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[Review]

ANTI-TUMOR IMMUNE RESPONSES INDUCED BY RADIOTHERAPY: A REVIEW

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Abstract: An anti-tumor immune response is one of the most important factors that can determine treatment response and prognosis of cancer patients. Recent studies have demonstrated that radiotherapy can activate tumor-specific immune responses and that these responses contribute to the therapeutic efficacy. However, the exact mechanisms underlying the radiation-induced immune responses remain unclear. Better understanding of the mechanisms could facilitate the application of immune-activating radiotherapy and provide new treatment strategies. We previously demonstrated that tumor-specific T cell responses could be induced in esophageal cancer patients during and after chemoradiotherapy. Furthermore, in a mouse model, immune responses played an important role in determining the local and systemic therapeutic efficacy of radiotherapy that could be augmented by the immune checkpoint blockade. In this review, radiotherapy-induced immune responses, the mechanisms underlying the induction of those responses, and a practical application of the therapy are discussed.

Key words : radiotherapy, anti-tumor immunity, immunotherapy, abscopal effect, cytotoxic T-lymphocyte

INTRODUCTION

The "immune surveillance" hypothesis postulates that immune cells recognize and eliminate the tumor cells¹⁾. The hypothesis has been supported by a study that found an increased incidence of cancer in organ transplanted patients treated with immunosuppressing agents²⁾. The study examined 905 patients who underwent heart, lung, or heartlung transplantation over a 15-year period and detected 102 newly diagnosed cases of cancer. The incidence was 7.1 times higher in the immunosuppressed transplant patients than in the general population, suggesting that immunity prevents tumor progression. Another study followed 3625 healthy people for 11 years and found that the incidence of cancer was lower in the subjects whose blood lymphocytes had high or medium natural cytotoxic activity compared with the subjects whose lymphocytes had low cytotoxic activity³⁾. Taken together with higher incidence of cancer observed in immune deficient mice⁴⁾, these observations support the immune surveillance hypothesis.

Immunity is involved not only in carcinogenesis but also in tumor growth and recurrence after treatment. However, for many years there was an intense debate about whether immunity can prevent tumor growth, because established tumors appeared to be resistant to anti-tumor immunity. Galon *et al.* demonstrated that the type, density, and location of tumor-infiltrating immune cells were strongly correlated with survival in large cohorts of human patients with colorectal cancers⁵). The differentiation of Th1 cells with cytotoxic and memory components was especially important^{6,7)}. The density and location of infiltrating CD3⁺ and CD45RO⁺ cells used as an "immunoscore" were a better predictor of the patient's survival than the currently used histopatho-

Corresponding author : Yoshiyuki Suzuki, MD, PhD E-mail : ysuzu@fmu.ac.jp https://www.jstage.jst.go.jp/browse/fms http://www.fmu.ac.jp/home/lib/F-igaku/ logical methods such as TNM classification and Duke's classification⁵⁾. Correlation between immune infiltrates and prognosis has also been observed in other human cancers such as melanoma⁸⁾, ovary^{9,10)}, breast¹¹⁻¹³⁾, lung¹⁴⁻¹⁸⁾, esophageal^{19,20)}, and prostate cancer²¹⁾. Therefore, anti-tumor immunity is an important factor that determines the prognosis of cancer. Accordingly, novel anti-cancer immuno-therapies are now emerging^{22,23)}.

Furthermore, it has been shown that anti-tumor immunity contributes to the efficacy of anti-tumor therapies, such as chemotherapy and radiother apy^{24-27} . We have previously reported that the anti-cancer agent doxorubicin induces Fas on the tumor cells and that its anti-tumor effect depends on the presence of functional ligand (FasL) in the hosts, suggesting that the effect was exerted via the interaction of Fas on the tumor and FasL on the immune cells²⁴⁾. Casares et al. also reported that doxorubicin was more effective against established CT26 mouse colon cancer in immune-competent mice than in immune-deficient nude mice²⁵⁾. These results indicate that the therapeutic efficacy of doxorubicin is, surprisingly, the result of its effect on antitumor immunity. Additionally, Apetoh et al. demonstrated that the anti-tumor therapies induce immunogenic tumor cell death (ICD), resulting in the activation of anti-tumor immunity^{26,27)}. Thus, the therapies trigger an activation of immunity, and the immunity plays a role in the efficacy of the therapies. In a clinical setting, it was reported that platinum-based chemotherapy is more effective in advanced ovarian cancer patients who carry CD3⁺ tumor-infiltrating lymphocytes (TILs)^{9,28)}. In breast cancer patients treated with neoadjuvant chemotherapy, TILs and dendritic cells (DCs) in biopsies independently predict complete pathological responses²⁹⁻³¹⁾. Additionally, in gastrointestinal stromal tumor (GIST), imatinib exerts its anti-cancer efficacy via immune cells³²⁻³⁴⁾.

In the field of radiotherapy, Nakano *et al.* reported that tumor-infiltrating antigen presenting cells (Langerhans' cells) are positively correlated to successful radiotherapy and good prognosis in cervical cancer patients³⁵). This result suggests that radiotherapy induces anti-tumor immunity and that the immunity contributes to the therapeutic efficacy of radiotherapy. An extreme example of immune activation by radiotherapy is the phenomenon known as the "abscopal effect," the regression of a metastatic tumor located at a distance from the irradiated tumor^{36,37)}. This phenomenon is rarely observed but has been reported in several types of cancer. These observations strongly suggest that radiotherapy and anti-tumor immunity are closely related. However, the detailed mechanisms of radiotherapy-induced immunity and its contribution to therapeutic efficacy and prognosis are still unclear. In this review, we discuss radiation-induced immune responses, the mechanisms underlying the induction of those responses, and the practical application of immune responses induced by radiotherapy.

RADIOTHERAPY-INDUCED ANTI-TUMOR IMMUNITY IN ANIMAL EXPERIMENTS

Recently, it was reported that anti-tumor therapies induce ICD that can activate the immune cells^{27,37,38)}. Furthermore, it was proposed that the therapy-induced anti-tumor immunity contributes, at least in part, to the therapeutic efficacy of the anti-tumor therapies. A hypothetical schema of the mechanisms of radiotherapy-induced anti-tumor immunity is shown in Figure 1. To prove the hypothesis, we established an animal model for radiotherapy and investigated immunity in radiotherapy as follows³⁹⁾.

In the C57BL/6 mouse model, hind limb inoculation of syngeneic EL4 lymphoma cells causes death from systemic metastasis. Local radiotherapy suppresses growth of the EL4 tumor and the mice survive. The mice seem to obtain EL4-specific immunity because EL4 lymphoma cells were rejected on re-inoculation. However, in mice previously inoculated with B16 lymphoma cells, EL4 lymphoma cells were not rejected. In the EL4treated mice, EL4-specific IgG1 was detected and splenocytes produced IFN γ when co-cultured with EL4 as an antigen, indicating that the mice obtained EL4-specific humoral and cellular immunity. Therefore, radiotherapy can induce anti-tumor specific immunity in the animal model.

The therapeutic potential of the immunity was also examined, again using the C57BL/6 mouse model. We inoculated EL4 lymphoma cells in bilateral hind limbs and waited 10 days until the tumor became measurable. After the tumor reached approximately 100 mm³, only right limb tumors were irradiated. The irradiated tumor shrank rapidly and became almost undetectable 4 days after irradiation. Notably, the growth of non-irradiated left limb tumors was also inhibited. After 9 days of irradiation the volume was half, compared with the volume of tumor in non-irradiated mice. It was proposed that irradiation to the right limb tumor induced antitumor immunity and that immunity inhibited the



Fig. 1. A schematic illustration of the mechanism of irradiation-induced anti-tumor specific immunity. Irradiationinduced cell death involves a release of "danger signals" such as HMGB1, which can activate the dendritic cells. The dendritic cells stimulate tumor-specific T cells and activated T cells attack the tumor. The irradiation also causes the tumor to be susceptible to the immunity, for example, via the increased expression of HLA class I molecules.

growth of the left limb tumor. This phenomenon was an experimental example of the abscopal effect. Furthermore, immunity is essential for successful radiotherapy because the radiotherapy failed in immune-deficient nude mice.

The immune contribution to the therapeutic effect was also examined in the Lewis lung carcinoma model. Depleting CD8⁺ cells using an anti-CD8 antibody significantly reduced the therapeutic efficacy of irradiation in terms of both tumor growth and survival time. These results demonstrate that local irradiation induces systemic tumor-specific immune responses, which are also essential for the local control of tumors. We emphasize that irradiation delayed tumor growth, as a result of tumor-specific immune responses not solely because of DNA breakage caused by the irradiation. Lee et al. recently reported that irradiation increases T cell priming, leading to tumor reduction in syngeneic models of B16 melanoma⁴⁰⁾. Takeshima et al. reported that CD8⁺ cell depletion decreases the therapeutic efficacy of irradiation in a C57BL/6 mouse tumor model that used ovalbumin-transfected cells⁴¹. Taken together, these reports demonstrate that radiotherapy-induced anti-tumor immunity contributes to the therapeutic efficacy of irradiation in the animal models.

A key event that induces the activation of antitumor immunity is ICD. ICD involves changes in the expression of cell-surface molecules as well as the release of soluble mediators, called damage-associated molecular pattern molecules (DAMPs). Apetoh et al. have shown that DAMPs are released by the cells treated with DNA-damaging agents such as doxorubicin, oxaliplatin, and γ -irradiation and can stimulate DCs via a toll-like receptor (TLR), resulting in the activation of T cells²⁶⁾. They also showed that among various types of TLRs, TLR4 is essential because T cell activation did not occur in TLR4^{-/-} mice $^{26)}$. The DAMP molecule in the pathway was high mobility group box 1 (HMGB1), which binds to TLR4 on the surface of DCs and activates the downstream immune reactions. We reported that X-ray irradiation as well as carbon-ion beam irradiation induced *in vitro* release of HMGB1 from human cancer cell lines originating from different organs, suggesting that ionizing irradiation is capable of inducing immune reactions $^{42)}$.

These observations, which show that the activation of anti-tumor immunity is involved in the effi-

cacy of irradiation, suggest that the therapeutic efficacy is enhanced by augmenting the immune response. Demaria et al. reported that irradiation caused the abscopal effect in their mouse model and that the effect was augmented by cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) blockade^{43,44)}. We also found that the administration of CTLA-4 antibody enhanced the therapeutic efficacy of irradiation in our animal model³⁹⁾. CTLA-4 is an immune-modulatory molecule, expressed on the surface of activated T cells where it acts as an "immune checkpoint"^{45,46}. The checkpoint is an inhibitory pathway and blocking the CTLA-4 receptors on T cells may boost anti-tumor immunity. Therefore, we propose that CTLA-4 blockade could boost irradiation-induced tumor specific T cells, resulting in further therapeutic efficiency.

RADIOTHERAPY-INDUCED ANTI-TUMOR IMMUNITY IN THE PATIENTS TREATED WITH RADIOTHERAPY

Translational research to detect radiotherapyinduced anti-tumor immunity is necessary to develop new therapies to induce immunity. However, to date, such studies are limited because quantifying immunity in the human patients is difficult and complex. Therefore, we investigated a method to detect tumor-specific T cells in the patients receiving chemoradiotherapy to treat esophageal cancer⁴⁷. To detect tumor-specific T cells, it is necessary to identify tumor-specific antigens that are recognized by T cell receptors. In recent studies by Nakamura et al., a number of tumor-associated antigens (TAAs) have been identified in genome-wide screening using cDNA microarray technologies. Among TAAs, we have shown that TTK protein kinase (TTK), lymphocyte antigen 6 complex locus 6 (LY6K), and IGF-II mRNA binding protein 3 (IMP-3) are expressed in almost all the esophageal squamous cell carcinomas (ESCCs) and peptides derived from these TAAs can stimulate cytotoxic T lymphocytes (CTLs) that recognize and kill the ESCC cells⁴⁸⁻⁵¹). In addition, other tumor-specific TAAs, cell division cycle associated 1 (CDCA1)52,53), 34 kDa translocase of the outer mitochondrial membrane (TOMM34)⁵⁴⁾, and hypoxia-inducible protein 2 (HIG2)⁵⁵⁾, were also chosen. In total, six TAAs were used for our detection system for tumor-specific T cells. We collected peripheral blood mononuclear cells (PBMCs) from the patients with ESCC before chemoradiotherapy treatment, during treatment, and three days and 30 days after the end of treatment. The patients with HLA-A*0201 and/or HLA-A*2402 were eligible because the detection uses HLA-restricted recognition. The PBMCs were cultured with COS7 cells transfected with one of the TAAs and HLA-A*0201 or -A*2402. TAAs are processed inside the COS7 cells and are presented on the HLA molecules. TAA-specific T cells recognize the cells in a HLA-restricted manner, start proliferating and produce IFNy, which is detected by IFNy-ELISPOT assay. For example, in one patient, there was no specific T-cell response before chemoradiotherapy, but antigen-specific T-cell responses (IFNy production) were induced against COS7 cells transfected with IMP3, CDCA1, TOMM34, and HIG2 in combination with HLA-A0201 during chemoradiotherapy. No response was observed in COS7 cells transfected with HLA-A0201 alone. In line with the patient's status (HLA-A0201+ but HLA-A2402-), there was no specific T-cell response against COS7 cells transfected with target genes plus HLA-A2402. The induction of tumor-specific T cells was observed in six of the 16 patients (38%), indicating that the tumor antigen-specific T-cell response was induced by chemoradiotherapy in the patients with ESCC.

To examine the role of DAMPs in the activation of immune response, we measured HMGB1 protein levels in the serum of 14 of the 16 ESCC patients treated with chemoradiotherapy. The HMGB1 levels were significantly increased after the therapy whereas only the marginal levels were detected before the therapy. In addition, the levels in the six patients who showed specific T cell responses were significantly elevated compared with the other eight patients. Therefore, it was proposed that the HMGB1 induced by the chemoradiotherapy triggered the systemic T cell responses. HMGB1-expression levels in resected specimens from another group of ESCC patients who underwent surgical resection with or without neoadjuvant chemoradiotherapy were examined by immunohistochemistry. The patients were classified into HMBG1-strong or -weak groups based on the median value of counts of the cancer cells. The proportion of HMGB1strong patients was higher in the chemoradiotherapy group (29 of 45 patients) compared with the surgery alone group (17 of 43 patients, p=0.04). In the HMGB1-strong patients, there tended to be a higher number of infiltrating CD8⁺ cells, but the difference did not reach the significance. Moreover, compared with the HMBG1-weak group, the prognosis was better in the HMGB1-strong group (p < 0.05). We also examined the levels of calreticulin, which is known to be DAMP, but there were no differences in the prognosis between calreticulin-strong and -weak groups. Therefore, the up-regulation of HMGB1 may be involved in anti-tumor immune responses and may improve patient survival.

Although evidence is limited, anti-tumor immune responses could be involved in human malignancies. Apetoh *et al.* reported that the activation of TLR4 on DCs was essential for anti-tumor immunity because the patients with breast cancer who carry a TLR4 loss-of-function allele relapsed more quickly after radiotherapy and chemotherapy²⁶⁾. As described above, both natural killer (NK) cells and T cell activation are involved in GIST patients treated with imatinib³²⁻³⁴⁾. In breast cancer patients treated with neoadjuvant chemotherapy, the number of TILs and DCs in biopsies independently predicted the complete pathological responses²⁹⁻³¹⁾.

Irradiation also activates the immune responses in another way. We have reported that the number of HLA class I molecules, which are essential for the recognition of T cells, is elevated in resected specimens from the patients with rectal cancer who received chemoradiotherapy with hyperthermia⁵⁶⁾. Among the 78 patients, HLA class I expression on most of the tumors before treatment was categorized as "None" (19 patients, 24%) or "Weak" (58 patients, 74%), and only one patient was classified as "Moderate" (1%). However, after treatment, six (8%), 27 (35%), seven (9%), and 12 (15%) patients were categorized as "None", "Weak", "Moderate", and "Strong", respectively, and the up-regulation of HLA class I molecules was statistically significant (p < 0.01). In addition to the induction of HLA molecules, it has been reported that radiotherapy can induce immunostimulatory and cytotoxic cytokines, such as TNF α and IL-18^{57,58)}. Therefore, radiotherapy can induce or support anti-tumor immune responses in the cancer patients.

COMBINATION OF RADIOTHERAPY AND IMMUNOTHERAPY AND RESULTS OF CLINICAL TRIALS

As mentioned above, an extreme example of immune activation in radiotherapy is the abscopal effect. We have previously reported a case of the abscopal effect in a patient with lymphoma treated with radiotherapy³⁷⁾. In that patient, a metastatic tumor decreased in size after radiotherapy. Although the abscopal effect is a rare phenomenon, it has recently been reported that the effect is seen in the patients undergoing radiotherapy combined with immuneboosting therapies such as TLR agonists⁵⁹⁾, transforming growth factor β (TGF β) blockade⁶⁰, and CTLA-4 blockade. Among the immune-boosting agents, the anti-CTLA-4 antibody ipilimumab has been the most widely studied.

Ipilimumab, a fully human anti-CTLA-4 antibody, was approved by the FDA for use in unresectable and metastatic melanoma in 2011^{45,46,61)}. Postow et al. reported a case of the abscopal effect in a patient with melanoma treated with radiotherapy and ipilimumab⁶²⁾. The tumor regression in this patient was associated with antibody responses against the tumor-specific cancer-testis antigen, indicating that augmentation of tumor-specific immune responses had occurred and demonstrating the promise of the combination of radiotherapy and CTLA-4 blockade. Moreover, Grimaldi et al. reported that an abscopal effect was seen in 11 of the 21 melanoma patients (52%) who had experienced the disease progression after ipilimumab and received subsequent radiotherapy⁶³⁾. An abscopal effect in combination with ipilimumab was also observed in a patient with lung adenocarcinoma⁶⁴⁾, suggesting the benefit of combination therapy for the other types of malignancies. The findings of a trial of ipilimumab after radiotherapy in the patients with metastatic castration-resistant prostate cancer indicated that ipilimumab can reduce PSA concentration and improve progression-free survival, but the trial did not meet its primary endpoint of the improved overall survival⁶⁵⁾. Currently, a number of clinical trials of radiotherapy combined with ipilimumab are ongoing⁶⁶⁾.

Programmed death-1 (PD-1) receptor is also an important immune checkpoint molecule expressed on activated T cells and its ligation to programmed death-ligand 1 (PD-L1) leads to peripheral tolerance^{45,46)}. Monoclonal antibodies against PD-1 or PD-L1 developed for treatment of melanoma, nonsmall cell lung cancer, and renal cell carcinoma resulted in improved outcomes in clinical trials⁴⁶⁾. In 2014, a fully human IgG4 anti-PD-1 antibody, nivolumab, was approved for the treatment of unresectable malignant melanoma in Japan⁶⁷⁾. Blockade of the PD-1/PD-L1 pathway is also a good candidate for combination with radiotherapy because a number of animal experiments have demonstrated significant efficacy of the combination⁶⁸⁻⁷⁰⁾.

Vaccination with DCs is another therapy that has shown promise in combination with radiotherapy. DCs are professional antigen-presenting cells that most effectively activate naive T cells and initiate T cell responses⁷¹⁾. Immature DCs (iDCs) that distribute in the tissues engulf extracellular material



Fig. 2. The treatment regimen for the clinical trial of a combination of iDC vaccination, LAK, and chemoradiotherapy. An injection of iDCs under endoscopy (days 11 and 18) and LAK-infusion (days 11, 18, and 39) are combined with standard chemoradiotherapy.

and on maturation, present the materials on the HLA molecules as antigens with co-stimulatory molecules such as CD40, CD70, and CD86. Several stimuli can promote maturation of iDCs, including microbe-associated molecular patterns (MAMPs), DAMPs, cytokines, and chemokines. For DC vaccination, autologous DCs loaded with autologous tumor lysate are matured with various maturationprotocols and are administered to the patients⁷². Sipuleucel-T is the first therapeutic cancer vaccine that can prolong the median survival of the patients with metastatic castration-resistant prostate cancer^{73,74}). In some clinical trials of advanced melanoma, treatment with sipuleucel-T resulted in a better prognosis than treatment with ipilimumab, suggesting the potential of the DC vaccination⁷⁵⁾.

We propose that a combination of radiotherapy and injection of iDCs is attractive because radiotherapy induces HMGB1, one of the DAMP signals capable of promoting the maturation of iDCs. Therefore, we conducted a phase I/II clinical study of the combination of DC vaccination, adoptive activated T lymphocyte infusion, which can restore impaired and imbalanced T cell immune status⁷⁶, and standard chemoradiotherapy for Stage IB-IIIC ESCC (UMIN000014099). After the patient enrollment and clinical verification of the disease status, leukopheresis to obtain monocytes for DC generation was performed. The lymphocytes were also used to expand lymphokine-activated killer (LAK) cells. The patients then received standard chemoradiotherapy with 60 Gy in 30 fractions (2 Gy per fraction) over six weeks (days 1-40) of radiotherapy and two cycles of cis-diamminedichloroplatinum (CDDP) 70 mg/m² (days 1 and 29) plus 5-fluorouracil (5-FU) 700 mg/m² (days 1-4 and days 29-32). Injection of iDCs was performed endoscopically directly into the tumor on days 11 and 18, by which time tumor tissues should express HMGB1. LAK was infused on days 11, 18, and 39, and should support effective anti-tumor immunity. Figure 2 presents an overview of the study protocol. We will evaluate the safety of the treatment as the primary endpoint and then will proceed to further evaluations.

As we discussed above, radiotherapy has an anti-tumor vaccine-like effect that can be augmented by, for example, immune-boosting checkpoint inhibitory antibodies or autologous immune cells. Therefore, these new approaches may lead to the development of novel treatment strategies.

CONCLUSION

For many years it has been thought that radio-

therapy exerts its therapeutic effect via its ability to cause DNA breakage. However, recent studies have demonstrated that radiotherapy induces tumorspecific immunity and its therapeutic efficacy can be augmented by modification of the immunity. These finding could potentially change the treatment strategy of radiotherapy and in future, new "immune–radiotherapy" strategies will be developed based on radiobiology and tumor immunology.

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CONFLICT OF INTEREST NOTIFICATION

The authors have no conflicts of interest to declare.

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