Antitumor abscopal effects in mice induced by normal tissue irradiation using pulsed streamer discharge plasma

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Abstract
An antitumor abscopal effect is occasionally observed in radiotherapy and plasma treatment. It is a remote antitumor effect induced by tumor irradiation that delays the growth of other distant, nonirradiated tumors. In this study, it was demonstrated that the plasma irradiation of normal tissues (not tumors) also leads to an abscopal effect. When a pulsed streamer discharge was irradiated to the left flanks of mice where no tumor existed, the growth of murine colorectal carcinoma CT26 tumors in their right limbs was delayed. This abscopal effect was significant for mice with small tumors before plasma irradiation, whereas it was not significant for those with large tumors before plasma irradiation. The abscopal effect induced by normal tissue irradiation was compared to the antitumor effect induced by direct tumor irradiation. Contrary to our expectation, normal tissue irradiation delayed the tumor growth equally or more than the direct tumor irradiation under the present experimental conditions.

Supplementary material for this article is available online

Keywords: antitumor abscopal effect, normal tissue irradiation, plasma medicine, streamer discharge, CT26 tumor, in vivo

(Some figures may appear in colour only in the online journal)
1. Introduction

Atmospheric pressure nonthermal plasma is an emerging technology used for cancer treatment. The plasma irradiation of tumors can reduce their growth in various mouse models [1–4]. Plasma is a partially ionized gas generated by applying a high voltage to the gas. It produces reactive oxygen and nitrogen species (RONS) via collisions of gaseous molecules/atoms with electrons accelerated by the high electric field in the plasma. The plasma-generated RONS acting on cancer cells are considered to be responsible for the antitumor effects [4, 5].

Recent studies suggested that plasma irradiation also activates antitumor immune responses [6–10]. Our previous study [11] suggested that the plasma irradiation of murine melanoma B16F10 tumors in mice may activate an adaptive immune response specific to B16F10. This was supported by the high secretion level of inflammatory cytokine interferon-γ from the splenocytes of plasma-irradiated mice when co-cultured with B16F10 cells. Our following study [12] showed that plasma irradiation induces a long-term, systemic antitumor effects on mice. In that experiment, plasma-irradiated B16F10 tumors were resected from mice; then 2 weeks later, fresh B16F10 cells were reirradiated at sites distant from the resected sites. The plasma irradiation of primary tumors delayed the growth of reirradiated tumors. The increased level of cytotoxic T cells (CD8+ T cells) observed in the reirradiated tumors of plasma-irradiated mice suggested the activation of adaptive immune responses. Other researchers also reported the possibility of plasma-induced antitumor immunity by vaccinating mice with plasma-treated cancer cells [13], irradiating tumors with plasma [14], and applying plasma-treated saline to tumors [15]. In [12], it was also suggested that the plasma-induced immunity may inhibit recurrence after tumor resection.

Antitumor immunity is a well-known phenomenon in radiotherapy (RT) and photodynamic therapy [16–19]. Damaged cancer cells partially undergo immunogenic cell death; then, they express and secrete damage-associated molecular patterns (DAMPs) to activate immune cells. A similar mechanism is expected in plasma-induced antitumor immunity, as demonstrated in vitro [6, 8, 9, 20].

Our previous study [11] also showed a plasma-induced abscopal effect, which is an antitumor effect on distant nonirradiated tumors. The plasma irradiation of one tumor in mice with two distant B16F10 tumors reduced the growth of another nonirradiated tumor. The plasma-induced abscopal effect was also observed for murine breast cancer 4T1 in another group [14]. The abscopal effect has been occasionally observed in RT [16, 17], but its mechanism is not clear. Immune system activation is considered to be responsible for this effect.

In the experiments of plasma-induced abscopal effects described above [11, 14], the tumors were irradiated with plasma. However, our recent experiments showed that the abscopal effect is also observed when the plasma is irradiated to normal tissues of tumor-bearing mice. In this study, the abscopal effect induced by normal tissue irradiation is reported using a murine colorectal carcinoma CT26 tumor mouse model. For the irradiation, a pulsed streamer discharge was used. The streamer discharge is a branching, filamentary shaped nonthermal plasma typically generated in atmospheric-pressure air or oxygen-nitrogen mixtures [21]. It produces many types of RONS which may be effective for the cancer treatment [22].

2. Experiments

2.1. Plasma device

The plasma device for generating the streamer discharge consisted of a high-voltage rod electrode with a hemispherical tip and a grounded plate electrode to put a mouse on [11, 12]. A streamer discharge was generated between the hemispherical tip and mouse skin. The plate electrode was heated to 37 °C to prevent the mouse from losing heat. The rod 3 mm in diameter was concentrically placed inside a glass tube with 4 mm inner diameter. The hemispherical tip was protruded 1 mm from the glass tube end. Oxygen gas humidified with a water bubbler was flowed through a glass tube at a rate of 0.5 L min⁻¹ (relative humidity ≥ 90%). Humid oxygen was used because a large amount of reactive oxygen species production was expected. A 5 mm width grounded metal tape was wrapped around the glass tube to 20 mm from the tube end to generate a dielectric barrier discharge inside it, which stabilized the streamer generation. More details of the plasma device and streamer discharge characteristics are given in section S1 of supplementary materials (available online at stacks.iop.org/JPhysD/55/17LT01/mmedia).

2.2. Cells and animals

CT26 cells purchased from RIKEN BioResource Center (Ibaraki, Japan) were cultured in RPMI media supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum at 37 °C with 5% CO₂. The experiments were performed in two different laboratories at Hongo and Komaba. In Hongo experiments, BALB/c mice (female, 7 weeks old) were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan), while in Komaba experiments, the mice (female, 6 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan). The mice were maintained in animal facilities for 1 week before starting the experiments. The hair at tumor inoculation and plasma irradiation sites was removed by applying depilatory cream. The hairless mice were inoculated subcutaneously with 2 × 10⁶ cells in a 100 μL phosphate-buffered saline (figure 1(a)). In Hongo experiments, 10% matrigel (BD, NJ, USA) was mixed with saline. The mice were anesthetized using isoflurane during plasma irradiation. All animal experiments were approved by and conducted in accordance with the guidelines of the Animal Ethics Committee of the University of Tokyo.

2.3. Plasma irradiation

In the present experiments, the abscopal effect was not stably observed. To obtain a significant result, six experiments were...
performed in total, as listed in table 1. Some parameters were not fixed in the series of experiments because the experiments were performed by trial and error. To investigate the effect of pulse width, three different high-voltage pulses were used: 20, 60, and 90 ns. The peak voltage and pulse repetition rate were fixed in all experiments to 25 ± 1 kV and 100 pulses s⁻¹, respectively. The increase in pulse width increased the discharge intensity. To generate discharges with approximately equivalent intensities but with different pulse widths, the gap between the hemispherical tip and mouse skin was set to 3 mm larger than the threshold gap distance at which a spark discharge occurred. The threshold gap distance was approximately 5 mm for the 20 ns pulse and 10 mm for the 90 ns pulse. Thus, discharges of not the same but with similar intensities were obtained with different pulse widths.

Six days after cell injection (day 6), the mice were randomized into three groups: (1) normal tissue irradiation, (2) tumor irradiation, and (3) control groups. There were 4–8 mice in each group, depending on the experiment. The left flank of mice in the normal tissue irradiation group was irradiated with plasma (figure 1), whereas it was directly irradiated to tumors in the tumor irradiation group. The distance between the plasma irradiation point and cell injection point was 2–3 cm. Plasma irradiation was done for 10 min/day and was kept from days 6 to 10. Tumor sizes were measured using calipers and tumor volumes were calculated as \( \frac{4}{3} \pi \times \left(\frac{\text{length}}{2}\right) \times \left(\frac{\text{width}}{2}\right)^2 \).

Skin surface temperature of mice was measured using infrared thermography (Avio, H2640, NEC). The increase in skin temperature during plasma irradiation was approximately 1 °C (figure S2 in supplementary materials). Additionally, plasma irradiation did not cause any visible damage to the mouse skin. Therefore, it can be assumed that the streamer discharge did not have considerable thermal effects on the treatment of mice.

In Komaba experiments, immune cells in tumors were measured using flow cytometry, but the results are not shown because it was a preliminary analysis. Results of flow cytometry analysis will be presented in future works.

3. Results

3.1. Effect of plasma irradiation on tumor growth

The effect of plasma irradiation on tumor growth suppression was significant in some experiments, but it was marginal in others. The tumor growth curves in figure 2 show that in experiment #2, (A) exhibits a significant effect, whereas in experiment #3, (B) only exhibits a marginal effect. The tumor growth curves in all experiments (#1–#6) are shown in figure S3 in supplementary materials. The antitumor effect caused by normal tissue irradiation is an abscopal effect, whereas that caused by tumor irradiation is not, since the plasma was directly irradiated to the tumors. Although both antitumor effects may...
Figure 2. Tumor growth in (A) experiment #2 and (B) #3 for the (a) control, (b) normal tissue irradiation, and (c) tumor irradiation groups.

have been caused by different mechanisms, they show similar tumor growth suppression.

The tumor volumes on the final day, \( V_f \), were used to examine the efficacy of plasma treatment. For example, the tumor volumes on day 27 were used for experiment #1, and those on day 21 were used for experiment #2 (table 1). To compare the \( V_f \) in experiments #1–#6 with different final days, \( V_f \) was normalized by dividing it by the average tumor volume of the control mice on the final day, \( \bar{V}_{f, \text{ctrl}} \), in each experiment. Figure 3 shows the cumulative frequency of normalized tumor volumes on the final day, \( V_{f,n} (= V_f / \bar{V}_{f, \text{ctrl}}) \), including all mice in experiments #1–#6. It appears that normal tissue irradiation delayed the tumor growth. Contrary to our expectations, the tumor irradiation caused less antitumor effects than the normal tissue irradiation.

3.2. Effect of tumor volumes before plasma irradiation

To examine the efficacy of plasma irradiation, the plasma-irradiated mice were classified into three groups:

(a) Efficacious group, in which the tumor volume on the final day was much smaller than the average of the control group, satisfying \( V_f \leq \bar{V}_{f, \text{ctrl}} - 0.5\sigma_{f, \text{ctrl}} \), where \( \sigma_{f, \text{ctrl}} \) is the standard deviation in the tumor volume of the control mice on the final day of each experiment.

(b) Marginally efficacious group, not satisfying (i) but satisfying \( V_f \leq \bar{V}_{f, \text{ctrl}} - 0.3\sigma_{f, \text{ctrl}} \).

(c) Inefficacious group, satisfying \( V_f > \bar{V}_{f, \text{ctrl}} - 0.3\sigma_{f, \text{ctrl}} \).
Figure 4. Tumor volumes on day 6 of (a) normal tissue irradiated and (b) tumor irradiated mice for experiments #1–#6 labeled with efficacious, marginal efficacious, and inefficacious groups. The plot of experiment #6 is missing in (b) because tumor irradiation was not performed in this experiment.

Figure 5. $V_{f,n}$ of all experiments #1–#6 as a function of $V_6$ for (a) normal tissue irradiation and (b) tumor irradiation.

The analysis showed that the mice in the efficacious group had smaller tumor volumes on day 6, which was the starting day of plasma irradiation, than those in the inefficacious group, as shown in figure 4. The mice with small tumor volumes on day 6 had a high efficacy both in the normal tissue irradiation and tumor irradiation groups.

To further investigate the influence of tumor volume on day 6, $V_6$, on plasma irradiation efficacy, the $V_{f,n}$ of all experiments #1–#6 were plotted together as a function of $V_6$. Figure 5(a) shows the plots for the normal tissue irradiation and control groups. The $V_{f,n}$ of control mice was distributed higher and lower than 1.0 ($= \text{normalized average volume of control mice for each experiment}$), irrespective of $V_6$. However, the $V_{f,n}$ of normal tissue-irradiated mice tends to be distributed lower than 1.0 for small $V_6$ (e.g. $V_6 \leq 70$ mm$^3$), whereas it was distributed higher and lower than 1.0 for large $V_6$ (e.g. $V_6 > 70$ mm$^3$). This suggests that normal tissue irradiation is effective for mice with small $V_6$. Tumor irradiation also showed a similar tendency, as demonstrated in figure 5(b).

To statistically analyze this hypothesis, the plots in figures 5(a) and (b) were divided into two groups based on the value of $V_6$: $V_6 \leq 70$ mm$^3$ and $V_6 > 70$ mm$^3$. Figure 6(a) shows the average and standard deviation of $V_{f,n}$ for all mice satisfying $V_6 \leq 70$ mm$^3$. Both normal tissue and tumor irradiation showed significant effects in reducing tumor growth. In contrast, for mice satisfying $V_6 > 70$ mm$^3$, almost no effects were observed (figure 6(b)). Thus, normal tissue and tumor irradiation significantly reduced the tumor growth in mice with a small $V_6$, whereas no significant effect was observed in mice with a large $V_6$. The threshold value of $V_6$ seems to be at around 70–90 mm$^3$ in this series of experiments.

In experiment #1, four of the eight mice in the normal tissue irradiation group had tumors on left limbs, which was the opposite side of other mice in experiments #1–#6, and the plasma was irradiated to the right flanks. All of the four right
flank-irradiated mice were in the efficacious group. This suggests that normal tissue irradiation to either the left or right flank was effective, but the number of mice for right flank irradiation \((N = 4)\) was not large enough to obtain a significant result.

In this study, high-voltage pulse widths of 20, 60, and 90 ns were used. However, figure 4(a) suggests that the pulse width does not have significant effects on the abscopal effect.

4. Discussion

In RT, there are few studies on the abscopal effect induced by normal tissue irradiation. Camphausen et al [23] observed a dose-dependent abscopal effect induced by normal tissue irradiation using Lewis lung carcinoma and T241 fibrosarcoma tumor mouse models. Irradiation with 10 Gy \(\times\) 5 and 2 Gy \(\times\) 12 showed abscopal effects, but the effect of the former irradiation was more marked. The abscopal effect was found to be mediated by p53, as shown in using p53 knockout mice and p53 inhibitor. It was hypothesized that local inflammation caused by normal tissue irradiation makes p53 to produce cytokines and other factors, which may exert some effects on distant tumors. The p53-mediated abscopal effect was also reported in tumor-irradiated mice [24].

Contrary, there are also some studies in RT reporting that normal tissue irradiation has no positive abscopal effects. Shiraishi et al [25] reported that normal tissue irradiation (6 Gy) in mice with CT26 tumors has no abscopal effects. Yasuda et al [26] reported that normal tissue irradiation (2 Gy \(\times\) 10) in mice with subcutaneous and liver metastasis CT26 tumors enhanced the growth of metastatic tumors.

Thus, this phenomenon is not well-understood. It is unclear whether the effect is positive or negative. This study showed the positive abscopal effect of normal tissue irradiation. Here, tumor volume before plasma irradiation (day 6) was considered as a factor that determines the efficacy of this treatment.

The even or less efficacy of tumor irradiation compared to normal tissue irradiation was contrary to our expectation. Tumor irradiation was expected to be more effective because it may produce DAMPs that trigger the immune response. However, this study implies that normal tissue irradiation might be more efficacious at least under the present conditions. For clinical use, normal tissue irradiation is preferable because it is easier than irradiating tumors. This difference between normal tissue and tumor irradiation should be clarified in future studies.

Some questions that need to be solved in future studies arise from our results.

(a) The mechanism of this phenomenon should be examined. It might be the p53-mediated local inflammation, as suggested in RT [23], or other possible mechanisms.
(b) It should be examined why the abscopal effects were preferably observed in mice with small tumor volumes before plasma irradiation.
(c) The abscopal effect was also caused by tumor irradiation using plasma [11, 14]. It should be examined whether the mechanisms of the abscopal effects caused by normal tissue and tumor irradiation are the same or not.
(d) Adaptive immune activation may occur simultaneously with the abscopal effects caused by tumor irradiation [11]. It should be determined if the abscopal effect caused by normal tissue irradiation also leads to adaptive immune activation.

This study supports the existence of abscopal effects caused by normal tissue irradiation with significant difference, which was only reported in few studies. The efficacy of this treatment was shown to depend on the tumor volume before plasma irradiation. The elucidation of the mechanism indicated in (a) is currently ongoing. So far, immune cells in tumors have been analyzed using flow cytometry as a preliminary analysis. The mechanism will be discussed in future studies.

5. Conclusions

The abscopal effect caused by normal tissue irradiation was demonstrated using pulsed streamer discharge in CT26...
tumor-bearing mice. It supports the existence of abscopal effects caused by normal tissue irradiation, while few studies have reported this phenomenon for RT. The abscopal effect was observed with significant difference in mice with small tumor volumes on day 6, whereas it was not observed in those with large tumor volumes on day 6. The threshold tumor volume on day 6 seemed to be at around 70–90 mm³ in this series of experiments. Direct tumor irradiation was also performed to compare it with normal tissue irradiation. Contrary to our expectations, tumor irradiation caused less antitumor effects than normal tissue irradiation.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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References

[1] Ratovitski E A, Cheng X Q, Yan D Y, Sherman J H, Canady J, Trink B and Keidar M 2014 Anti-cancer therapies of 21st century: novel approach to treat human cancers using cold atmospheric plasma Plasma Process. Polym. 11 1128–37
[2] Hirst A M, Frame F M, Arya M, Maitland N J and O’Connell D 2016 Low temperature plasmas as emerging cancer therapeutics: the state of play and thoughts for the future Tumor Biol. 37 7021–31
[3] Dubuc A, Monsarrat P, Virard F, Merbahi N, Sarrette J P, Laurencin-Dalicieux S and Cousty S 2018 Use of cold-atmospheric plasma in oncology: a concise systematic review Ther. Adv. Med. Oncol. 10 1–12
[4] Sennmmer M L et al 2020 Molecular mechanisms of the efficacy of cold atmospheric pressure plasma (CAP) in cancer treatment Cancers 12 269
[5] Graves D B 2012 The emerging role of reactive oxygen and nitrogen species in redox biology and some implications for plasma applications to medicine and biology J. Phys. D: Appl. Phys. 45 263001
[6] Bekesches S, Favia P, Robert E and von Woedtke T 2018 White paper on plasma for medicine and hygiene: future in plasma health sciences Plasma Process. Polym. 16 e1800033
[7] Bekesches S, Clemen R and Metelmann H R 2018 Potentiating anti-tumor immunity with physical plasma Clin. Plasma Med. 12 17–22
[8] Tanaka H, Mizuno M, Ishikawa K, Toyokuni S, Kajiyama H, Kikkawa F and Hori M 2018 New hopes for plasma-based cancer treatment Plasma 1 150–5
[9] Khalili M, Daniels L, Lin A, Krebs F C, Snook A E, Bekesches S, Bownel W B and Miller V 2019 Non-thermal plasma-induced immunogenic cell death in cancer J. Phys. D: Appl. Phys. 52 423001
[10] Bekesches S, Seebauer C, Wende K and Schmidt A 2019 Physical plasma and leukocytes—immune or reactive? Biol. Chem. 400 63–75
[11] Mizuno K, Yonetamari K, Shirakawa Y, Akiyama T and Ono R 2017 Anti-tumor immune response induced by nanosecond pulsed streamer discharge in mice J. Phys. D: Appl. Phys. 50 12LT01
[12] Mizuno K, Shirakawa Y, Sakamoto T, Ishizaki H, Nishijima Y and Ono R 2018 Plasma-induced suppression of recurrent and renoculated melanoma tumors in mice IEEE Trans. Radiat. Plasma Med. Sci. 2 353–9
[13] Lin A G, Xiang B, Merlino D J, Baybutt T R, Sahu J, Fridman A, Snook A E and Miller V 2018 Nonthermal plasma induces immunogenic cell death in vivo in murine CT26 colorectal tumors Oncoimmunology 7 e1484978
[14] Mahdikia H, Saadati F, Freund E, Gaip U S, Majidzadeh K, Shokri B and Bekesches S 2020 Gas plasma irradiation of breast cancers promotes immunogenicity, tumor reduction and an abscopal effect in vivo Oncoimmunology 10 1859731
[15] Freund E, Liedtke K R, van der Linde J, Metelmann H R, Heidecke C D, Partecke L I and Bekesches S 2019 Physical plasma-treated saline promotes an immunogenic phenotype in CT26 colon cancer cells in vitro and in vivo Sci. Rep. 9 634
[16] Formenti S C and Demaria S 2013 Combining radiotherapy and cancer immunotherapy: A paradigm shift J. Natl. Cancer Inst. 105 256–65
[17] Reynkers E, Illidge T, Siva S, Chang J Y and De Ruysscher D 2015 The abscopal effect of local radiotherapy: using immunotherapy to make a rare event clinically relevant Cancer Treat. Rev. 41 503–10
[18] Castano A P, Mroz P and Hamblin M R 2006 Photodynamic therapy and anti-tumour immunity Nat. Rev. Cancer 6 535–45
[19] Dabrowski J M and Arnaut L G 2015 Photodynamic therapy (PDT) of cancer: from local to systemic treatment Photochem. Photobiol. Sci. 14 1765–80
[20] Miller V, Lin A and Fridman A 2016 Why target immune cells for plasma treatment of cancer Plasma Chem. Plasma Process. 36 259–68
[21] Nijdam S, Teunissen J and Ebert U 2020 The physics of streamer discharge phenomena Plasma Sources Sci. Technol. 29 103001
[22] Ono R 2016 Optical diagnostics of reactive species in atmospheric-pressure nonthermal plasma J. Phys. D: Appl. Phys. 49 083001
[23] Camphausen K, Moses M A, Menard C, Sproull M, Beecken W D, Folkman J and O’Reilly M S 2003 Radiation abscopal antitumor effect is mediated through p53 Cancer Res. 63 1990–3
[24] Tesei A et al 2021 TP53 drives abscopal effect by secretion of senescence-associated molecular signals in non-small cell lung cancer J. Exp. Clin. Cancer Res. 40 89
[25] Shiraiishi K et al 2008 Enhancement of antitumor radiation efficacy and consistent induction of the abscopal effect in mice by ECI301, an active variant of macrophage inflammatory protein-1α Clin. Cancer Res. 14 1159–66
[26] Yasuda K, Nirei T, Tsuno N H, Nagawa H and Kitayama J 2011 Intratumoral injection of interleukin-2 augments the local and abscopal effects of radiotherapy in murine rectal cancer Cancer Sci. 102 1257–63