and the study of Ashley *et al* (1997) utilized human *EGFRvIII* gene transfected cells syngeneic to the challenged host, it remains to be established whether the observed reactivity is EGFRvIII-specific. Studies are currently in progress with a murine genetic EGFRvIII construct transfected cell line in a syngeneic model system to examine the specificity of the observed responses and their induction.

Summary and prospects

The tumor-associated variant transmembrane glycoprotein of the EGFR, EGFRvIII, is unique among tumor associated cell surface antigens in that: (1) it has yet to be detected in normal tissue; (2) it is present at relatively high density on the cell surface of tumor cells expressing the amplified, rearranged *EGFRvIII* gene; (3) it has been demonstrated in animal model systems to elicit both a B- and a T-cell response, and (4) the target receptor is internalizable following interaction with specific Mab or Mab fragments (Reist *et al*, 1995; manuscript in progress). As has been demonstrated in the studies cited above, immunotargeting approaches in model systems have been successful whether the effectors are intact radioactive Mabs, intact Mab-toxin constructs, Mab fragment-toxin constructs, or purposefully elicited T or NK cells. For approaches utilizing Mabs or Mab fragments, the constantly evolving chemistries for radiolabeling, and the genetic engineering techniques for generating Mab-toxin, drug, or other effector constructs which can be optimized for internalizing complexes show great promise. The preliminary success with the induction of cytotoxic cells by preimmunization with EGFRvIII expressing cells suggests that vaccination protocols involving synthetic epitope-containing peptides or EGFRvIII protein fragments may be an exploitable approach, not requiring syngeneic presentation. Thus, for the approximately 150 000 cases of GBM, breast, and non-small cell lung cancers a year which express this antigen, a specific, therapeutic target exists, the exploitation of which is just beginning.

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