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SIGNAL TRANSDUCTION BY THE *neu/erb*B-2 RECEPTOR: A POTENTIAL TARGET FOR ANTI-TUMOR THERAPY

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Summary—The *neu/erb*B-2 protooncogene encodes a transmembrane tyrosine kinase homologous to receptors for polypeptide growth factors. The oncogenic potential of the presumed receptor is released through multiple genetic mechanisms including a point mutation, truncation of non-catalytic sequences and overexpression. The latter mechanism appears to be relevant to human cancers as elevated expression of the *neu/erb*B-2 gene is frequently observed in solid tumors of various adenocarcinomas. It is therefore conceivable that strategies aimed at the biochemical mechanism of action of the *neu/erb*B-2 tyrosine kinase may contribute to the treatment of certain human cancers.

To this aim we undertook a multiple research approach consisting of the following directions: (i) The *neu/erb*B-2 ligand—a systematic screening of potential biological sources of the hypothetical hormone molecule, that presumably binds to the *neu/erb*B-2 protein, resulted in detection of a candidate activity in the medium of certain cultured transformed cells. Partial purification indicated that the factor is a 30–35 kDa glycoprotein. Further studies revealed several biochemical characteristics of the factor that may be helpful for complete purification and structural analysis of this novel hormone. (ii) Signal transduction by *neu/erb*B-2—using a chimeric receptor approach and various mutants we found that all the oncogenic forms of the *neu/erb*B-2 are constitutively coupled, both physically and functionally, to a multi-protein complex of signaling molecules. The latter includes the phosphatidylinositol-specific phospholipase Cγ and a phosphatidylinositol kinase. Thus, the metabolism of inositol lipids is probably a major biochemical pathway utilized by the *neu/erb*B-2 tyrosine kinase. (iii) Tumor inhibitory antibodies—we generated a panel of monoclonal antibodies to the presumed receptor. Surprisingly, some antibodies almost completely inhibited the growth of tumor cells in athymic mice, whereas one antibody significantly accelerated the rate of tumor growth in animals. Interestingly, the inhibitory antibodies conferred a mature phenotype to cultured breast cancer cells, implicating terminal differentiation in tumor retardation.

RECEPTORS FOR POLYPEPTIDE GROWTH FACTORS: A FAMILY OF TRANSMEMBRANE TYROSINE KINASES

To achieve tight regulation of mitotic cell division in multi-cellular organisms, intercellular communication mechanisms have evolved. These include surface- and matrix-anchored cell adhesion molecules as well as soluble polypeptide growth factors. The latter are usually secreted proteins of low molecular weight that operate in paracrine or autocrine fashions [1]. Although their major function involves entry of resting cells into the cell cycle, growth factors have also been implicated in differentiation and morphogenic processes [2]. All the biological actions of growth factors are mediated by specific cell surface receptors that transduce the biochemical signal through stimulatory associations with cytoplasmic proteins. Fundamental to the function of the receptors for growth factors is a shared catalytic function that phosphorylates tyrosine residues [3]. The extracellular ligand binding domains display variable structures as expected from their distinct ligand specificities. The cytoplasmic catalytic core of receptor tyrosine kinases (RTKs) is shared not only by receptors for different growth factors, but also with a group of non-membrane tyrosine kinases, the prototypic one being the *src* protein [4, Fig. 1]. Perhaps the best exemplification of the major role that tyrosine kinases play in

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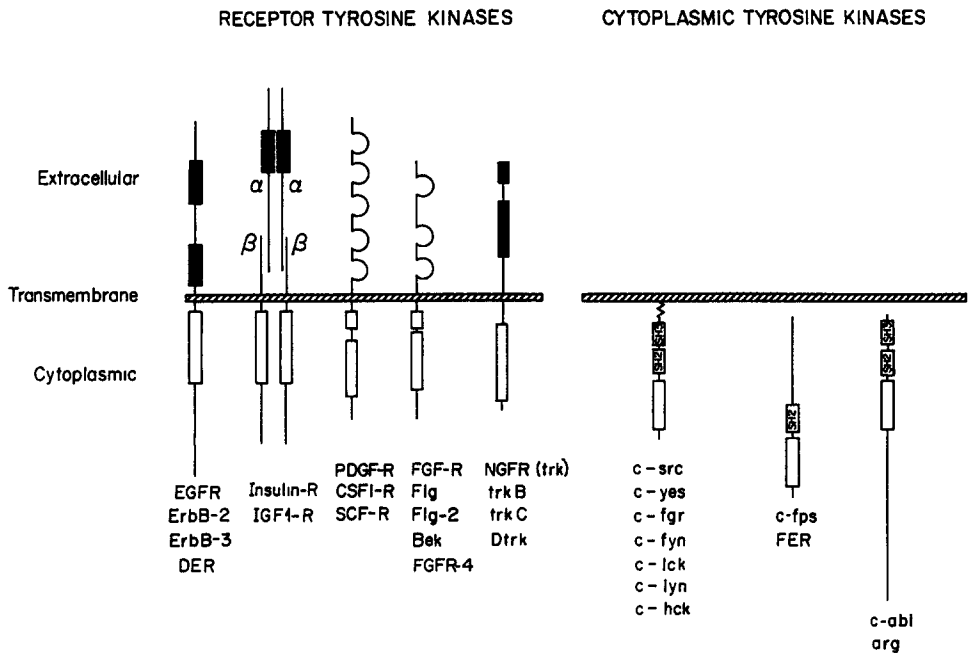

Fig. 1. Protein tyrosine kinases. The major groups of RTKs and cytoplasmic tyrosine kinases are schematically presented and the names of members of each group are indicated. The tyrosine kinase domains are shown by open boxes, cysteine-rich regions are shown by closed boxes and open circles denote immunoglobulin like loops. *Src* homology domains (SH2 and SH3) are indicated.

growth control is the existence of viral and other structurally modified kinases that confer a transformed phenotype to normal cells.

A variety of growth factors bind to RTKs. The current list includes the epidermal growth factor (EGF), insulin, insulin-like growth factor-1 (IGF-1), the platelet-derived growth factors (PDGFs), the stem cell factor (SCF), the macrophage growth factor (CSF-1), heparin-binding fibroblast growth factors (FGFs), the nerve growth factor (NGF), the brain derived neurotrophic factor (BDNF) and neurotrophin-3. In contrast to the heterogeneity of the ligand molecules, all the RTKs appear to induce cellular activation by a shared biochemical mechanism. This involves receptor dimerization that leads to activation of the catalytical function [5]. As a result, the receptors undergo self-phosphorylation on tyrosine residues. The sites of phosphorylation function as anchoring points for a group of cytoplasmic proteins [6], some of them carry domains homologous to non-catalytic portions of *src* [7]. Phosphorylation of the latter is thought to induce their catalytic activation or cellular redistribution leading to enhanced production of second messenger molecules [8]. It may otherwise mediate activation of a cascade of protein kinases which eventually results in specific gene expression and cell cycle progression.

The present report will concentrate on the *neu* RTK and will describe recent results obtained by us and other groups. These results demonstrate the implications of signal transduction mechanisms on oncogenesis.

THE *neu*/*erb*B-2 ONCOGENE

The *neu* oncogene was first detected as a transfectable transforming gene in DNA derived from ethylnitrosourea (ENU)-induced tumors in BDIX rats [9, Fig. 2]. Cell lines established from such tumors displayed neuronal and glial phenotypes [10]. Both normal and transforming alleles of the rat gene were isolated and found to have identical restriction maps [11], yet they differed by several orders of magnitude in their ability to transform fibroblasts. Recombinants of normal and transforming cDNA clones localized the transforming potential to a single nucleotide change, an A to T transversion [12]. This results in the substitution of a valine residue at the transmembrane domain of the RTK for a glutamic acid (schematically illustrated in Fig. 3). The human counterpart of the *neu* gene was independently isolated by several groups by virtue of hybridization to an *erb*B probe [13–15], and termed HER-2 or c-*erb*B-2. The protein product of *neu* is most closely related to the EGF-receptor and the *erb*B-3

protein [16]. These proteins have distinct extracellular ligand binding domains that include two cysteine-rich regions. The cytoplasmic tyrosine kinase portion is connected to the ectodomain through a single transmembrane stretch of hydrophobic amino acids. One of these is the target for the carcinogen-induced oncogenic activation of *neu*. Although such replacement does not occur in the human gene [17], ectopic overexpression of the gene in rodent fibroblasts causes phenotypic transformation and tumorigenicity [18, 19]. The transforming property is unique to the *neu* gene as the closely related receptor for EGF is at least 200-fold less active [20]. This intrinsic characteristic of *neu* is apparently relevant to human adenocarcinomas that often display remarkable amplification and/or overexpression of the oncogene [21 and references therein]. Importantly, breast cancer patients having tumors with multiple copies of *neu* had a poorer disease outcome with shorter time to relapse and overall survival [22]. Theoretically, receptor overexpression at the cell surface may lead to elevated basal tyrosine kinase

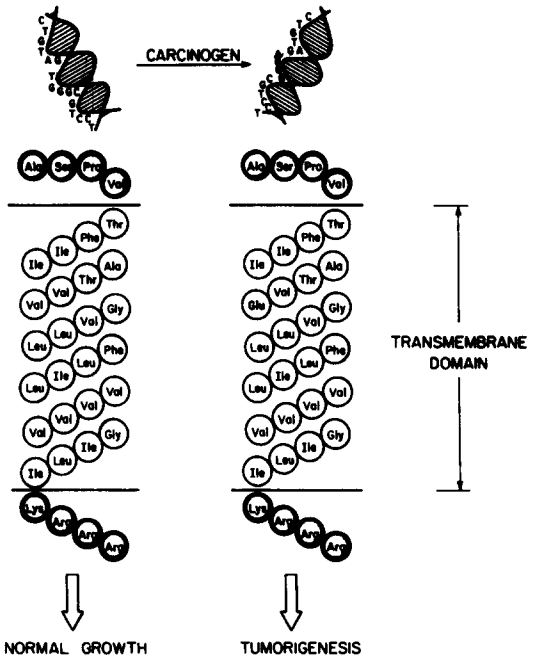

Fig. 3. Mutational activiation of the *neu* protooncogene. The cartoon depicts the carcinogen-induced mutation within the coding sequence of *neu* (upper part) and the resulting change in the amino acid sequence of the transmembrane domain of the protein.

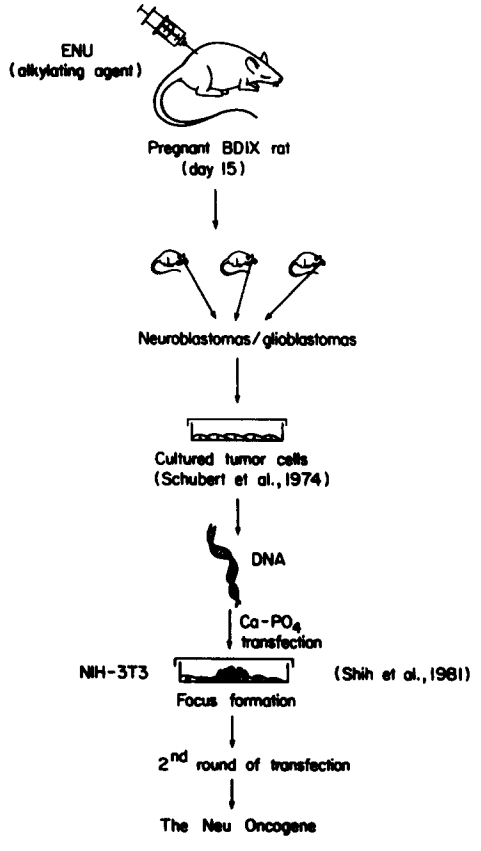

Fig. 2. Detection and isolation of the *neu* oncogene. The flow diagram schematically presents the major steps involved in the isolation of the *neu* oncogene. References to the original papers are also given.

activity that would exceed a threshold needed for cell stimulation. Otherwise, overexpression of the *neu*-encoded receptor may be coupled to an autocrine or a paracrine loop that involves synthesis and secretion of the corresponding ligand. Such a mechanism may confer a selective advantage to *neu* overexpressing clones, and eventually brings about tumor formation (Fig. 4). These mutually non-exclusive possibilities underscore the importance of in-depth understanding of the mechanism by which the *Neu* protein transduces the mitogenic signal.

PERMANENT RECEPTOR DIMERIZATION OF ONCOGENIC *Neu* PROTEINS

The oncogenic potential of the *neu*-encoded tyrosine kinase is released by multiple genetic and biochemical mechanisms. These include certain single amino acid substitutions at the transmembrane domain [23], overexpression of the wild-type protein [18, 19, 24] truncation of non-catalytic portions of it [18, 23] and ligand-induced stimulation in the context of chimeric molecules [25, 26]. These different mechanisms could converge on a single pathway of cell activation. Alternatively, each oncogenic form may utilize a distinct signaling pathway (Fig. 5). As will be shown here, apparently the

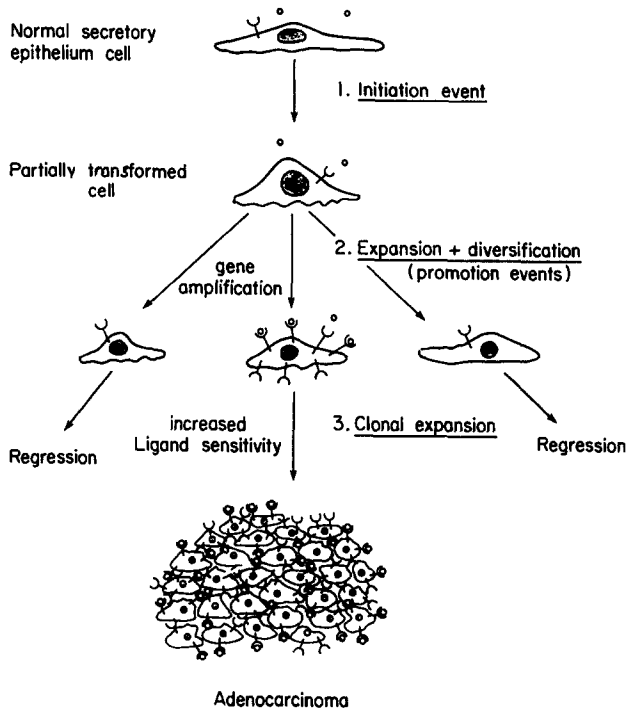

Fig. 4. Clonal tumorigenesis by an overexpression-autocrine mechanism. A hypothetical model is schematically illustrated Accordingly, overexpression of a surface receptor, in conjunction with self-production of the corresponding growth factor, leads to tumorigenic clonal expansion.

converging model is correct, namely: oncogenically activated forms of *Neu* are permanently active as tyrosine kinases, and are therefore constitutively coupled to their effector pathways. The latter include enzymes involved in phospholipid turnover. This means that all the transforming proteins are functionally equivalent to ligand-occupied receptors for growth factors.

A logical consequence of this model is that the activated *Neu* proteins have a strong bias to form oligomerized receptors. Indeed, protein

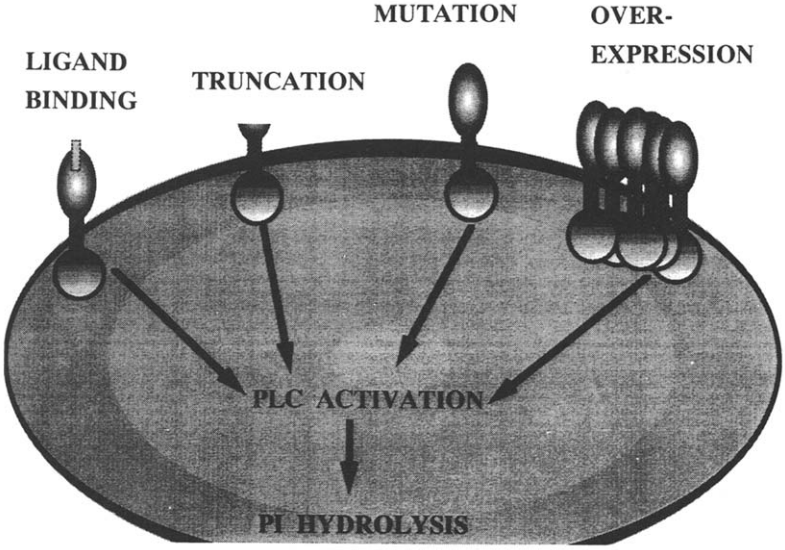

Fig. 5. Multiplicity of oncogenic *Neu* proteins. The transforming forms of the *Neu* protein are schematically depicted along with their biochemical convergence on the metabolism of phosphoinositide lipids

modeling predicted that the point-mutated oncogenic *Neu* forms dimers as opposed to the monomers of the wild-type protein [27, 28]. Employing stimulatory monoclonal antibodies to the *Neu* protein we came to the conclusion that receptor dimerization is essential for kinase activation [29]. The oncogenic protein, however, was constitutively active as if permanent receptor dimerization took place [29, 30]. Covalent cross-linking experiments confirmed the existence of dimeric mutant *Neu* proteins; immunoprecipitation of the receptor from cells that express either the mutant (B104-1-1 cells) or the wild-type protein (DHFR-G8 cells or their equivalent that were transformed by the *ras* protein for appropriate control) revealed the existence of dimers only in B104-1-1 cells [31] [Fig. 6(a)]. Importantly, dimers were also observed in cells that express very high levels (8×10^5 molecules/cell) of the human *Neu* protein [Fig. 6(b)], but not cells that overexpress the EGF-receptor (results not shown). Presumably, the *Neu* protein has some tendency to form dimers and this is statistically enhanced by overexpression. However, *Neu* can form heterodimers with the EGF-receptor where only the latter is ligand occupied [32, 33]. These heterodimers are characterized by elevated tyrosine kinase activity and very high ligand affinity.

ONCOGENIC *Neu* PROTEINS ARE PERMANENTLY COUPLED TO SIGNALING COMPLEXES

In analogy with other receptors that associate with signal generating molecules upon their self-phosphorylation on tyrosine residues [6], we examined the possibility that oncogenic forms

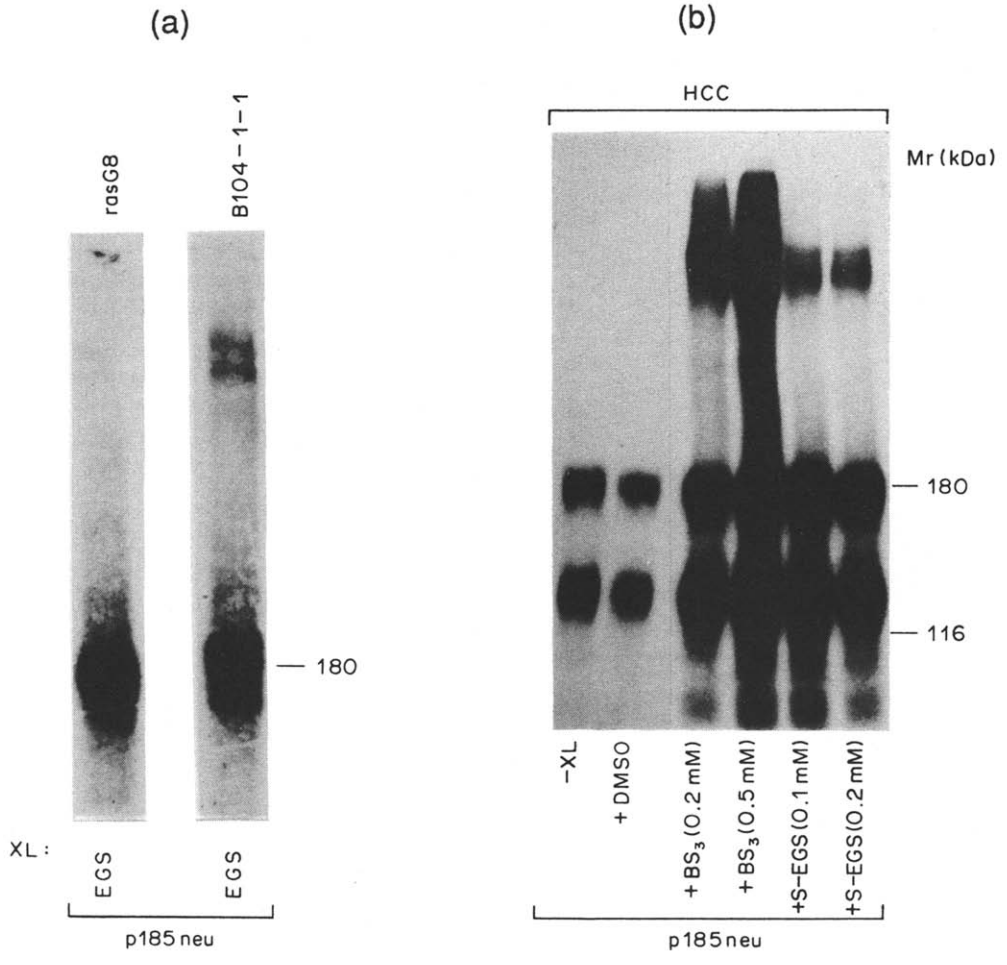

Fig. 6. Dimerization of oncogenic forms of the *Neu* protein. Cells that express either the normal (*ras*-G8), or the transforming rat *Neu* protein (B104-1-1, panel a) or the human receptor (HCC cells, panel b) were exposed to bifunctional chemical cross-linking agents: EGS—ethylene glycol bis(succinimidyl succinate); S-EGS—sulfo-EGS; BS^3—bissulfosuccinimidyl-suberate. The *Neu* proteins were then subjected to immunoprecipitation and autophosphorylation *in vitro* according to our published procedures [32]. The immunocomplexes were resolved by gel electrophoresis and autoradiography.

of *Neu* will also be found in such complexes. By using Western blot analysis with antibodies to phosphotyrosine we could demonstrate that multiple proteins undergo elevated tyrosine phosphorylation as a result of overexpression of the mutant *Neu* (Fig. 7). Some of these proteins associate non-covalently with the transforming *Neu* protein but not with the wild-type receptor. Very similar patterns of tyrosine phosphorylated proteins were observed upon ligand-mediated stimulation of the tyrosine kinase, as indicated by using a chimera between EGF-receptor (extracellular domain) and the *Neu* protein (transmembrane and tyrosine kinase domains, Ref. [34]). Identification of the individual proteins that constitute the *Neu*-signaling complex is critical to the elucidation of the signal transduction pathways utilized by the protein. Furthermore, specific inhibitors of these pathways may offer strategies to block *Neu*-mediated tumorigenesis. To this end we have started analysis of the signaling complex. One major protein of the complex is the phosphoinositide-specific phospholipase, PLCγ [34].

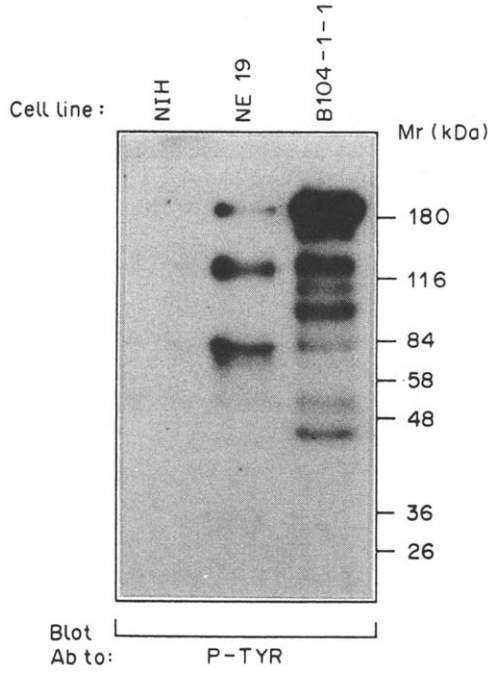

Fig. 7 Tyrosine phosphorylated proteins in *Neu*-transformed cells Whole cell lysates were prepared from murine fibroblasts that ectopically express the normal (NE19 cells) or the transforming rat *Neu* protein (B104-1-1 cells). The lysates were electrophoresed and analyzed by Western blotting with antibodies to phosphotyrosine as we have described previously [34]. For control we included also lysates prepared from untransfected murine fibroblasts (labeled NIH). The resulting autoradiogram is shown and the locations of molecular weight marker proteins are indicated in kilodaltons (kDa)

This protein undergoes elevated tyrosine phosphorylation and physically associates with all the oncogenic forms of *Neu* including a point mutant, overexpressed or truncated wild-type receptor, and ligand-stimulated receptor. Biochemically, this association is linked to enhanced phosphatidylinositol breakdown that is probably coupled to activation of protein kinase C and calcium fluxes [35]. We are currently analyzing other signaling proteins that physically couple to oncogenic forms of *Neu* in the hope of obtaining a detailed biochemical picture of the *Neu* mitogenic signal. Our preliminary results show that a phosphatidylinositol kinase activity also associates with *Neu*.

THE *Neu* LIGAND

Based on evolutionary, biochemical and genetic lines of evidence, we came to the conclusion that the *Neu* protein may have a distinct ligand molecule, that probably belongs to the EGF family [36]. To detect the ligand we concentrated on well known sources of EGF-like molecules, and identified *ras*-transformed fibroblasts as a possible source of the *Neu* ligand [37]. This very rich source of transforming growth factor-α (TGF-α) secretes an activity that stimulates the *Neu* tyrosine kinase, induces down-regulation of the receptor and transmits a mitogenic signal. By using medium conditioned by *ras* transformed fibroblasts we were able to partially purify the ligand molecule [38]. The factor is a heat-stable glycoprotein of 30–35 kDa M_r and contains disulfide bonds that are essential for its biological activity. In addition, the molecule binds to heparin and undergoes precipitation by either high salt concentrations or acidic alcohol. These properties may facilitate protein purification, which is yet incomplete. Nevertheless, our most purified fraction appears to simultaneously activate both *Neu* and the EGF-receptor, implying redundancy in ligand specificity. In this respect, our protein resembles a TGF-α-related factor that was isolated from breast cancer cells and is also a candidate *Neu* ligand [39]. Further studies, however, will require a homogeneously purified preparation of the ligand.

TUMOR INHIBITORY ANTIBODIES DIRECTED TO THE *Neu* PROTEIN

The association between *neu* and tumorigenesis in animal model systems and in human

adenocarcinomas, identified the cognate protein as a potential target for antibody therapy. Indeed, antibodies to the human protein have been raised and shown to inhibit the growth of tumor cells *in vitro* [40–42], whereas antibodies to the rat *Neu* displayed anti-tumor activity both *in vitro* and *in vivo* [43–45]. To further explore the potential clinical use of murine monoclonal antibodies and humanized antibodies to *Neu* we have raised a panel of mouse monoclonal antibodies and studied their effect on tumor growth. Whereas some antibodies (most notably the N29 antibody) when injected intraperitoneally (i.p.) or intravenously (i.v.) displayed significant anti-tumor effects (Fig. 8), one of our antibodies, denoted N28, significantly accelerated tumor growth in athymic mice [46]. The latter effect was attributable to a strong kinase stimulatory action that N28 induced on living cells. However, the tumor inhibitory effects observed with other antibodies could not be correlated with various cellular and biochemical parameters, including receptor turnover, tyrosine phosphorylation and cellular cytotoxicity [46]. Our most recent experiments indicate that the tumor inhibitory antibodies, unlike the N28 or control antibodies, specifically induced terminal differentiation of cultured breast cancer cells. The differentiated phenotype resembles in many aspects a state that is induced by well known differentiation inducers like retinoic acid and phorbol esters [47]. These include synthesis and secretion of milk components (casein and lipids), induction of mature cell morphology, cell cycle arrest at the G_2 phase and translocation of the *Neu* protein from the membrane to perinuclear sites [S. Bacus *et al.*, manuscript in preparation]. Taken together, our results suggest that antibody-induced differentiation of carcinoma cells holds promise for therapy of cancers that involve overexpression of the oncogene.

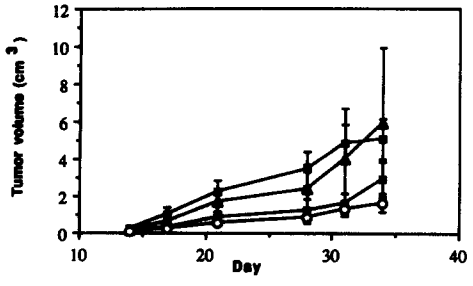

Fig. 8. The effect of monoclonal antibody N29 on tumor growth. Murine fibroblasts that overexpress the human *Neu* protein were injected subcutaneously into groups of 5 CD1/nude mice. The N29 murine monoclonal antibody to *Neu* was injected i.p. on days 3, 7 and 10, at three doses: 0.5 mg/mouse (triangles), 1 mg/mouse (closed squares) and 2 mg/mouse (circles). For control phosphate buffered saline was also injected (open squares). Tumor growth was followed and tumor volumes determined according to our published protocol [46].

PERSPECTIVES

Despite multiplicity of genetic and biochemical modes of oncogenic activation of *neu*, it appears that they all converge on the same biochemical pathway that ultimately leads to de-regulated cell growth. Based on the data currently available, the oncogenic forms of *neu* are biochemically equivalent to a ligand-occupied receptor, whereas the wild-type protein is functionally silent unless overexpressed. The active state of the protein, which is analogous to other stimulated hormone receptors, is probably released from allosteric conformational constraints that repress the catalytic function. Derepression is apparently achieved by constitutive receptor dimerization, that results in elevated auto-phosphorylation and permanent coupling to signal transducing proteins. The latter include enzymes involved in phosphoinositide metabolism, but the complete content of the *Neu* signaling complex is yet to be determined. Once fully characterized, pharmacological strategies aimed at critical steps of the *Neu* pathway may be combined with specific differentiation-inducing antibodies to achieve tumor retardation.

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