Letter

Anti-tumor immune response induced by nanosecond pulsed streamer discharge in mice

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Abstract
Plasma is known to activate immune cells in vitro; however, its effect on cancer immunotherapy is not well understood in vivo. In this study, we report B16–F10 tumor growth suppression at a non-irradiated site on a mouse leg after a nanosecond pulsed streamer discharge was applied to the tumor on the other leg. The tumor growth suppression at non-irradiated remote sites was observed from the day next to that of plasma irradiation: the rapid abscopal effect suggests innate immune response activation. Additionally, the production of inflammatory cytokines from splenocytes was enhanced after plasma irradiation. This suggests the activation of adaptive immune response specific to B16–F10 melanoma by plasma irradiation.

Keywords: plasma medicine, mouse melanoma tumor, innate immune response, adaptive immune response, in vivo, pulsed streamer discharge, cancer treatment

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

1. Introduction
Cancer treatment using cold atmospheric-pressure plasma has recently gained attention. A number of in vitro and in vivo studies have validated plasma treatment for cancer therapy [1–5]. Vandamme et al [6–8] showed that a dielectric barrier discharge (DBD) treatment of human malignant glioma (U87MG) tumors xenografted onto mice increased the lifespan of mice by 60%. Keidar et al [9] showed that treatment using a helium plasma jet has anti-tumor effect on B16–F10 melanoma and subcutaneous bladder cancer tumors in mice. Similarly, certain types of tumors have been efficiently treated in vivo using helium plasma jets and DBDs [10–13]. Utsumi et al [14] indirectly treated ovarian xenograft tumors using a plasma-activated medium and observed an anti-tumor effect. The success of these anti-tumor treatments suggests a potential for using plasma in cancer treatments.

During radiotherapy, irradiation of tumors induces tumor-specific immune responses [15]. Yoshimoto et al [16] applied 30 Gy of x-rays to EL4 tumors in C57BL/6 mice and reduced the tumor size. The mice were then re-inoculated with EL4 and B16 melanoma cells, resulting in the rejection of only the EL4
Figure 1. Equipment and photograph of nanosecond pulsed streamer discharge. The photograph is an average of 10 pulses, in which the ground plate was replaced with liquid water and the gap distance was 5 mm. Mice to be treated were placed on the ground plate.

cells owing to the specific activation of the immune system. Such anti-tumor immune response is also observed in photodynamic therapy [17], and thus, it is expected that plasma could also induce a similar response [18–20]. The effects of plasma irradiation on immune cells have been studied in vitro. Immune cell viability and immunological reactions have been measured after plasma irradiation [21–24]. Furthermore, immune cell migration and anti-tumor cytotoxicity induced by plasma have been investigated [25–27].

In the present study, we evaluated the use of plasma treatment for B16–F10 melanoma in mice in vivo and examined the anti-tumor immunity stimulated by it. B16–F10 cells were subcutaneously injected into the right and left hind legs of CD2F1 and C57BL/6 mice, and the tumors on the right hind leg were treated using a nanosecond pulsed streamer discharge. The plasma treatment suppressed the tumor growth not only on the irradiated right leg but also on the non-irradiated left leg of the mice. Additionally, splenocytes from mice treated with plasma irradiation produced more inflammatory cytokines when cocultured with B16–F10 cells. These results suggest that use of plasma strengthened the anti-tumor immune response.

2. Experiments

2.1. Pulsed streamer discharge

Figure 1 shows the setup for the nanosecond pulsed streamer discharge. The discharge was generated by applying a nanosecond high-voltage pulse (24 kV, 8 ns, 30–100 pps) to the copper-rod hemisphere electrode. The tip of the hemisphere electrode protrudes 1 mm from the nozzle of the glass tube. The nanosecond pulsed streamer discharge is rarely used for cancer treatment; however, it can cause apoptosis of B16–F10 in vitro [28]. Oxygen or nitrogen was flowed through the glass tube at a rate of 0.5 l min⁻¹ and was used as the working gas of the discharge. In most of the experiments, the working gas was humidified using a water bubbler up to above 90% of relative humidity.

2.2. Cells and animals

CD2F1 and C57BL/6 mice were subcutaneously inoculated with murine melanoma B16–F10 cells. Although the B16–F10 syngeneic model C57BL/6 is desirable, the CD2F1 model was also used as the latter was easy for the authors to handle. The CD2F1 and C57BL/6 mice (female; 5 weeks old) were purchased from Charles River Co. (Kanagawa, Japan) and housed for 1 week at our animal facility prior to starting the experiments. The B16–F10 cells were cultured in DMEM medium containing 10% fetal bovine serum and 1% antibiotics at 37 °C and at 5% CO₂. Upon conclusion of experiments, mice were euthanized with CO₂. All the animal experiments in this study followed the guidelines of the Animal Ethics Committee of the University of Tokyo.

2.3. Tumor inoculation and plasma irradiation

We used four treatment schedules for the CD2F1 mice and two treatment schedules for the C57BL/6 mice. Table 1 summarizes the conditions used for all the experiments. Experiments #1–#4 were conducted in CD2F1 mice and experiments #5–#6 were conducted in C57BL/6 mice. Because the experimental parameters used were determined by trial and error, they differed throughout experiments #1–#6. The mice were inoculated with B16–F10 cells suspended in 0.1 mL of phosphate-buffered saline. The number of cells used for experiments #1–#2 was 1 × 10⁶ and that for experiments #3–#6 was 2 × 10⁵. When the average tumor volume reached approximately 50–100 mm³, the mice were randomized into irradiation and control groups (N = 4–7 each) so that both groups had an equivalent average tumor volume. Before the randomization, some mice having too large or too small tumor volumes were excluded. As a result, the number of mice was not necessarily sufficient (i.e. N = 4). The tumor size was measured daily using calipers, and tumor volumes were calculated using the following formula: V = 4π × (length × width²)/3, assuming that the tumor is ellipsoid. The correlation between the tumor volumes on the left and right legs was not good, as indicated by the correlation coefficients shown in table 1, making randomization difficult and resulting in some disagreement regarding average tumor volume among the groups for each treatment schedule.

The plasma treatment was started on the day of randomization (day 0). Mice in the irradiation group were placed on the ground plate (figure 1), and tumors on the right legs were irradiated with plasma for 10 min per day for 3–7 consecutive days (see ‘period of treatment’ in table 1); tumors on the left legs were not irradiated. The discharge pulse repetition rate was 30 pps for experiments #1–#4 and was increased to 100 pps for experiments #5–#6. The gap distance between the tumor surface and the end of electrode was adjusted using a z-stage.
was plotted because the initial

- $\pm 0.84$
- $\pm 0.56$
- $\pm 0.19$
- $\pm 0.86$
- $\pm 0.37$
- $\pm 0.67$
- $\pm 0.04$
- $\pm 0.05$

than in the control mice. Tumor growth suppression was showed that tumor growth in plasma-treated mice was slower depending on the treatment schedule. The results and reached endpoints on day 5 to day 9, when the mice were euthanized, depending on the treatment schedule. The results showed that tumor growth in plasma-treated mice was slower than in the control mice. Tumor growth suppression was observed even for the left-side tumor, although only the right-side tumor was treated with plasma.

2.4. Cytokine production from splenocytes

The cytokine production from splenocytes of the plasma-treated CD2F1 mice was measured in experiments #2 and #4 to analyze the induction of anti-tumor immunity [29]. The mice were euthanized on day 9 (9 days after the first treatment day) in experiment #2 and on days 3 and 7 in experiment #4, and their spleens were collected. The splenocytes were isolated by filtering through a cell strainer in RPMI media; $6.0 \times 10^6$ cells were co-cultured with $6.0 \times 10^4$ B16–F10 cells in 96 well plates for 24h. IFN-γ and TNF-α concentrations in the supernatants were measured using ELISA kits (R&D Systems, Inc., Minnesota, USA) following the manufacturer’s protocol.

3. Results and discussion

In all the experiments, tumor volumes increased over time and reached endpoints on day 5 to day 9, when the mice were euthanized, depending on the treatment schedule. The results showed that tumor growth in plasma-treated mice was slower than in the control mice. Tumor growth suppression was observed even for the left-side tumor, although only the right-side tumor was treated with plasma.

3.1. Suppression of tumor growth on CD2F1 mice

Figures 2(a) and (b) show the cumulative frequency of left- and right-side tumor volumes on day 3 ($V_3$), including all the experiments conducted in CD2F1 mice (experiments #1–#4). The ratio of $V_9/V_0$ was plotted because the initial tumor volumes on day 0 ($V_0$) displayed a large dispersion. The results show that plasma treatment suppressed the growth of tumors on both legs, suggesting that an abscopal anti-tumor effect is induced by plasma irradiation.

On performing the unpaired Student’s t-test between the irradiation and control groups (results shown in figure 2), small $P$ values were obtained: 0.00031 and 0.044 for the left- and right-side tumors, respectively, suggesting a significant difference between the two groups. However, this is not an accurate statistical analysis because the conditions for experiments #1–#4 were not identical. In addition, normality, which is needed for the Student’s t-test, is rejected in most of the four data sets (shown in figure 2) with Shapiro-Wilk test at 0.05 level. For these reasons, the Student’s t-test was not used in the present work.

There are two possible reasons for the abscopal effect that suppresses tumor growth: innate and adaptive immune responses. The adaptive immune response requires 7–10 d to be activated [16, 30], while the innate immune response is rapidly induced. In the present experiments, the plasma treatment rapidly induced the tumor suppression as these effects were observed from day 1 (figure 3). Therefore, the abscopal

| Parameters                              | Values                              |
|-----------------------------------------|-------------------------------------|
| Number of inoculated B16–F10 cells      | $1 \times 10^6$ cells for #1–#2 and $2 \times 10^4$ cells for #3–#6 |
| Discharge pulse repetition rate          | 30 pps for #1–#4 and 100 pps for #5–#6 |
| Gap distance between the tumor and the electrode | 4 mm for #1–#4 and 6 mm for #5–#6 |

| (a) CD2F1 mice | Plasma-treated mice | Control mice |
|---------------|---------------------|--------------|
| No. | Tumor volumes on day 0 (mm³) | Working gas | Period of treatment | ELISA | Tumor volumes on day 0 (mm³) | No. | Tumor volumes on day 0 (mm³) |
|----|-------------------------------|-------------|---------------------|-------|-------------------------------|----|-------------------------------|
|    | Right | Left | $r$ | Wet O₂ | 5 d | — | 5 | Right | Left | $r$ | Wet O₂ | 5 d | — |
| #1 | 5     | 80±18 | 132±56 | 0.67 | — | 5 | 84±51 | 126±48 | 0.84 |
| #2 | 7     | 46±10 | 34±11 | —0.49 | 5 d | — | 7 | 45±14 | 43±15 | —0.42 |
| #3 | 5     | 92±27 | 117±13 | 0.37 | 5 d | — | 5 | 89±19 | 117±39 | —0.55 |
| #4 | 5     | 134±43 | 132±57 | 0.56 | 3 d | — | 5 | 134±52 | 141±67 | 0.19 |
|    | 5     | 132±44 | 132±19 | —0.57 | 5 d | — | 5 | 60±10 | 71±10 | 0.86 |

| (b) C57BL/6 mice | Plasma-treated mice | Control mice |
|----------------|---------------------|--------------|
| No. | Tumor volumes on day 0 (mm³) | Working gas | Period of treatment | ELISA | Tumor volumes on day 0 (mm³) | No. | Tumor volumes on day 0 (mm³) |
|----|-------------------------------|-------------|---------------------|-------|-------------------------------|----|-------------------------------|
| #5 | 4     | 49±7  | 69±27 | 0.90 | 5 d | — | 4 | 62±14 | 81±13 | —0.23 |
| #6 | 5     | 61±29 | 73±15 | —0.92 | 5 d | — | 5 | 60±10 | 71±10 | 0.86 |

| (c) Other parameters | Values |
|----------------------|--------|
| Gap distance between the tumor and the electrode | 4 mm for #1–#4 and 6 mm for #5–#6 |

Table 1. List of experimental conditions as follows: number of mice ($N$), volumes of right- and left-side tumors the day when plasma treatment started (day 0), the correlation coefficient between the right- and left-side tumor volumes ($r$), working gas of the plasma, period of treatment, and day of ELISA experiments. The error of tumor volume indicates standard deviation.
anti-tumor effect observed here is likely due to an innate immune response. The effect of adaptive immune response is discussed in section 3.2.

We expected the tumor growth suppression by plasma treatment to be more pronounced on the right-side than on the left because the right-side tumor was suppressed both by the direct plasma irradiation and abscopal effect. However, figure 2 shows that tumor suppression was not much different between the two sides, suggesting that the effect of direct plasma treatment on the right-side tumor was not dominant.

No correlation was found between the anti-tumor effects on the right and left legs. The correlation coefficient between the ratios $V_l/V_0$ of the left and right legs of plasma-treated mice was $-0.03$, indicating that a strong anti-tumor effect on one side does not assure the same effect on the other side.

Most of the experiments shown in figure 2 (experiments #1–1, 2, 3, and 4) used wet $O_2$ plasma, and only experiment #1–2 used dry $N_2$ plasma because the wet $O_2$ plasma is expected to produce larger amount of reactive oxygen species (ROS) which are assumed to be effective for cancer treatment. However, it seems from figure 2 that there is no significant difference in the anti-tumor effects of the two plasmas. It suggests that (i) the dry $N_2$ plasma also produced much amount of ROS from water vapor evaporated from the mice [28], or (ii) some other factors other than ROS, such as electric field, caused the anti-tumor effects. Since the number of mice in experiment #1–2 ($N = 5$) was not sufficient to examine the difference between the two plasmas, further experiments are needed to discuss the effects of ROS on cancer treatment.

3.2. ELISA

Inflammatory cytokines were measured to determine whether or not an adaptive immune response specific to the inoculated cancer cells was induced by plasma irradiation. IFN-$\gamma$ and TNF-$\alpha$ are the major cytokines that are secreted from immune cells to regulate the immune response when exposed to antigens, including cancer-cell-derived antigens. IFN-$\gamma$ and TNF-$\alpha$ levels produced by splenocytes of the tumor-bearing mice with or without irradiation were measured after co-culture with B16–F10 cells. Figures 4(a) and (b) show the results of experiment #2, where the mice were euthanized on day 9. IFN-$\gamma$ levels increased after plasma exposure, whereas TNF-$\alpha$ levels remained the same. The enhanced production of IFN-$\gamma$ suggests the induction of a cancer-cell-specific immune response. We could not measure the elevation in IFN-$\alpha$ levels as the timing for TNF-$\alpha$ measurement may not have been optimal.

Figure 4(c) shows the result of experiment #4, where half of the plasma-treated mice were euthanized on day 3 and the remaining mice were euthanized on day 7. IFN-$\gamma$ levels were measured but TNF-$\alpha$ levels were not measured in this experiment. Only 2 of the 10 plasma-treated mice showed elevated IFN-$\gamma$ levels compared with control mice, and these values were only approximately two-fold higher than those in control mice. The adaptive immune response requires 7–10 d to be induced, explaining the lower IFN-$\gamma$ levels than those obtained on day 9 (figure 4(a)).

The high IFN-$\gamma$ levels shown in figure 4(a) showed no correlation with the previously discussed tumor growth suppression. If there was a correlation between these two, that is,
if the tumor growth in a mouse with high IFN-γ levels was strongly suppressed, the correlation coefficient between IFN-γ levels and the ratio of \( V_{60} \) would be negative. However, the correlation coefficient was 0.53 for the plasma-treated group in experiment #2, providing evidence that tumor growth suppression and high IFN-γ levels are caused by different mechanisms, which are assumed to be innate and adaptive immune responses, respectively.

3.3. Suppression of tumor growth in C57BL/6 mice

In the previous experiments, CD2F1 mice were used as a model. Because CD2F1 mice are not syngeneic to B16–F10 cells, it is possible that an allogeneic immune response was induced [31]. Thus, in the following experiments, a syngeneic mouse model, C57BL/6, was used to eliminate this possibility.

Figure 5 shows the cumulative frequency of tumor volume ratios on day 6 (\( V_{60} \)) for C57BL/6 mice (experiments #5–#6). Because the anti-tumor effect in the C57BL/6 mice was somewhat weaker than that in the CD2F1 mice, the difference between the irradiation and control groups on day 3 was marginal, and additional time was needed to observe a significant difference. The volume ratio on day 3 (\( V_{30} \)) was plotted for CD2F1 mice (figure 2), but it is not plotted in this experiment; \( V_{60} \) was plotted instead. Figure 5(a) shows tumor growth suppression that was observed in the C57BL/6 mice as well. This result supports the hypothesis that tumor growth suppression is caused by an innate immune response and not by an allogeneic immune response.

Figure 5(b) shows that there is no marked difference between the irradiation and control groups, even though the right-side tumor was suppressed by the innate immune response. This result could be due to the promotion of cell growth after exposure to plasma at low doses [32]; however, additional experiments are needed to clarify its underlying mechanism.

In the immunotherapy approach to the cancer treatment, the adaptive immune response is more important than the innate immune response. If plasma can induce an adaptive immune response, it could be used to treat tumors before surgically resecting them for inducing the adaptive immune response in the patient. It could cure a possible metastatic cancer and prevent cancer recurrence. To test the adaptive immune response,
ELISA and immunization-challenge experiments with the C57BL/6 model are currently underway.

One of the most challenging problems in plasma medicine has been determining the depth of penetration of the effect of plasma from the surface [8, 33–35]; it may be of the order of 10 μm–1 mm or more. However, the present work suggests that in some cases, plasma has an effect that is much farther from the irradiated surface as previously predicted [18, 19].

4. Conclusions

We demonstrated the possibility of abscopal effects of cold plasma treatment on tumor growth in vivo. Tumor growth suppression at non-irradiated remote sites was observed in both CD2F1 and C57BL/6 mouse models, and the rapid induction of the tumor growth suppression suggests that the anti-tumor effect was caused by an innate immune response. The production of pro-inflammatory cytokines by splenocytes was increased after plasma irradiation, suggesting the induction of adaptive immunity as well.

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