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Increased Epidermal Growth Factor Receptor in Multidrug-Resistant Human Neuroblastoma Cells

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Multidrug-resistant human neuroblastoma cell lines obtained by selection with vincristine or actinomycin D from two independent clonal lines, SH-SY5Y and MC-IXC, have 3- to 30-fold more cell surface epidermal growth factor (EGF) receptors than the drug-sensitive parental cells as indicated by EGF binding assays and immunoprecipitation, affinity-labeling, and phosphorylation studies. Reversion to drug sensitivity in one line was accompanied by a return to the parental level of EGF receptor. SH-EP cells, a clone derived from the same neuroblastoma cell line as SH-SY5Y but which displays melanocyte rather than neuronal lineage markers, also express significantly more EGF receptor than SH-SY5Y cells. By nucleic acid hybridization analysis with a molecularly cloned probe, increased receptor level in multidrug-resistant cells was shown to be the result of higher levels of EGF receptor mRNA in drug-resistant than in drug-sensitive cells. The increased steady state amount of specific RNA did not result from amplification of receptor-encoding genes. A small difference was observed in the electrophoretic mobility under denaturing conditions of EGF receptor immunoprecipitated from drug-resistant and drug-sensitive cells. Quantitative and qualitative modulation of the EGF receptor might reflect alterations in the transformation and/or differentiation phenotype of the resistant cells or might result from unknown selective pressures associated with the development of multidrug resistance.

Key words: EGF receptor, multidrug-resistance, human neuroblastoma, binding assay, immunoprecipitation, transformation/differentiation

Cultured cells selected for resistance to vincristine or actinomycin D are termed multidrug-resistant because they are cross-resistant to a wide variety of natural product agents, many of which are used in the treatment of cancer [1–3]. This type of resistance may develop in tumors of drug-treated patients. A major concomitant of multidrug-

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resistance is overexpression of a plasma membrane phosphoglycoprotein, named P-glycoprotein, with a molecular weight of 150–180 kilodaltons and a pI of about 7.0 [1–8]. The similarity between the physicochemical characteristics of this protein species and those of the epidermal growth factor (EGF) receptor [9,10] prompted a study of EGF receptors in resistant rodent cells. This earlier study indicated that the resistant sublines, selected with vincristine or actinomycin D, consistently express higher numbers of EGF receptors than drug-sensitive cells and that the resistance-related P-glycoprotein and EGF receptor are separate species [11]. The *mdr1* gene encoding P-glycoprotein has recently been sequenced and the complete primary structure of human P-glycoprotein determined [12], demonstrating that it is distinct from the EGF receptor gene. The current study was undertaken to determine whether human cells also manifest EGF receptor modulation as a concomitant of multidrug resistance and to understand further the molecular basis for the observed modulation in receptor level. Of significance in this regard are the findings that the P-glycoprotein gene(s) maps to human chromosome 7 [13] and is amplified in many multidrug-resistant cells, including the two vincristine-selected human neuroblastoma lines, SH-SY5Y/VCR and MC-IXC/VCR [14], studied here. The EGF receptor gene is also on human chromosome 7 [15]. Appropriate questions to be asked are whether multidrug-resistant cells may amplify the latter gene, also, and whether there is interaction between these two genes or their gene products. The first question is addressed in this report (the genes are not coamplified); the second remains to be investigated. The data show that human neuroblastoma cells selected for resistance to vincristine or actinomycin D have increased numbers of EGF receptors compared to drug-sensitive parental cells. We do not as yet know whether this increase is associated with normalized cell growth and decreased tumorigenic potential, a phenomenon termed reverse transformation, which has been observed in multidrug-resistant Chinese hamster and mouse tumor cells in this laboratory [2,11]. The correlation of increased receptor with state of transformation is of particular interest because EGF receptor and the related *neu* receptor genes are proto-oncogenes for the retroviral *erbB* and the transforming gene *neu* [16–19]. In addition, the EGF receptor gene is amplified and overexpressed in several human tumors [20–22]. A brief report of the present study has been published [23].

METHODS

Cell Lines

The development and maintenance of SH-SY5Y, SH-SY5Y/VCR, SH-SY5Y/VCR-U, MC-IXC, MC-IXC/VCR, and SH-EP have been described [1,24,25]. SH-SY5Y/VCR and MC-IXC/VCR are vincristine-resistant sublines of the SH-SY5Y and MC-IXC clones isolated from the neuroblastoma line SK-N-SH and the neuroepithelioma line SK-N-MC, respectively. SH-SY5Y/VCR-U, a revertant of SH-SY5Y/VCR, was cultured for 8 months (100 doublings) in drug-free medium before characterization [1]. Vincristine for these studies was a gift from Eli Lilly and Company (Indianapolis, IN). SH-EP is a drug-sensitive cloned variant of SK-N-SH, the human neuroblastoma line from which SH-SY5Y was also cloned [26]. SH-EP cells are nonneuroblastic, substrate-adherent cells that exhibit a loss of tumorigenic capacity in nude mice and of colony-forming efficiency in soft agar as compared to neuroblastic SH-SY5Y cells [27]. SH-SY5Y/ACT was derived from thrice-cloned SH-SY5Y cells by growth in stepwise increases in concentration of actinomycin D (Sigma Chemical Co., St. Louis, MO);

cells are maintained in standard medium containing 50 ng/ml of drug and 100 ng/ml of EGF (Collaborative Research, Inc., Lexington, MA). SH-SY5Y/ACT is an EGF-dependent subline, unlike the other lines in this study which do not require supplemental EGF for growth in a 1:1 mixture of Eagle's Minimum Essential Medium and Ham's F12 medium containing 15% fetal calf serum. SH-SY5Y/ACT cells exhibit polysomy of chromosome 7. Prior to experiments cells were cultured in the absence of drug, and in the case of SH-SY5Y/ACT in the absence of EGF, for 2–5 days. A431 cells (CRL 1555), which have high EGF receptor levels [28], were obtained from the American Type Culture Collection (Rockville, MD) and used as a control line.

EGF Binding Studies

Procedures for EGF binding were essentially those described by Das et al. [29] and previously used in this laboratory [11]. Binding of [^{125}I]EGF to monolayer cells was measured at 22°C after a 1-hr incubation in the presence and absence of 100 nM unlabeled EGF. Specific binding was calculated as total minus nonspecific cell-associated radioactivity. Incubations for each experiment were performed in duplicate. The number of receptors and K_D for binding were determined by Scatchard plot analyses.

Radioactive Labeling, Immunoprecipitation, Affinity-Labeling, and Phosphorylation

Cells were grown for 4 hr in methionine-free medium containing 50 μCi/ml [^{35}S]methionine (New England Nuclear Corp., Boston, MA) in the presence or absence of 50 nM EGF. After removal of the medium and one wash with phosphate-buffered saline, cells were lysed in 20 mM Hepes (pH 7.4) containing 10% glycerol and 1% Triton X-100. Aliquots of the 13,000g supernatants containing 10^6 trichloroacetic acid-precipitable cpm were incubated with monoclonal antibody 528 raised against A431 cell EGF receptor. The antibody was a gift from Dr. Gordon Sato [30]. The antigen-antibody complex was precipitated with *Staphlococcus aureus* cells (The Enzyme Center, Malden, MA) and prepared for electrophoretic separation of EGF receptor as described by Xu et al. [31]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out with 7.5% acrylamide gels (0.075 × 14 × 11 cm) [32]. Molecular weight markers were purchased from Bethesda Research Laboratories (Gaithersburg, MD). After electrophoresis gels were dried and exposed to X-OMAT AR X-ray film (Eastman Kodak Co., Rochester, NY) at −70°C for 3 weeks. For measurement of radioactivity incorporated into EGF receptor, gel slices containing EGF receptor were excised and treated with 0.5 ml of 30% hydrogen peroxide for 16 hr at 60°C. The eluted radioactivity was quantitated by liquid scintillation spectrometry.

Iodinated EGF was cross-linked to EGF receptor with the use of 1-ethyl-3-(dimethylaminopropyl)carbodiimide (Sigma) according to published procedures [33]. Briefly, cell monolayers were incubated with 1 nM [^{125}I]EGF for 1 hr at 22°C, as for binding assays, then treated with the cross-linking agent for 15 min. Labeled cells were solubilized with Laemmli sample buffer [32] and aliquots containing 100 μg of protein, determined by the Lowry procedure [34], were subjected to electrophoresis. Dried gels were exposed to X-ray film at −70°C for 2 weeks.

Phosphorylation of immunoprecipitated EGF receptor was accomplished according to procedures described by Carlin and Knowles [35]. Essentially, unlabeled cells were lysed in 1% Triton X-100 as described above and aliquots containing 100 μg of protein were incubated with monoclonal antibody 528 for 1 hr at 4°C. After treatment with

Staphlococcus aureus cells for 1 hr at 4°C, the pelleted complex was suspended in 20 mM Hepes (pH 7.4)/0.1% bovine serum albumin/10% glycerol/30mM NaCl/50 mM $MgCl_2$ and then incubated with [γ-^{32}P]ATP (New England Nuclear, 3000 Ci/mmol). Phosphorylated proteins were solubilized by boiling the pelleted complex in Laemmli buffer [32] and separated by electrophoresis. Dried gels were exposed to X-ray film at −70°C for 1 hr. Electrophoresis supplies were purchased from Bio-Rad (Cambridge, MA) and other chemicals from Sigma.

Southern and Northern Transfer and Cytodot Analyses

The cloned EGF receptor probe, pE7 [22,36], provided by Dr. Ira Pastan, was used for evaluation of EGF receptor DNA and RNA. The 2.4-kb human EGF receptor cDNA insert (pE7 Cla I fragment) was labeled by nick translation [37] with [α-^{32}P]dCTP (New England Nuclear). Standard procedures were employed for Southern hybridization analysis [38]. Genomic DNA from A431 cells, known to contain amplified EGF receptor genes [21,22], was used as a positive control. A human globin cDNA probe served as an internal control of DNA amount (data not shown). Twenty micrograms of genomic DNA from each of the cell lines was digested with EcoR1 and separated on 1% agarose (FMC Corp., Rockland, ME) gels. Hybridization was done under stringent conditions. Prehybridization was done at 42°C for 16 hr with 50% formamide, 5 X SSC, 5 X Denhardt's solution, 10 mM Tris-HCl (pH 7.5), 1% glycine, and 100 μg/ml denatured salmon sperm DNA. Hybridization was carried out at 42°C for 16 hr with 50% formamide, 5 X SSC, 1 X Denhardt's, 10 mM Tris-HCl (pH 7.5), 10% dextran sulfate, and 25 μg/ml denatured salmon sperm DNA. The blots were washed several times in 2 X SSC, 0.1% SDS at room temperature and four times in 0.1 X SSC, 0.1% SDS at 50°C, and the filters were exposed to X-ray film with intensifying screen for 42 hr. For analysis of EGF receptor RNA, total cellular RNA was isolated from cells by guanidine isothiocyanate solubilization and centrifugation over a 5.7 M cesium chloride cushion [39]. For Northern analysis 20 or 2 μg of RNA were electrophoresed on 1% agarose/formaldehyde gels and transferred to nitrocellulose (Bio-Rad) [40]. For cytodot analysis RNA was denatured with formaldehyde and serial twofold dilutions of RNA samples were spotted on nitrocellulose paper using a "minifold" apparatus [41]. The filters were prehybridized in 50% formamide, 5 X Denhardt's, 50 mM sodium phosphate (pH 7.4), 5 X SSC, 0.5 mg/ml salmon sperm DNA at 42°C for 16 hr and then hybridized at 42°C for 16 hr in 50% formamide, 1 X Denhardt's, 20 mM sodium phosphate (pH 7.4), 5 X SSC, and 0.1% SDS. The blots were washed and exposed to film as described above. A human HLA-B7 cDNA probe was used to control for RNA amount and integrity (data not shown).

RESULTS

EGF Binding Studies

Resistant neuroblastoma cells in this study specifically bound [^{125}I]EGF to a greater extent than sensitive cells. Figure 1 displays representative binding curves and Scatchard analyses from which EGF B_{max} values were obtained (Table I). We calculate that from 3- to 30-fold more EGF receptors are present on multidrug-resistant than on parental sensitive cells. SH-SY5Y/VCR-U, a revertant of SH-SY5Y/VCR in terms of decreased drug resistance (see Methods), has also reverted to low EGF receptor level (Table I). SH-EP, a clone derived from the same neuroblastoma line (SK-N-SH) as clone SH-

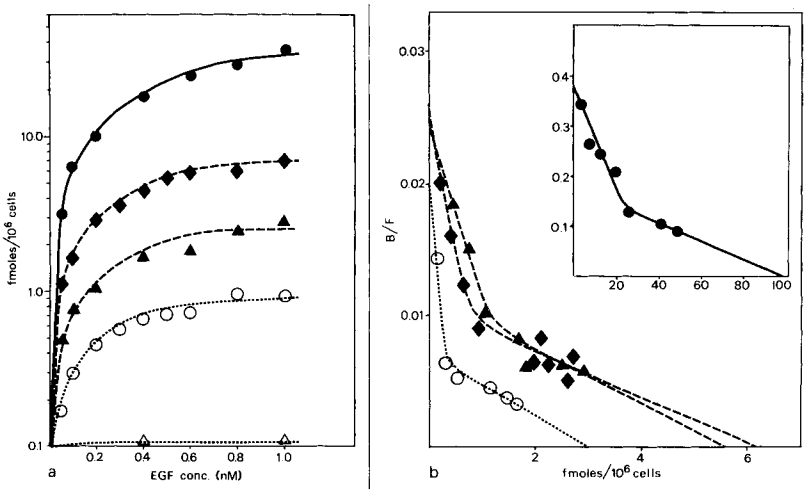

Fig. 1. Representative EGF binding curves (**a**) and Scatchard analyses (**b**). Cell lines: ●, SH-SY5Y/ACT; ◆, SH-SY5Y/VCR; ▲, MC-IXC/VCR; ○, SH-SY5Y; △, MC-IXC. Binding of [^{125}I]EGF to monolayer cells was measured at 22°C after a 1-hr incubation in the presence and absence of 100 nM cold EGF. The SH-SY5Y/ACT Scatchard plot (b) is shown in an inset. The expanded axes in the inset are proportional to the smaller-valued counterparts in the main figure (ratio of 25:1) in order to permit comparison of the slopes.

TABLE I. Characteristics of Multidrug-Resistant and Multidrug-Sensitive Human Neuroblastoma Cells

Cell line	ED_{50} (μg/ml)	Resistance increase	EGF B_{max} ± SEM (fmol/10^6 cells)[a]	Receptor no./cell	Receptor increase[b]	K_D (nM)
SH-SY5Y	(VCR)0.0039[c] (ACT)0.00070	1	2.4 ± 0.1	1,400	1	0.6
SH-SY5Y/VCR	5.5	1,420	6.9 ± 0.6	4,100	2.9 (5.1)	0.6
SH-SY5Y/VCR-U	0.031	8	1.1	700	0.5	0.6
SH-SY5Y/ACT	0.021	30	72 ± 7	43,000	30 (26)	0.9
SH-EP	0.00092	0.2	156 ± 97	91,000	65	0.6
MC-IXC	0.00026	1	0.3 ± 0.1	150	1	1.4
MC-IXC/VCR	5.7	21,920	3.6 ± 0.6	2,100	14 (15)	1.1

[a]Mean B_{max} ± the standard error of the mean (SEM) of low-affinity class receptors was calculated from four to five experiments per cell line, with one exception; the value for SH-SY5Y/VCR-U was derived from a single assay. Very low levels of specific binding were demonstrable in two of five experiments with MC-IXC cells.

[b]Ratio of B_{max} values and, in parentheses, of radioactivity in gel slices containing the immunoprecipitated EGF receptor of resistant:sensitive cells.

[c]VCR, vincristine; ACT, actinomycin D.

SY5Y, but which displays another differentiation phenotype [42], has even more available EGF receptors than the multidrug-resistant lines (Table I) [27,42]. That there are increased numbers of binding sites rather than altered affinity of receptor for EGF is indicated by the similarity in affinity constants calculated from slopes of the Scatchard plots. (The low affinity K_D values are listed in Table I.) Scatchard plots were generally curvilinear (Fig. 1b), as is observed with other types of cells [43]. Such plots are compatible with the presence of heterogeneous receptors with different but fixed affinities for ligand but

may also reflect the existence of occupancy-dependent interactions among sites [44]. High-affinity binding constants determined by computer analysis were 0.07 nM (SH-SY5Y), 0.11 nM (SH-SY5Y/VCR), 0.28 nM (SH-SY5Y/ACT), 0.06 nM (MC-IXC), and 0.15 nM (MC-IXC/VCR).

Immunoprecipitation, Affinity-Labeling, and Phosphorylation of EGF Receptor

Procedures that assay the structure and activity of the EGF receptor confirmed that resistant cells have increased amounts of functional receptor protein (Fig. 2). These procedures were: immunoprecipitation of [^{35}S]methionine-labeled receptor by monoclonal antibody 528 [30] (Fig. 2a, b, Table I); affinity-labeling of the receptor (Fig. 2c); and in vitro phosphorylation of immunoprecipitated EGF receptor (Fig. 2d). Immunoprecipitation studies also show increased receptor protein in SH-EP cells (Fig. 2b, lane 3).

Immunoprecipitation of receptor was conducted with cells labeled in the presence or absence of 50 nM EGF to verify the identity of the immunoprecipitated protein. Receptor amounts were quantitated by counting radioactivity in excised gel bands containing receptor protein (Table I). Down-regulation as a result of ligand binding may explain the apparent decrease in amount of radiolabeled EGF receptor in SH-SY5Y/ACT and SH-EP cells labeled in the presence of EGF (Fig. 2b, lanes 1,2 and lanes 3,4). As yet unexplained is the detection of more EGF receptor in SH-SY5Y cells labeled in the presence of EGF than in the absence (Fig. 2a, lanes 1, 5). Immunoprecipitation analysis of receptors in revertant SH-SY5Y/VCR-U cells produced a signal level similar to that for SH-SY5Y cells labeled in the absence of EGF (data not shown), thereby confirming the binding data.

Receptors immunoprecipitated from multidrug-resistant SH-SY5Y variants (closed arrows) consistently show a slightly lower apparent molecular weight, as judged by electrophoretic mobility, than receptors from control SH-SY5Y, SH-EP, or A431 cells (open arrows) (Fig. 2a, b). Immunoprecipitated MC-IXC/VCR receptor has the same M_r as receptor from SH-SY5Y resistant variants (data not shown). Receptor in MC-IXC cells was not detected by these techniques. Affinity-labeling of receptor with [^{125}I] EGF yields two bands from SH-SY5Y and SH-SY5Y/VCR (Fig. 2c). Only the upper component was observed for MC-IXC/VCR cells. The two bands in control SH-SY5Y samples are of nearly equal intensity whereas an unequal intensity between the two SH-SY5Y/VCR bands is observed. Phosphorylated receptor is seen as two bands in SH-SY5Y/VCR samples (Fig. 2d); the upper band (open arrow) is not observed in SH-SY5Y lanes.

Thus, three analytical methods demonstrate not only the presence of augmented numbers of receptors in resistant cells but also an apparent structural difference between the receptors of resistant and control cells.

Increased EGF Receptor Protein Is Not the Result of Gene Amplification

Because P-glycoprotein genes are known to be amplified in multidrug-resistant cell lines, including SH-SY5Y/VCR and MC-IXC/VCR [14], we tested the possibility that increased EGF receptor expression results from amplification of this gene as well. Southern hybridization analysis of DNA with a cloned EGF receptor gene probe, pE7 [22,36], revealed that receptor genes are not amplified ($\leq$twofold) in SH-SY5Y/VCR compared to SH-SY5Y or SH-SY5Y/VCR-U (Fig. 3a). The small increase in receptor DNA observed in SH-SY5Y/ACT cells (Fig. 3a, lane 5) is commensurate with the presence in these near-diploid cells of five copies of chromosome 7 as compared to three copies in the SH-SY5Y parent.

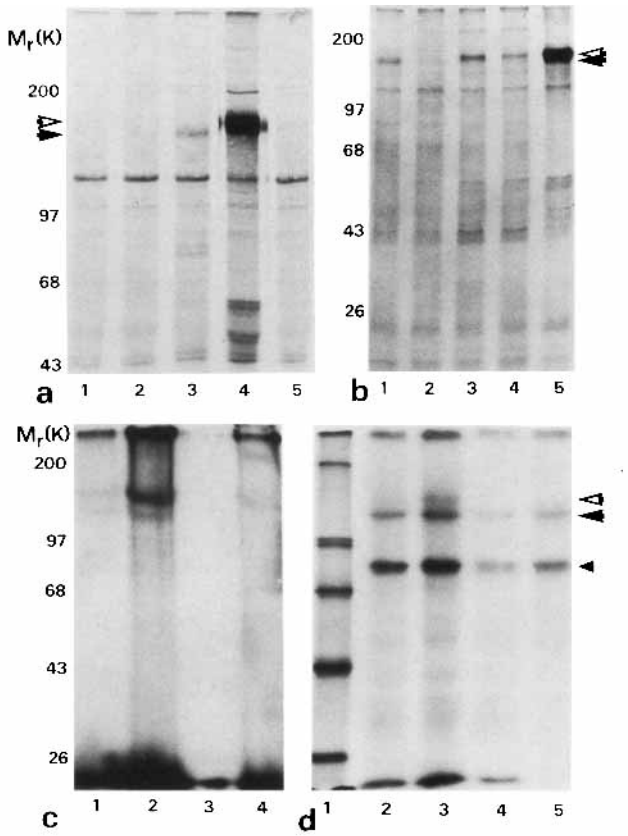

Fig. 2. Demonstration of increased EGF receptor protein amount in resistant variants by immunoprecipitation (**a,b**), affinity-labeling (**c**), and phosphorylation of receptor immunoprecipitates (**d**). Photographs depict representative autoradiograms of proteins examined by $NaDodSO_4$-polyacrylamide gel electrophoresis on 7.5% (0.075 × 14 × 11 cm) acrylamide gels. **a:** Lane 1, SH-SY5Y; lane 2, SH-SY5Y/VCR; lane 3, SH-SY5Y/ACT; lane 4, A431; lane 5, SH-SY5Y labeled in the presence of 50 nM EGF. Monoclonal antibody 528 raised against A431 cell EGF receptor was a gift from Dr. Gordon Sato. **b:** Lane 1, SH-SY5Y/ACT; lane 2, SH-SY5Y/ACT labeled in the presence of 50 nM EGF; lane 3, SH-EP; lane 4, SH-EP labeled in the presence of 50 nM EGF; lane 5, A431. **c:** Lane 1, SH-SY5Y; lane 2, SH-SY5Y/VCR; lane 3, MC-IXC; lane 4, MC-IXC/VCR. Cross-linking of [^{125}I]EGF (1 nM) to its receptor on cells growing in monolayers was carried out according to published procedures with the use of 1-ethyl-3-(dimethylaminopropyl)carbodiimide as cross-linking agent [32]. **d:** Phosphorylation of immunoprecipitated EGF receptor from SH-SY5Y (lane 2), SH-SY5Y/VCR (lane 3), MC-IXC (lane 4), and MC-IXC/VCR (lane 5) cells with [γ-^{32}P]ATP was accomplished according to procedures described by Carlin and Knowles [35]. Lane 1 contains molecular weight marker proteins. The prominent phosphoprotein band of 80 kilodaltons in Figure 2d (small arrowhead) has not been identified, although the higher intensity of label in resistant cells compared to control suggests an association with EGF receptor and drug resistance. Open and closed arrows indicate forms of EGF receptor.

Increased Receptor RNA Expression Is the Basis of Increased EGF Receptor Number

The increase in amount of receptor protein in resistant cells correlates with increased receptor mRNA amount, as demonstrated by Northern and cytodot hybridization analyses with the pE7 probe (Fig. 3b, c). There is a low level of EGF receptor RNA in SH-SY5Y cells, about a twofold reproducible increase in amount in SH-SY5Y/VCR, and

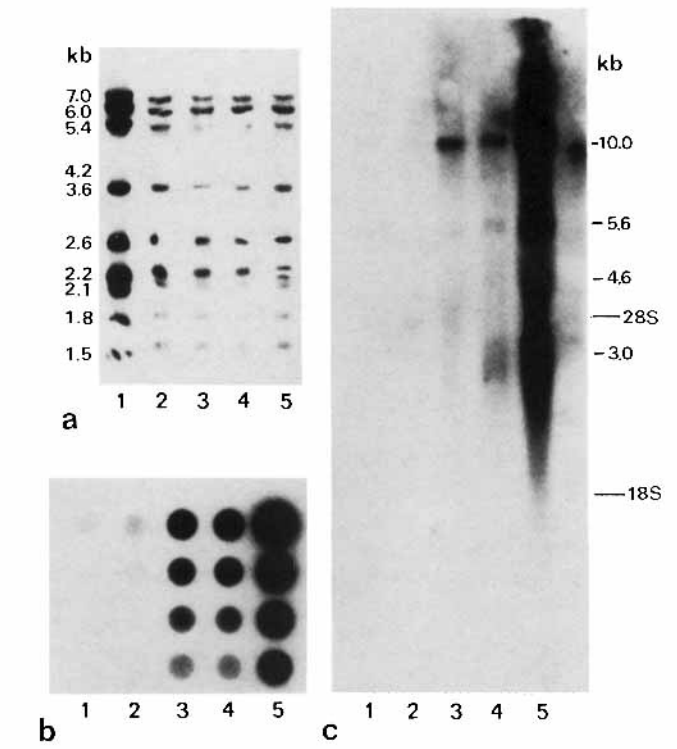

Fig. 3. Southern, cytodot, and Northern analysis of DNA and RNA with pE7 [22,36], a cloned EGF receptor probe provided by Dr. Ira Pastan. **a**:Twenty micrograms of genomic DNA from each of the cell lines was digested with EcoR1 and separated on 1% agarose gels. The lanes show DNA from (1) A431, (2) SH-SY5Y, (3) SH-SY5Y/VCR, (4) SH-SY5Y/VCR-U, and (5) SH-SY5Y/ ACT. Hind III-digested lambda DNA was used as a size marker. **b**: Cytodot analysis of total cellular RNA from (1) SH-SY5Y, (2) SH-SY5Y/VCR, (3) SH-SY5Y/ACT, (4) A431 (one-tenth amount of RNA), and (5) A431. Except for lane 4, RNA amounts ranged from 4 μg in the top row to 0.5 μg in the bottom row. **c**: Twenty or 2 μg of RNA were fractionated on 1% agarose/formaldehyde gels and then transferred to nitrocellulose [40]. Northern analysis of RNA from (1) SH-SY5Y (20 μg), (2) SH-SY5Y/VCR (20 μg), (3) SH-SY5Y/ ACT (20 μg), (4) A431 (2 μg), and (5) A431 (20 μg). Sizes of A431 RNA transcripts are reported values [22].

an apparent 32-fold increase in receptor RNA in SH-SY5Y/ACT. The latter cells contain about one-tenth the level of receptor RNA in control A431 cells (Fig. 3b). The size of the message in SH-SY5Y/ACT and SH-SY5Y/VCR cells was determined by Northern hybridization analysis to be the same as the complete transcript in A431 cells (Fig. 3c). The 3.0-kb fragment in A431 cells, encoding a truncated, secreted form of EGF receptor [45], is not present in SH-SY5Y resistant variants. The level of increase of receptor RNA transcripts (about twofold in SH-SY5Y/VCR and about 30-fold in SH-SY5Y/ACT) is directly proportional to the observed increases in ligand binding and immunoprecipitated receptor protein in resistant cells. Receptor mRNA is increased in SH-EP cells as well (unpublished observations).

DISCUSSION

Increased EGF binding to the multidrug-resistant human neuroblastoma cells is due to increased numbers of EGF receptor molecules resulting, in the two resistant sublines examined, from increased amounts of receptor mRNA, but not from amplification of the receptor gene.

Immunoprecipitation studies indicate that the increased levels of EGF binding and the increased amounts of specific mRNA are associated with an increase in EGF receptor protein in the resistant cells. In these experiments, decreased precipitable receptor in cells metabolically labeled in the presence of EGF can be interpreted as receptor down-regulation (Fig. 2b). The cross-linking experiments (Fig. 2c) indicate possible differences in receptor type between SH-SY5Y/VCR and MC-IXC/VCR. SH-SY5Y and SH-SY5Y/VCR cells contain two cross-linked species, MC-IXC/VCR cells only one. The two bands in SH-SY5Y and its drug-resistant subline may represent different affinity types (represented in differing amounts in the two cell lines), a possibiltiy to be examined in the future. Two receptor types are not visualized by immunoprecipitation. The two phosphorylated receptor bands in SH-SY5Y/VCR (Fig. 2d, lane 3) may also represent two affinity types. Presumably, the upper cross-linked band (Fig. 2c, lane 2), the upper phosphorylated band (Fig. 2d, lane 3), and the immunoprecipitated band (Fig. 2a, lane 2) in SH-SY5Y/VCR samples represent the same species, although this is not certain. Overall, the results of the experiments depicted in Figure 2 demonstrate that the receptor molecules in resistant cells are functional in terms of specific known properties of EGF receptor. The full nature and variety of these properties and whether increased receptor amount results in increased EGF responsiveness are areas of future study.

What role the increased number of EGF receptors plays in the development or maintenance of the multidrug-resistant phenotype is not clear. Perhaps receptor increase is a compensatory response to altered or deficient nutrient transport in resistant cells. Clearly there is no direct correspondence between level of resistance and degree of receptor increase, suggesting a lack of direct effect of receptor modulation on resistance development. That the two phenomena are associated, however, is dramatically supported by the finding of decreased receptors in revertant cells (Table I).

The observed increase in EGF receptor expression may be associated with the altered state of transformation exhibited by many multidrug-resistant cells. Support for this idea is found in previous studies of resistant Chinese hamster lung and mouse tumor cells all of which manifest a reverse transformed phenotype and express elevated levels of EGF receptor when compared to parental control lines [11]. SH-EP cells also display a less transformed phenotype than SH-SY5Y as judged by decreased tumorigenic potential in nude mice and decreased plating efficiency in soft agar [27]. Future studies of the human multidrug-resistant cells will include investigation of whether they also become reverse transformed. The investigation will include analysis of transforming growth factor-α (TGF-α) synthesis and secretion. TGF-α competitively binds to and causes down-regulation of EGF receptor [46]. It is possible that the tumorigenic SH-SY5Y and MC-IXC cells synthesize more TGF-α or other EGF-like factors than drug-resistant sublines and bind less EGF for that reason.

On the other hand, increased EGF receptor may play a role in the differentiation phenotype as observed in a comparison of SH-EP and SH-SY5Y cells [42,47]. SH-EP is a flat, substrate-adherent cell that expresses tyrosinase (a melanocyte marker) but not tyrosine hydroxylase (a neuronal cell marker) [48]. SH-SY5Y cells are poorly substrate-

adherent and neuroblast-like and express tyrosine hydroxylase. SH-EP and SH-SY5Y cells can undergo morphological and biochemical interconversion [47], which has been shown to represent an ordered transition between two neuroectodermal differentiaion programs [42]. Whether multidrug-resistant human neuroblastoma cells display differentiation characteristics distinct from their drug-sensitive counterparts is a question for future investigation.

The basis of the apparent qualitative difference between resistant and sensitive cell EGF receptors is not known. Whether the difference reflects a primary translation product alteration or posttranslational modification and whether the difference has functional signficance are subjects of future study. Of possible relevance is the recent report [49] that EGF receptor from EGF-treated A431 cells had a slightly lower mobility on gels than receptor from untreated control cells, presumably owing to its increased phosphorylation.

Zuckier and Tritton [50] have demonstrated that treatment of HeLa and 3T3 cells with adriamycin (a drug to which the resistant sublines described in this report are cross-resistant) results in an increase in EGF receptor in those cells after a 3- or 4-day exposure. This result was reversible over a similar period. The authors suggest that this response may be related to the mechanism of cytotoxic action of the drug. This finding supports the thesis that EGF receptor expression is affected by drugs such as vincristine and adriamycin and that elevated receptor levels may play a role in some aspect of the resistant phenotype.

This report expands on the original study of the phenomenon of EGF receptor increase in multidrug-resistant cells [11] by (a) increasing the number of sublines in which the phenomenon is found, (b) showing that human as well as hamster and mouse sublines can undergo the modulation, and (c) demonstrating that the increase in receptor number is the result of increase in steady state amount of receptor RNA rather than of gene amplification. Further investigation of EGF receptor in resistant lines may lead to new information about mechanisms or consequences of resistance development. In any event, the use of EGF receptor antibody as a cytotoxic agent for receptor-positive cells has been reported [51]. If resistant tumor cell populations in some patients manifest EGF receptor increase (possibly of a type qualitatively distinguishable from other EGF receptors), specific monoclonal antibodies could be prepared for selective recognition and removal of resistant cells in patients. The possibility then exists that the findings reported here are potentially therapeutically exploitable, either directly or through a more complete understanding of mechanisms of multidrug-resistance development.

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