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Original Article

Plasma-activated medium selectively eliminates undifferentiated human induced pluripotent stem cells

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ABSTRACT

Human pluripotent stem cells, including human induced pluripotent stem cells (hiPSCs), are promising materials for regenerative medicine and cell transplantation therapy. However, tumorigenic potential of residual undifferentiated stem cells hampers their use in these therapies. Therefore, it is important to develop methods that selectively eliminate undifferentiated stem cells from a population of differentiated cells before their transplantation. In the present study, we investigated whether plasma-activated medium (PAM) selectively eliminated undifferentiated hiPSCs by inducing external oxidative stress. PAM was prepared by irradiating cell culture medium with non-thermal atmospheric pressure plasma. We observed that PAM selectively and efficiently killed undifferentiated hiPSCs cocultured with normal human dermal fibroblasts (NHDFs), which were used as differentiated cells. We also observed that undifferentiated hiPSCs were more sensitive to PAM than hiPSC-derived differentiated cells. Gene expression analysis suggested that lower expression of oxidative stress-related genes, including those encoding enzymes involved in hydrogen peroxide (H_2O_2) degradation, in undifferentiated hiPSCs was one of the mechanisms underlying PAM-induced selective cell death. PAM killed undifferentiated hiPSCs more efficiently than a medium containing the same concentration of H_2O_2 as that in PAM, suggesting that H_2O_2 and various reactive oxygen/nitrogen species in PAM selectively eliminated undifferentiated hiPSCs. Thus, our results indicate that PAM has a great potential to eliminate tumorigenic hiPSCs from a population of differentiated cells and that it may be a very useful tool in regenerative medicine and cell transplantation therapy.

1. Introduction

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), are potential sources of cells for use in regenerative medicine, drug screening, and cell transplantation therapy [1–4]. In 2014, the first human trial on hiPSC-derived retinal pigment epithelium [5] was performed by the Riken Center for Developmental Biology, Kobe, Japan. Thus, innovative therapy by using hiPSCs-derived differentiated cells is becoming an increasingly realistic prospect. However, hPSCs-based therapy is associated with some risks such as tumor and teratoma formation because of the residual tumorigenic potential of

Abbreviations: hPSCs, human pluripotent stem cells; hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; PAM, plasma-activated medium; NHDFs, normal human dermal fibroblasts; SOD, superoxide dismutase; GPX1, glutathione peroxidase 1; CAT, catalase; ATM, ataxia telangiectasia mutated; ROS, reactive oxygen species; RONS, reactive oxygen/nitrogen species; PI, Propidium Iodide.

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undifferentiated stem cells that may be present in a population of differentiated cells [6]. Therefore, it is important to develop methods that selectively eliminate undifferentiated cells before transplanting differentiated cells to realize the safety of hPSC-based therapy.

Several methods have been developed to eliminate residual hPSCs from a population of differentiated cells, including induction of selective cell death by using cytotoxic antibodies [7,8], use of lectin-conjugated cytotoxic proteins [9], chemical inhibitors [10–12], use of conditionally replicating adenoviruses [13], selective separation by performing cell sorting with hPSC-specific antibodies [14], and metabolic properties [15]. However, these methods are associated with some limitations with respect to specificity, cost, efficacy, safety, and throughput. Therefore, alternative novel methods based on different mechanisms should be developed.

Plasma is an ionized gas containing positive and negative ions, radicals, electrons, uncharged (neutral) atoms and molecules, and UV photons [16]. In recent years, non-thermal atmospheric pressure plasma (NEAPP) has been used in various biological applications [17–19] and has emerged as a novel technology for medical applications such as cancer therapy. Moreover, both direct irradiation of cancer cells and indirect irradiation of medium or water with NEAPP affects cancer cells because of the presence of various reactive oxygen species (ROS), including superoxide anions, hydroxyl radicals, singlet oxygen, nitric oxide (NO), nitrate/nitrite (NOx), hydrogen peroxide (H_2O_2), and other uncertain species. Plasma-activated medium (PAM) or plasma-activated water exerts cytotoxic effects on various cancer cells compared with those on normal cells [20–22] but exerts a curative effect on age-related macular degeneration [23]. Moreover, recent studies have suggested that PAM or plasma-activated water is a new tool for treating various diseases and can be used in various biological researches.

Compared to differentiated cells, hPSCs respond differently to oxidative stress [24,25]. Prigione et al. [25] reported that (1) expression levels of some oxidative stress-related genes were lower in hPSCs than in differentiated cells and that (2) expression level of glutathione peroxidase-1 (GPX1), the most abundant glutathione peroxidase isozyme in mammalian cells [26], significantly decreased in hPSCs compared with that in differentiated cells. Therefore, we investigated whether hPSCs could be selectively eliminated by using PAM because it induces oxidative stress in cells. Results of the present study indicated that PAM could be a potential tool for eliminating residual hPSCs present in a population of differentiated cells.

2. Results

2.1. Differential sensitivities of undifferentiated and differentiated cells toward PAM

To determine whether PAM eliminated undifferentiated cells, we investigated the sensitivities of undifferentiated 201B7 hiPSCs and NHDFs toward PAM. Different dilutions of PAM were added to hiPSC and NHDF cultures, and viabilities of these cells were evaluated after 24 h. Undifferentiated hiPSCs were completely killed after treatment with 1- to 16-fold diluted PAM (Fig. 1). In contrast, NHDFs were not killed even after treatment with 8-fold diluted PAM (cell viability, 97.6% ± 10.4%). These results indicated that undifferentiated hiPSCs were more sensitive to PAM than NHDFs and suggested that PAM could selectively eliminate undifferentiated cells.

Since we previously reported that the effect of PAM varied with cell density or its volume, using a cancer cell line [21], we

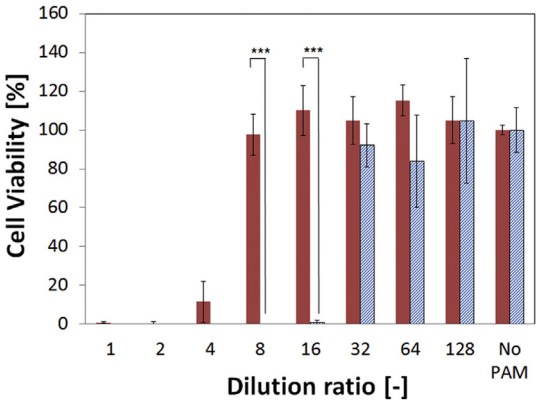

Fig. 1. Quantitative effects of PAM on undifferentiated 201B7 hiPSCs and differentiated NHDFs. PAM was diluted to an optimal concentration by using fresh Stemfit AK03 medium containing A, B, and C solutions and was added each wells (n = 3). After 24 h, cell viabilities (%) were evaluated using the Cell Counting Kit-8 by following the manufacturer's instructions. Red bars denote NHDFs, and blue diagonal bars denote 201B7 hiPSCs. The means ± SD of three experiments are shown; ***$p < 0.005$.

investigated whether these parameters affected the sensitivity of hiPSCs to PAM. The viability of iPSCs varied with cell density (Fig. 2A), with values of 113.9% ± 7.1% for 2.0×10^4 cells/well, 81.9% ± 5.8% for 1.0×10^4 cells/well, and 42.0% ± 2.8% for 5.0×10^3 cells/well. The viability of iPSCs also varied with PAM volume (Fig. 2B), with values of 96.7% ± 9.0% for 60 μL, 81.9% ± 5.8% for 90 μL, and 51.0% ± 12.3% for 120 μL. Considering these results, in subsequent experiments with different experimental conditions, the effective dilution ratio of PAM for each experiment was obtained and used to eliminate undifferentiated hiPSCs.

2.2. Selective elimination of undifferentiated cells by PAM

We next investigated whether PAM selectively eliminated undifferentiated hiPSCs. We cocultured undifferentiated hiPSCs and NHDFs. HiPSCs were first seeded in a multi-well plate and were cultured. After 48 h, fluorescently labeled NHDFs were seeded into the same plate and were cultured for 24 h. Next, the cells were treated with PAM for 24 h, stained with PI, and observed under a fluorescence microscope (Fig. 3). We observed that hiPSCs that were treated with undiluted PAM detached from the surface and

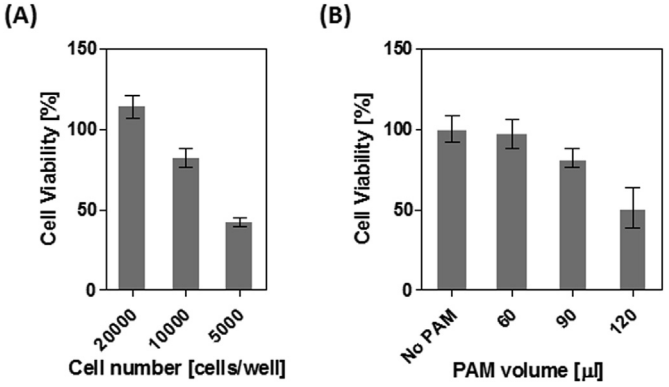

Fig. 2. Effects of cell density or PAM volume on the viability of undifferentiated 201B7 hiPSCs. (A) HiPSCs were seeded at densities of 2.0×10^4, 1.0×10^4, and 5.0×10^3 cells/well and were treated with 90 μL of 8-fold diluted PAM. (B) HiPSCs were seeded at a density of 1.0×10^4 cells/well and were treated with 60, 90, and 120 μL of 8-fold diluted PAM. After 24 h, cell viability (%) was evaluated using the Cell Counting Kit-8 in accordance with the manufacturer's instructions. The means ± SD of three experiments are shown.