Effect of nitric oxide in promoting proliferation of radical-irradiated fibroblasts

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We investigated whether nitric oxide radical (NO•) in plasma contributes to the promotion of fibroblast proliferation. The results showed the proliferation promoting ratio of fibroblasts in suspension with C-PTIO was reduced compared to that in suspension without C-PTIO. In addition, fluorescence observation of intracellular NO• showed that intracellular NO• increased with irradiation and decreased in the presence of C-PTIO. These results suggest that NO• contributes to the promotion of fibroblast proliferation through radical irradiation.

Keywords: Nitric Oxide, Fibroblasts, Proliferation, Atmospheric-Pressure Plasma

1. Introduction

Medical applications of non-equilibrium atmospheric pressure plasma (NEAPP) [1] for selective killing of cancer cells [2], fast-acting wound healing [3], and promoting cell proliferation [4] currently attract great attention. In our previous study, mouse fetal fibroblast cells NIH3T3 suspended in Dulbecco's saline solution (DPBS) were treated using a non-equilibrium atmospheric pressure radical source that can irradiate only electrically neutral species in NEAPP. The species and densities irradiated from radical source was investigated with a quadrupole mass spectrometer. Nitric oxide radical (NO•), nitric dioxide radical (NO_2•) and ozone (O_3) were the main irradiation-species from radical source. As a result, the proliferation rate of radical irradiated fibroblast was promoted. The proliferation of fibroblast depended on the dose of NO• irradiated from radical source and not on the dose of NO_2• and O_3. Therefore, NO• is identified as the proliferation-promoting-substance in the gas phase.[5] Fibroblasts play an important role to maintain the structural integrity of skin tissue. This technique is considered to be useful to accelerate preparation of skin for graft. Nitric oxide (NO•) is identified as a promotion species in gas phase. However, it has been widely reported that NO• reacted with water molecules and produced longer-lived reactive oxygen and nitrogen species (RONS) such as nitrite (NO_2^-) and nitrate (NO_3^-). Therefore, the direct promotion species for the fibroblast was still unclear.

In this study, we have evaluated the promoting effect of NO• in solutions on fibroblast cells using a NO• scavenger (Carboxy-PTIO: C-PTIO), and measured a relative amount of intracellular NO• using a fluorescent probe (DAF-FM DA).

2. Experimental procedure

Figure 1 show a schematic diagram of experimental procedure. C-PTIO was dissolved to fibroblast suspension (3.0×10^5 cell/mL) with a concentration of 100 μM. 3 mL of the suspension in φ38-mm dish was treated using an atmospheric-pressure radical source (Tough plasma, FPA10, Fuji Co., Ltd.). [6] The gas flow rates were set at 4.0 slm, 0.3 slm, and 0.7 slm for Ar, O_2, and N_2, respectively. The treated suspension was replaced with 3 mL of cell culture medium and adjusted to concentration of 1.0×10^5 cells/mL. The concentration-adjusted cell suspension was seeded in 96-well plate at 160 μL and incubated for 24 hour at 37 ℃ and 5% of CO_2 concentration. 10 μL of MTS reagent was dropped into each well and absorbance was measured after 1 hour using a plate reader (Multiskan, Thermo Fisher Scientific). Cell viability was evaluated using the ratio of the absorbance of radical-treated fibroblasts to the absorbance of radical-unirradiated fibroblasts.

DAF-FM DA was employed for investigating the absorption of NO• in fibroblast cells. Fibroblasts were suspended in DPBS at a concentration of 1.6×10^6 cells/mL and mixed with 5 μL of 5 mM DAF-FM DA. Dropped cell suspensions were incubated for 20 min at 37 ℃ and 5% of CO_2 concentration. Then, cell suspensions were adjusted to a concentration of 3.0×10^5 cells/mL using a hemocytometer. 3 mL of cell suspension with/without 100 μM of C-PTIO was added to a cell suspension in a ϕ 38-mm dish. The cell suspensions with/without C-PTIO were irradiated using the radical source. The supernatant of treated suspension was replaced with fresh 3mL-DPBS. 200 μL of the replaced suspension was dripped into a glass bottom dish. After incubation for 20 min at 37 ℃ and 5% of CO_2 concentration, RONS and NO· fluorescences were observed using a confocal laser microscopy. DAF-FM DA is excited at 488 nm and fluorescence is observed at 530 nm.

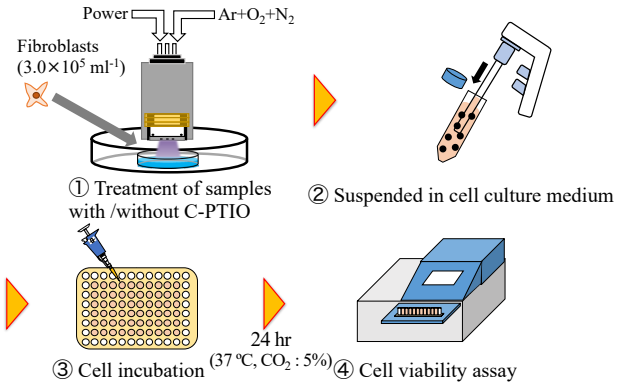

Fig. 1. Schematic diagram of the experimental procedure.

3. Results and discussion

Figure 2 shows the proliferation-promoting rates of the cell suspensions with/without C-PTIO. When fibroblast suspensions without C-PTIO are treated, fibroblast proliferation was promoted by 35.5% after radical irradiation of 15 s. On the other hand, when fibroblast suspensions with C-PTIO were treated, the proliferation-promoting effect was reduced to be only 14% at 15 s irradiation. This result suggests that the proliferation-promoting species generated in the liquid phase using the radical source are scavenged by C-PTIO and the proliferation-promoting effect is reduced.

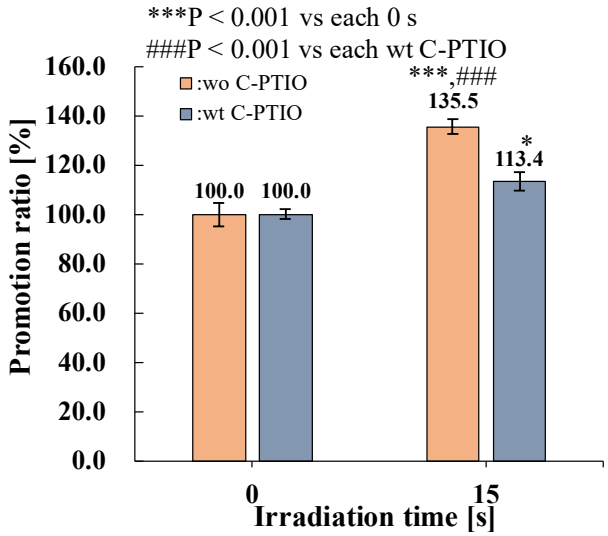

Fig. 2. Proliferation-promoting effect of radical irradiation of cell suspensions.

Figure 3 shows the fluorescence intensities of intracellular NO• after the radical irradiation. When without C-PTIO, the fluorescence intensity of NO• in fibroblasts is increased by radical irradiation. After 60 s in radical irradiation, the fluorescence intensity is increased by 28.8% compared with control. This result suggests that NO• produced in the liquid phase by radical irradiation is absorbed into fibroblasts. Even with c-PTIO, the fluorescence intensity of NO• in fibroblasts is increased by radical irradiation. However, the increase of fluorescence intensity is lower than that without c-PTIO. After 60 s in radical irradiation, the fluorescence intensity is increased by only 10.5% compared with control. This result suggests that some of NO• produced in the liquid phase by radical irradiation are scavenged by c-PTIO and inhibit its absorption into fibroblasts.

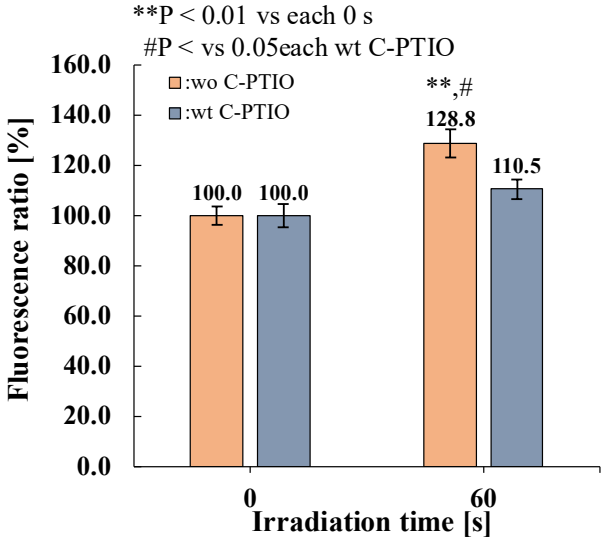

Fig. 3. The fluorescence intensity of NO• in fibroblasts after radical irradiation.

4. Conclusion

We investigate the contribution of NO• in proliferation promotion of radical-irradiated fibroblasts. Radical irradiation to fibroblast suspensions without c-PTIO promotes proliferation of 35.5%, whereas cell suspensions with c-PTIO reduce the promotion rate to 13.4%. Fluorescence observation of NO• in fibroblasts confirmed that NO• produced by radical irradiation are absorbed into fibroblasts. In the cell suspension with c-PTIO, NO• absorption is significantly reduced compared to the suspension without c-PTIO. From these results, we speculate that NO• is a significant cell-proliferation-promoting species in this study.

Acknowledgements

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References

[1] T. Shimizu, *Jpn. J. Appl. Phys* **59**, 120501 (2020).
[2] G. Fridman, *et al., Plasma Chem. Plasma Process* **27**, 163-176 (2007).
[3] K. Miyamoto, *et al., J. Clin. Biochem. Nutr* **60**, 25-28 (2017).
[4] M. Yamato, *et al., Appl. Phys. Express* **14**, 067002 (2021).
[5] Y. Hori, *et al., Plasma Process Polym.* **18**, 2000225 (2021).
[6] H. Hashizume, *et al., Appl. Phys. Lett.* **103**, 153708 (2013).