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SHORT COMMUNICATION

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Differential resistance of human embryonic stem cells and somatic cell types to hydrogen peroxide-induced genotoxicity may be dependent on innate basal intracellular ROS levels

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Abstract
Previously, we demonstrated that undifferentiated human embryonic stem cells (hESC) displayed higher resistance to oxidative and genotoxic stress compared to somatic cells, but did not further probe the underlying mechanisms. Using H_2O_2-induced genotoxicity as a model, this study investigated whether higher resistance of hESC to oxidative and genotoxic stress could be due to lower innate basal intracellular levels of reactive oxygen species (ROS), as compared to their differentiated fibroblastic progenies (H1F) and two other somatic cell types — human embryonic palatal mesenchymal (HEPM) cells and peripheral blood lymphocytes (PBL). Comet assay demonstrated that undifferentiated hESC consistently sustained lower levels of DNA damage upon acute exposure to H_2O_2 for 30 min, compared to somatic cells. DCFDA and HE staining with flow cytometry showed

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that undifferentiated hESC had lower innate basal intracellular levels of reactive oxygen species compared to somatic cells, which could lead to their higher resistance to genotoxic stress upon acute exposure to H_2O_2. *(Folia Histochemica et Cytobiologica 2015, Vol. 53, No. 2, 169–174)*

Key words: human ESC; genotoxicity; H_2O_2; reactive oxygen species; comet assay; flow cytometry

Introduction

Oxidative stress and genotoxicity are known to be closely intertwined, with abundant evidence in the scientific literatures linking oxidative stress to DNA damage and mutations [1, 2]. Moreover, several toxicological studies have conclusively demonstrated that many genotoxic agents in fact cause an elevation in intracellular levels of reactive oxygen species (ROS), which in turn contribute to DNA damage [3, 4]. Our previous studies have shown that undifferentiated human embryonic stem cells (hESC) displayed higher resistance to both oxidative and genotoxic stress compared to somatic cell types [5, 6], but the underlying molecular mechanisms for this observation have not been well documented.

The aim of the study, based on H_2O_2-induced genotoxicity as a model system, was to investigate whether the observed higher resistance of undifferentiated hESC to oxidative and genotoxic stress could be due to lower innate basal intracellular levels of reactive oxygen species within undifferentiated hESC, as compared to somatic cell types. Three different somatic cell types were examined in this study — human embryonic palatal mesenchymal (HEPM) cells, peripheral blood lymphocytes (PBL), and differentiated fibroblastic progenies (H1F) of hESC.

Material and methods

Human embryonic stem cell culture. Undifferentiated hESC of the H1 line (Wicell Inc., Madison, WI, USA) were cultured on feeder layers of mitotically-inactivated mouse embryonic fibroblasts (MEF) in DMEM-F12 medium supplemented with 20% (v/v) knockout serum replacement, 1 mM glutamine, 4 ng/mL basic fibroblast growth factor (bFGF), 1% (v/v) non-essential amino acids (all purchased from Gibco BRL Inc., Grand Island, NY, USA), and 0.1 mM β-mercaptoethanol (DMSO, SigmaAldrich, St Louis, MO, USA), within a humidified 5% CO_2 incubator at 37°C. The hESC cultures were serially passaged every 5–7 days at a split ratio of 1:6, through treatment with 1 mg/mL collagenase type IV (Gibco) followed by manual dissociation of the hESC colonies by pipetting. The resulting hESC clumps were re-plated on fresh MEF feeder layers seeded on culture plates pre-coated with 0.1% porcine gelatine (Gibco). For microarray analysis that required a pure population of hESCs without contaminating MEFs, the hESCs were subjected to 2 passages under feeder-free conditions with Matrigel (BD Bioscience, Franklin Lakes, NJ, USA), before being utilized for experiments.

Differentiation of human embryonic stem cells into fibroblastic progenies (H1F). Dissociated hESC clumps were initially seeded in T-75 flasks that were pre-coated with 0.1% porcine gelatine, and were induced to differentiate into fibroblasts by culturing in Dulbecco's Modified Essential Medium (DMEM; NUMI Media Preparation Facility NUS, Singapore) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, USA), within a humidified 5% CO_2 incubator. Subsequent passages of cells were plated directly in T-75 flasks without the gelatine coating. The differentiated fibroblastic progenies obtained were referred to as H1 hESC derived progenies (H1F). Cells were cultured to the 5th passage, before being utilized in experiments.

Culture of human embryonic palatal mesenchymal cells and peripheral blood lymphocytes. To isolate human peripheral blood lymphocytes, heparinized venous blood was subjected to Ficoll-Hypaque (GE Healthcare Bio-Sciences, Singapore) density gradient centrifugation at 500 *g*, without using brakes for 20 min at room temperature. After collection of the lymphocyte layer above the Ficoll gradient, the cells were washed three times in phosphate-buffered saline (PBS), followed by cell counting with a hemocytometer. The culture milieu utilized for cultures of human peripheral lymphocytes was Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) supplemented with 10% (v/v) FBS (Hyclone) and 1% (v/v) Penicillin/Streptomycin (Gibco).

Human embryonic palatal mesenchymal cells (HEPM, ATCC Catalogue no: CRL1486) were cultured in DMEM (NUMI Media Preparation Facility, Singapore) supplemented with 10% (v/v) FBS (Hyclone, South Logan, UT, USA). All cell cultures were carried out within a humidified 5% CO_2 incubator at 37°C.

Exposure of cells to hydrogen peroxide. Stock solutions of hydrogen peroxide (H_2O_2; Kanto Chemical Co. Inc., Japan) were freshly prepared in PBS before each experiment. The four different cell types (hESC, H1F, PBL & HEPM) were then exposed to doses of 50 μM, 100 μM and 200 μM H_2O_2 for 30 min, and were subjected to analyses by comet assay and DCFDA/HE flow cytometry.

Alkaline single cell gel electrophoresis assay (Comet Assay). Comet assay was utilized to quantify the extent of DNA damage. Alkaline conditions at pH greater than 13 allow for the detection of single stranded, double-stranded breaks, apurinic and apyridiminic sites as well as alkali adducts. Cells were lysed and then denatured under alkaline conditions to unwind the DNA and hydrolyze the damaged sites. The cell lysate was then subjected to electrophoresis. Electrophoresis induced the cleaved DNA fragments to migrate further under the electric field, as compared to undamaged DNA that remained in the nucleoidal shape. After treatment, cells were harvested and washed in PBS, followed by suspension in Hank's Balanced Salt Solution (HBSS; Gibco) supplemented with 10% (v/v) DMSO (SigmaAldrich) and 0.5 M ethylenediaminetetraacetic acid (EDTA, SigmaAldrich). Ten percent of the cell suspension volume was suspended in liquid agarose (Trevigen, Gaithersburg, MD, USA) and 50 μl of this suspension was added to the comet assay slides (Trevigen). After solidification of the agarose at 4°C, the slides were then left in lysis solution (Trevigen) overnight at 4°C. Denaturation of the slides was carried out in the dark for 40 min, in chilled alkaline electrophoresis buffer (pH 13.0–13.7) consisting of 24 g sodium hydroxide pellets (Merck Chemicals, Singapore), 0.5 M EDTA and distilled water. Subsequently, electrophoresis was carried at 25 V for 25 min. The slides were dried and then stained with SYBR green dye (Molecular Probes, Eugene, OR, USA) prior to being observed under a Zeiss Axioplan 2 imaging fluorescence microscope (Carl Zeiss, Wetzlar, Germany) equipped with triple band filter. Images were captured using Comet Analysis Software (Metasystems, Altlussheim, Germany). Fifty comets per sample were analyzed to obtain the mean tail DNA. All measurements were performed in duplicates.

Flow cytometry with DCFDA- and hydroethidine-stained cells. For DCFDA (dichlorofluorescin diacetate) staining, cells were washed with in PBS and exposed to 5 μM 5-(and-6)-chloromethyl-2,7-dichlorofluorescin diacetate (CM-H_2DCFDA; Molecular Probes) for 15 min at 37°C. Subsequently, the cells were washed again in PBS and analyzed by flow cytometry (Coulter EPICS Elite ESP; Beckman Coulter, Fullerton, CA, USA) utilizing excitation/emission wavelengths of 488 nm/525 nm respectively. At least 10,000 events were analyzed using the WinMDI (Windows multiple document interface for flow cytometry) software (Beckman Coulter Inc., Sunnyvale, CA).

For hydroethidine (HE) staining, we utilized MitoSox Red (Invitrogen, Carlsbad, CA, USA), a live-cell permeable dye with hydroethidine covalently linked to a triphosphonium cation through a hexyl carbon chain. The dye exhibits red fluorescence once it is oxidized by superoxide. Cells were incubated with MitoSox Red for 15 min, and then were washed twice with PBS, prior to being analyzed with an Epic Profile flow cytometer using an excitation wavelength of 510 nm and emission wavelength of 580 nm. Data were analyzed for at least 10,000 events using the WinMDI software.

Statistical analysis. Statistical analysis of the experimental data was carried out with Student's *t* test, utilizing Microsoft Excel 2003 (Microsoft Corporation, Richmond, WA, USA). The threshold of statistical significance was set at $p < 0.05$.

Results

DNA damage induced by hydrogen peroxide in the studied cell lines

Normal undamaged nuclei formed a nucleoidal shape whereas damaged nuclei formed a characteristic comet-like shape when subjected to electrophoresis (Figure 1A and B). The four studied cell types were exposed to an acute dose of H_2O_2 for 30 min and the level of DNA damage was quantified with the comet assay. It was observed that hESCs consistently sustained lower levels of DNA damage upon acute exposure to different dosages of H_2O_2 for 30 min, as compared to the three other cell types (Figure 1C). It was only at 200 μM of H_2O_2 that there was a significant change in the tail moment of the comet assay for hESCs, with no significant differences being observed at 50 and 100 μM of H_2O_2. In the case of H1F cells, significant changes in the tail moment of the comet assay were observed at 100 μM and 200 μM H_2O_2, but not at 50 μM H_2O_2 concentration. By contrast, HEPM and PBL displayed significant changes in the tail moment at all three concentrations of H_2O_2 (Figure 1C).

Intracellular H_2O_2 and superoxide levels as assessed by flow cytometry of DCFDA- and HE-stained cells

It was observed that hESCs had the lowest basal intracellular H_2O_2 levels (Figure 2A), followed by similar H_2O_2 levels in PBL and H1F (Figure 2A). Of the four cell types studied, HEPM cells had the highest basal level of H_2O_2 (Figure 2A). Similar trends in O^{2-} levels were observed (Figure 2B). hESCs were found to have the lowest basal intracellular O^{2-} levels (Figure 2B). PBL and H1F cells had similar basal levels of O^{2-} (Figure 2B). Again, of the 4 cell types, HEPM cells had the highest basal levels of O^{2-} (Figure 2B).

Discussion

Alkaline single-cell gel electrophoresis (comet assay) is a sensitive indicator of DNA damage incurred. The comet assay was carried out under alkaline conditions (pH > 13), to detect all types of DNA damage, including double-strand breaks, single-strand breaks, and

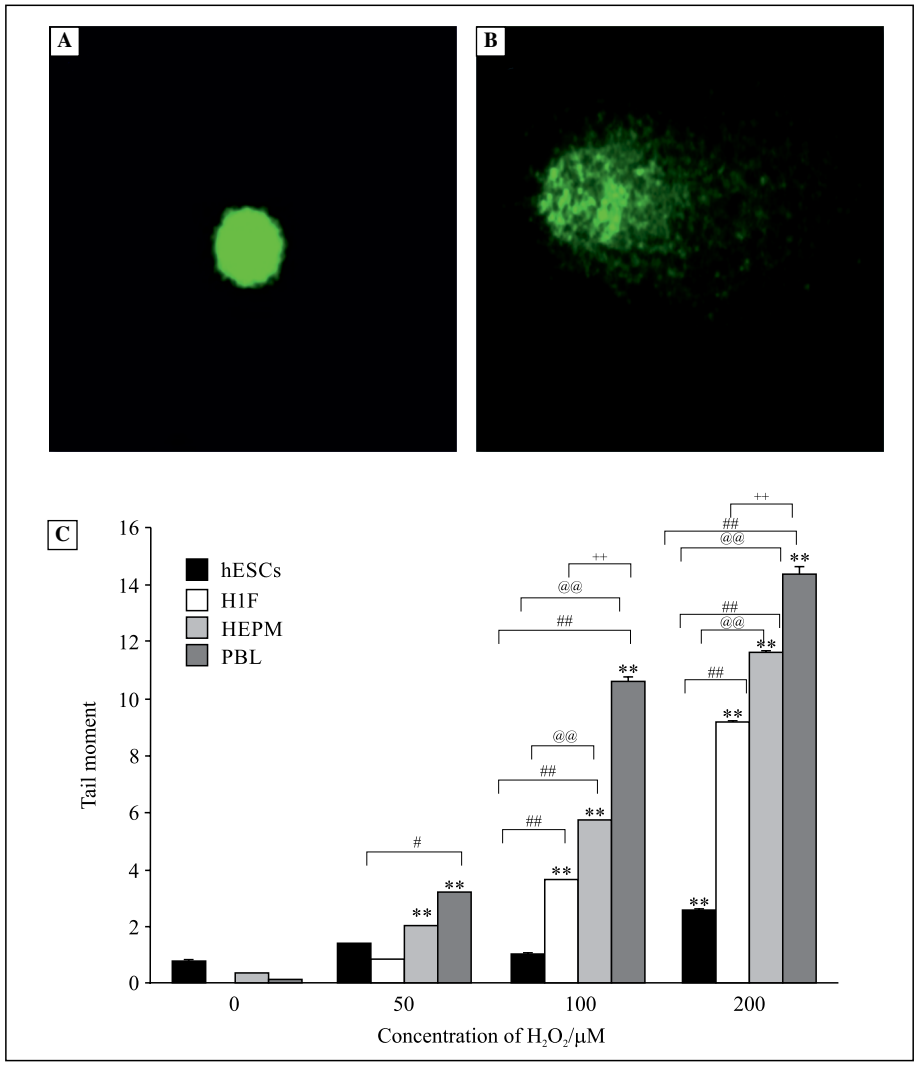

Figure 1. Extent of DNA damage of hESC, HEPM, and H1F cells as well as PBL following H_2O_2 treatment as assessed by comet assay. Representative images of (**A**) SYBR Green-stained undamaged nuclei of PBL cells and (**B**) damaged nuclei of PBL cells with characteristic comet-like shape. (**C**) X-axis: hESC, HEPM, PBL and H1F cells following H_2O_2 treatment. hESC (black bars), H1F (white bars), HEPM (horizontal bars) and PBL (vertical bars) were treated with 50 μM, 100 μM or 200 μM of H_2O_2 for 30 min. Y-axis: tail moment (product of tail length and fraction of DNA) corresponds to the extent of acute DNA damage as measured by the comet assay. Data represent mean value and error bars indicate standard error. Two sets of 50 comets per sample were analyzed. Abbreviations of cell type names as for Figure 1. Cell symbols: hESC — human embryonic stem cells; HEPM — human embryonic palatal mesenchymal cells; H1F — differentiated fibroblastic progenies cells; PBL — peripheral blood lymphocytes. $^{*,**}p < 0.05$ and $p < 0.01$, respectively, as compared to control (no H_2O_2 treatment) cells of the same cell type; $^{\#,\#\#}p < 0.05$ and $p < 0.01$, respectively, as compared to H_2O_2-treated hESC cells; $^{@@}p < 0.01$ as compared to H_2O_2-treated H1F cells; $^{+,++}p < 0.05$ and $p < 0.01$, respectively, as compared to H_2O_2-treated HEPM cells (Student's *t*-test)

alkali labile sites [7, 8]. Consistent with our previous studies, the results of the comet assay showed that hESCs exhibited the lowest level of DNA damage upon acute exposure to H_2O_2, as compared to the three somatic cell types. We wanted to determine if the lower levels of genotoxicity observed in hESCs could be due to their lower innate basal intracellular ROS levels, as compared to somatic cells. Indeed, oxygen metabolism itself has been demonstrated to contribute to increased levels of double strand DNA breaks [9].

To evaluate whether the increased levels of DNA damage observed under comet assay were a product of increased intracellular ROS levels, flow cytometry with DCFDA and hydroxyethidine staining was applied to elucidate innate intracellular hydrogen

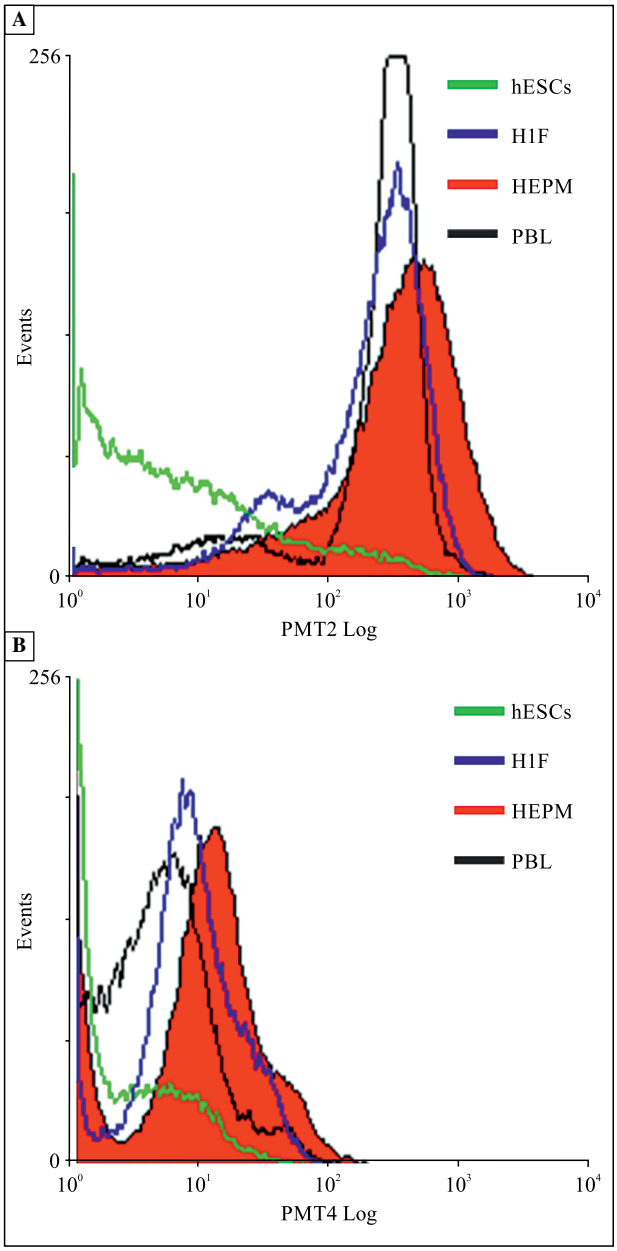

Figure 2. Endogenous hydrogen peroxide (**A**) and superoxide (**B**) levels in the studied cell types. hESC, H1F, HEPM and PBLs were treated with 100 μM hydrogen peroxide for 30 min. Then the cells were analyzed with flow cytometry for intracellular H_2O_2 formation by DCFDA staining and intracellular superoxide levels by hydroxyethidine stainin as described in Material and methods. hESCs (Green), H1F (Blue), HEPM (Red) and PBLs (Black). Fifty thousand cells were analyzed per run. Figures are representative of 3 replicate experiments. The X-axes of the graphs represent light signal being detected by a photomultiplier tube (PMT) that is converted *via* a pre-amplifier to an electrical voltage output signal that is proportional to the original fluorescence intensity. This data is then subjected to log transformation at either the base 2 (PMT2 Log) or base 4 (PMT4 Log)

peroxide and superoxide levels. A higher ROS level is indicated by the shift of the specific ROS profile towards the right (Figure 2). The results conclusively demonstrated that hESC had the lowest innate basal intracellular levels of hydrogen peroxide and superoxide among the four different cell types examined. Hence, the observed higher resistance of undifferentiated hESC to oxidative stress and genotoxic stress compared to somatic cell types, as reported by our previous studies [5, 6], could be due to lower innate basal intracellular levels of ROS within hESCs compared to somatic cell types.

Our findings are consistent with previous studies which showed that ROS play crucial roles as secondary messengers during stem cell differentiation into various somatic lineages [10, 11], so that an increase in ROS levels in differentiated stem cell progenies is therefore expected. Hirano and Tamae [12] and Kuboyama et al. [13], also reported that undifferentiated embryonic stem cells were more resistant to DNA damage induced by the ROS — 8-oxoguanine (which is known to generate GC-to-TA point mutations in genomic DNA), as compared to their differentiated progenies. These findings are thus consistent with the results of this study, as well as our previous studies [5, 6].

Future studies will be needed to investigate the mechanism by which hESCs maintains lower innate basal intracellular ROS levels compared to somatic cell types, *e.g.* due to higher levels of antioxidants and ROS protective enzymes such as superoxide dismutase.

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