Effects of Anti-erbB-2 (HER-2/neu) Recombinant Oncotoxin AR209 on Human Non-Small Cell Lung Carcinoma Grown Orthotopically in Athymic Nude Mice

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ABSTRACT

The recombinant oncotoxin AR209 [e23(Fv)PE38KDEL; formerly OLX-209] was developed to treat neoplasia that expresses the c-erbB-2 (HER-2/neu) protein product p185erbB-2. The AR209 compound contains a single-chain antibody domain specific for p185erbB-2, coupled with a portion of the Pseudomonas exotoxin. The drug has been shown to be effective in inhibiting cells that overexpress erbB-2 due to gene amplification and in cells that do not contain amplified erbB-2 but express slightly higher levels of the protein than normal cells do. To test the efficacy of AR209 on human lung tumors, athymic nude mice were inoculated intrathoracically with a cell line derived from a poorly differentiated lung adenocarcinoma. This cell line, termed 201T, expresses moderately elevated levels of p185erbB-2 7.6-fold over normal bronchial epithelium. Mice treated with i.v. injections of AR209 for 5 weeks after orthotopic tumor implantation had smaller tumors and in 20% of cases showed no evidence of disease. The data from this study indicate that AR209 may be an effective treatment for patients with non-small cell lung cancers that express p185erbB-2.

INTRODUCTION

Lung cancer is the leading cause of death from neoplasia for both men and women in the United States (1, 2). Because of the large number of aging smokers and ex-smokers in the U.S. population, the incidence of lung cancer will remain high for the next 20–30 years, despite the overall decline in smoking (2–5). Current therapies for lung cancer are not adequate; the 5-year survival rate is only about 13% for all lung cancer patients regardless of stage (1), a rate unchanged in 50 years. NSCLC accounts for about 75% of all lung neoplasia and is not responsive to conventional chemotherapy or radiotherapy (6, 7).

Modern experimental therapeutics have attempted to exploit recently acquired knowledge of the biology of lung cancer (8). The proto-oncogene c-erbB-2 (HER-2/neu) codes for a Mr 185,000 transmembrane glycoprotein (p185erbB-2) that is a membrane-bound receptor with tyrosine kinase activity (9). The c-erbB-2 protein product has significant homology with the epidermal growth factor receptor, especially to a small section of the extracellular domain, the transmembrane domain, and the entire cytoplasmic domain, excluding the final 32 carboxy-terminal residues (10). Approximately 30% of human lung cancer cell lines express detectable levels of the erbB-2 protein product that are higher than the levels observed in normal lung epithelia (11, 12). In one report from China, 50% of primary NSCLC expressed p185erbB-2 (13). Overexpression of erbB-2 has been observed in a subset of NSCLC (primarily in adenocarcinoma), but not in small cell lung cancer. High levels of p185erbB-2 have been linked to shortened survival (12) and are associated with intrinsic multiple drug resistance (14). Frequent overexpression of erbB-2 has also been observed in carcinoma of the breast (15–17), stomach (18), and ovaries (17).

We have previously demonstrated that the recombinant oncotoxin AR209 (formerly OLX-209) is not only effective in targeting cells that bear large quantities of surface p185erbB-2 (19) but is also effective in targeting cell lines without gene amplification of c-erbB-2 (20). In AR209 [e23(Fv)PE38KDEL], the specificity of an anti-erbB-2 antibody contained within a single-chain antibody domain (e23Fv) was coupled with a portion of Pseudomonas exotoxin (PE38KDEL), which has the ability to kill cells through inactivating the synthesis of cellular proteins (19). The drug has previously been tested using s.c. injections of tumor cells in athymic nude mice (20). Although mice injected with AR209 showed a significant reduction in the size of tumors, the human lung cancer cells were grown s.c. on the backs of nude mice; this site probably does not approximate the microenvironment of a de novo tumor in human patients. According to the “seed and soil” hypothesis of Paget (21), organ site-specific implantation of tumor cells is essential for optimal growth and progression of xenografts in vivo. A comparison of orthotopic and s.c. models showed that NSCLC tumors implanted intrathoracically into nude mice were almost universally fatal (92%), whereas those implanted s.c. had minimal effect (5%; Ref. 22). It has been proposed that the lack of an optimal animal model has impeded the development of new treatment.

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4 The abbreviations used are: NSCLC, non-small cell lung carcinoma; FBS, fetal bovine serum; t.t., intrathoracic.
modalities (23, 24). In this study, we have implanted a poorly differentiated human adenocarcinoma of the lung orthotopically into nude mice. Subsequent treatment of animals with i.v. injections of AR209 significantly reduced the growth of i.t. tumors and resulted in the cure of 20% of animals.

**MATERIALS AND METHODS**

**Cell Lines and Culture Conditions.** Human tumor cell line 201T was established from a poorly differentiated human lung adenocarcinoma using the method of Siegfried and Owens (25). This cell line has been described previously (20, 26, 27). Cells were cultivated in culture medium conditioned by the bronchioloalveolar carcinoma cell line A549 (28). Culture medium was produced by blending conditioned basal Eagle’s medium (Life Technologies, Inc., Grand Island, NY) supplemented with 1% FBS (HyClone Laboratories, Logan, UT) with fresh Ham’s F-12 medium (Life Technologies) supplemented with 1% FBS. The basal Eagle’s medium was conditioned for 48 h on a confluent monolayer of A549 cells in an 850-cm² roller bottle (100 ml medium/bottle). Medium was sterilized by filtration through a 0.22-μm filter. Cells were maintained in humidified incubators at 37°C in an atmosphere of 7.5% CO₂. 201T cell monolayers approaching 75% confluency were harvested using the De Larco formulation of trypsin-EDTA (Life Technologies) and were subcultured for serial passage. For orthotopic implantation, cells were isolated by centrifugation at 500 × g for 15 min, washed once in medium supplemented with 1% FBS, and washed an additional two times with PBS (Life Technologies). Cells were suspended in HBSS (Life Technologies). For injections, tumor cells were mixed with a Matrigel basement membrane matrix (Becton Dickinson Labware, Bedford, MA; Refs. 29 and 30) and Omnipaque (Sanofi Winthrop Pharmaceuticals, New York, NY) at a ratio of 1:1:1, to a final concentration of 2 × 10⁶ cells/0.15 ml. The Omnipaque was added for visualization of i.t. injections by fluoroscopy.

**Experimental Animals.** Specific pathogen-free, 4–6-week-old female nude mice obtained from Harlan-Sprague-Dawley (Indianapolis, IN) were housed in sterilized, filter-topped cages kept in laminar flow isolators (Forma Scientific). All studies were approved by the Louisiana State University Medical Center Institutional Animal Care and Use Committee.

**Tumor Cell Implantation.** Animals were fully anesthetized in a Harvard small animal anesthesia chamber using nebulized Metaflane (Pitman Moore, Inc., Washington Crossing, NJ). i.t. injections were performed at the lateral dorsal medullary line just below the inferior border of the scapula using a 1.2-cm, 27-gauge needle under fluoroscopic guidance, as described below (Fig. 1; Ref. 22). The needle was advanced through the chest wall into the lung tissue, and the tumor cell inoculum of 2 × 10⁶ cells was then dispersed in a final volume of 0.15 ml HBSS/Matrigel/Omnipaque (1:1:1 ratio). The procedure requires approximately 1 min to complete and is the most accurate described to date. Animals were allowed to recover from anesthesia for approximately 10 min under heat lamps to maintain body temperature.

**Tumor Development and Drug Therapy.** Take rates were measured in the mice by determining the fraction of mice implanted with tumor cells that had evidence of gross tumors after 3 weeks. This time point has been shown to allow for the development of a sizable (>0.3 g) tumor with 201T cells. The mice were inspected five times/week for evidence of tumor development. To determine whether tumor development was subsequently followed by regression, each animal was radiographed (see below) at 3 weeks after implantation and thereafter every 2–3 weeks. Therapy with AR209 was initiated on day 21 after implantation. The mice were randomly segregated into three groups: group A mice (n = 31) were implanted with tumors and were treated with PBS (see below); group B mice (n = 10) were implanted with tumors but were treated with PBS; and group C mice (n = 7) were not implanted with tumors but were treated with PBS. All mice were injected with either AR209 at 86 μg/kg (approximately 0.1 ml/mouse in saline) or PBS (0.1 ml) in the lateral tail vein on days 21, 23, 25, 35, 45, and 53. No animals died during the study. All sacrificed animals were autopsied.

**Histopathology.** To characterize the growth patterns of tumors, heart and lung mediastinal blocks were removed from all euthanized animals with or without gross evidence of tumor. The blocks were fixed with 20 ml of 10% buffered formalin and
Two of the mice implanted with tumors and treated with AR209 examined by a pathologist (J. C. A.) in a double-blind fashion. Sections from these mice were also examined for evidence of inflammatory or immunological reactions or microscopic tumors at the implantation site.

Chest Radiography. Animals were anesthetized as previously described and radiographed in the anteroposterior position, using 1:9 magnification on a General Electric Senographe DMR unit (General Electric Medical Systems, Milwaukee, WI). The distance between X-ray source and image was 660 mm, and source-to-magnification plate distance was 397 mm. The images were obtained by using 0.1 focal spot coupling with Kodak MIN-R2 cassettes (Eastman Kodak, Windsor, CO), Kodak MIN-R medium screens, and compatible Kodak MIN-R film (18 × 24 cm). Exposures with 25 kilovolt potential and 18 milliamperes were used. Fluoroscopic images were obtained by means of a General Electric Fluoricon 300 with an MSI 1250 IV control panel (General Electric Medical Systems; Fig. 1) by inclusion of Omnipaque in the injection solution (see above).

Statistical Analyses. Means are shown ± 1 SE. The data were analyzed using repeated-measures ANOVA and the Tukey method for comparing contrasts. When time (either week 8 or 9) was used in the repeated-measures analysis, it was found not to be a significant variable. Moreover, contrasts between pairs of means (using ANOVA) were used to find out whether any of the three levels of intervention (PBS, control, or drug) were significantly related with time. All contrasts turned out to be not statistically significant. Simple t tests were also used to compare whether the effect of the drug at week 8 was significantly different from the effect of the drug at week 9, excluding everything else. The result was also negative. These findings support pooling the data from weeks 8 and 9. Therefore, all analyses, graphs, and figures contain only data pooled in that manner.

RESULTS

The human adenocarcinoma cell line 201T was previously shown to express p185<sub>erbB</sub>-2 at an amount 7.6-fold greater than normal bronchial epithelium (20). This cell line was chosen for the present study because it expressed the least amount of p185<sub>erbB</sub>-2 of the four NSCLC cell lines used in that previous study. 201T cells (2 × 10<sup>6</sup>) were injected intrathoracically into 41 nude mice as described in “Materials and Methods.” After 3 weeks to allow for tumor growth (see Fig. 2), mice were examined by radiography. At 3 weeks, 30% of the mice showed suspicion of tumor growth (data not shown).

Mice were divided into three separate groups and injected with either AR209 or PBS as described in “Materials and Methods.” The mice showed no apparent side effects of the drug and grew robustly (see Table 1). After 8 weeks of i.v. treatment with AR209, the mean whole body weight of the mice was comparable to that of the control mice not containing tumors. However, mice that contained tumors and that were treated with PBS alone were smaller than those treated with AR209 (P = 0.0075), likely because they contained large i.t. tumors that occluded the esophagus, leading to reduced feeding and dehydration. After 9 weeks, the mice treated with AR209 remained larger than those treated with PBS alone, although they were smaller than those with no tumors. The combined data collected for body weight demonstrate that those mice containing i.t. tumors treated with PBS alone were significantly smaller than those treated with AR209 (ANOVA, P = 0.014). The data collected for each group are summarized in Table 1.

After 56 days (8 weeks) of tumor growth, 20 of 31 mice injected with PBS alone were sacrificed along with 5 of 10 randomly selected animals that had been injected with AR209. One week later (day 63, 9 weeks), the remaining 5 of 10 animals treated with AR209 and 11 of 31 treated with PBS were sacrificed. Tumors were observed throughout the lungs of mice treated only with PBS (Fig. 3). Often the tumors remained in the lung; however, in two mice treated with PBS, the tumors grew back along the needle track and were contiguous with the rib cage, growing s.c. along the lateral right quadrant. Histology on all tumors showed them to be adenocarcinoma of the lung (Fig. 4). Tumors were also found in AR209-treated mice; however, these tumors were smaller and never extended back through the needle track (see below).

Mediastinal blocks were resected from all sacrificed animals. The weight of the combination of the lung, heart, and tumor (when present) was measured for each animal. The tumors in animals treated with PBS alone drastically increased the weight of their mediastinal blocks compared with those treated with AR209 after 8 weeks (Table 1). In contrast, the mediastinal blocks of mice treated with AR209 were only slightly larger than those resected from healthy mice that did not receive tumor cells. Each tumor that was visible on resection was carefully embedded, sectioned, and microscopically inspected for the presence of tumors. No tumors were observed (data not shown).

In addition, no evidence of inflammatory or immunological reactions was observed. Although these animals may never have contained tumors, the fact that 31 of 31 mice treated with injections of PBS contained tumors implies a 100% take rate. Therefore, it is likely that these mice were “cured” of their tumors with this dose of AR209.
Table 1  
Average of data collected from mice by group

<table>
<thead>
<tr>
<th>No. of mice</th>
<th>Day sacrificed</th>
<th>Average body weight (g)</th>
<th>TLH weight (mg)</th>
<th>Tumor weight (mg)</th>
<th>Tumor volume (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A, i.t. tumor, PBS i.v.</td>
<td>20</td>
<td>56</td>
<td>22.2 ± 0.80</td>
<td>765 ± 79.1¹</td>
<td>355 ± 79.1</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>63</td>
<td>22.1 ± 1.01</td>
<td>856 ± 113.0</td>
<td>449 ± 113.0</td>
</tr>
<tr>
<td></td>
<td>Total, 31</td>
<td></td>
<td>22.2 ± 0.62</td>
<td>797 ± 64.3</td>
<td>389 ± 64.3</td>
</tr>
<tr>
<td>Group B, i.t. tumor, AR209 i.v.</td>
<td>5</td>
<td>56</td>
<td>27.2 ± 0.56</td>
<td>448 ± 28.7</td>
<td>44.8 ± 25.4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>63</td>
<td>24.1 ± 1.62</td>
<td>702 ± 134.1</td>
<td>295 ± 134.1</td>
</tr>
<tr>
<td></td>
<td>Total, 10</td>
<td></td>
<td>25.7 ± 0.96</td>
<td>575 ± 77.2</td>
<td>170 ± 76.7</td>
</tr>
<tr>
<td>Group C, no tumor implantation, PBS i.v.</td>
<td>4</td>
<td>56</td>
<td>n/d</td>
<td>410 ± 18.7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>63</td>
<td>27.7 ± 0.49</td>
<td>407 ± 4.6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Total, 7</td>
<td></td>
<td>27.7 ± 0.49²</td>
<td>409 ± 10.1</td>
<td>0</td>
</tr>
</tbody>
</table>

¹ TLH, weight of tumor + lung + heart.
² Tumor weight = TLH weight - mean weight of lung + heart in group C.
³ All data are mean ± SE.
⁴ n/d, not determined.
⁵ n = 3 for calculation of average body weight.

DISCUSSION

Immunotherapy is rooted in the assumption that antigens expressed on tumor cells allow the host immune system to recognize tumors and distinguish them from normal cells (31). There has been considerable interest in developing immunotherapy for treatment of tumors that are traditionally unresponsive to standard chemotherapy or radiotherapy. Recently, limited success has been demonstrated with immunotherapy, most notably with the use of recombinant vaccines (32–36). A multifaceted approach to immunotherapy will likely increase efficacy. Recombinant oncotoxins, which are based on our knowledge of the biology of tumor cells, are another approach to immunotherapy. Because some tumor cells overexpress p185erbB-2, it is possible to elucidate a dose of AR209 that will kill tumor cells without concomitant significant damage to normal cells. This therapeutic dosage window is particularly useful for the treatment of lung tumors, because normal bronchial epithelial cells express very low levels of p185erbB-2, whereas certain NSCLCs have been shown to express up to 3300-fold excess protein (20). Because the presence of high levels of p185erbB-2 in tumors has been linked to poor prognosis (12), patients with aggressive disease may be particularly good candidates for therapy with AR209.

Not surprisingly, AR209 has been shown in vitro to inhibit protein synthesis in target cells. Likewise, in s.c. tumors grown in nude mice, AR209 causes a significant reduction in the sizes of tumors, not only in those with overexpression of p185erbB-2, but also in those cells that express low levels of protein (20). In this present study, we describe a lung cancer model by the propagation of a poorly differentiated human lung adenocarcinoma in an orthotopic (organ-specific) manner. Using Matrigel and the i.t. injection of 2 × 10⁶ tumor cells produced tumors in 31 of 31 (100%) control (PBS-injected) animals. This orthotopic model approximates the microenvironment present in a de novo human tumor of the lung much more than with the s.c. model. Of particular importance in this model is the ability of the tumor to cause angiogenesis and recruit stromal support that might not otherwise occur in a s.c. model. In addition, the ability of the tumor to expand in the confines of the thoracic cavity may limit growth of certain clonal elements of the tumor. This model more closely predicts the potential for the drug to reach and destroy all of the cells in a large tumor located in the lung.

There was a significant decrease in the sizes of tumors in animals treated with AR209, as measured by tumor weight and tumor volume. However, we have only tested one dose of AR209 (86 µg/kg) using a schedule designed to test the nature of the effect. We have previously demonstrated that three injections separated by 1 day between injections resulted in the reduction of tumors but not in their complete elimination. We hypothesized that an additional three injections separated by 10 days between injections would likewise result in the reduction
Fig. 4 Histological examination of tumors present in the lungs of mice. Adenocarcinomas were observed in all animals that had tumors detected by gross inspection.

Fig. 5 The volume of each tumor found was calculated using the formula (weight $\times$ height $\times$ length)/2. The average tumor volume for all animals is significantly higher in saline-treated than in drug-treated animals (ANOVA, $P = 0.0362$ with separate variances). Values are mean volumes. Bars, SE.

of the sizes of tumors but not in their elimination. We have confirmed this hypothesis in this study. In addition, we did not sacrifice all of the mice at the termination of injections to determine whether the tumors would continue to grow. Our data indicate that the tumors would continue to grow if therapy was stopped (Table 1). Surprisingly, only 8 of 10 mice treated with AR209 demonstrated the presence of tumors after 8 weeks, versus 31 of 31 mice treated with PBS, indicating that two animals had been cured of tumors. These data may also predict the efficacy of AR209 on metastatic tumors to the lung. Given the results of this study, the most effective use of the drug may be a continuous administration over a sustained but finite period (e.g., 1–2 weeks). There is an abundance of clinical data suggesting that continuous infusion of recombinant toxins over a 1-week period is a viable option (37–40).

Because AR209 is based on the Fv region of a monoclonal antibody of murine origin and contains a $M_r$ 38,000 Pseudomonas exotoxin, it is likely that human patients will develop an immune response to the drug. However, clinical trials have demonstrated that not all patients develop human antimonouse antibody or human antitoxin antibody (40–42). Therefore, its most efficacious use will probably be in combination with traditional therapy and other immunotherapy. Again, a continuous infusion over a 2-week period will allow for the use of the drug before the patient can raise a primary immune response to the drug. However, continued use on a patient that has previously had a primary immune response and has developed human antitoxin antibody will not be effective. In patients with operable NSCLC, surgical resection is commonly followed by radiotherapy to destroy residual local disease (6), although recurrence of their tumors is essentially inevitable and is universally fatal. Obviously, undetected micrometastasis has occurred prior to surgery. Treatment of these patients with AR209 may significantly reduce the likelihood of recurrence by destroying single cells or metastatic foci. The combination of cancer vaccines specifically made for the tumor (32) may further increase survival. In addition, in those patients with inoperable tumors, the combination of radiotherapy and AR209 may increase overall survival by reducing tumor volume in the primary and metastatic foci.

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REFERENCES


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