Beneficial Effects of Radiation-Induced Autoantibody on Clinical Outcomes after Stereotactic Radiotherapy for Metastatic Breast Cancer

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ABSTRACT

**Purpose:** Recent observations suggest that irradiation activates host immunologic response, resulting in potential clinical benefits. The aim of this study is to assess whether autoimmunity induced by stereotactic radiotherapy (SRT) has beneficial impacts on the treatment outcomes for metastatic breast cancer patients.

**Methods and Materials:** Plasma serially corrected before, during, and after SRT on a prospective protocol from 28 patients with metastatic breast cancer were analyzed. Western blot experiments were performed to detect antibody formation to tumor antigens extracted from human breast cancer cell lines, and 13 cytokines were also measured by cytokine array.

**Results:** Tumor-specific antibody of IgM or IgG binding was detected in 50% (14/28) and 57% (16/28) of the patients, respectively, and median time to appearance of IgM and IgG was 9 days (range, 4-15 days) and 24 days (range, 10-111 days) from the start of RT, respectively. The immuno-responsive group had significantly improved overall survival. The 3-year OS rates of the IgM positive (n=14) and negative (n=14) groups were 68.8% and 22.2%, respectively (p=0.027). Similarly, the corresponding rates of IgG positive (n=16) and negative (n=12) groups were 65.6% and 22.2%, respectively (p=0.33). Furthermore, the immuno-responsive patients typically had elevated levels of granulocyte macrophage colony-stimulating factor, interferon-gamma, and tumor necrosis factor-alpha.

**Conclusion:** These data suggest that radiation-induced autoimmunity works synergistically with local effects of radiation, with endogenous cytokines acting as immune adjuvants, ultimately translating to improved survival in patients with metastatic breast cancer.

**Key Words:** Immune response, Autoantibody, Radiotherapy, Breast cancer, Oligometastasis
INTRODUCTION

Radiotherapy (RT) is a localized treatment, but recent advances in our understanding of cancer immunology provide evidence for its systemic effects, as well (1, 2). Cancer cell death by irradiation may activate immune system recognition or release signals for dendritic cell (DC) activation (3, 4). Additionally, nonlethal doses of radiation may alter the tumor cell phenotype and increase expression of tumor-associated antigens (TAA) and MHC class I on the tumor cell surface to facilitate increased cytotoxic tumor-specific T cell activity (5, 6). While radiation exposure alone makes tumor cells more amenable to immune-mediated kill, preclinical studies have shown that combining local RT with immunotherapy promotes tumor-specific immunity better than with either modality alone (7-9). Furthermore, recent clinical trials have exploited the immunostimulatory effect of RT used in concert with therapeutic cancer vaccines, leading to greater tumor reduction (10, 11).

Stereotactic body RT (SBRT) uses novel technology for more accurate tumor localization, thereby allowing for delivery of larger fractional doses of RT,(12, 13). We propose that RT given with this approach induces autoimmunity and subsequently augments the local tumoricidal effects of SBRT while potentially hindering the development of metastatic lesions elsewhere. Here, we demonstrate the appearance of a RT-induced autoantibody (RIAA) and document the increase in granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) in these patients. These are the first results to correlate RIAA induction in metastatic cancer patients treated with hypofractionated SBRT with a positive impact on clinical outcome.
MATERIALS AND METHODS

Patients and sample collection

Between February 2001 and December 2007, 51 women with metastases from primary breast cancer were enrolled on one of two prospective pilot studies to investigate the efficacy of SBRT in treating metastatic disease. The details of patient eligibility were previously described (13). Plasma from 28 of 51 patients was serially collected more than 3 times within 3 months, and Table 1 summarizes characteristics of the subjects in this study.

The sampling included 1-7 days before RT, 1-2 weeks after initiation of RT, and at several points after RT completion. The median plasma sample number obtained from these patients was 6 (range, 3-8), and 21 patients (75%) had five or more blood sample collections. The venous blood samples were centrifuged at -4°C within 30 minutes of collection. The separated plasma was stored at -80°C until analysis.

Treatment and follow up

Treatment approach for SBRT in these protocols has been outlined elsewhere (12, 13). Briefly, three-dimensional treatment planning was performed using BrainSCAN software (BrainLAB AG, Heimstetten, Germany). Subjects were immobilized with a custom vacuum bag, and verification of the RT field was performed with the Novalis ExacTrac® positioning platform (BrainLAB AG, Heimstetten, Germany) using a relaxed end-expiratory breath hold. The gross target volume (GTV) was contoured on the planning computed tomography (CT) scan. The planning target volume was obtained by adding a 10 mm craniocaudal margin and other directional margins of 7 mm to the GTV. SBRT was delivered using conformal arcs. The fraction size and total doses were determined with consideration of the dose-volume histogram.
of the organ at risk, with a preferred schedule of 50 Gy in 10 fractions given over 2 weeks. If subjects had 5 or fewer tumors, SBRT was simultaneously delivered to all the tumors with a curative intent, whereas it was performed selectively with a palliative intent when subjects had 6 or more metastases (Table 1). Nobody received concurrent chemotherapy during SBRT.

The last patient follow-up was performed in March 2009, and median follow-up was 52 months (range: 24-91). The follow-up examinations included physical examination, blood collections, and imaging studies at 1 month after completion of SBRT, then 3-month intervals during the first 2 years, and at 3-6 month intervals thereafter.

Autoantibody detection

Western blot technique, adopted from Nesslinger et al. (14), was used for assessment of tumor-specific antibody appearance. Protein extracted from 10 breast cancer cell lines (BT-549, HBL-100, MDA-MB-157, MDA-MB-330, MDA-MB-436, MDA-MB-453, MDA-MB-468, T-47D, MCF-7, and ZR-75-1) served as tumor-associated antigens for immunoblot analysis. These tumor cells were lysed on ice for 30 minutes in a buffer containing 50mM Tris-HCl (pH 7.4), 150mM NaCl, 1% Triton, 1mM Na₂-EDTA, 2.5mM sodium pyrophosphate, 1mM-β-glycerophosphate, 100μM PMSF (Sigma-Aldrich, St. Louis, MO, USA), 1mM Na₃VO₄, and 25ng aprotinin and leupitin (Sigma-Aldrich) and then centrifuged. Forty µg of protein from each cell line were combined for a total loading sample of 400µg (14). This mixture was loaded onto 8% and 12% polyacrylamide gels after denaturing at 95°C in 5% β-mercaptoethanol. The proteins fractionated by SDS-PAGE were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA) in a transfer buffer consisting of 48mM Tris base, 20% methanol, 0.04% SDS and 30mM glycine. The membranes were blocked for non-specific
binding with a blocking buffer consisting of TBST (50mM Tris, 150mM NaCl (pH 7.6), 0.05% Tween-20) and 5% (v/v) skim milk for 1 hour at room temperature. Then, the membranes were cut into 6-7mm strips and individually incubated with 4ml aliquots of patient plasma (diluted 1:1000 with TBST) in a 5ml tube (Becton Dickinson, Franklin Lakes, NJ, USA) and shaken overnight at 4°C. The strips were washed 4 times with TBST, and incubated for 1 hour at room temperature with peroxidase-conjugated goat anti-human IgG (KPL, Gaithersburg, MD, USA) diluted 1/20,000 in TBST or with rabbit anti-human IgM (Jackson ImmunoResearch Laboratory, West Grove, PA, USA) diluted 1/10,000 in TBST. After 4 additional washings, they were examined with Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA).

To validate breast tumor-specific binding of the detected antibodies, 2 sets of negative control experiments were performed. Protein extracted from MCA-10A, a normal breast cell line, was incubated with plasma from antibody positive patients to assess for nonspecific antibody binding. Further, previously stocked plasma obtained from 8 males and 8 females with primary brain tumors treated with brain RT on IRB approved protocol URC1395 were used as the primary antibody against antigens from our human breast cancer cell lines.

**Cytokine assay**

Fresh plasma samples were centrifuged at 1,000g for 5 min at -4°C immediately before analyzing using the supernatant as test samples for the cytokine assay. GM-CSF, IFN-γ, TNF-α, monocyte chemotactic protein-1 (MCP-1), Eotaxin, IL-1α, IL-1β, IL-2, IL-3, IL-6, IL-10, IL-12p70, and IL-13 levels in the samples were measured using a Milliplex™ cytokine array system (Millipore, Billerica, MA, USA) according to manufacturer instructions, with the Luminex® 200™ (Luminex, Austin, TX, USA) (15).
The values of GM-CSF, IFN-γ, and TNF-α were further tested with our institutional radioimmunoassay kit. For this experiment, 50µl of premixed antibody beads (R&D systems. Inc., Minneapolis, MN, USA) was applied to each well after pre-wetting the filter plate. The beads were washed twice with washing buffer (0.05% tween twenty in PBS) with removal of the buffer by vacuum filtration. 50µl of assay buffer with 25µl of standard (BioLegend, San Diego, CA, USA) or plasma samples were applied to each well. The plate was wrapped with aluminium foil and shaken overnight at 4°C for antibody binding. After the plate was washed 3 times, Biotin-labeled detection antibodies (BioLegend) were incubated for 1 hour at 500 rpm at room temperature. After washing, 50µl of Streptavidin was incubated for 30 minutes at 500 rpm at room temperature, and then further washing was performed. 125µl of reading buffer (Luminex Xmap Sheath Fluid, LumineX) was added, and the plate was shaken for 5 minutes at 500 rpm at room temperature. Cytokine values were measured using Luminex® 200™ (Luminex).

Statistical analysis

Analysis was performed using Student’s t-test, Yates’s continuity-corrected Chi-square test or Fisher’s exact t-test. Rates of overall survival (OS), progression free survival (PFS), new distant metastasis free survival (N-DMFS), and local control of irradiated metastatic tumors (LC) were calculated from date of RT start to death date or the last follow-up using Kaplan-Meier method (16). OS included deaths attributed to any cause, and PFS accounted for both cancer deaths and any tumor progression. N-DMFS included appearance of new metastatic tumors which were not present at the time of enrollment. Survival curves were compared by log rank test.
RESULTS

Autoantibody reactivity and clinical outcomes

IgM or IgG binding was detected in 50% (14/28) and 57% (16/28) of patients, respectively (Fig. 1 and Table 2). Median time to appearance of IgM and IgG was 9 days (range, 4-15 days) and 24 days (range, 10-111 days) from the start of RT, respectively. Furthermore, there was a significant positive correlation between IgM and IgG RIAA responses among all patients (p<0.001). Thirteen patients developed both positive IgM and IgG antibodies (IgM/IgG positive group), whereas 11 developed neither IgM nor IgG binding (IgM/IgG negative group). Antibody binding was frequently noted at or near 45-50 kDa in these patients.

Table 3 summarizes clinical outcomes according to RIAA reactivity. The 3-year OS in IgM or IgG positive patients were significantly superior to those in the negative group. Similarly, OS in the IgM/IgG positive group was greater than the negative group (p=0.017) (Fig. 1B), but the improvement in N-DMFS did not reach significance (Fig. 1C). There were no significant disparities in subject and treatment characteristics between these 2 groups (Table 4) to account for differences in outcome.

Among 21 subjects with 5 or fewer metastases treated with curative-intent, 11 subjects were IgM/IgG positive, and 6 were IgM/IgG negative (Table 2). These patients demonstrated a similar positive correlation between IgM and IgG RIAA responses (p=0.018). In the IgM/IgG positive group, 9 of 11 (82%) subjects were alive at the last follow-up, whereas 4 of 6 (67%) in the IgM/IgG negative group died of disease progression; the difference in OS was significant (Fig. 3). The 3-year OS in IgM or IgG positive patients treated with curative-intent was also superior to those in the negative group (Fig. 3). Moreover, there were no significant differences in characteristics of patients or treatment intensiveness such as total dose and other combination
therapy between IgM/IgG positive and negative groups (Table 4). Negative control studies supported our assertion that the detected antibodies were breast cancer-specific.

**Relationship between RIAA response and cytokine levels**

The levels of 13 cytokines in collected samples were evaluated according to the time points collected (pre-RT, during RT, end of RT, and post RT). The samples collected 1-14 days after RT were defined as “end of RT” and those collected 1-3 months after RT were defined as “post RT”.

A half value of cytokine threshold was substituted for the value under the threshold, but a comparison according to autoantibody reactivity was done only when 85% of these values were above the threshold to avoid inaccuracy. Consequently, GM-CSF, IFN-γ, MCP-1, Eotaxin, and TNF-α met these criteria for comparison in the present study.

Table 5 summarizes the cytokine levels of IgM/IgG positive and negative groups at each sampling point. A similar tendency was observed in GM-CSF, IFN-γ and TNF-α; the values of these cytokines in the IgM/IgG positive group were up-regulated during RT and almost returned to the baseline levels post RT, but there were no obvious changes of the cytokine values in the negative group (Fig. 4). Furthermore, the values of the cytokines in the positive group were significantly higher than those in the negative group at several sampling points. On the other hand, there were no significant differences in the values of MCP-1 and Eotaxin between the two groups (Table 5).

**DISCUSSION**
Despite advances in diagnostic techniques and treatment methods, breast cancer remains a leading cause of cancer death in women worldwide, and an estimated 40,480 women died of breast cancer in 2008 (17). Given that the natural course of breast cancer is to spread systematically even when the tumors are diagnosed at an early stage, systemic chemotherapy and hormone therapy in combination with local treatment are often necessary for disease control. Therefore, exploring innovative strategies for patients with locally advanced or metastatic breast cancers remains an important goal.

To address this problem successfully, the immunostimulatory effects of focal radiation with immune adjuvant may be one of encouraging challenges for leading to greater control of the disease. Although RT has been generally considered immunosuppressive in the past, the beneficial effects of RT in enhancing the immune system in the presence of cancer cells have been described due to recent remarkable advances in understanding mechanisms of the immune system in cancer patients (4, 7-11, 18,19). Furthermore, preclinical studies suggest that the efficacy of immunotherapy on immune-based tumor cell kill is much enhanced when radiation exposure is combined (6, 20-23). A strategy that augments the RT effects via activating patient autoimmunity is promising, especially modern conformal RT technique such as SBRT are given.

Nevertheless, there is little available data the immunological effects of RT itself clinically contribute to outcomes of cancer patients. A previous study reported by Nesslinger et al. showed that 29% of patients with prostate cancer treated with RT and hormone therapy mounted tumor-specific immune response resulting in new antibody formation (14). In the present study, >50% of subjects demonstrated RIAA, a remarkable finding when considering that our patients included those who previously received multiple immunosuppressive chemotherapeutic regimens. Importantly, those patients who exhibited RIAA generally had a
prolonged interval to developing new metastatic lesions, and RIAA formation conferred superior OS (Fig. 2 and Table 3), although they did not evaluated the effects of RIAA on the clinical outcomes. Furthermore, no significant difference in characteristics of patients according to IgM/IgG reactivity in this study validates the predictive value of RIAA observation (Table 4).

To define potential immunomodulatory mechanisms leading to RIAA formation, radioimmunoassay experiments were performed to detect variations in cytokine levels in those patients who mounted an antibody response. Most patients with elevated GM-CSF, IFN-γ or TNF-α levels above their baseline benefited from an immune response, suggesting an interaction between these cytokines and radiation-induced immunologic signaling (Fig. 4) (24). These cytokines have critical roles in both innate and adaptive immunity, and notably, these have proven to be effective cancer treatments for immune sensitive solid tumors including renal cell carcinoma and melanoma (25-27). Conversely, cytokines such as MCP-1 and Eotaxin which are not currently used for immunotherapy, showed no significant correlation with anti-tumor immunity.

We present the first clinical evidence for autoimmune response induced by SBRT for metastatic breast cancer patients, and RIAA predicts superior treatment outcomes in the patients evaluated in this study. Thus, these results would be useful for exploring a novel treatment modality utilizing RT combined with immune therapy for not only metastatic but also advanced breast cancers as advanced disease also has a high potential to develop occult metastatic tumors. However, there are some limitations in this study.

Firstly, our study included some variations in patient characteristics between IgM/IgG RIAA positive group and negative groups. We previously reported better survival in patients with ≤5 metastatic tumors than in patients with >5 after SBRT (12,13). In this study, 2 (15%) of
13 patients in the positive group had >5 tumors compared with 5 (45%) of 11 in the negative group (Table 4). Similarly, 3 (27%) patients in the IgM/IgG positive group had metastases in multiple organs, whereas 5 (45%) in the negative group had multiple organ involvement. These differences may impact patient survival outcome, but there was no statistical difference in the number of lesions between the groups (Table 4). Of the 21 patients with ≤5 metastatic tumors who were treated with a curative intent in the present study, 11 and 6 patients were considered as IgM/IgG positive and negative, respectively. Among them, only 2 (18%) of 11 IgM/IgG positive patients died of progressive disease, whereas 4 (67%) of 6 IgM/IgG negative patients had died at the last follow-up. Similarly, 1 (14%) of 7 in the IgM/IgG positive group and 2 (50%) of 4 in the negative group died of breast cancer recurrences in 12 patients with single metastasis. Although the number of patients analyzed in the study was small, the autoantibody reactivity may be a useful tool in predicting outcomes of metastatic breast cancer patients after SBRT.

Secondly, there was a slight difference between IgM and IgG detections in this study. Using Western blot technique, antibodies to human IgM and IgG were detected in 14 and 16 patients, respectively (Table 2). Although a significant correlation between IgM and IgG detection was observed with earlier appearance of IgM (median time to appearance; IgM: 9 days, IgG: 24 days), the immune-blot failed to detect IgM reactivity in 3 of 16 IgG positive patients. The reasons for this discrepancy may be due to comparatively low sensitivity of the blot and short half-time of IgM. As IgM has a much lower concentration and shorter half-life (approximately 5 days) than IgG in human blood, we may have not collected blood sample at a suitable time point regardless of serial sampling. It is beyond our scope of this study to assert which type of RIAA is a reliable predictor, but evaluating both IgM and IgG could ensure that RIAA is detected and is cancer-specific.
Lastly, it may be questioned whether RT actually induced antibodies in our breast cancer patients. Our data support that RT likely stimulated “cancer immunosurveillance” and induced the antibody formation by making “danger signals”. Again, the differences in cytokine levels of GM-CSF, IFN-γ and TNF-α between the IgM/IgG positive and negative groups were consistent with the antibody results (Table 5). In addition, these cytokines increased after SBRT in the positive group only. Furthermore, early detection of IgM, which had a significant correlation with IgG detection, can support that SBRT induces antibody expression as IgM immediately responds to stimuli in the host.

CONCLUSION

Our observations are compelling for the assertion that radiation-induced autoimmunity works synergistically with local tumor-killing effects of RT, and ultimately, this translates to an improvement in OS. A larger clinical trial is necessary to validate our results, and a multi-institutional study to assess the efficacy of SBRT and RIAA induction in oligometastatic breast cancer patients is underway. Our current work focuses on characterization of the antigens which have exhibited specific binding to RIAA. Identification of breast cancer-associated antigens such as BRCA and NY-ESO-1 (28) may lead to development of a vaccine that enhances the tumor-specific immune activation conferred by focal RT. We suggest that GM-CSF, IFN-γ or TNF-α may be given adjuvantly to enhance the RIAA response, especially for patients who have low endogenous levels of these cytokines at RT initiation. Therefore, novel approaches to systemic cancer treatment may be developed by harnessing an individual’s immune response, thereby holding promise for incurable cancer patients.
REFERENCES


FIGURE LEGENDS

Figure 1
Evidence for autoantibody induced by SBRT in patients with metastatic breast cancer.
Multiple IgM and IgG bands newly or gradually appeared at different time points after SBRT (P: pre-treatment, 1: 6th day, 2: 12th day, 3: 67th day, 4: 111th day after SBRT start) by Western blotting.

Figure 2
Treatment outcomes for metastatic breast cancer according to autoantibody reactivity.
(a) The 3-year overall survival rates of the IgM/IgG positive (n=13) and negative (n=11) groups were 65.9% (95% CI: 38.0-93.9%) and 13.6 % (95% CI: 0-36.6), respectively (p=0.017; log rank test). (b) The new metastasis-free survival rates of IgM/IgG positive and negative groups at 3 years after SBRT were 53.9% (95% CI: 26.8%-81.0%) and 18.2% (95% CI: 0-39.1%), respectively (p=0.236; log rank test).

Figure 3
Overall survival curves according to antibody reactivity for 21 subjects treated with a curative-intent. (a) The 3-years overall survival rates of the IgM/IgG positive (n=11) and negative (n=6) groups were 77.9% (95% CI: 50.2-100%) and 25.0 % (95% CI: 0-65.0%), respectively. The difference between these groups for the overall survival curve was statistically significant (p=0.043, log rank test). (b) The 3-years overall survival rates of the IgM positive (n=12) and negative (n=9) groups were 80.2% (95% CI: 44.3-100%) and 34.6 % (95% CI: 0-70.5%), respectively. The difference between these groups for the overall survival curve was statistically
significant (p=0.044, log rank test). (c) The 3-years overall survival rates of the IgG positive 
(n=14) and negative (n=7) groups were 75.0% (95% CI: 49.7-100%) and 38.1 % (95% CI: 0-
77.2%), respectively. The difference between these groups for the overall survival curve did not 
reach a significant level (p=0.133, log rank test).

Figure 4

Plasma GM-CSF (a), IFN-γ (b), and TNF-α (c) levels using two assay kits according to 
autoantibody reactivity.

The levels of the IgM/IgG positive group (mean ± s.e.) were higher than those of the negative 
group before, during and after SBRT (* P<0.05, **P<0.10; Student’s t-test). A similar tendency 
difference between the groups was observed for the assay kits (Assay kit 1: Milliplex™ cytokine 
array system, Assay kit 2: institutional kit).