ABSTRACT

Purpose: The monoclonal antibody (MAb) trastuzumab (Herceptin) effectively treats HER2-overexpressing extracerebral breast neoplasms. Delivery of such macromolecule therapeutic agents to intracerebral metastases, however, is limited by the tight junctions characteristic of the cerebral vasculature. Direct intracerebral microinfusion (ICM) is a technique that bypasses this blood-brain barrier and allows for a greater delivery of drugs directly into intracerebral tumors.

Experimental Design: A human breast cancer cell line transfected to overexpress HER2, MCF-7/HER2–18, was transplanted into the cerebrum of athymic rats. Saline, trastuzumab, or an isotype-matched control MAb was delivered systemically or by ICM to assess toxicity and efficacy.

Results: No clinical or histological toxicity related to trastuzumab was evident under any of the conditions studied. Delivery of trastuzumab (2 mg/kg) i.p. led to a median survival of 26.5 days, whereas treatment with trastuzumab (2 mg/kg) by ICM increased the median survival by 96% to 52 days, with two of nine rats surviving >120 days (P = 0.009). Treatment with an isotype-matched control MAb (16 mg/kg) resulted in a median survival of 21 days, which did not differ significantly from the survival of rats treated by ICM with saline (16 days; P = 0.42). Treatment by ICM with trastuzumab (16 mg/kg) led to a median survival of 45 days, with 2 of 10 rats surviving >120 days. These results represent 181% and 114% increases in median survival over the saline and MAb controls, respectively (P < 0.001).

Conclusion: ICM of trastuzumab is safe and superior to systemic delivery as therapy for HER2-overexpressing intracerebral neoplasms in an athymic rat model.

INTRODUCTION

Metastatic breast cancer is a frequent cause of intracerebral tumors (1). In 1993, ~46,000 people in the United States died of breast cancer, of whom 20–30% were found to have brain metastases at the time of autopsy (2). Brain metastases imply a poor prognosis and are frequently the cause of death in these patients. Even with aggressive treatment, including radiation, chemotherapy, and surgery, the average patient diagnosed with intracerebral metastatic breast cancer survives only 14 months (3).

Trastuzumab (Herceptin; Genentech, Inc., South San Francisco, CA) has been shown to be efficacious against extracranial metastatic breast cancers that overexpress the HER2 antigen (4). Although the survival of patients with HER2-positive brain metastases has not been examined, the survival of patients with HER2-positive metastatic breast cancer is significantly shorter than HER2-negative patients (32 months versus 62.6 months, respectively; P < 0.0001; Ref. 5). It is, thus, logical to assume that the survival of patients with HER2-positive intracerebral brain metastases may be even shorter than 14 months. Systemic therapy of intracerebral tumors with relatively large therapeutic agents, like monoclonal antibodies MAbs\(^4\) such as trastuzumab, may not be as effective because the capillary barrier of the cerebral vasculature restricts the entry into the brain of such large molecules (6). For example, our human imaging studies using an antigen-specific radiolabeled MAb demonstrated that after systemic i.v. administration, only 0.0006–0.0043% of the total injected dose localized to the intracerebral tumor (7). In accordance with this, it has also been reported that after systemic administration of trastuzumab, cerebrospinal fluid levels of the MAbs are 300-fold lower than systemic levels (8). As expected from these data, a frequent cause of treatment failure in the trials of trastuzumab for metastatic breast cancer is the development of intracerebral metastases. Because of their increased survival, patients whose systemic metastases respond to trastuzumab may develop and die from intracerebral metastatic disease (9). These data suggest that enhancing the delivery of

\(^4\)The abbreviations used are: MAb, monoclonal antibody; BBB, blood brain barrier; CNS, central nervous system; ICM, intracerebral microinfusion; HPF, high-powered field; CI, confidence interval.
trastuzumab to the brain might enhance its efficacy against intracerebral metastases.

Regional intracerebral drug delivery has the potential to bypass the BBB, deliver high concentrations of the therapeutic agent directly to the site of the tumor, and reduce systemic exposure to any drug-induced toxicity. Our studies with bolus injection of radionabeled antitenascin MAb 81C6 into surgically created resection cavities have demonstrated the safety and potential advantage of regional intracerebral administration in humans (10). Unlike radionabeled MAbs, however, unconjugated MAbs like trastuzumab require direct cell contact to be effective. This requires distribution of the therapeutic agent beyond the site of regional injection. Conventional techniques of regional intracerebral delivery, such as impregnated polymer discs or bolus injection, however, depend on physical diffusion to distribute the therapeutic agent. Distribution of MAbs into the brain using such approaches is severely limited because the rate of diffusion is inversely related to the size of the agent and is slow relative to tissue clearance (10, 11). This limits the distribution of regionally delivered MAbs using conventional techniques to within a few millimeters of the injected area. Conversely, continuous ICM, an innovative technique of regional delivery of therapeutic agents directly into brain parenchyma, establishes a bulk flow current that has the potential to homogeneously distribute even large molecules much greater distances throughout the brain. ICM is capable of achieving concentrations of therapeutic agents within the brain several orders of magnitude greater than that obtainable after systemic delivery (12). Such enhancement of drug distribution has been demonstrated in experimental animal models (12) and preliminary human trials (13). Thus, ICM should allow delivery of therapeutic MAbs, like trastuzumab, to a greater portion of the tumor and should saturate invasive neoplastic cells distant from the site of infusion. This study demonstrates that ICM delivery of trastuzumab is safe and superior to systemic delivery in the treatment of HER2-overexpressing intracerebral breast cancer metastases.

MATERIALS AND METHODS

Intracerebral Cannula Implantation. Athymic female rats were maintained in the Duke University Comprehensive Cancer Isolation Facility according to institutional policy. All rats were ~5 months of age and weighed 200–250 g at the time of surgery. Rats were anesthetized by i.p. injection of a mixture of ketamine (55 mg/ml) and xylazine (9 mg/ml) at a dose of 1 mg/kg and were placed into a stereotactic frame (Kopf Instruments, Tujunga, CA). A 25-gauge guide cannula (Plastics One, Inc., Roanoke, VA) was placed 1 mm anterior to bregma, 3 mm right of midline, and 3 mm deep to the outer table of the skull. The cannula was permanently secured to the calvaria using cranioplast cement (Plastics One, Inc.). The catheter system was closed with a dummy cannula (Plastics One, Inc.), and the incision was stapled closed. The rats were allowed to recover for a minimum of 7 days before tumor cell implantation. Only rats showing normal weight and neurological function and no evidence of infection were randomized into experiments.

Xenografts and Tumor Implantation. The MCF-7/HER2–18 cell line is a subclone of MCF-7, a human breast cancer cell line, stably transfected with a full-length HER2 cDNA coding region (14). The cell line was provided by Genentech, Inc., and was grown as a monolayer in culture in high-glucose DMEM:F-12 (50:50), 10% heat-inactivated fetal bovine serum, insulin, 2 mm l-glutamine, and 400 μg/ml Genticin.

Cells for implantation were harvested from 150-cm² culture flasks when they reached ~70% confluency. Cells were harvested with rubber cell scrapers and washed three times in sterile Dulbecco’s PBS. After the final wash, the cells were suspended in an appropriate volume of sterile 0.9% saline for injection to yield a concentration of 8 × 10⁷ cells/ml.

The homogeneous cell suspension was loaded into a 500-μl Hamilton syringe and injector (Hamilton Co., Reno, NV), and 10 μl of suspension (8 × 10⁵ cells) were implanted intracerebrally via a 25-gauge blunt needle 7 mm deep to the outer table of the skull through the guide cannula.

Seven days before cell implantation, a 1.6-mg, 60-day continuous release, 17β-estradiol pellet (Innovative Research, Toledo, OH) was implanted s.c. into each rat.

Trastuzumab Preparation. Trastuzumab, a fully humanized IgG1 kappa antibody directed against the HER2 protein, was obtained from Genentech, Inc. The drug was first diluted to 22 mg/ml with sterile water for injection to maintain the optimal pH. This suspension was then diluted further to 2 mg/ml or 0.25 mg/ml using sterile 0.9% saline for injection on the day of administration. Campath-1H, a fully humanized antibody to CD52 was used as an isotype control. Campath-1H, like trastuzumab, is a fully humanized IgG1 kappa antibody.

ICM. ICM was performed using an Alzet osmotic pump (Product 2ML1; ALZA Corp., Palo Alto, CA) connected by silicone tubing (Molded Rubber and Plastics, Butler, WI) to a 33-gauge infusion cannula (Plastics One, Inc.), which fit into the 25-gauge intracerebral guide cannula and projected 7 mm deep to the outer table of the skull into the area where the tumor was implanted. The Alzet pump was primed for 8 h at 37°C and then implanted s.c. Rats underwent a total infusion of 2 ml over a 7-day period. Systemic administration was performed by implantation of the same pump i.p. All pumps were explanted after the completion of the infusion.

Assessment of Toxicity. Toxicity was monitored by daily weights and daily neurological function tests (consisting of stepping and placing reflex, incline ramp climbing ability), and a complete autopsy was performed, including histological review of organs, after death.

Serum Trastuzumab Levels. Serum levels of trastuzumab were determined during and after ICM and systemic administration of trastuzumab (2 mg/ml). One milliliter of blood was obtained via cannulation of the tail veins of control and experimental rats before infusion, day 3 of infusion, day 8 (immediately after the discontinuation of treatment), and day 14. Serum level analysis was performed by Susan Brignoli at Genentech, Inc. using an in vitro-labeled antibody plate-binding assay.

 Autoradiographic Distribution Studies. Autoradiography was used to quantify the distribution of infused trastuzumab. Radiolabeled trastuzumab (10 μCi of 125I/0.5 mg of trastuzumab/rat) was infused intratumorally, peritumorally, and i.p. for 7 days via Alzet osmotic pumps, as described for all other experiments. At the end of each infusion, all rats were sacrificed and the brains were extracted. These brains were soaked in 10% formalin for 48 h, embedded in paraffin, and then serially sectioned in the coronal plane at 10-μm intervals. Brain sections were then exposed to photographic films (Kodak Biomax MS, Eastman Kodak Co., NY) for 6 days at 20°C. The films were then developed and coregistered with H&E histological sections.

After this macro-autoradiography processing, these brain H&E sections were coated with LM-1 emulsion (Amersham Biosciences, Piscataway, NJ) and exposed for 7 days at 4°C to visualize the distribution of trastuzumab at a microscopic level (×40). These brain sections were then exposed and developed.

 Autoradiographic Image Analysis. The volume of distribution of 125I-trastuzumab was analyzed using a computer image analysis system (Image J 1.28; NIH, Bethesda, MD). Sections were scanned into the computer, and the volume of distribution was calculated using 10% of the maximum signal as a threshold. The area analyzed spanned from 5 mm rostral to 5 mm caudal to the infusion point. Coregistration of autoradiographic image and H&E-stained histological sections was performed using commercial image-analysis software (Adobe Photoshop 5.0; Adobe Systems, Inc.).

 Statistical Analysis. Survival estimates and median survival was determined by using the method of Kaplan and Meier (15). Survival data were compared using nonparametric test statistics. Data from autoradiography analyses were expressed as mean ± SD. Volume of distribution data and quantitative autoradiography were also compared using nonparametric test statistics. The criterion for statistical significance was considered to be P < 0.05 in all statistical evaluations.

 RESULTS

 Tumor Growth. On histological examination, tumor was consistently evident microscopically 3 days after tumor challenge. Furthermore, tumor challenge with 8 × 10⁶ cells intracerebrally consistently resulted in tumor growth in all rats challenged, and all untreated rats died from intracerebral tumors.

 Toxicity. During all trials, neither weight loss >10% nor neurological deficits were observed. Complete gross pathological examination of the neuroaxis and all systemic organs as well as microscopic histological examination of the brain, spinal cord, and hearts of each rat were performed. Histological examination of the brain revealed the presence of intracerebral tumors in all rats that died during the studies. In the surviving rats, histological examination at the level of the infusion cannula revealed evidence of minor focal gliosis surrounding the cannula tract resulting from cannula implantation, but no histological evidence of tumor cells. Antibody infusion was not associated with any acute neurological changes or seizures. No evidence of hemorrhage, necrosis, edema, or demyelination was identified after treatment with trastuzumab. No histological changes were noted in the spinal cords of rats in any of the treatment groups. Additionally, histological examination of the hearts of rats treated with trastuzumab (2 mg/ml) by ICM revealed only rare perivascular collections of mast cells in 6 of 10 rats. This frequency did not differ from that for saline-treated control rats, and there was no evidence of cardiomyopathy.

 Plasma Drug Levels. During a 1-week infusion of trastuzumab (2 mg/ml), average serum levels of trastuzumab ranged from 29 to 102 μg/ml throughout the treatment period in rats treated by ICM and from 56 to 109 μg/ml in rats treated systemically. Average serum levels of trastuzumab were twice as high in rats treated systemically as they were in rats treated by ICM 1 week after the discontinuation of treatment (8 versus 40 μg/ml). Serum trastuzumab levels in saline-treated control rats were less than the assay detection limit of 0.15 μg/ml in all rats tested.

 Intracerebral Distribution of 125I-Radiolabeled Trastuzumab. To analyze differences in intracerebral distribution based on route of delivery, autoradiographic analysis was used to detect 125I-trastuzumab within the cerebral hemisphere after intratumoral, peritumoral, or i.p. infusion (Fig. 1A). After i.p. infusion, no 125I-trastuzumab was detected intracerebrally at the 10% threshold level using the macroscopic autoradiographic imaging technique described in “Methods and Methods.” In contrast, after ICM, the volume of distribution was 0.26 ± 0.02 cm³ after intratumoral infusion, and 0.26 ± 0.01 cm³ after peritumoral infusion. There was a significant difference in the volume of distribution obtained after either intratumoral or peritumoral infusion when compared with i.p. infusion (P = 0.013), but there was no significant difference in volume distribution between intratumoral infusion and peritumoral infusion (P = 0.613).

 Using microscopic visualization, large amounts of 125I-trastuzumab were detected within the tumor and surrounding normal brain after ICM (Fig. 1B). After i.p. infusion, however, only a small amount of 125I-trastuzumab was detected within the tumor and surrounding normal brain (Fig. 1B). Overall, the number of 125I tracks within the tumor (P = 0.0273) and within the surrounding normal brain (P = 0.0257) after intratumoral or peritumoral infusion was significantly greater than after i.p. infusion. After intratumoral infusion, the mean number of tracks per HPF within the central necrotic portions of the tumor was 724.6 ± 49.2. This was significantly higher than within areas of solid tumor (P = 0.049) or within areas of the brain surrounding the tumor (P = 0.039), in which the mean number of tracks was 323.0 ± 98.0 and 473 ± 163.3, respectively. These data suggest, in addition, that necrotic areas of the tumor may serve as “sinks” for agents delivered by this technique. After peritumoral infusion, the mean number of tracks per HPF within the tumor was 218.6 ± 38.0. After i.p. infusion, only 66.3 ± 16.0 tracks per HPF were seen within the tumor, and only 27.7 ± 16.0 tracks per HPF were seen in the surrounding normal brain, confirming that ICM was significantly more effective at delivering the trastuzumab to the tumor than systemic administration.

 Efficacy. To determine whether ICM of trastuzumab provided an advantage over systemic administration, the efficacy of trastuzumab delivered by ICM was compared with that of trastuzumab delivered systemically in an equivalent dose via the same infusion pumps. Nine athymic rats were treated by ICM
with 2 ml of trastuzumab directly into the tumor over 7 days via a 33-gauge infusion cannula. Another 10 rats received an equivalent volume and concentration of trastuzumab i.p. via the same microinfusion pump. Trastuzumab (0.25 mg/ml, 2–2.5 mg/kg) delivered i.p. led to a median survival of 26.5 days (95% CI, 25.42–27.58 days), whereas ICM of trastuzumab (0.25 mg/ml, 2–2.5 mg/kg) led to a median survival of 52 days (95% CI, 42.61–61.39 days), with two of nine rats surviving >120 days.
without evidence of tumor (Fig. 2). This represents a 96% increase in median survival for ICM over i.p. treatment ($P < 0.009$).

To demonstrate that the survival benefit of ICM of trastuzumab was a specific function of trastuzumab and that ICM alone was not responsible for the results, ICM delivery of trastuzumab was compared with ICM delivery of an isotype-matched control MAb, Campath-1H, delivered in an equivalent manner and concentration (Fig. 3). Treatment via ICM in both groups consisted of 2 ml of 2 mg/ml MAb (total treatment, $16–20$ mg/kg) or saline. Saline treatment ($n = 9$) led to a median survival of 16 days (95% CI, 14.54–17.46 days). Treatment with Campath-1H ($n = 9$) resulted in a median survival of 21 days (95% CI, 16.62–25.38 days), which did not differ significantly from that for saline treatment (16 days; $P = 0.42$). Treatment with ICM of trastuzumab ($n = 10$) led to a median survival of 45 days (95% CI, 27.96–62.04 days) with 2 of 10 rats surviving >120 days after tumor implantation. This represents a 181% increase in median survival compared with saline control survival and a 114% increase in survival compared with Campath-1H control survival ($P < 0.001$).

**DISCUSSION**

Intracerebral metastatic breast cancer has a dismal prognosis. Despite current therapy, the average survival is <14 months after diagnosis (3). The survival of patients with HER2-positive intracerebral brain metastases may be even shorter because HER2 overexpression is associated with a decreased survival in patients with metastatic breast cancer (5). This has prompted the search for novel therapeutic strategies. Although trastuzumab is clinically successful against systemic metastatic breast cancers that overexpress the HER2 antigen, (4) clinical reports have noted that the antibody seems to be relatively ineffective against intracerebral metastatic lesions (9). Because trastuzumab does not cross the BBB, patients who respond systemically to trastuzumab are susceptible to intracerebral disease. Because it is generally accepted that the BBB restricts entry into the brain of larger proteins, it is presumed that trastuzumab, a $M_r$ 148,000 antibody, is relatively restricted. This restriction by the BBB may lead to suboptimal dosing of trastuzumab when intracerebral metastases are targeted. Accordingly, i.v. infusion of trastuzumab in human patients results in a 300-fold lower concentration in cerebrospinal fluid compared with serum concentration (8).

In addition to the BBB, systemic treatment of solid tumors with large molecular weight therapeutic agents faces several other barriers, including altered vascular permeability, vascular heterogeneity within the tumor, increased transvascular and interstitial pressure, and large intravascular distances, all of which result in an unpredictable and heterogeneous distribution of the MAb throughout the tumor (16). Because of these physiological barriers, systemic administration of trastuzumab for intracerebral metastases would require the delivery of extremely high concentrations of drug over prolonged intervals to attain effective drug concentrations within the tumor.

Direct administration of therapeutic agents into the interstitium of the tumor bypasses the BBB and provides high concentrations of the therapeutic agent directly at the tumor site while minimizing systemic exposure. Regional therapy is par-
particularly attractive as a modality for the treatment of intracerebral brain metastases for two reasons. First, these tumors have proven to be resistant to systemic chemotherapeutic agents. Second, intracerebral metastases are often fatal despite aggressive and even successful treatment of systemic disease (17).

Several methods of delivering therapeutic agents directly into an intracerebral tumor have been used, including drug-impregnated polymers, direct bolus infusions, and intrathecal or intraventricular infusion. These methods of administration, however, rely on diffusion to deliver the therapeutic agent and are, therefore, highly dependent on the molecular weight of the agent. ICM, which uses a pressure gradient to produce a bulk flow infusion, has been shown to deliver large molecules throughout the tumor and brain in a more homogeneous fashion over a significantly greater area (18). In the brains of larger animals, ICMs have perfused volumes on the order of $10^2$–$10^3$ cm$^3$ with macromolecules even larger than trastuzumab, which represents a 10-fold greater area of distribution over simple distribution (11, 19).

Our initial human studies are also confirming the delivery advantages that can be obtained with ICM.5 In our rat model, we were able to demonstrate that ICM delivery of trastuzumab provides a significant survival advantage over an equivalent dose of trastuzumab delivered systemically (20, 21). In addition, trastuzumab via ICM induced complete responses in several rats with intracerebral neoplasms without the induction of any significant systemic or intracerebral toxicity. In addition to the studies presented here, we have also confirmed the efficacy of ICM of trastuzumab against intracerebral breast neoplasms in other rodent models and with other HER2-expressing cell lines, including BT-474, a breast cancer cell line that naturally overexpresses HER2.5

Although MAbs have been used intracerebrally as a delivery agent for radioisotopes (10, 22) and recombinant bacterial toxins (13), these constructs are limited by the inherent nonspecific local toxicity of the immunoconjugate. The data presented herein confirm previous findings in our laboratory and by others that unarmed MAbs that target tumor-specific antigens can mediate a potent therapeutic response in the CNS (23). Previous studies performed by our laboratory suggest that this passive immunotherapy with unarmed MAbs may mediate this effect in the CNS by recruiting Fc receptor-containing macrophages, microglia, and astroglial cells that are rich throughout the substance of the brain (23).

In conclusion, the data presented here suggest that direct ICM delivery of trastuzumab may prove to be an efficacious and safe treatment in human clinical trials. Future studies further defining the pharmacokinetics and distribution of MAbs, such as trastuzumab, in large animal models or humans will help optimize the delivery of the drug. Furthermore, these data suggest the possibility that other MAbs currently used for systemic cancers, such as lymphoma and colon cancer, may also be efficacious against intracerebral neoplasms with other histologies or that MAbs delivered by ICM may be a viable mechanism of defense against other infective or degenerative pathological processes in the CNS.

Fig. 3  Survival benefit of ICM is dependent on trastuzumab. Infusion cannulas were implanted in the cerebrum of athymic rats. Rats were challenged with 800,000 MCF-7/HER-2 clone 18 cells implanted via the infusion cannula. Treatment consisted of ICM of 2 ml of trastuzumab (2 mg/ml), Campath-1H (2 mg/ml), or saline infused directly into the tumor via osmotic infusion pumps over 7 days starting 3 days after tumor implantation. Treatment with Campath-1H (n = 9) resulted in a median survival of 21 days (95% CI, 16.62–25.38) and did not differ significantly from saline (n = 9) treatment (16 days; $P = 0.42$). Treatment with ICM of trastuzumab (n = 10) led to a median survival of 45 days (95% CI, 27.96–62.04) with 2 of 10 rats surviving >120 days after tumor implantation. This represents a 181% increase in median survival compared with saline and a 114% increase compared with the Campath-1H control ($P < 0.001$).
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