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The combined effect of resveratrol and diphenyleneiodonium on irradiation-induced injury to the hematopoietic system

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ABSTRACT

Both resveratrol(Res) and diphenyleneiodonium(DPI) have been shown to have radioprotective effects on hematopoietic system injury. However, the cooperative effect of Res and DPI are unknown. In this study, we explored the radioprotective effect of the combination of Res and DPI both in vitro and in vivo. Our results showed that the combined treatment of Res and DPI was more effective in protecting irradiated BMMNCs in terms of cell viability, colony-forming ability, and reconstitution ability in vitro compared with Res or DPI treatment alone. However, in mice, the combination of Res and DPI had no enhanced protection on 4 Gy total body irradiation (TBI)-induced hematopoietic system injury, including TBI-induced myelosuppression, induction of the splenic index, and increases in HSC/HPC numbers and the colony-forming ability of BMCs,compared to Res or DPI alone. An exception was the number of BMCs. These studies illustrated the inconsistency between experiments carried out in vitro and in vivo and suggest an interaction between Res or DPI in vivo.

1. Introduction

Total body irradiation(TBI) may induce hematopoietic system injury,including myelosuppression, hematopoietic stem cell(HSC)injury, and hematopoietic progenitor cell(HPC) injury.HSC is one of the best-characterized somatic stem cell types among all tissue stem cells, and they reside in a specialized niches with low levels of oxygen and have limited levels of reactive oxygen species(ROS) [1]. One of the important mechanisms by which TBI induces hematopoietic system injury is mediated by ROS, which can activate numerous oxidative stress related pathways. Studies have shown that NADPH oxidases 4(NOX4) play an important role in regulating the production of TBI-induced ROS and HSC function [2].TBI may upregulate the expression of NOX4 and relevant NOX4 inhibitors,such as diphenyleneiodonium(DPI) or resveratrol, which may alleviate TBI-induced hematopoietic stem cell injury.

There are five members (NOX1-NOX5) in the NOX family. They are expressed in different tissues and cells and regulatethe production of ROS [3,4]. It has been reported that NOX1,NOX2,and NOX4 are expressed in HSC [5,6]. More specifically, NOX4 is expressed in LSK($Lineage^-sca1^+c\text{-}kit^+$) cells but not in HPCs($Lineage^-sca1^-c\text{-}kit^+$),$Lineage^-$ cells, or bone marrow mononuclear cells(BMMNCs) [2].DPI administration attenuates TBI-induced HSC injury by inhibiting NOX4 regulated oxidative stress.

Our previous study suggested that Resveratrol could protect TBI-induced HSC injury [7]. In addition to inhibiting NOX4, Res also specifically activates sirtuin1(Sirt1),which can regulate the expression of many genes through downstream transcriptional factors, including FoxO3 [8,9]. Res ameliorates TBI-induced hematopoietic cells injury and HSC senescence by down-regulating the expression of NOX4 and up regulating the expression of Sirt1, Sod2, and Gpx1 [7].

Furthermore, DPI and Res regulate the same protein (NOX4) as well as many other different proteins in the hematopoietic system when mice are exposed to ionizing radiation (IR). Thus, in this study, we combined Res and DPI to block multiple oxidative stress regulated targets and to explore their radioprotective effect on IR-induced hematopoietic cell injury both in vivo and in vitro. Our results showed that compared to a single agent treatment,the combination of Res and DPIenhanced protection in IR-induced BMMNCs injury in vitro but not in TBI-induced hematopoietic system injuryin vivo.

2. Materials and methods

2.1. Mice

Male C57BL/6 (CD45.2) mice were purchased from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College (ILAS-PUMC, Beijing, China). Male C57BL/6 (CD45.1) mice were purchased from the Institute of Hematology and Blood Disease Hospital, Chinese Academy of Medical Sciences & Peking

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Union Medical College (IHBDH-PUMC, Tianjin, China). Male C57BL/6 (CD45.1/5.2) mice were bred in the Experimental Animal Center of the Institute of Radiation Medicine (IRM-PUMC, Tianjin, China). The mice were used at the age of approximately 6–8 weeks. All animal experiments in our study were approved by the Animal Care and Ethics Committee at IRM-PUMC.

2.2. Antibodies

The following antibodies were purchased from eBioscience(San Diego, CA, USA): anti-mouse CD34 (clone RAM34)anti-mouse CD117 (c-kit)-APC (clone 2B8), and anti-mouse Sca-1-PE (clone D7). The following antibodies were purchased from BioLegend(San Diego, CA, USA): PerCP-conjugated streptavidin, anti-mouse CD45.1-FITC(cloneA20), anti-mouse CD45.2-PE(clone104), biotin-conjugated anti-mouseTer119 (cloneTER119), anti-mouse B220 (cloneRA3-6B2), anti-mouse Gr1 (cloneRB6-8C5), anti-mouse CD11b (cloneM1/70), anti-mouse CD4 (cloneGK1.5), and anti-mouseCD8 (clone53-6.7).

2.3. In vitro culture

BMMNCs were isolated as described previously [10]. A total of 1×10^6 BMMNCs were cultured in 1 ml RPMI-1640 plus 10%FBS Medium supplied with Res or DPI 30 min before IR. Then, the cells were collected and used for CFU-GM culture and competitive transplantation assays. For the analysis of cell viability in vitro, BMMNCs were treated with Res or DPI for 30 min before IR, after which the cells were cultured for 18 h.

2.4. TBI and Res, DPI administration

Two different experimental conditions were used in this study, with 5 groups in each experiment, as follows: control, 4 Gy, 4 Gy + 20 mg/kg Res group, 4 Gy + 1 mg/kg DPI, and 4 Gy + 20 mg/kg Res + 1 mg/kg DPI. In the first experimental condition (FEC), the mice received Res by gavage 30 min before TBI and once a day on the following 7 days after TBI. The mice received DPI by intraperitoneal injection (i.p.) 6 h after TBI and once a day on the following 7 days after TBI. The mice were sacrificed 22 days after TBI. In the second experimental condition (SEC), the mice received Res by gavage 30 min before TBI and once a day on the following 30 days after TBI. The mice received DPI by intraperitoneal injection (i.p.) 6 h after TBI and once every other day on the following 30 days after TBI. The mice were sacrificed 30 days after TBI. All the mice in TBI groups received a 4 Gy γ-ray at a dose rate of 0.99 Gy/min.

2.5. Peripheral blood cell and bone marrow cell counts

Blood was obtained from the mice via the orbital sinus and was collected in a micro-pipette coated with K_3EDTA. The blood parameters measured included white blood cells (WBCs), red blood cells (RBCs), platelets (PLT), and hemoglobin (HGB). Bone marrow cells were isolated by flushing both the tibias and femurs with sterile PBS, and the number of cells was counted using a Celltac E hemocytometer (Nihon Kohden, Japan).

2.6. Flow cytometry analysis

For HPC and HSC analysis, bone marrow cells were isolated as described above. The cells were filtered and counted prior to staining with the antibodies. Then, 5×10^6 bone marrow cells were stained with biotin-labeled antibodies against Ter119, B220, Gr1, CD11b, CD4, and CD8 and then stained with streptavidin, c-kit, and sca1. To measure donor chimerism, 50 μl of peripheral blood was first stained with CD45.1and CD45.2 antibodies at room temperature, after which the red blood cells were removed with BD FACS™ Lysing Solution (BD Bioscience, San Jose, CA, USA). Data acquisition was performed on a BD Accuri C6 instrument and analyzed using the BDAccuri C6 software (BD Bioscience, San Jose, CA, USA).

2.7. Colony formation units of granulocyte macrophage cells (CFU-GM)

A total of 1×10^4 bone marrow cells (1×10^3BMMNCs in vitro)from the control group and 1×10^5 bone marrow cells(1×10^4BMMNCsin vitro) from the TBI groups were cultured in M3534 methylcellulose medium (Stem Cell Technologies, California, America) for 5 days. The colonies of CFU-GM with >30 cells were counted according to the instructions. The results are expressed as the numbers of CFU-GM per 10^5 cells.

2.8. Competitive transplantation assay

A total of 1×10^5 BMMNCs from C57BL/6(CD45.2) mice and 1×10^6 bone marrow cells from C57BL/6(CD45.1/45.2) mice were transplanted into lethally irradiated C57BL/6 mice (CD45.1). The percentage of donor-derived (CD45.2) cells in the peripheral blood of recipients was examined 2 months after transplantation.

2.9. Statistical analysis

We performed the statistical analyses using GraphPad Prism 5 software. Welch's correction *t*-test (significantly different at $P < 0.05$) was used, and in all figures, the error bars represent the standard deviation of the mean.

3. Results

3.1. Enhanced protection ofcells exposed to irradiation by the combination treatment of Res and DPI in vitro

To explore whether the application of Res and DPI in combination could elevate the viability of irradiated BMMNCs compared to the application of Res or DPI separately, BMMNCs were co-cultured with the relevant agent or agent combination 30 min before IR. After IR,the cells were cultured for 18 h . Then cell viability was measured using a Multimode Reader. The results showed that compared to the 0 Gy control group,the cell viability of BMMNCs in the 4 Gy group was significantly decreased. The application of Res or DPI separately or in combination rescued the viability of the irradiated BMMNCs,and the combination of Res and DPI showed enhanced protection compared to Res or DPI applied separately (Table 1). These results indicate that the combination of Res and DPI has a better protective effect on the viability of irradiated BMMNCs in vitro than individual treatments alone.

3.2. Enhanced protection of the colony formation ability of BMMNCs by the combination of Res and DPI in vitro after exposure to irradiation

To explore whether the application of Res and DPI in combination could increase the number of colonies formed by irradiated BMMNCs, compared to application of Res or DPI separately, we conducted CFU-GM assays. BMMNCs were co-cultured with Res, DPI or a combination of Res and DPI 30 min before IR,and BMMNCs were cultured in M3534 methylcellulose medium for 5 days. Our result showed that compared to 0 Gy control group, the number of colonies in the 4 Gy IR group decreased significantly. The application of Res or DPI separately or in combination mitigated the decrease in the number of colonies formed by irradiated BMMNCs,and there were more colonies in the group treated with the combination of Res and DPI compared to the groups in which Res or DPI were applied separately (Table 2). These results indicate that the combination of Res and DPI had a better protective effect on the colony-forming ability of irradiated BMMNCs in vitro than the individual treatments alone.