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Growth inhibition in a brain metastasis model by antibody delivery using focused ultrasound-mediated blood-brain barrier disruption

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ABSTRACT

HER2-targeting antibodies (*i.e.* trastuzumab and pertuzumab) prolong survival in HER2-positive breast cancer patients with extracranial metastases. However, the response of brain metastases to these drugs is poor, and it is hypothesized that the blood-brain barrier (BBB) limits drug delivery to the brain. We investigated whether we could improve the response by temporary disruption of the BBB using focused ultrasound in combination with microbubbles. To study this, we inoculated 30 nude rats with HER2-positive cells derived from a brain metastasis of a breast cancer patient (MDA-MB-361). The animals were divided into three groups: a control-group that received no treatment; an antibody-only group that received six weekly treatments of trastuzumab and pertuzumab; and an ultrasound + antibody group that received trastuzumab and pertuzumab in combination with six weekly sessions of BBB disruption using focused ultrasound. In two animals, the leakiness of the tumors before disruption was evaluated using contrast-enhanced T1-weighted magnetic resonance imaging and found that the tumors were not leaky. The same technique was used to evaluate the effectiveness of BBB disruption, which was successful in all sessions.

The tumor in the control animals grew exponentially with a growth constant of 0.042 ± 0.011 mm^3/day. None of the antibody-only animals responded to the treatment and the growth constant was 0.033 ± 0.009 mm^3/day during the treatment period. Four of the ten animals in the ultrasound + antibody-group showed a response to the treatment with an average growth constant of 0.010 ± 0.007 mm^3/day, compared to a growth constant 0.043 ± 0.013 mm^3/day for the six non-responders. After the treatment period, the tumors in all groups grew at similar rates. As the tumors were not leaky before BBB disruption and there were no responders in the antibody-only group, these results show that at least in some cases disruption of the BBB is necessary for a response to the antibodies in these brain metastases. Interestingly, only some of the rats responded to the treatment. We did not observe a difference in tumor volume at the start of the treatment, nor in HER2 expression or in contrast-enhancement on MRI between the responders and non-responders to explain this. Better understanding of why certain animals respond is needed and will help in translating this technique to the clinic. In conclusion, we demonstrate that BBB disruption using focused ultrasound in combination with antibody therapy can inhibit growth of breast cancer brain metastasis.

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Chemical substances studied in this article

Trastuzumab (PubChem SID:521856)
Pertuzumab (PubChem SID: 91145207)

1. Introduction

Of the patients with breast cancer, 5–15% develop metastases in the central nervous system [11]. The prognosis for patients with metastases in the brain is poor: for patients with multiple metastases the one-year survival rate is around 25% [8], and neurologic disease is the cause of death, or a major factor, in 68% of these patients [4]. The incidence of brain metastases seems to be higher in patients that overexpress human epidermal growth factor receptor 2 (HER2) and who have been treated with trastuzumab [23]. This HER2-targeting antibody is effective in extracranial metastases and prolongs survival, which might be the reason for the higher incidence of brain metastases [17]. It is hypothesized that the blood-brain barrier (BBB), or the blood-tumor barrier (BTB), is

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the reason for the poor response of brain metastases to drugs that are effective extra-cranially, such as trastuzumab. Most small molecule and essentially all large molecule drugs are prevented from reaching the brain parenchyma due to this barrier [15]. Although blood vessels in brain metastases are somewhat leaky, this permeability is heterogeneous and mice studies have shown that the delivery of chemotherapeutics in breast cancer brain metastases stays below therapeutic levels in the vast majority of the brain metastases [12].

Focused ultrasound (FUS) in combination with microbubbles, small gas bubbles used as ultrasound contrast agents, has been shown to enable temporarily and focal disruption of the BBB [9]. Preclinical studies have shown that the interaction between acoustic pressure waves and microbubbles leads to temporary disassembly of the tight junction proteins, making drug delivery past the BBB possible [19–21]. The advantage of this technique is that it is non-invasive, repeatable, and targets only specific regions of the brain and is compatible with approved drugs. Two approved therapeutic agents that are effective for HER2-positive breast cancer and extracranial metastases are trastuzumab and pertuzumab, which are monoclonal HER2-receptor targeting antibody therapies. For these drugs to be effective in brain metastases, they will need to pass the BBB/BTB. Previously, it has been demonstrated that the delivery of trastuzumab to the mouse brain can be enhanced by BBB disruption using FUS in combination with microbubbles [10]. Furthermore, in an animal study using nude rats that were injected with human breast cancer cells (BT-474), it was demonstrated that median survival time increased by at least 32% in animals that received trastuzumab in combination with ultrasound-mediated disruption of the BBB compared to animals that were not treated [16]. In four of ten animals treated with FUS and trastuzumab, the tumor appeared to be completely resolved in the follow-up MRI. However, in that study a cell line was used that was derived from a primary breast tumor that is highly sensitive to trastuzumab [16]. Therefore, it might not be the most appropriate model for translational studies. Here we evaluate the treatment effect of FUS-induced BBB disruption using a HER2-overexpressing human cancer cell line that has been derived from a brain metastasis of a breast cancer patient. Similar to the clinic, we use trastuzumab in combination with pertuzumab and we kept all animals alive until they met the criteria for euthanasia in order to perform a complete survival analysis.

2. Materials and methods

2.1. Study design

In this prospective study the treatment benefit of trastuzumab and pertuzumab in combination with FUS-mediated BBB disruption was determined in a breast cancer brain metastasis model. The experiments were approved by the Harvard Medical Area Standing Committee on Animals. The brain metastasis model was obtained by implanting MDA-MB-361 HER2-positive human cancer cells in the right brain hemisphere of nude rats. The animals were divided in three treatment groups of 10 animals each: group 1 received no treatment; group 2 received trastuzumab and pertuzumab treatment; and group 3 received trastuzumab and pertuzumab in combination with FUS-mediated BBB disruption. Previous studies have demonstrated that FUS-mediated disruption of the blood-brain barrier without administration of the drugs has no therapeutic effect on different brain tumor models [2,5,16,22]. For that reason, we did not include an arm with FUS mediated BBB-disruption-only. The weekly treatments started five weeks after tumor implantation, when the maximum tumor diameter was around 2 mm, and lasted six weeks. Comparable to the clinical protocol, trastuzumab and pertuzumab (provided by Genentech, South San Francisco, CA) were administered intravenously at a dose of 4 mg/kg (loading dose) in the first treatment week and 2 mg/kg during the following weeks (maintenance dose). Every other week, the tumor size was monitored with high-resolution MR-imaging. The animals were euthanized if the tumor diameter exceeded 13 mm, the animal showed excessive weight loss, or if there were signs of suffering or poor condition. Fig. 1 shows the time-line of the experiments. Cell growth assays were performed to compare the response of the MDA-MB-361 and BT-474 cell lines to trastuzumab and pertuzumab therapy.

2.2. Cell growth assays

The HER2-positive human cancer cells BT-474 and MDA-MB-361 were obtained from ATCC (Manassas, VA). Variants of these cell lines transfected with the GFP-labeled histone H2B (MDA-MB-361-H2B-GFP and BT-474-H2B-GFP) were used to evaluate drug-induced growth inhibition over time using a laser scanning cytometer (TTP Labtech, Cambridge, MA). Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37 °C in 5% CO_2. Forty-eight hours before adding the HER2 targeting antibodies, $5 \cdot 10^3$ tumor cells/well were seeded in 96-well plates. Trastuzumab, pertuzumab or both antibodies were added to the wells at concentrations of 0, 7.47, 22.22, 66.67 or 200 μg/ml, that were selected to match the maximum concentration measured in patients [11]. To measure cell growth in the presence of each drug and their combination we measured cell numbers at day 0, day 3 and day 6 for the MDA-MB-361-H2B-GFP and BT-474-H2B-GFP cells. Specifically, we quantified the total area of nuclei (A_{nuclei}) at each time point, and next calculated normalized cell growth by dividing A_{nuclei}(day x) by A_{nuclei}(day 0). These experiments were executed in duplicates and used to determine which duration of antibody therapy was necessary to observe treatment effects. Based on these studies, we decided to treat MDA-MB-361 and BT-474 cells for six days. At day 6, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and Hoechst and Propidium Iodide (PI, Sigma Adrich) were added to stain cell nuclei and dead cell, respectively. To estimate the number of viable cells (total cells – dead cells) we computed the differences between the area of all cells (A_{nuclei}) from the Hoechst stain and the area of dead cells (A_{dead}) from the PI stain. Using this method we determined growth inhibition due to HER2-targeting antibodies for each well as follows:

$$\text{Growth inhibition} = \frac{(A_{nuclei} - A_{dead})}{A_{control}},$$

where $A_{control}$ is the average area of viable cells in the wells that did not receive treatment. These experiments were performed in six replicates and for each concentration and drug combination the average growth inhibition was determined. The Spearman's rank correlation coefficient between the concentration and inhibition was determined. The differences between the growth inhibition at each concentration of the different treatments were evaluated using one-way ANOVA with a Tukey's post-test.

2.3. Cell culture and tumor implantation

MDA-MB-361 cells were cultured in Leibovitz's L-15 Medium (ATCC) with 20% fetal bovine serum and 5% penicillin streptomycin at 37 °C without additional CO_2. For tumor implantations, cells were suspended in cell culture medium at $1 \cdot 10^6$ cells per 2 μl medium. Thirty male

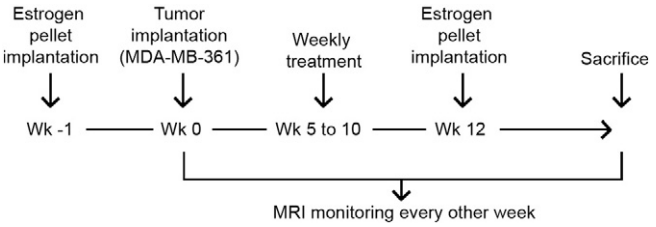

Fig. 1. Study design.

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