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Advances in Brief

Hepatotoxicity in Cancer Patients Receiving erb-38, a Recombinant Immunotoxin That Targets the erbB2 Receptor

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Abstract

To exploit overexpression of erbB2 in human cancers, we constructed a single-chain immunotoxin (erb-38) that contains the Fv portion of monoclonal antibody e23 fused to a truncated form of *Pseudomonas* exotoxin A. In a Phase I study, five breast cancer patients and one esophageal cancer patient received three doses of erb-38 at 1.0 and 2.0 μg/kg. Hepatotoxicity was observed in all patients. Immunohistochemistry showed the presence of erbB2 on hepatocytes explaining the liver toxicity of erb-38. We suggest that targeting of tumors with antibodies to erbB2 armed with radioisotopes or other toxic agents may result in unexpected organ toxicities due to erbB2 on normal cells.

Introduction

erbB2/HER2/neu encodes an M_r 185,000 cell membrane glycoprotein with tyrosine kinase activity. Overexpression of the erbB2 protein and its inherent tyrosine kinase activity causes loss of growth control and has an important role in the development of breast cancer and several other human cancers (1–3). erbB2 has been reported previously to be minimally expressed in normal adult tissues (4). For this reason, erbB2 is an attractive target for antibody-directed therapies. To exploit the overexpression of erbB2 in many human cancers (breast, stomach, lung, and ovary), we made a single-chain immunotoxin erb-38 using MAb[2] e23 (5), which reacts with erbB2.

erb-38 is a M_r 63,000 recombinant protein composed of the Fv portion of MAb e23 fused to PE38, a truncated form of PE with a molecular weight of 38,000. MAb e23 was selected from a panel of antibodies to erbB2 because it produced very active conventional immunotoxins when it was chemically linked to PE (6). PE is a M_r 66,000 protein secreted by *Pseudomonas aeruginosa*. PE is composed of three major structural domains (7): an NH_2-terminal cell binding domain (domain Ia), a central translocation domain (domain II), and a COOH-terminal catalytically active domain (domain III). Domain III catalyzes the ADP ribosylation and inactivation of elongation factor 2, which inhibits protein synthesis and leads to cell death. Deletion of domain Ia of PE (amino acids 1–252) and part of domain Ib produces a M_r 38,000 protein (PE38) that has low cellular and animal cytotoxicity because it cannot bind to PE-specific cellular receptors. The gene encoding PE38 was fused to a gene encoding the Fv portion of MAb e23 to form erb-38 [e23(dsFv)PE38]. In this recombinant immunotoxin, the Fv fragment contains a disulfide bond connecting the light and heavy chains of MAb e23 (8). The IC_{50} of erb-38 is 0.2–4 ng/ml on the various tumor cells lines that express the erbB2 antigen. erb-38 is capable of causing complete remission in nude mice bearing epidermoid carcinoma (A431) and breast cancer (MCF-7; Ref. 9). Preclinical toxicity experiments indicated that the LD_{50} of erb-38 in mice was 450 μg/kg every other day times three doses. Death was caused by acute hepatic necrosis. Hepatic and renal injury was observed in cynomolgus monkeys at three doses of ≥500 μg/kg.[3]

On the basis of the biological activity and safety testing results, we concluded that erb-38 had excellent antitumor activity and acceptable animal toxicities. We initiated a Phase I study of erb-38 in patients with advanced carcinoma who had failed standard therapy.

Patients and Methods

Eligibility. Adult patients with metastatic carcinoma known to express erbB2 were eligible for this study. Tumors were considered positive if they expressed erbB2 on ≥30% of the tumor cells, as determined by immunohistochemistry on formaldehyde-fixed, paraffin-embedded tumor blocks. Tumor histology was confirmed by a NIH pathologist. Other eligibility criteria included: advanced unresectable disease, failed conventional therapy, Eastern Cooperative Oncology Group performance status of 0 or 1, AST and ALT levels of <1.5 times the upper limits of normal, total bilirubin levels within normal limits, absolute granulocyte counts of >2,000/mm^3, and platelet counts of >100,000/mm^3. Patients with positive hepatitis B antigen, history of any other prior liver disease (*e.g.*, alcohol liver disease), central nervous system metastasis, or seizure disorders were excluded. Patients with positive antibodies to erb-38 were excluded. The clinical protocol and the consent form were approved by the Institutional Review Board of the NCI. Prior to therapy, patients underwent complete physical examination,

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[2] The abbreviations used are: MAb, monoclonal antibody; PE, *Pseudomonas* exotoxin A; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NCI, National Cancer Institute; MTD, maximum tolerated dose.

[3] Unpublished data.

Table 1 Patient characteristics

Patient no.	Age/sex	Diagnosis	erbB2 expression (%)	Prior therapy	Sites of metastasis
1	49/F	Breast cancer	80	XRT[a], Doxo, cyclophosphamide, paclitaxel	Lung, pleura, bone, skin, LN
2	36/F	Breast cancer	80	Surgery, XRT, CMF, Doxo, 5-FU, taxotere, navalbine	Pleura, pericardium, skin, LN
3	58/M	Esophageal cancer	80	XRT, cisplatin, paclitaxel	Liver, lung, LN
4	35/F	Breast cancer	80	Surgery, CAF, Taxol, Ctx, etoposide/melphalan (PBSC)	Lung, skin, LN
5	57/F	Breast cancer	100	Surgery, XRT, CAF, TAM, arimidex, magace, cyclophosphamide/novantrone/5-FU Cyclophosphamide/thiotepa/carboplatin (PBSC)	LN
6	40/F	Breast cancer	100	Surgery, XRT, CAF, paclitaxel, Etoposide/melphalan (PBSC)	Lung, bone, skin

[a] XRT, radiation therapy; Doxo, doxorubicin; CMF, cyclophosphamide, methotrexate and 5-FU; 5-FU, 5-fluorouracil; CAF, cyclophosphamide, Doxo, and 5-FU; Ctx, cyclophosphamide; PBSC, peripheral blood stem cell support; TAM, tamoxifen; LN, lymph node.

Table 2 Dosing and drug-related toxicity

Patient no.	Dose (μg/kg)	Toxicity grade		
		AST/ALT	Nausea/vomiting	Skin rash
1	2.0	2		
2	2.0	3		2
3	2.0	2	1	
4	2.0	3		
5	1.0	3		
6	1.0	3	3	

complete blood count, determination of chemistry profile, urinalysis, chest X-ray, electrocardiogram, tumor staging work-up, and tumor measurement. Informed consent was obtained from all patients.

erb-38. erb-38 (NSC 683039) was produced and placed in vials by NCI-Frederick Cancer Research & Development Center. erb-38 was supplied through the Cancer Therapy Evaluation Program, NCI, under Investigational New Drug No. 7373. erb-38 in phosphate buffer solution was supplied as a sterile solution at 0.5 mg/ml in 2-ml vials containing 1.0 mg of erb-38. Test and treatment doses were diluted in 0.9% normal saline and 0.2% albumin.

Trial Design. erb-38 was administered by i.v. infusion over 30 min on days 1, 3, and 5. All patients received a 10-μg test dose on day 1 given as a bolus over 2 min. The second and third doses were delayed or withheld if any measure of toxicity was not less than grade II on the scheduled day of administration. A cycle could be repeated every 28 days, provided that the patient had not developed neutralizing antibodies against erb-38 and had no disease progression. Patients were to be entered in groups of three. Dose escalation was to based on the modified Fibonacci series.

While on study, patients were followed with complete blood count, blood chemistry and urine. Tumor imaging was repeated 1 month after therapy. Toxicity and response to treatment were graded by the NCI Common Toxicity Criteria. Limiting toxicity was defined as any grade III or grade IV toxicity.

Pharmacokinetics. Blood samples were collected at the following times for pharmacokinetics: pretreatment; 2, 15, 30, 45, 60, and 90 min; and 2, 4, 8, 12, 24, and 48 h. After the second and third doses of erb-38, samples were collected at 2 min, 30 min, and 4 h. The concentration of erb-38 was determined by incubating various dilutions of serum with N87 cells, a gastric cancer cell line known to express erbB2, and measuring its ability to inhibit protein synthesis. A standard curve was used to determine the amount of erb-38 in each sample. Data were weighted inversely, and the fit program used was RSTRIP (MicroMath Scientific Inc., Salt Lake City, UT).

Immunogenicity of erb-38. Antibodies against erb-38 were assessed pretreatment, on day 21, and then bimonthly thereafter by a serum neutralization assay: erb-38 was added to serum samples at 0.0 (control), 0.1, 0.5, and 1.0 μg/ml and incubated at 37°C for 15 min. The activity of erb-38 was assayed by incubating the samples with MCF-7 cells and measuring its ability to inhibit protein synthesis. A standard curve with erb-38 was used to determine IC_{50}. A serum sample was considered negative for antibodies against erb-38 when the cytotoxic activity of erb-38 was not neutralized when incubated with sera at concentrations of >0.1 μg/ml erb-38.

Immunohistochemistry of Normal Liver. Fresh normal human liver samples were frozen by immersion in liquid nitrogen. Cryostat sections (~6 μm) were thaw-mounted on slides and fixed in 3.7% formaldehyde in PBS for 10 min at 23°C, followed by washing in PBS. Sections were not treated with H_2O_2 or any organic solvents. After blocking in 1% BSA in PBS for 10 min, sections were incubated in mouse monoclonal anti-erbB2 (CB11; Novocastra Laboratories, Novocastra antibody, Vector Laboratories, Burlingame, CA) at 10 μg/ml in BSA-PBS for 30 min at 23°C. Alternatively, sections were incubated in affinity-purified rabbit anti-erbB2 (code A 0485; Dako Corporation, Carpinteria, CA) at 7 μg/ml in BSA-PBS. After further washing in PBS, sections were incubated, as appropriate, with either goat antimouse IgG or goat antirabbit IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch) at 25 μg/ml in BSA-PBS for 30 min. Peroxidase was detected using diaminobenzidine (1 ng/ml) with 0.01% H_2O_2 in PBS for 10 min. Controls included deletion of the first antibody step. Sections were mounted using dehydration and Permount without further counterstains and viewed using bright-field microscopy.

Results

Six patients were enrolled in this trial. Patient characteristics are shown in Table 1. The initial dose level was 2 μg/kg times three, which was 1/250 of the MTD in monkeys and 1/225

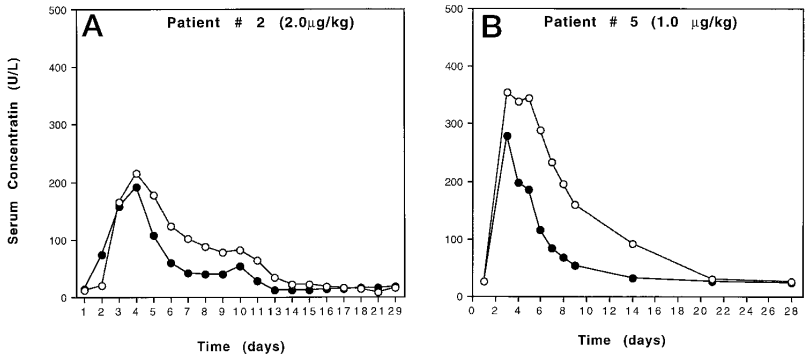

Fig. 1 Time course of AST (○) and ALT (●) of patient 2, who received 2.0 μg/kg (*A*) and patient 5, who received 1.0 μg/kg erb-38 (*B*).

of the LD_{50} in mice. This conservative starting dose was chosen due to the fact that the toxicity of immunotoxins in humans has thus far been found to be unpredictable. Furthermore, 2.0 μg/kg times three has been shown to be safe with two other recombinant immunotoxins made with PE (LMB-7 and LMB-2).[3]

Toxicity. Transient elevation of AST and ALT was observed in all patients. At 2.0 μg/kg, two patients had grade II and two patients had grade III elevations of AST and ALT. The toxicity observed in the first cohort prompted us to amend the protocol to decrease the dose by 50%. Two patients were treated at the 1.0 μg/kg dose level. Both patients had a grade 3 transaminase elevation after one cycle of erb-38 (Table 2).

Increases in AST and ALT usually occurred within 24 h after the first or second dose of erb-38, and peak levels were reached 24–48 h after the last dose. Two patients required a 50% dose reduction for the third dose (patients 2 and 6), and in two patients, the third dose was held (patients 4 and 5). Hepatic transaminases returned to baseline levels 14–21 days following therapy. Fig. 1 shows the time course of AST and ALT of patient 2, who received 2.0 μg/kg erb-38, and patient 5, who received 1.0 μg/kg erb-38. There was no evidence of liver enlargement or right upper quadrant tenderness on exam. Two patients had symptoms of nausea and vomiting during therapy, with unclear relation to the hepatic injury. No significant drug-related changes in bilirubin, alkaline phosphatase, serum albumin, or prothrombin time were observed. Other erb-38-related toxicities include a skin rash in one patient 4 days after erb-38 and nausea and vomiting in two patients.

As expected in a group of patients with advanced carcinoma, several untoward events occurred while the patients were on study: pain ($n = 3$), malignant pleural effusion ($n = 2$), malignant pericardial effusion ($n = 1$), catheter infection ($n = 2$), venous thrombosis ($n = 1$), and proteinuria ($n = 1$). One patient (patient 6) died of acute respiratory failure in her hometown 10 days after discharge, due to pulmonary emboli. An autopsy was not performed. These events were attributed to the patient's underlying disease and not related to erb-38.

Localization of erbB2 in Normal Liver. Although prior literature had suggested that erbB2 was not expressed in normal liver (4), we used a sensitive immunohistochemical method with antibodies to erbB2 that were not available until recently. Rapidly frozen normal human liver samples were sectioned with a cryostat and postfixed using formaldehyde but were not exposed to H_2O_2 or any organic solvents. Mouse monoclonal CB11 and rabbit polyclonal A0485 were used for the detection of erbB2 using affinity-purified antiglobulins labeled with horseradish peroxidase. When viewed without counterstains, the localization of erbB2 to the sinusoidal surface of normal hepatocytes was clearly visible using either primary antibody (CB11 and AD485; Fig. 2). The same result was observed from samples of normal liver derived from at least three different patients. Similar results were also obtained with antibody E23 (data not shown).

Pharmacokinetics. Blood samples were collected for pharmacokinetics in all patients. erb-38 is cleared monoexponentially from the circulation, with a $T_{1/2}$ that varies from 2.4 to 10.3 (mean, 3.6 h). A high degree of variation was observed from patient to patient. The peak concentration achieved at the end of infusion ranged from <20 ng/ml (patient 1) to 105 ng/ml (patient 3). At 1.0 μg/kg, the peak plasma levels were 38.1 and 39.4 ng/ml. The volumes of distribution of central compartment and area under the concentration *versus* time curve were found to be 28.0 ± 8.6 ml/kg and 248.7 ± 139.6 ng/ml·h, respectively. Although full pharmacokinetic analysis was not possible due to limited sampling after second and third doses, clearance rates appeared to be similar to that after the first dose.

Immunogenicity. Patients had no neutralizing antibodies against erb-38 prior to therapy, as required by protocol. At the end of one cycle, only one patient (patient 3) developed neutralizing antibodies (>1.0 μg/ml) against erb-38 14 days after treatment. Retreatment was not possible in this patient, due to disease progression.

Response. No objective responses were observed in the trial. One patient had stable disease for 3 months, four patients progressed after one cycle of therapy, and one patient was not evaluable for response. Patient 2 had improvement of chest wall pain after erb-38 that lasted for 3 weeks. Fentanyl transdermal patch requirement decreased from 225 to 25 μg/h in this patient.

Discussion

We have shown that, when a recombinant immunotoxin, erb-38, which targets erbB2, is given to cancer patients, it causes hepatic injury, manifested by transient elevation of transami-

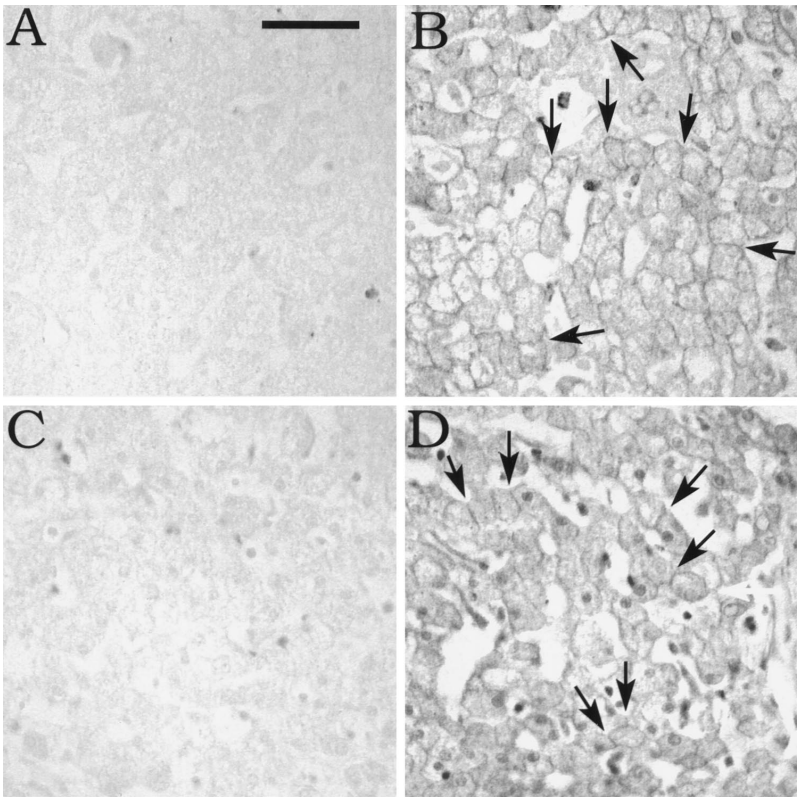

Fig. 2 Localization of erbB2 to the surface of normal human hepatocytes. Frozen sections of normal human liver were incubated with either a mouse MAb to erbB2 (CB11; *B*) or a rabbit polyclonal affinity-purified anti-erbB2 (A0485; *D*). Corresponding controls with deletion of the first step but with the second step included are shown for antimouse IgG (*A*) or antirabbit IgG (*C*). The characteristic pattern, especially at intercellular junctions, of the sinusoidal surface of hepatocytes is evident (*arrows*) with both antibodies (*B* and *D*). These sections were not counterstained, and the nuclear localization seen in *D* represents a nonspecific pattern seen only with the rabbit anti-erbB2 antibody. (Magnification, ×375; *scale bar*, 50 μm).

nases. The dose-limiting toxicities of erb-38 occurred at 1.0 and 2.0 μg/kg when it was given i.v. every other day times three. These doses are significantly below the doses necessary to show antitumor effects in animals. Although the MTD was not determined, based on our preclinical experience with this agent, effective serum levels cannot be achieved at doses <1.0 μg/kg (6). In addition, we found that the erb-38 was cleared monoexponentially from the circulation, with a $T_{1/2}$ that varied from 2.4 to 10.3 h. As compared with other biological agents of this nature, a great deal of variation was observed from patient to patient. At 2.0 μg/kg, the maximum concentration varied from <20 to 105 ng/ml. No clear correlation can be made between the pharmacokinetic findings and the tumor burden, toxicity, or expression of erb-38 on tumor cells (all patients had tumors that expressed erbB2 on ≥80% of the cells).

The toxicity of erb-38 is most likely due to the presence of erbB2 on hepatocytes, not detected by immunohistochemical staining in earlier publications (4). We have now repeated these assays using newly available antibody to erbB2 (affinity-purified rabbit anti-erbB2, code A 0485; Dako Corp.) and have shown here that erbB2 is clearly present on the surface of normal hepatocytes. The expression of erbB2 is known to be very high in ~30% of breast cancers, often due to gene amplification. Despite the fact that there is a very large difference in the amount of erbB2 on the surface of cancer cells relative to the small amount present on liver cells, liver toxicity was the first biological effect seen in this study. Several factors contribute to this finding. One is that hepatocytes are more rapidly exposed to agents injected into the circulation than tumor cells. There is a barrier to proteins entering tumors because tumors do not have lymphatics and there is no connective flow. As a consequence, mixing within tumors is solely by diffusion and, therefore, very slow. In addition, tumors are often poorly vascularized (10).

There are several ways in which antibodies are being used as antitumor agents. Antibodies have been used by themselves to produce antitumor activity, taking advantage of their ability to carry out complement-mediated cell killing or ADCC (antibody dependent cell- and complement-mediated cytotoxicity) or to induce apoptosis directly. One or more of these effects explains how Rituximab, which binds to CD20, causes regression of lymphomas (11). Recently, clinical data concerning the safety and efficacy of a humanized antibody to erbB2 termed Herceptin were presented (12, 13). The antibody alone has been found to produce objective responses in breast cancer and when combined with chemotherapy results in an increased response rate. It is likely that the antitumor activity of the antibody in this

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