「ヒト造血幹/前駆細胞の放射線応答における
ミトコンドリア機能及び遺伝子の関与」

The involvement of mitochondrial function and genes
in the radiation response of
human hematopoietic stem/progenitor cells

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略 語 一 覧

BFU-E	burst-forming unit erythroid
BSA	bovine serum albumin
CB	cord blood
CD	cluster of differentiation
cDNA	complementary DNA
CFC	colony-forming cells
CFU-GM	colony-forming unit granulocyte-macrophage
CFU-Mix	colony-forming unit granulocyte, erythroid, macrophage, megakaryocyte
EDTA	ethylenediamine –N, N, N’, N’-tetraacetic acid
EPO	erythropoietin
ETC	electron transport chain
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBSS	Hanks’ Balanced Salt Solution
HSPCs	hematopoietic stem and progenitor cells
IL-3	interleukin-3
IMDM	Iscove’s modified Dulbecco’s medium
LDL	low density lipoprotein
mtDNA	mitochondrial DNA
NADPH	nicotinamide adenine dinucleotide phosphate
PBS(-)	calcium- and magnesium-free phosphate buffered saline
qRT-PCR	quantitative real-time reverse transcription polymerase chain reaction
ROS	reactive oxygen species
SCF	stem cell factor

序　論

個体への放射線ばく露では，造血組織や腸管粘膜などの再生能の高い組織への障害が重大な問題となってくる．その中で造血組織内にごく僅かに存在する造血幹細胞は，数日から数十日しか機能しない成熟血球を生涯に渡って産生し続ける生体の恒常性維持に必須の細胞であり，高い増殖能と未分化性のため，放射線などの体外・体内酸化ストレスに対して高い感受性を示す細胞であるとされている．しかしながらヒト造血幹細胞は生体内に極めて少数しか存在しない為，その採取や分離精製及び培養評価等には困難を伴い，造血幹細胞の放射線感受性に関する詳細は不明な点が多い．これまでの報告で，造血幹細胞は造血幹細胞ニッチと呼ばれる特殊な微小環境において静止期で存在し，自己の幹細胞特性を維持するためミトコンドリアを脱共役させ細胞老化に繋がる活性酸素種産生を抑制しており，実際に成熟細胞と比べミトコンドリア含有量は少ない．一方，分化/増殖過程では，ミトコンドリア生合成及び酸化的リン酸化が促進し，生理的レベルの活性酸素種が造血サイトカインと共に内因性の分化/増殖シグナルとなり，ミトコンドリア生合成と同時に幹細胞特性が喪失する現象が起こる．造血幹細胞の幹細胞特性の維持や分化の進行におけるミトコンドリアの重要性は近年明らかにされつつあるが，その造血幹細胞の分化/増殖過程に及ぼす電離放射線の影響と細胞内ミトコンドリアの関連性についての多くは不明のままである．

東日本大震災に伴う東京電力・福島第一原子力発電所からの核分裂生成物の環境への拡散は，放射能汚染や放射線被ばくという深刻な問題を生み出した．福島第一原発事故に伴う廃炉作業や高汚染エリアの除染作業に伴う放射線被ばくのリスクは高く，さらに核関連施設での事故や核テロの脅威は依然存在する．福島原発事故後，放射線の生物影響研究の重要性が様々な分野で求められており，広島・長崎の被ばく者やチェルノブイリ事故での被災住民の白血病発症では放射線の寄与が高い事からも，とりわけヒト造血幹細胞に対する放射線の影響研究は将来の発がんリスク評価にも直結する極めて重要な課題である．

本研究では，未だ詳細が不明な放射線のヒト造血幹細胞へ分化/増殖への影響

を*in vitro*で評価することを目的とし，ヒト胎盤/臍帯血由来CD34陽性造血幹/前駆細胞の分化/増殖過程における放射線応答と，活性酸素種やミトコンドリア機能との関連性について評価した．本研究に際し，弘前大学大学院医学研究科倫理委員会より承認を得，また，インフォームド・コンセントはそれぞれの研究対象である全ての妊婦に対し臍帯血の採取前（受診後の妊娠後期）に十分な説明を行った後に承諾を得ている．本論文は以下に述べる第1章及び第2章で構成される．

《第 1 章》「ヒト造血幹/前駆細胞の放射線応答における活性酸素種の役割」

電離放射線及び造血サイトカインで刺激したヒト臍帯血由来 CD34 陽性造血幹/前駆細胞のクローナル増殖能及び細胞内活性酸素種産生，ミトコンドリア応答の関連性を評価した．これらの細胞が電離放射線にばく露されると，細胞増殖及びクローナル増殖能の低下，細胞内活性酸素種の過剰産生が引き起こされる一方，ミトコンドリア含有量及びミトコンドリア由来活性酸素種の産生量に関しては変化がみられないことを明らかにした．

《第 2 章》「放射線ばく露ヒト造血幹/前駆細胞の分化・増殖におけるミトコンドリア関連遺伝子の発現変化」

ミトコンドリア内膜に存在する電子伝達系を構成する13のタンパク質をコードしているミトコンドリアDNAのうち，電子伝達系の律速段階となっている複合体1におけるND1のmRNA発現は放射線の有無によって差は認められなかった．一方，ミトコンドリア転写関連遺伝子のうちミトコンドリアDNAのゲノム安定性に関わる遺伝子及び抗酸化酵素遺伝子のmRNA発現が放射線によって優位に増加していた．即ち，電離放射線で刺激したヒト臍帯血由来CD34陽性造血幹/前駆細胞ではミトコンドリア由来の酸化ストレスが抑制される可能性を示唆している．

以上のことから，放射線ばく露ヒト胎盤/臍帯血由来 CD34 陽性造血幹/前駆細

胞の分化/増殖過程において，ミトコンドリア生合成及びミトコンドリア由来ROS の異常生成は抑制されており, 放射線による細胞の DNA 損傷及び持続的な細胞内 ROS の異常産生が幹細胞機能破綻を引き起こしている可能性が示唆された．放射線ばく露ヒト造血幹細胞の分化・増殖における新たな機構解明等の新たな展開が期待される．

Chapter 1

Role of Reactive Oxygen Species in the Radiation Response of Human Hematopoietic Stem/Progenitor Cells

ABSTRACT

Hematopoietic stem/progenitor cells (HSPCs), which are present in small numbers in hematopoietic tissues, can differentiate into all hematopoietic lineages and self-renew to maintain their undifferentiated phenotype. HSPCs are extremely sensitive to oxidative stressors such as anti-cancer agents, radiation, and the extensive accumulation of reactive oxygen species (ROS). The quiescence and stemness of HSPCs are maintained by the regulation of mitochondrial biogenesis, ROS, and energy homeostasis in a special microenvironment called the stem cell niche. The present study evaluated the relationship between the production of intracellular ROS and mitochondrial function during the proliferation and differentiation of X-irradiated $CD34^+$ cells prepared from human placental/umbilical cord blood HSPCs. Highly purified $CD34^+$ HSPCs exposed to X-rays were cultured in liquid and semi-solid medium supplemented with hematopoietic cytokines. X-irradiated $CD34^+$ HSPCs treated with hematopoietic cytokines, which promote their proliferation and differentiation, exhibited dramatically suppressed cell growth and clonogenic potential. The amount of intracellular ROS in X-irradiated $CD34^+$ HSPCs was significantly higher than that in non-irradiated cells during the culture period. However, neither the intracellular mitochondrial content nor the mitochondrial superoxide production was elevated in X-irradiated $CD34^+$ HSPCs compared with non-irradiated cells. Radiation-induced gamma-H2AX expression was observed immediately following exposure to 4 Gy of X-rays and gradually decreased during the culture period. This study reveals that X-irradiation can increase persistent intracellular ROS in human $CD34^+$ HSPCs, which may not result from mitochondrial

ROS due to mitochondrial dysfunction, and indicates that substantial DNA double-strand breakage can critically reduce the stem cell function.

INTRODUCTION

Mitochondria, the organelles that produce the energy molecule adenosine triphosphate (ATP) by oxidative phosphorylation, are also a source of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide, which are generated during respiratory metabolism *in vivo*. Mitochondria contain extranuclear genomes that encode proteins constituting the mitochondrial electron transport chain (ETC). This series of proteins produces ATP by electron transfer and generates ROS as a byproduct[1-3)]. The abnormal accumulation of endogenous or exogenous ROS by the mitochondrial ETC or in response to low linear energy transfer ionizing radiation, such as X-rays, may cause lipid peroxidation, protein denaturation, or DNA mutations[4-6)].

Hematopoietic stem/progenitor cells (HSPCs), which are present in small numbers in hematopoietic tissues, can self-renew to maintain their own undifferentiated phenotype and can differentiate into all functional mature hematopoietic cells. Hematopoietic stem cells are usually present in a special microenvironment called the stem cell niche, which maintains the stemness by controlling the ROS generation[7-10)]. In this niche, physiological levels of intracellular ROS affect the endogenous growth signals, cell survival, proliferation, and differentiation of HSPCs with the production of many cytokines[2, 11, 12)]. When the ROS are elevated beyond physiological levels, oxidative stress can cause HSPC dysfunction and aging. These cells are extremely sensitive to oxidative stressors, such as anti-cancer agents, radiation, and the extensive accumulation of ROS[13-19)]. In addition, because hematopoietic stem cells generally reside in the G0 phase of the cell cycle and require very little energy, they possess lower intracellular mitochondrial contents than other functional mature cells[20-24)]. Recently, Charlie et al. reported that $CD34^+$ hematopoietic stem cells with a low mitochondrial mass are enriched in hematopoietic repopulating stem cell function, and that the upregulation of nascent mitochondrial biogenesis in $CD34^+$ HSPCs parallels the loss of pluripotency[25)]. Thus, the response of mitochondrial function and intracellular ROS to

ionizing radiation may also depend on the radiosensitivity of $CD34^{+}$ HSPCs. However, information about the relationship between the radiosensitivity of HSPCs and mitochondrial function is limited.

To determine the contributions of mitochondria and intracellular ROS to the radiosensitivity of human HSPCs, the present study investigated the features of X-irradiated $CD34^{+}$ HSPCs prepared from human placental/umbilical cord blood and cultured with hematopoietic cytokines. We herein describe the influence of radiation on the HSPC proliferation and differentiation from the perspective of mitochondrial function.

MATERIALS AND METHODS

Growth factors and fluorescent antibodies

Recombinant human interleukin-3 (IL-3) and human stem cell factor (SCF) were purchased from BioSource (Tokyo, Japan). Recombinant human erythropoietin (EPO) and granulocyte colony stimulating factor (G-CSF) were purchased from Sankyo Co. Ltd. (Tokyo, Japan). Recombinant human granulocyte/macrophage colony stimulating factor (GM-CSF was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). These growth factors were administered at the following concentrations: IL-3, 100 ng/ml; SCF, 100 ng/ml; EPO, 4 U/ml; G-CSF, 10 ng/ml and GM-CSF, 10 ng/ml medium. The following fluorescence-labeled monoclonal antibodies (mAbs) were purchased from Beckman Coulter Immunotech (Marseille, France): fluorescein isothiocyanate (FITC)-conjugated anti-human CD34 (CD34-FITC), phycoerythrin (PE)-conjugated anti-human CD34 (CD34-PE) and mouse IgG_1-FITC and IgG_1-PE, which were used as isotype controls. The ROS detection reagent, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA); the MitoSOX™ Red mitochondrial superoxide indicator and the MitoTracker Green FM mitochondrion-selective probe reagent (specially packaged) were purchased from Molecular Probes (CA, USA). Anti-phospho-histone H2AX monoclonal antibodies (JBW301) were purchased from Upstate Biotechnology (NY, USA), and Alexa Fluor 488®-conjugated anti-mouse IgG secondary antibodies were purchased from Molecular Probes.

Collection, purification and cryopreservation of CD34$^+$ HSPCs

This study was approved by the Committee of Medical Ethics of Hirosaki University Graduate School of Medicine (Hirosaki, Japan). Informed consent was obtained from mothers who had delivered full-term infants after written and verbal explanations were provided before delivery. Signatures were obtained from the mothers, as well as from a

relative, such as the husband or the patient's mother/father, etc. All documents related to the informed consent process were reviewed and approved. After delivery, placental/umbilical cord blood samples were collected into sterile collection bags (CBC-20; Nipro, Osaka, Japan) containing the anticoagulant, citrate phosphate dextrose, according to the guidelines of the Tokyo Cord Blood Bank (Tokyo, Japan). The samples were isolated and maintained separately prior to each experiment. Within 24 hours of cord blood collection, light-density mononuclear cells were separated by centrifugation in a Limphosepar I (1.077 g/ml; Immuno-Biological Laboratories, Takasaki, Japan) instrument for 30 min at 300×*g* and were washed three times with calcium- and magnesium-free phosphate-buffered saline (PBS (–); Sigma-Aldrich, Stockholm, Sweden) containing 5 mM ethylenediamine-N,N,N′,N′-tetraacetic acid (Wako, Tokyo, Japan). The cells were then processed for $CD34^+$ enrichment according to the manufacturer's instructions. An autoMACS™ Pro Separator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used for the sorting of $CD34^+$ cells. The CD34-enriched cell population is referred to as HSPCs in this study. The $CD34^+$ HSPCs were suspended in serum-free Cell Banker 3 reagent (Juji Field, Tokyo) and were frozen at −150°C prior to experimentation.

In vitro irradiation

$CD34^+$ HSPCs were exposed to X-rays in serum-free medium within 24 hours of isolation. Radiation (2 or 4 Gy, 150 kVp, 20 mA; 0.5 mm aluminum and 0.3 mm copper filters) was delivered using an X-ray generator (MBR-1520R; Hitachi Medical Co., Tokyo, Japan) with a distance of 45 cm between the focus and the target. The dosage was monitored using a thimble ionization chamber placed next to the sample during irradiation. The dose rate was approximately 1 Gy/min.

Methylcellulose culture

The lineage-committed myeloid hematopoietic progenitor cells included

colony-forming unit-granulocyte macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E) and colony-forming unit-granulocyte erythroid, macrophage, and megakaryocyte (CFU-Mix) cells. $CD34^+$ HSPCs were assayed in methylcellulose culture as described previously[26] by suspending them in 1 ml of methylcellulose medium (MethoCult H4230, StemCell Technologies Inc.) supplemented with a hematopoietic cytokine mixture that contained five growth factors (GFs; IL-3, SCF, EPO, G-SCF, and GM-SCF). This mixture was transferred to 24 well cell culture plates (Falcon, Becton Dickinson Biosciences, Franklin Lakes, NJ) at 0.3 ml/well and then was incubated for 14 days at 37°C in a humidified atmosphere with 5% CO_2. Colonies consisting of more than 50 cells were counted using an inversion microscope (Olympus, Tokyo, Japan).

Liquid culture

Frozen cells were thawed rapidly at 37°C, suspended in Cell Lotion (Juji Field, Tokyo, Japan) and centrifuged at 400×*g* at 10°C for 10 min. The supernatant was removed and resuspended in serum-free Iscove's Modified Dulbecco's Medium (Gibco Invitrogen, Grand Island, NY, USA) supplemented with BIT 9500 serum substitute (StemCell Technologies, Vancouver, Canada) and low density lipoprotein (Calbiochem®, US and Canada). $CD34^+$ HSPCs were treated with the hematopoietic cytokine combination of five GFs in γ-ray–sterilized assist tubes (Assist, Tokyo, Japan) at a concentration of 3×10^4 cells/0.5 ml/tube, and were incubated at 37°C in a humidified atmosphere with 5% CO_2. The number of viable cells was counted on days 0, 1, 3 and 7 using the trypan blue dye (Sigma-Aldrich Co. Ltd., St. Louis, MO, USA) exclusion method.

Flow cytometric analysis

The expression of specific cell surface antigens was analyzed by direct immunofluorescence flow cytometry (FC500, Beckman Coulter Inc., Fullerton, CA,

USA) using triple staining combinations of mAbs. In brief, cells were incubated with saturated concentrations of the relevant mAbs for 20 min at room temperature, washed and then subjected to flow cytometry. For each experiment, the isotype-matched control mAb was used as a negative control.

Measurement of the intracellular ROS generation

The fluorescent probe, CM-H_2DCFDA, and the mitochondrial superoxide indicator, MitoSOX Red, were used for the assessment of intracellular ROS and for mitochondrial superoxide generation, respectively. $CD34^+$ HSPCs were incubated for 15 min with 5 μM CM-H_2DCFDA in PBS (–) and for 10 min with 2.5 μM MitoSOX Red in Hanks' balanced salt solution (HBSS) (+) at 37°C in a humidified atmosphere with 5% CO_2. Unincorporated CM-H_2DCFDA or MitoSOX Red was removed by washing with PBS (–) or HBSS (+). Each sample was resuspended in PBS (–) or HBSS (+) and was analyzed by flow cytometry as described above.

Measurement of the intracellular mitochondrial fluorescence

The intercellular mitochondrial contents were measured by staining cells with a membrane potential-independent mitochondrial dye; MitoTracker Green FM[27-29]. $CD34^+$ HSPCs were incubated with 5 nM Mitotracker Green FM in PBS (–) for 15 min at 37°C in a humidified atmosphere containing 5% CO_2. Unincorporated MitoTracker Green FM was removed by washing the cells with PBS (–). Each sample was resuspended in PBS (–) and then analyzed by flow cytometry as described above.

Immunofluorescent detection of gamma-H2AX

Following treatment of the cells with hematopoietic cytokines and X-irradiation for the indicated periods, $CD34^+$ HSPCs (2×10^5 cells/sample) were harvested, washed with PBS (–) and fixed with ice-cold 75% ethanol for 10 min at room temperature. Fixed cells were washed with PBS (–), permeabilized in 0.5% Triton X-100 (Wako,

Osaka, Japan) on ice for 5 min, and washed twice with PBS (–). The cells were then incubated with an anti-phospho-histone H2AX monoclonal antibody diluted 1:300 with 20 mM Tris-HCl [pH 7.4], 137 mM NaCl, 0.1% Tween-20 (TBST) containing 5% skim milk at 37°C for 120 min, and were subsequently washed with PBS (–) and incubated with Alexa Fluor 488-conjugated anti-mouse IgG secondary antibody diluted 1:400-fold with TBST containing 5% skim milk at 37°C for 60 min. Following a second wash with PBS (–), the cells were adhered to microscope glass slides (Matsunami Glass Ind., Osaka, Japan) using a StatSpin® CytoFuge 2 (Iris Sample Processing, Inc., MA, USA), and mounted with Vectashield® Mounting Medium with DAPI (Vector Laboratories, Inc., CA, USA). For the quantitative analysis, the gamma-H2AX foci were counted per cell using a LSM 710 laser scanning microscope (Carl Zeiss Microscopy Co., Ltd., Tokyo, Japan) with a Z-stack function that scans by changing the depth for thick samples and storing the data as layers. Under blinded conditions, the gamma-H2AX foci per cell were counted for at least 50 cells in every sample.

Statistical analysis

The statistical analysis was performed using the Origin software package (OriginLab® Pro v8.1; Northampton, MA, USA) for the Windows operating system. Dose–survival curves were fitted using the Levenberg–Marquardt algorithm, and the values for D_0 (37% survival dose) and n (number of targets) were determined using a single-hit multi-target equation. Data were obtained from three independent experiments and were compared between control and experimental groups by the paired t-test. The data were analyzed by two-sided Student's t-tests and the Mann–Whitney U-test. $*P < 0.05$ and $**P < 0.01$ were considered to be statistically significant.

RESULTS

Characteristics and radiosensitivity of myeloid hematopoietic progenitors

To evaluate the radiosensitivity of myeloid hematopoietic progenitors, $CD34^+$ HSPCs exposed to 0.5–7 Gy were assayed for BFU-E, CFU-GM, CFU-Mix, and colony-forming cells (CFCs) using methylcellulose semisolid culture supplemented with a hematopoietic cytokine combination comprising recombinant human EPO, G-CSF, GM-CSF, IL-3 and SCF. The radiation survival curves for each type of progenitor cell are shown in Fig. 1. In addition, a single-hit multitarget equation was used to determine the values for D_0 and n, which are important parameters of these curves that characterize the radiosensitivity, and these are summarized in Table 1. The parameter D_0 is the dose that reduces the survival rate up to 37%, and n is the number of targets in the cell under the single-hit multi-target theory. In general, the values for D_0 and n range from 1 to 2 Gy and from 1 to 10, respectively. Progenitor-derived colony formation was found to decrease with dose. The D_0 values ranged from 0.95 to 1.19. No shoulders were observed on the dose–response curves, and the n values ranged from 1.01 to 1.20, suggesting that $CD34^+$ HSPCs are highly radiosensitive.

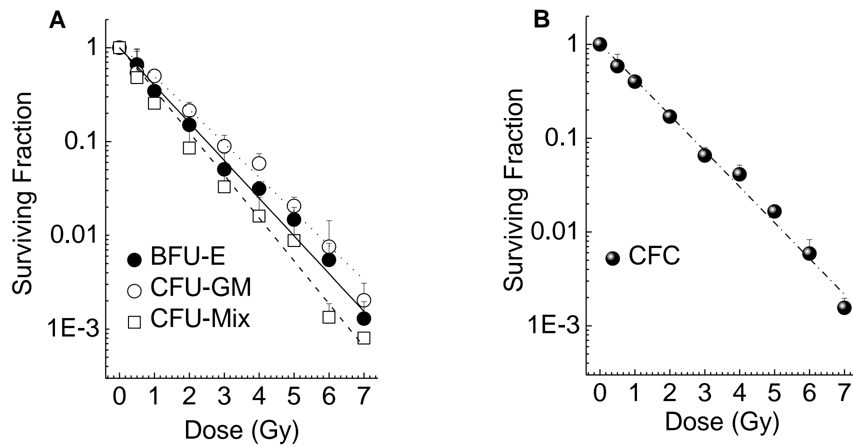

Fig. 1 The radiation dose–response curves of human umbilical myeloid hematopoietic progenitors. $CD34^+$ HSPCs were X-irradiated and assayed in methylcellulose cultures for 14 days. The values represent the means ± SD of three separate experiments performed in three wells. Curves were fitted as described in the Materials and Methods. Panel A and B: Radiation dose-response curves for each of the cell types and for the total hematopoietic myeloid progenitor cells were shown, respectively.

Table 1. The radiosensitivity of the hematopoietic myeloid progenitor cells

	D_0	n
CFU-GM	1.19 ± 0.11	1.20 ± 0.13
BFU-E	1.08 ± 0.08	1.01 ± 0.14
CFU-Mix	0.95 ± 0.08	1.03 ± 0.14
CFC	1.14 ± 0.10	1.03 ± 0.10

Note. The values for D_0 and n are the 37% survival dose and number of targets, respectively.

Characteristics and radiosensitivity of HSPCs

CD34$^+$ HSPCs were cultured in serum-free liquid medium as described in the Materials and Methods to investigate the effects of X-irradiation on the cell proliferation and differentiation. Non-irradiated CD34$^+$ HSPCs exhibited an increase in the total number of cells with the duration of culture, proliferating approximately 18-fold from the initial input to day 7 (Fig. 2). Although slight proliferation was observed in the CD34$^+$ HSPCs exposed to 2 or 4 Gy of X-rays, the treated cells displayed significant differences in proliferation compared with non-irradiated CD34$^+$ HSPCs on days 3 and 7. The total number of myeloid hematopoietic progenitors generated in both cultures was evaluated by calculating the total cell numbers and colony numbers in the cultures at each incubation time. The total number of CFCs generated in the non-irradiated CD34$^+$ HSPCs increased in the range from 1.5- to 9.3-fold with increasing culture time (Table 2). The CD34$^+$ HSPCs exposed to 2 Gy of X-rays exhibited a slight increase on days 3 and 7, showing 1.6- and 2.8-fold increases, respectively. No increase was observed in CD34$^+$ HSPCs exposed to 4 Gy.

In addition, the effects of radiation exposure on the composition ratios of hematopoietic progenitors generated in liquid culture are summarized in Table 3. With increasing culture time, the BFU-E ratio exhibited an increase similar to that of CFU-GM for each day and dose. In contrast, compared to the initial input, the ratio of CFU-Mix to CFU-GM in non-irradiated and 2 and 4 Gy irradiated cultures exhibited increases of 7.5-, 17- and 6-fold, respectively, on day 7. This suggests that X-irradiated CD34$^+$ HSPCs treated with hematopoietic cytokines had dramatically suppressed cell growth and clonogenic potential.

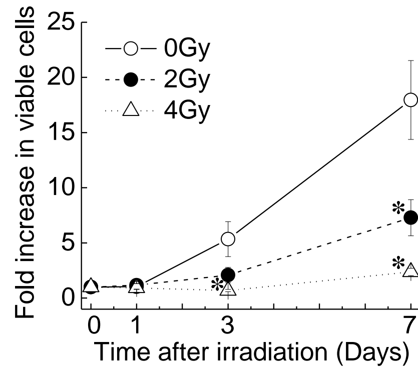

Fig. 2 The relationship between the incubation period and ratio of viable cells. CD34+ HSPCs exposed to 0, 2 or 4 Gy of X-rays were cultured in liquid medium supplemented with a combination of G-CSF, GM-CSF, IL3, SCF and EPO. Cells were harvested from the culture on days 1, 3 and 7. The viable cells were counted using trypan blue dye exclusion and the results are relative to the initial value. The values represent the means ± SD of three separate experiments. * $P < 0.05$ vs. non-irradiated controls (0 Gy).

Table 2. The total numbers of myeloid hematopoietic progenitors generated in liquid culture

Dose (Gy)		Day 0	Day 1	Day 3	Day 7
0 Gy	CFU-GM	778 (1.0)	1,043 (1.3)	4,173 (5.4)	4,565 (5.9)
	BFU-E	913 (1.0)	1,447 (1.6)	9,835 (10)	10,915 (12)
	CFU-Mix	15 (1.0)	72 (4.7)	418 (27)	362 (24)
	CFC	1,706 (1.0)	2,562 (1.5)	14,426 (8.5)	15,842 (9.3)
2 Gy	CFU-GM	-	381 (0.5)	671 (0.9)	892 (1.1)
	BFU-E	-	589 (0.7)	1,920 (2.1)	3,501 (3.8)
	CFU-Mix	-	28 (1.9)	138 (9.1)	404 (26)
	CFC	-	998 (0.6)	2,729 (1.6)	4,797 (2.8)
4 Gy	CFU-GM	-	97 (0.1)	78 (0.1)	81 (0.1)
	BFU-E	-	159 (0.2)	233 (0.3)	253 (0.3)
	CFU-Mix	-	6 (0.4)	13 (0.9)	28 (1.8)
	CFC	-	262 (0.2)	323 (0.2)	362 (0.2)

Note. The values in the parentheses indicate the rates of the number of each myeloid hematopoietic progenitor compared to Day 0.

Table 3. The composition ratios of myeloid hematopoietic progenitors compared to CFU-GM

Dose (Gy)		Day 0	Day 1	Day 3	Day 7
0 Gy	BFU-E	1.28 (1.0)	1.57 (1.2)	2.70 (2.1)	2.96 (2.3)
	CFU-Mix	0.02 (1.0)	0.09 (4.5)	0.10 (5.0)	0.15 (7.5)
2 Gy	BFU-E	-	1.77 (1.4)	3.61 (2.8)	3.98 (3.1)
	CFU-Mix	-	0.09 (4.5)	0.20 (10)	0.35 (17)
4 Gy	BFU-E	-	1.75 (1.4)	3.06 (2.4)	2.72 (2.1)
	CFU-Mix	-	0.06 (3.0)	0.17 (8.5)	0.12 (6.0)

Note. The values in the parentheses indicate the composition ratios compared to Day 0.

Detection of intracellular ROS and mitochondrial superoxide production

The expression levels of intracellular ROS and mitochondrial superoxide were analyzed by flow cytometry to investigate the effects of X-irradiation on the generation of various ROS (Fig. 3). The intracellular ROS generation in non-irradiated CD34$^+$ HSPCs reached a maximum level on day 1 and then gradually decreased with increasing culture time (Fig. 3A). CD34$^+$ HSPCs exposed to 4 Gy of X-rays displayed a significant increase in intracellular ROS compared with controls on days 1 and 3, with values peaking on day 3 (an approximately 4-fold increase compared to the initial value). Mitochondrial superoxide production did not differ by culture treatment or culture time from days 0 to 7 (Fig. 3B). On day 3, an approximately 7-fold increase compared to the initial input was observed in the culture exposed to 4 Gy. These findings show that the overproduction of intracellular ROS was induced following X-irradiation of CD34$^+$ HSPCs, but that this was not derived from the mitochondria.

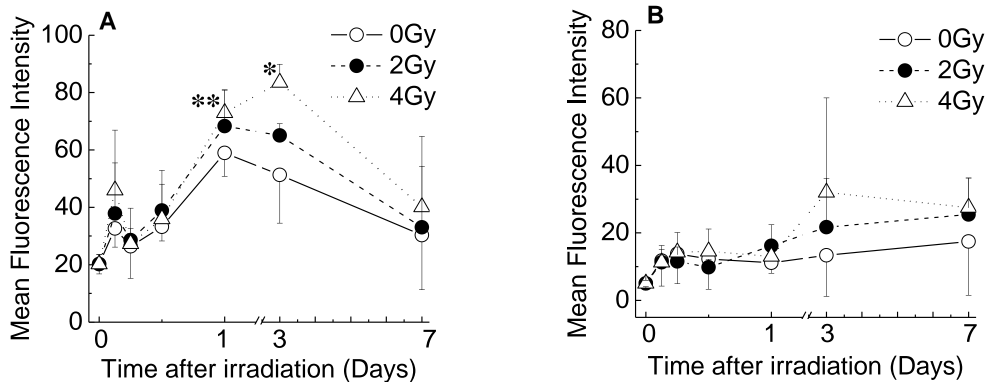

Fig. 3 The relationships among the incubation periods, intracellular ROS and mitochondrial superoxide detected in CD34$^+$ HSPCs. Cells were cultured for 3 h, 6 h, 12 h, 1 day, 3 days or 7 days. The intracellular ROS (panel A) and mitochondrial superoxide levels (panel B) were analyzed by flow cytometry. The values represent the means ± SD of three separate experiments. * $P < 0.05$ or ** $P < 0.01$ vs. non-irradiated controls (0 Gy).

Intracellular mitochondrial contents

The intracellular mitochondrial contents in the cells were measured by flow cytometry to investigate the effects of X-irradiation on nascent mitochondrial biogenesis (Fig. 4). The intracellular mitochondria levels in non-irradiated $CD34^+$ HSPCs peaked on day 3 (an approximately 2.5-fold increase compared to the initial input) then decreased slightly thereafter. The X-irradiated cultures exhibited similar levels of mitochondrial contents; no significant differences were observed at two exposure levels, suggesting that X-irradiation does not affect the mitochondrial contents in $CD34^+$ HSPCs.

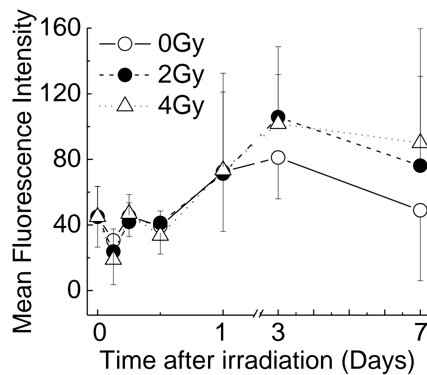

Fig. 4 The relationship between the incubation period and intracellular mitochondrial contents detected in $CD34^+$ HSPCs. Cells were cultured for 3 h, 6 h, 12 h, 1 day, 3 days or 7 days. After cultivation, the intracellular mitochondrial contents were analyzed by flow cytometry. The values represent the means ± SD of three separate experiments.

Gamma-H2AX expression

To monitor the repair of DNA double-strand breaks induced by ionizing radiation, the expression of gamma-H2AX, a marker of the DNA double-strand break response[30, 31)], was measured in cells harvested from liquid culture. Radiation-induced gamma-H2AX expression was observed immediately after exposure to 4 Gy of X-rays and gradually decreased over 72 hours (Fig. 5), suggesting that the DNA double-strand breaks induced immediately after irradiation were gradually repaired during the culture period, but that DNA damage remained.

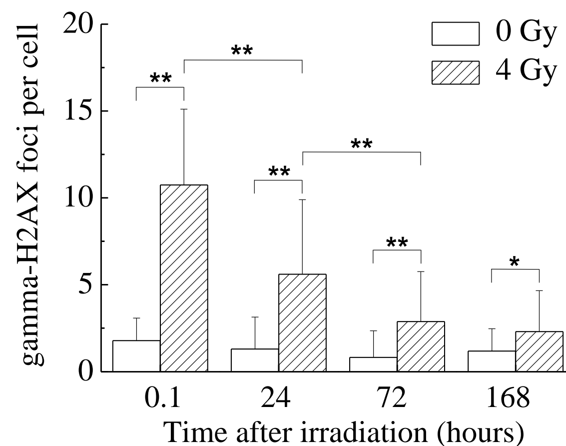

Fig. 5 The effects of X-irradiation on DNA double-strand breaks in CD34$^+$ HSPCs. CD34$^+$ HSPCs exposed to 0 or 4 Gy of X-rays were cultured in liquid medium supplemented with a combination of G-CSF, GM-CSF, IL3, SCF and EPO. Cells were harvested at 24, 72 and 168 hours, and the expression of gamma-H2AX was evaluated as described in the Materials and Methods. * $P < 0.05$ or ** $P < 0.01$ vs. non-irradiated controls (0 Gy) and vs. X-ray irradiated cells.

DISCUSSION

The present study investigated the involvement of intracellular ROS and mitochondria in the proliferation and differentiation of X-irradiated human CD34$^+$ HSPCs. The survival curve of progenitor cells demonstrated that HSPCs are sensitive to ionizing radiation (Fig. 1). Similarly, the generation of hematopoietic progenitor CFCs from primitive HSPCs decreased with the radiation dose in liquid culture (Table 2) suggesting that CD34$^+$ HSPCs are highly radiosensitive. However, no dose dependence of the composition ratios of hematopoietic progenitors was observed.

The hematopoietic system is generally regulated by several factors in the niche microenvironment, including hematopoietic cytokines and the physiological level of ROS. In the present study, the intracellular ROS production peaked in non-irradiated CD34$^+$ HSPCs on day 1 (Fig. 3A). Given that hematopoietic cytokines induce intracellular ROS production, the findings of the present study are consistent with those of previous studies [2, 11)]. The physiological level of ROS can lead to activation of the Janus kinase 2, signal transducers activator of transcription 5, Ras/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase, and phosphoinositide 3-kinase/Akt signaling pathways and to the transactivation of cytokine receptors and cell cycle progression, and can promote cell proliferation and differentiation[11, 32-35)]. However, the production of excessive ROS triggered by ionizing radiation or chemotherapeutic agents can indirectly damage cellular components and DNA[13-16, 36)].

In the present study, CD34$^+$ HSPCs exposed to 4 Gy of X-rays showed a significant increase in ROS production on days 1 and 3 in liquid culture (Fig. 3A). However, the cultures irradiated with 2 Gy were similar to non-irradiated CD34$^+$ HSPCs with respect to ROS generation (Fig. 3A). In addition, the mitochondrial superoxide levels were similar for all cultures (Fig. 3B). The mitochondrial contents gradually increased in all cultures until day 3, and no significant differences were observed (Fig. 4). However, the increases of the mitochondrial contents were not related to the cell growth (Fig. 1). In

addition, although a statistically significant increase of gamma-H2AX expression was observed in the irradiated cells, it gradually decreased with the time after irradiation (Fig. 5). We did not check whether DNA repair occurred promptly after irradiation or whether gamma-H2AX expression was observed only in the living cells. However, it is generally thought that DNA double-strand breaks that are induced immediately after irradiation are gradually repaired during the culture period[37)], while DNA damage remains. In any case, since the contents of hematopoietic progenitors in the cells generated in the liquid culture were decreased in a radiation dose-dependent manner (Table 2), our findings suggest that the DNA damage caused by ionizing irradiation leads to a critical loss of clonogenic potential. Further studies will be required to determine the precise mechanisms underlying these issues. The findings regarding the mitochondrial contents were consistent with the production of mitochondrial superoxide, and it is considered that the intracellular ROS induced by X-irradiation may not be derived from mitochondria.

The DNA double-strand break response in cultures was assessed by measuring the gamma-H2AX expression in $CD34^+$ HSPCs exposed to 4 Gy of X-rays in liquid culture (Fig. 5). It was not possible to detect gamma-H2AX expression in non-irradiated $CD34^+$ HSPCs, but the expression increased to detectable levels immediately after exposure to 4 Gy of X-rays and then decreased gradually over 72 hours (Fig. 5). Yahata et al. reported that the induced elevation of ROS within $CD34^+$ HSPCs, as determined using a glutathione synthesis inhibitor (buthionine sulfoximine), was associated with extensive DNA damage[14)]. Elevated DNA damage activates the expression of cell cycle inhibitors in HSPCs, leading to premature senescence and the eventual loss of stem cell function[14, 38)]. Wang et al. reported that total body irradiation in mice induces selective and persistent oxidative stress in hematopoietic stem cells, partly via the upregulation of the membrane-bound NADPH oxidase 4[39)]. In addition, Yamamori et al. reported that ionizing radiation induces mitochondrial ROS production in human lung carcinoma A549 cells, which is accompanied by elevated mitochondrial contents and upregulation

of the mitochondrial ETC function[40]. However, we observed no increase in the mitochondrial contents or ROS with the increase in cell numbers generated in liquid culture (Figs. 2, 5), suggesting that the DNA damage induced by the X-irradiation of $CD34^+$ HSPCs accounts for much of the cellular damage observed in the presence of hematopoietic cytokines. Moreover, uncontrolled overproduction of intracellular ROS in $CD34^+$ HSPCs leads to abnormal hematopoiesis and HSPC dysfunction[14, 38], suggesting that an increase in the physiological level of ROS by X-irradiation is involved in the suppression of HSPC proliferation and differentiation (Fig. 2, Table 2). We accordingly propose that the NADPH oxidase family is primarily responsible for the X-irradiation-induced persistent and prolonged intracellular ROS generation in human $CD34^+$ HSPCs, and that the resulting oxidative stress is associated with inhibition of the clonogenic potential of HSPCs. Our results indicate that damaging effects induced by mitochondrial ROS are limited.

The present study has demonstrated that X-irradiation-mediated damage to $CD34^+$ HSPCs, as measured by decreases in the clonogenic and proliferative potential, is induced to some extent by intracellular ROS generation. Substantial DNA damage may lead directly to a loss of HSPC function. Although physiological levels of intracellular ROS play an important role in endogenous growth signaling, cell survival, and the proliferation and differentiation of HSPCs, complete elucidation of the role of ROS in hematopoiesis awaits additional investigation of the DNA repair mechanisms that are active in HSPCs.

Chapter 2

The expression of mitochondrial DNA related genes in the Radiation Response of Human Hematopoietic Stem/Progenitor Cells

ABSTRACT

HSPCs are characterized by two essential functions, which are self-renewal ability and to differentiate into all functional mature hematopoietic cells throughout the lifetime of an organism. These quiescence and stemness of HSPCs are maintained by the regulation of mitochondrial biogenesis, reactive oxygen species, and energy homeostasis. In this study, the expression of mitochondrial DNA (mtDNA), its transcriptional genes and anti-oxidant enzyme genes, was analyzed by using the quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) examination during the proliferation and differentiation of X-irradiated $CD34^+$ cells that were highly purified from human placental/umbilical cord blood HSPCs. The qRT-PCR results revealed that hematopoietic cytokine treatment for 3 days significantly decreased the expression of mtDNA transcription related genes (*TFB1M*, *TFB2M*, *TEFM*, *POLG*, *OGG1*, *APEX1*, *APEX2*, *NUDT1*, *UNG*, *MUTYH*), antioxidant enzyme related genes (*SOD1*, *SOD2*), and *MT-ND1* compared with the day 0 control. X-irradiation, 4 Gy, significantly increased the expression of *PEO1* and significantly down-regulated the expression of the same genes except *APEX2* as 0 Gy at day 3 compared with the day 0 control. In particular, *PEO1*, *APEX2*, and *SOD2* exhibited a significantly higher expression in irradiated $CD34^+$ HSPCs compared with the non-irradiated control at day 3. These results suggest that X-irradiated human $CD34^+$ HSPCs with hematopoietic cytokines may potentially decrease oxidative stress in mitochondria.

INTRODUCTION

Mitochondria, the organelles that produce the energy molecule ATP, are also thought to act as mediators of cell metabolism during important processes in a cell's life. Mitochondria adjust their number and function in different cell types in response to different energy demands. These changes are effected by mitochondrial biogenesis, which must be accomplish through the coordinated expression of proteins encoded by nuclear DNA as well as by mitochondrial DNA (mtDNA)[41)]. Mitochondria contain extranuclear genomes that encode 13 proteins of the approximately 100 proteins constituting the mitochondrial ETC, which is located at the inner membrane of mitochondria. This series of proteins produces ATP by oxidative phosphorylation and also generates ROS as a result of regular bioenergetics metabolism[1-3)]. ROS levels must be tightly regulated in the cells as they play a key role as second messengers, but are detrimental when their levels are too high[4-6)].

HSPCs are characterized by two essential functions, which are self-renewal ability and to differentiate into all functional mature hematopoietic cells throughout the lifetime of an organism. Hematopoietic stem cells are usually present in a special microenvironment called the stem cell niche, which maintains the stemness by controlling the ROS generation[7-10)]. In fact, these cells are extremely sensitive to oxidative stressors, such as anti-cancer agents, radiation, and the extensive accumulation of ROS[13-19)], and oxidative stress can cause HSPC dysfunction and senescence[42)]. Therefore, the comparatively lesser mitochondrial content in murine and human hematopoietic stem cells might contribute to protect the cells from mitochondrial dysfunction and subsequent apoptosis and/or senescence driven by ROS overexpression[20-24)]. Recently, Mantel *et al*. have shown that $CD34^+$ hematopoietic stem cells with a low mitochondrial mass are enriched in hematopoietic repopulating stem cell function and that the upregulation of nascent mitochondrial biogenesis in $CD34^+$ HSPCs parallels the loss of pluripotency[25)]. Thus, the biogenesis and functions of

mitochondria are important in the homeostasis of cell physiology, but information about the expression of mtDNA and its related genes during proliferation and differentiation of non-irradiated or irradiated HSPCs is limited.

To determine the contributions of mtDNA and its related genes to the radiation response of human HSPCs, the present study investigated the expression of mitochondrial DNA, its transcriptional genes, and anti-oxidant enzyme genes in X-irradiated $CD34^+$ HSPCs prepared from human placental/umbilical cord blood and cultured with recombinant human hematopoietic cytokines. We herein describe the influence of radiation on the HSPC proliferation and differentiation from the perspective of mitochondrial function.

MATERIALS AND METHODS

Growth factors and fluorescent antibodies

Recombinant human IL-3 and human SCF were purchased from BioSource (Tokyo, Japan). Recombinant human EPO and G-CSF were purchased from Sankyo Co. Ltd. (Tokyo, Japan). Recombinant human GM-CSF was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). These growth factors were administered at the following concentrations: IL-3, 100 ng/ml; SCF, 100 ng/ml; EPO, 4 U/ml; G-CSF, 10 ng/ml and GM-CSF, 10 ng/ml medium. The following fluorescence-labeled monoclonal antibodies (mAbs) were purchased from Beckman Coulter Immunotech (Marseille, France): fluorescein isothiocyanate (FITC)-conjugated anti-human CD34 (CD34-FITC), phycoerythrin (PE)-conjugated anti-human CD34 (CD34-PE) and mouse IgG_1-FITC and IgG_1-PE, which were used as isotype controls.

Collection, purification and cryopreservation of $CD34^+$ HSPCs

This study was approved by the Committee of Medical Ethics of Hirosaki University Graduate School of Medicine (Hirosaki, Japan). Informed consent was obtained from mothers who had delivered full-term infants after written and verbal explanations were provided before delivery. Signatures were obtained from the mothers, as well as from a relative, such as the husband or the patient's mother/father etc. All documents related to the informed consent process were reviewed and approved. After delivery, placental/umbilical cord blood samples were collected into sterile collection bags (CBC-20; Nipro, Osaka, Japan) containing the anticoagulant, citrate phosphate dextrose, according to the guidelines of the Tokyo Cord Blood Bank (Tokyo, Japan). The samples were isolated and maintained separately prior to each experiment. Within 24 hours of cord blood collection, light-density mononuclear cells were separated by centrifugation in a Limphosepar I (1.077 g/ml; Immuno-Biological Laboratories, Takasaki, Japan) instrument for 30 min at $300\times g$ and were washed three times with calcium- and

magnesium-free phosphate-buffered saline (PBS (–); Sigma-Aldrich, Stockholm, Sweden) containing 5 mM ethylenediamine-N,N,N′,N′-tetraacetic acid (Wako, Tokyo, Japan). The cells were then processed for $CD34^+$ enrichment according to the manufacturer's instructions. An autoMACS™ Pro Separator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used for the sorting of $CD34^+$ cells. The CD34-enriched cell population is referred to as HSPCs in this study. The $CD34^+$ HSPCs were suspended in serum-free Cell Banker 3 reagent (Juji Field, Tokyo) and were frozen at −150°C prior to experimentation.

Flow cytometric analysis

The expression of specific cell surface antigens was analyzed by direct immunofluorescence flow cytometry (FC500, Beckman Coulter Inc., Fullerton, CA, USA) using triple staining combinations of mAbs. In brief, cells were incubated with saturated concentrations of the relevant mAbs for 20 min at room temperature, washed and then subjected to flow cytometry. For each experiment, the isotype-matched control mAb was used as a negative control.

Liquid culture

Frozen cells were thawed rapidly at 37°C, suspended in Cell Lotion (Juji Field, Tokyo, Japan) and centrifuged at $400\times g$ at 10°C for 10 min. The supernatant was removed and resuspended in serum-free Iscove's Modified Dulbecco's Medium (Gibco Invitrogen, Grand Island, NY, USA) supplemented with BIT 9500 serum substitute (StemCell Technologies, Vancouver, Canada) and low density lipoprotein (Calbiochem®, US and Canada). Nonirradiated or irradiated $CD34^+$ HSPCs were treated with the hematopoietic cytokine combination of five growth factors (IL-3, SCF, EPO, G-SCF, and GM-SCF) in γ-ray–sterilized assist tubes (Assist, Tokyo, Japan) at a concentration of 1×10^6 cells/0.5 ml/tube, and were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 3 days.

***In vitro* irradiation**

CD34^{+} HSPCs were exposed to X-rays in serum-free medium within 24 hours of thawing. Radiation (4 Gy, 120 kVp, 4.2 mA; 0.5 mm aluminum filters) was delivered using an X-ray generator (MBR-1505R; Hitachi Medical Co., Tokyo, Japan) with a distance of 30 cm between the focus and the target. The dosage was monitored using a thimble ionization chamber placed next to the sample during irradiation. The dose rate was approximately 1.19 Gy/min.

Total RNA extraction

Total RNAs were extracted by ISOGEN (Nippon Gene, Toyama, Japan) as recommended by the manufacturer. RNA purity was checked by A260/280 ratio using Hitachi Spectrophotometer U-1900 (Hitachi High Technologies Corporation, Tokyo, Japan) and electrophoresis analysis. DNA was extracted using the rest of the extracted RNA samples according to the manufacturer's instructions.

Quantitative real-time reverse transcriptional polymerase chain reaction (qRT-PCR)

All cDNAs were prepared by reverse transcription of 1 μg total RNA using oligo dT(20) primer (0.4 μM/ 50 μl final volume), and ReverTra Ace (TOYOBO, Osaka, Japan) as recommended by the manufacturer. An equivalent volume of 0.1 μl of cDNA solution was used for quantification of specific cDNA by qRT-PCR. qRT-PCR was performed by ABI Prism 7000 (Thermo Fisher Scientific, Waltham, MA, USA) using QuantiTect SYBR Green PCR Kit (QIAGEN, Valencia, CA, USA). After qRT-PCR, data were analyzed using ΔΔCt method[43)]. The sequences of primers were compared with the available human genome and the EST database (http://blast.genome.jp/) in order to select primers that would produce a single amplification product. The oligonucleotide primer sets used in this study were purchased from Hokkaido System Science (Hokkaido, Japan, Table 4). The following genes were targeted: transcription

factor B1, mitochondrial (*TFB1M*); transcription factor B2, mitochondrial (*TFB2M*); transcription elongation factor, mitochondrial (*TEFM*); twinkle protein, mitochondrial (*PEO1*); polymerase (DNA directed), gamma (*POLG*); polymerase (RNA) mitochondrial (DNA directed) (*POLRMT*); 8-oxoguanine DNA glycosylase (*OGG1*); APEX nuclease (multifunctional DNA repair enzyme) 1 (*APEX1*); APEX nuclease (apurinic/apyrimidinic endonuclease) 2 (*APEX2*); flap structure-specific endonuclease 1 (*FEN1*); nudix hydrolase 1 (*NUDT1*); uracil DNA glycosylase (*UNG*); mutY DNA glycosylase (*MUTYH*); transcription factor A, mitochondrial (*TFAM*); peroxisome proliferator-activated receptor gamma, coactivator-related 1 (*PPRC1*); superoxide dismutase 1, soluble (*SOD1*); superoxide dismutase 2, mitochondrial (*SOD2*), and mitochondrially encoded NADH dehydrogenase 1 (*MT-ND1*). All data were normalized to an internal standard (glyceraldehyde-3-phosphate dehydrogenase; *GAPDH*).

Statistical analysis

The statistical analysis was performed using the Origin software package (OriginLab® Pro v8.1; Northampton, MA, USA) for the Windows operating system. Data were obtained from three independent experiments and were compared between all groups by the scheffe's F-test. All P values less than 0.05 were considered to be statistically significant. Data are presented as the mean ± SD.

Table 4　Sequences of synthetic oligonucleotide PCR forward and reverse primers

Gene	primer sequence
TFB1M	F: 5'-CTCTCCATCTCACACTTTAAGAGCCTC-3'
	R: 5'-GTCTGTAATTCTCTGCGTCATCCTCTTCTT-3'
TFB2M	F: 5'-CCCAAAGCGTAGGGAATTATTAGACCAATT-3'
	R: 5'-TATATCTCTCGCATCAAGTGGAGTCAATGA-3'
TEFM	F: 5'-CTCGGGTGTTCTTCCCATCAGATAAAATAG-3'
	R: 5'-GGCTGAGAGTCAAACACTGCTAATTCATAG-3'
PEO1	F: 5'-AGGAGGAGGTTCAGCTGGCTGATACAAT-3'
	R: 5'-TCGGGGAATAGTGGTTTCCTCGTAGCT-3'
POLG	F: 5'-TTACTAATGCAGTTTAACCACCGGCTCACA-3'
	R: 5'-ACTTCCTTGCAGTTTCTCTCTGGACCTT-3'
POLRMT	F: 5'-CTCATCTCCCACATGGGCTCTGTG-3'
	R: 5'-TTGGGCTTTCGGCTGATGTCTCCGTT-3'
OGG1	F: 5'-CATCGAATGCCTTTTCTCTT-3'
	R: 5'-GTGACATCATCAAGCTGGATGAGC-3'
APEX1	F: 5'-AGTGCCCACTCAAAGTTTCTTACGGCATA-3'
	R: 5'-TTAGGTACATATGCTGTTACCAGCACAAAC-3'
APEX2	F: 5'-TCATGACCCCGAAGACTCCAGAAGAGAA-3'
	R: 5'-ACAGACTTCCAGAATGAGGTCCGTAAC-3'
FEN1	F: 5'-AGTGACTACTGTGAGAGTATCCGGGGT-3'
	R: 5'-ATTTTCTGGCACAGGGTACTTGTTGGG-3'
NUDT1	F: 5'-ATGGACGTGCATGTCTTCTGCACAGACA-3'
	R: 5'-ACCCTGGAACTTGAAGTACCCGTGGAAT-3'
UNG	F: 5'-CAGTTGTGTCCTGGCTAAATCAGAACTC-3'
	R: 5'-TGTAGTACATGGTGCCGCTTCCTATCA-3'
MUTYH	F: 5'-TGACATATCAAGTATATGGGCTGGCCTTG-3'
	R: 5'-AAACAGCTGCGGTGTGAAATTCCTCCTG-3'
TFAM	F: 5'-TTCAGCATGCTAAAGAGGACGAAACTCG-3'
	R: 5'-CGACGTAGAAGATCCTTTCGTCCAACTT-3'
PPRC1	F: 5'-CTGAAAATGTACTTCCCTTGTCGATGGCT-3'
	R: 5'-TTCAGCAGACACACATGCTAGAGCCTTC-3'
SOD1	F: 5'-CATTGCATCATTGGCCGCACACTG-3'
	R: 5'-ACCACAAGCCAAACGACTTCCAGC-3'
SOD2	F: 5'-TTCTGGACAAACCTCAGCCCTAACGGT-3'
	R: 5'-AACAGATGCAGCCGTCAGCTTCTCCTTAAA-3'
MT-ND1	F: 5'-ACCCCCGATTCCGCTACGACCAAC-3'
	R: 5'-GGTTTGAGGGGGAATGCTGGAGAT-3'
GAPDH	F: 5'-TCTAGACGGCAGGTCAGGTCCACC-3'
	R: 5'-GGGCAAGGTCATCCCTGAGCTGAA-3'

RESULTS

Mitochondrial DNA related gene expression of HSPCs after X-irradiation

Non-irradiated or irradiated CD34$^+$ HSPCs were cultured in serum-free liquid medium supplemented with a hematopoietic cytokine combination composing recombinant human IL-3, SCF, EPO, G-SCF, and GM-SCF for 3 days. The expression levels of mtDNA related genes were then analyzed by qRT-PCR assay at day 0 (control) and day 3 (0 Gy and 4 Gy), and all data were normalized to an internal standard *GAPDH*. As shown in Table 5, hematopoietic cytokine treatment for 3 days significantly decreased the expression of mtDNA transcription related *TFB1M*, *TFB2M*, *TEFM*, *POLG*, *OGG1*, *APEX1*, *APEX2*, *NUDT1*, *UNG*, *MUTYH*, antioxidant enzyme related *SOD1*, *SOD2* and *MT-ND1* compared with the day 0 control. In addition, 4 Gy X-ray significantly increased the expression of *PEO1* and significantly down-regulated the expression of the same genes except *APEX2* as 0 Gy at day 3 compared with the day 0 control. In particular, *PEO1*, *APEX2*, and *SOD2* exhibited a significantly higher expression of 3.3-, 1.5-, 2.1-fold increase, respectively, in irradiated CD34$^+$ HSPCs compared with the non-irradiated control at day 3. Other genes investigated did not change significantly after 3 days periods of exposure of the CD34$^+$ HSPCs to hematopoietic cytokine or X-irradiation treatment.

Table 5 Results of gene expression analysis by qRT-PCR

	Control	0 Gy	4 Gy
TFB1M	0.0313 ± 0.0050	0.0222 ± 0.0097 [a]	0.0223 ± 0.0040 [a]
TFB2M	0.0095 ± 0.0009	0.0041 ± 0.0009 [a]	0.0033 ± 0.0004 [a]
TEFM	0.0193 ± 0.0018	0.0132 ± 0.0035 [a]	0.0142 ± 0.0041 [a]
PEO1	0.0023 ± 0.0009	0.0015 ± 0.0003	0.0050 ± 0.0034 [b,c]
POLG	0.0100 ± 0.0010	0.0034 ± 0.0006 [a]	0.0034 ± 0.0001 [a]
POLRMT	0.0253 ± 0.0030	0.0213 ± 0.0158	0.0299 ± 0.0159
OGG1	0.0012 ± 0.0001	0.0003 ± 0.00007 [a]	0.0004 ± 0.00009 [a]
APEX1	0.0251 ± 0.0106	0.0159 ± 0.0017 [a]	0.0108 ± 0.0029 [a]
APEX2	0.0079 ± 0.0011	0.0053 ± 0.0020 [a]	0.0080 ± 0.0023 [c]
FEN1	0.0126 ± 0.0010	0.0136 ± 0.0044	0.0153 ± 0.0040
NUDT1	0.0153 ± 0.0018	0.0088 ± 0.0015 [a]	0.0091 ± 0.0012 [a]
UNG	0.0037 ± 0.0010	0.0021 ± 0.0002 [a]	0.0020 ± 0.0003 [a]
MUTYH	0.0101 ± 0.0006	0.0051 ± 0.0029 [a]	0.0068 ± 0.0025 [a]
TFAM	0.0038 ± 0.0011	0.0026 ± 0.0007	0.0028 ± 0.0009
PPRC1	0.0078 ± 0.0031	0.0076 ± 0.0073	0.0090 ± 0.0058
SOD1	0.3692 ± 0.0990	0.1083 ± 0.0206 [a]	0.0858 ± 0.0214 [a]
SOD2	0.0146 ± 0.0020	0.0018 ± 0.0003 [a]	0.0038 ± 0.0010 [a,c]
MT-ND1	31.754 ± 6.0842	9.9437 ± 3.2924 [a]	13.067 ± 2.8676 [a]

[a] Decreased significantly vs. Day 0 control ($P < 0.05$).
[b] Increased significantly vs. Day 0 control ($P < 0.05$).
[c] Increased significantly vs. 0 Gy ($P < 0.05$).

DISCUSSION

In the present study, the response of mtDNA, its transcriptional related genes, and anti-oxidant enzyme related genes expression detected in highly purified human CD34$^+$ HSPCs exposed to 4 Gy X-irradiation were analyzed. The CD34$^+$ cells were treated with a hematopoietic cytokine combination composing recombinant human IL-3, SCF, EPO, G-SCF, and GM-SCF for 3 days. Non-irradiated cells without any treatment at day 0 were used as a control in this analysis. In a comparison of the gene expression obtained from cells irradiated or non-irradiated conditions, *APEX2*, *PEO1*, and *SOD2* exhibited a significantly higher expression in 4 Gy X-irradiated CD34$^+$ HSPCs compared with the non-irradiated control at day 3, and *MT-ND1* did not change significantly as shown in Table 5.

In general, more than 90% of the oxygen consumed by mammalian cells is utilized in mitochondria, and up to 4% of this oxygen is transformed into ROS[44]. It has been reported that superoxide is generated from complex I, complex III and also from complex II in ETC[45-47], and mitochondria are clearly a major source of intracellular ROS. It is not surprising that mitochondrial dysfunction, caused by mtDNA mutations, an abnormal mtDNA copy number, or defects in mtDNA expression and maintenance, is associated with numerous mitochondrial diseases, age-associated diseases and the ageing process in humans[48,49]. Therefore, it is equipped with base excision repair system that works against oxidative DNA damage in the mitochondria[50]. Damage base is removed by MUTYH and OGG1, abasic site-specific endonuclease and repair synthesis by DNA polymerases and ligases are performed as shown in Table 5, and then base excision repair reaction is completed. Thus, *APEX2* participates in mitochondrial base excision repair and these processes may promote in 4 Gy X-irradiated CD34$^+$ HSPCs [51]. Furthermore, mtDNA resides in the mitochondrial lumen, and its duplication requires the mtDNA replicative helicase, *PEO1*. In addition to *PEO1*, many DNA helicases, which are encoded by the nuclear genome and are crucial for nuclear genome

integrity, are transported into the mitochondrion to also function in mtDNA replication and repair[52,53]. Ikeda *et al*. have described the role of *PEO1*. Overexpression of *TFAM* or *PEO1* increases mtDNA copy number and facilitates cardioprotection associated with limited mitochondrial oxidative stress[54]. Moreover, the human MSH5 (MutS Homolog 5) protein localizes to mitochondria and protects the mitochondrial genome from oxidative damage. This protein binds to mtDNA and interacts with the Twinkle helicase and the DNA polymerase, and hMSH5 stimulates mtDNA repair in response to DNA damage induced by oxidative stress[55]. It is, therefore, suggested that mtDNA repair response may be promoted to some extent.

Production of ROS from the ETC results in oxidative stress in cells, and causes apoptotic cell death[56,57]. MnSOD is an essential enzyme, which scavenges superoxide in mitochondria[58]. The biological importance of MnSOD has been clearly demonstrated: expression of MnSOD is essential for the survival of aerobic life and the development of cellular resistance to oxygen radical-mediated toxicity[59]. Revious studies reported that mitochondrial localization of superoxide dismutase is required for decreasing radiation-induced cellular damage[59] and that overexpression of the MnSOD transgene product protects cryopreserved bone marrow hematopoietic progenitor cells from ionizing radiation[60]. Floratou et al. recently reported the effect of a range of radiation doses in HPCs and the possible protective mechanisms activated by insulin-like growth factor-1 (IGF-1)[61]. In their study, IGF-1 protects CD34$^+$ HPCs from radiation effects, by eliminating the oxidative microenvironment through the enhancement of MnSOD activation and by regulating the mitochondria-mediated pathway of apoptosis. Furthermore, our previous study has shown that X-irradiation can increase persistent intracellular ROS in human CD34$^+$ HSPCs stimulated with same hematopoietic cytokines, which may not result from mitochondrial ROS due to mitochondrial dysfunction or biogenesis[62]. Our present results *SOD2* exhibited a significantly higher expression in 4 Gy X-irradiated CD34$^+$ HSPCs but *MT-ND1* did not change significantly. Thus, it is consistent with previous study, and suggested that

hematopoietic cytokines may eliminate the oxidative stress through the enhancement of MnSOD activation.

The present study has demonstrated that up-regulation of *APEX2*, *PEO1*, and *SOD2* plays a key role in the radioresponse of $CD34^+$ HSPCs and that these genes have important functions in the suppressing the oxidative stress and damage from mitochondria of human hematopoietic cells. However, this study was performed under restricted conditions, such as an irradiation with 4 Gy and a 3 days' time-limit, because the number of $CD34^+$ cells obtained from one cord blood sample was very small. It will be necessary to perform more precise experimental approaches to confirm and build on our results.

CONCLUSIONS

All the results from Chapter 1 and Chapter 2 indicate as follows:

1. X-irradiation-mediated damage to $CD34^+$ HSPCs, as measured by decreases in the clonogenic and proliferative potential, is induced to some extent by intracellular ROS generation, which may not result from mitochondrial ROS due to mitochondrial dysfunction. In addition, Substantial DNA damage may lead directly to a loss of HSPC function.
2. The up-regulated genes resulting from irradiation might be involved in the suppressing the oxidative stress and damage derived from mitochondria of the $CD34^+$ HSPCs stimulated with combinations of human recombinant hematopoietic cytokines.

Taken together, all the results from Chapter 1 and Chapter 2 above-mentioned may contribute as useful information when knowing the influence of radiation on the HSPC proliferation and differentiation from the perspective of mitochondrial function. In addition, I expect the application for the field such as not only radiation therapy but also HSPC radiation-protection.

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和文要旨

ヒト造血幹/前駆細胞の放射線応答における

ミトコンドリア機能及び遺伝子の関与

山口　平

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造血組織内のニッチェと呼ばれる特殊な微小環境に存在している造血幹細胞は，自己複製能及び多分化能といった幹細胞特有の機能を有しており，放射線などの酸化ストレスに対して非常に高い感受性を示す．その幹細胞特性は細胞内ミトコンドリアの生合成，ミトコンドリア由来の活性酸素種を制御することで維持されている．造血幹細胞の幹細胞特性の維持や分化の進行におけるミトコンドリアの重要性は近年明らかにされつつあるが，その造血幹細胞の分化/増殖過程に及ぼす電離放射線の影響と細胞内ミトコンドリアの関連性についての多くは不明のままである．本研究では，X 線照射ヒト胎盤/臍帯血由来 CD34 陽性造血幹/前駆細胞の分化･増殖過程における放射線応答と, 細胞内活性酸素種、ミトコンドリア機能及びその関連遺伝子の発現について評価した．

先ず，放射線ばく露ヒト造血幹/前駆細胞の分化・増殖における ROS の役割について検討した(第一章)．造血幹/前駆細胞の分化・増殖を誘導するサイトカイン存在下では，X 線照射造血幹/前駆細胞は増殖能とクローン形成能を劇的に減少させた．その時の細胞内活性酸素種は，非照射造血幹細胞内と比較して培養時間経過とともに有意に増加した一方，細胞内ミトコンドリアやミトコンドリア由来の活性酸素種は非照射造血幹細胞内と比較して有意な増加は示さなかった．DNA 損傷は，X 線照射直後に最も多く確認され培養時間経過とともに減少したが損傷の残存が認められた．次に，ミトコンドリア DNA 及びその転写関連遺伝子，抗酸化酵素遺伝子の発現と造血幹/前駆細胞の放射線応答との関連を検

討した（第二章）．ミトコンドリア電子伝達系全体の律速段階となっている複合体 1 のタンパク質をコードする遺伝子の発現は，照射細胞と非照射細胞間で有意な差は認められず同等のレベルを示した一方，ミトコンドリア DNA のゲノム安定性や抗酸化ストレスに関わる遺伝子の発現が上昇しており，放射線によるミトコンドリアの機能異常を抑制，緩和している可能性が示唆された．

本研究の結果，放射線ばく露ヒト胎盤/臍帯血由来 CD34 陽性造血幹/前駆細胞の分化/増殖過程において，ミトコンドリア生合成及びミトコンドリア由来活性酸素種の異常生成は抑制されており，放射線による細胞の DNA 損傷及び持続的な細胞内活性酸素種の異常産生が幹細胞機能破綻を引き起こしている可能性が示唆された．

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