「生体幹細胞の分化・増殖に対する放射線の影響」

弘前大学大学院保健学研究科保健学専攻

提出者氏名: 林 直 樹

所 属: 放射線技術科学領域

指導教員: 柏 倉 幾 郎

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略語一覧

2-ME: 2-mercaptoethanol

7-AAD: 7-Aminoactinomycin D

α-MEM: α-modified Eagle's medium

BFU-E: burst forming unit-erythroid

BM: bone marrow

BSA: bovine serum albumin

CB: cord blood

CD: cluster of differentiation

cDNA: complementary DNA

CFC: colony-forming cells

CFU-GM: colony-forming unit-gramulocyte-macrophage

CFU-Meg: colony-forming unit-megakaryocyte

CFU-Mix: colony-forming unit-granulocyte-erythroid-macrophage-megakaryocyte

DMEM: Dulbecco's Modified Eagle's Medium

EDTA: ethylenediaminetetraacetic acid

EB: embryoid body

ES: embryonic stem

ES-FBS: ES cell-qualified fetal bovine serum

FBS: fatal bovine serum

FGF: fibroblast growth factor

FITC: fluorescein isothiocyanate

G-CSF: granulocyte colony-stimulating factor

GFP: green fluorescent protein

GM-CSF: granurocyte-macrophage colony-stimulating factor

GVHD: graft versus host disease

HLA: human leukocyte antigen

HSC: hematopoietic stem cell

HSPC: hematopoietic stem/progenitor cells

IFN-γ: interferon-γ

IL: interleukin

IMDM: Isocave's modified Dulbecco's medium

IP: interferonγ-inducible protein

iPS: induced pluripotent stem

LET: linear energy transfer

LIF: leukemia inhibitory factor

mAbs: monocleonal antibodies

MCP: monocyte chemoattractant protein

MEFs: mouse embryo fibroblasts

MEM: Modified Eagle's Medium

MIP: macrophage inflammatory protein

mRNA: messenger ribo nucleic acid

NEAA: nonessential amino acids

PBS: phosphate-buffered saline

PBS-B: PBS containing 0.5% BSA

PC5: phycoerythrin-cyanin-5-forochrome tandem

PDGF: platelet-derived growth factor

PE: phycoerythrin

PI: propidium iodine

RANTES: regulated on activation, normal T cell expressed and secreted

RNA: ribo nucleic acid

RT-PCR: reverse transcription polymerase chain reaction

SCF: stem cell factor

TNF: tumor necrosis factor

TPO: thrombopoietin

VEGF: vascular endothelial growth factor

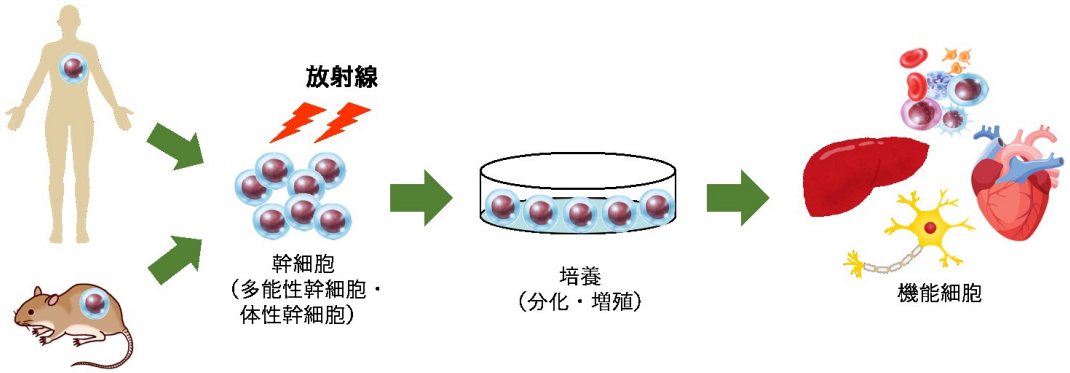
序　論

生体幹細胞は自己複製能と多分化能を有し，必要に応じて様々な機能細胞を生み出す．この幹細胞は胚性幹細胞や人工多能性幹（induced pluripotent stem; iPS）細胞のような多能性幹細胞と，造血幹細胞や間葉系幹細胞のような体性幹細胞に大別される．幹細胞が多量の放射線に曝露されると，分化・増殖能を失い個体の発生や機能阻害，場合によっては個体の死につながる．

多能性幹細胞は再生医療の材料として多いに期待され，例えば心筋細胞，神経細胞及び造血細胞など，様々な細胞に分化誘導されている．一般的に，未熟な細胞程放射線感受性が高いとされているが，その例外も報告されている．例えば，造血幹細胞の中でも，比較的成熟な細胞である末梢血由来造血幹細胞はより未熟である臍帯血由来造血幹細胞より放射線感受性が高いことが報告されている．そのような中，より未熟な細胞である多能性幹細胞への放射線の影響については不明な点が多い．

体性幹細胞である造血幹細胞は全ての血液細胞を産生する多分化能と，未分化性を維持したまま増殖する自己複製能を有しており，一方では放射線感受性の高い組織として広く知られている．そのため，放射線事故のように全身に多量の放射線を曝露されると造血幹細胞が分化・増殖能を失い，骨髄抑制を起こすことで個体を死に至らしめる可能性がある．その際，造血細胞の増殖を促すために G-CSF 等，生理活性因子であるサイトカイン投与による造血回復が試みられる．しかしながら，サイトカイン刺激による細胞増殖では，細胞の膨大な増殖は得られるが，幹細胞の未分化維持が困難である．長期的な造血組織の回復を想定するならば，造血幹細胞の未分化維持が必要不可欠である．造血幹細胞は生体内において，間葉系幹細胞や血管内皮細胞等により形成されるニッチと呼ばれる微小環境中に存在し，細胞間接触及び低濃度サイトカイン刺激によって絶妙にコントロールされ，未分化状態を維持しているとされる．その一方で，定常状態では個体や組織中に含まれる幹細胞は極めて少数であり，その為幹細胞の分化･増殖に対する放射線の影響についての詳細は不十分な点が多い．

本研究では多能性幹細胞としてマウス iPS 細胞，体性幹細胞としてヒト臍帯血から分離精製した造血幹細胞の分化・増殖に対する放射線の影響を検討すると共に，ヒト臍帯血より培養誘導した間葉系幹細胞様支持細胞による放射線曝露造血幹細胞の造血回復や未分化維持に対する効果について検討した．



本研究に際し，マウスを用いた実験は弘前大学動物実験委員会より承認を得ている．また，臍帯血を用いた研究においては，弘前大学大学院医学研究科倫理委員会より承認を得（整理番号：2008-155），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一章から第三章で構成される．

《第一章》「マウス多能性幹細胞の増殖及び分化に及ぼす電離放射線の影響」

多能性幹細胞であるマウス iPS 細胞に 1-7.5 Gy の X 線を曝露させた．その結果，マウス iPS 細胞の D_0 及び n 値は 1.85 ± 0.18 及び 1.00 ± 0.13 であった．一方，iPS 細胞と比較して成熟な細胞であるマウス $CD34^+$細胞の D_0 及び n 値は 1.11 ±0.55 及び 1.00 ± 0.13 であった．また，iPS 細胞からの初期分化形態である胚様体形成を検討したところ，放射線曝露の有無によって胚様体の形成に影響を示さなかったが，そのサイズは線量依存的に低下した．また，形成された胚様体

の分化は，放射線非曝露 iPS 細胞由来では内胚葉系マーカーである *Afp* の発現が劇的に up regulate したが，放射線曝露 iPS 細胞由来では *Afp* の発現が線量依存的に down regulate した．以上の結果より，マウス iPS 細胞は，造血幹/前駆細胞より放射線感受性が低いことが示された．また，分化能に対する放射線の影響は一様ではなく，内胚葉系への分化が抑制される可能性が示唆された．

《第二章》「臍帯血由来間葉系幹細胞様ストローマ細胞は放射線曝露ヒト $CD34^+$ 細胞の造血回復を支持する」

未熟な造血幹細胞の増殖及び未分化維持能を支持することが知られている臍帯血由来間葉系幹細胞様ストローマ細胞を樹立し，インターロイキン－3（IL-3），幹細胞因子（SCF）及びスロンボポエチン（TPO）のサイトカインコンビネーション存在下で放射線非曝露及び 2 Gy 曝露造血幹細胞と共培養した結果，骨髄球系前駆細胞数はサイトカインのみでの培養と比較してそれぞれ約 3.5 倍及び 2.5 倍の有意な増加を示した．加えて，未熟な造血幹/前駆細胞である $CD34^+$ 及び $CD34^+/CD38^-$ 細胞数も，共培養により放射線曝露細胞は約 6.9 倍及び 6.5 倍の有意な増加を示した．さらに，放射線曝露細胞をサイトカイン刺激前に 16 時間ストローマ細胞と共培養したところ，サイトカイン刺激を同時に行った場合と同等の細胞増殖を示した．また，培養液中のサイトカインを測定した結果，ストローマ細胞は IL-6，IL-9，G-CSF 及び GM-CSF のような，骨髄球系造血細胞の増殖に関係したサイトカインを産生した．以上の結果より，放射線曝露造血幹/前駆細胞からの造血再生に対する間葉系幹細胞様ストローマ細胞の造血支持効果があきらかになり，細胞間接触が重要な役割を果たしている可能性が示唆された．

《第三章》「凍結ヒト造血幹/前駆細胞の体外増殖における電離放射線の影響」

造血幹/前駆細胞は臨床使用及び実験使用のために凍結し，各機関によって保存されている．そこで，放射線照射や臍帯血由来間葉系幹細胞様ストローマ細胞との共培養に対する造血幹/前駆細胞の応答における凍結の影響について検討

した．凍結解凍処理後，2 Gy 曝露造血幹/前駆細胞を IL-3，SCF 及び TPO のサイトカインコンビネーションとストローマ非存在下もしくは存在下で培養した．凍結の前後で細胞の表面抗原の発現を測定した結果，未分化な細胞である $CD34^+/CD38^-$細胞の割合は凍結前の 3.4±1.1%と比較して，凍結後では 5.9±1.3%と有意に増加した．一方で，造血前駆細胞である $CD34^+/CD45RA^+$及び $CD34^+/CD117^+$細胞は凍結前の 16.0±5.2%及び 14.4±9.2%と比較して，凍結後では 9.3±3.3%及び 6.9±5.1%と有意に減少した．放射線曝露された凍結細胞の培養において，放射線非曝露細胞の増殖率に対する $CD34^+$，$CD34^+/CD38^-$及び造血前駆細胞のそれぞれの増殖率は 12.6%，11.8%及び 14.9%であり，放射線曝露された新鮮な細胞のそれぞれの増殖率 9.9%，9.3%及び 14.6%より高かった．驚くことに，ストローマ細胞との共培養における造血支持効果が凍結後の放射線曝露造血幹/前駆細胞において確認できなかった．以上の結果より，凍結処理はストローマ細胞の造血支持効果を低下させるが，未熟及び放射線抵抗性造血幹/前駆細胞の割合を増すことが示された．このように，凍結処理は造血幹/前駆細胞の性質を変化することが示唆された．

本研究から，多能性幹細胞のように非常に未分化な幹細胞では放射線感受性が低く，放射線に対する防御機構が備わっている可能性が示唆された．また，分化能に対する放射線の影響は一様ではなく，内胚葉系組織への分化誘導は抑制されるが，その他の細胞への誘導には影響しないことが示唆された．一方，体性幹細胞である造血幹細胞は放射線曝露した場合も，間葉系幹細胞様ストローマ細胞が構築する造血微小環境での cell to cell 相互作用が大きく影響し，造血幹細胞の放射線損傷が緩和され，その後の増殖能及び未分化維持能力を所有することが示唆された．一方，細胞バンク等で行われる凍結保存により，放射線曝露造血幹細胞の分化・増殖能維持に対する間葉系幹細胞等の作用が低下することが示唆された．本研究の結果から，生体幹細胞の分化・増殖に対する放射線の影響は，未熟や分化の程度に大きく依存し，その修復や再生にはサイトカインを含めた多様な刺激因子を必要とすることが明らかとなった．今後はこ

うした細胞由来の成熟細胞の機能や将来の発がん等のリスク検証も必要であるが，放射線曝露された生体幹細胞の分化誘導及び機能評価の詳細な検証で，再生医学への利用の可能性が期待できる．

Chapter 1

Effects of Ionizing Radiation on Proliferation and Differentiation of Mouse Induced Pluripotent Stem Cells

Abstract

The present study aimed to estimate the clonogenic and differentiation potential of induced pluripotent stem (iPS) cells exposed to ionizing radiation. Compared with mouse hematopoietic stem/progenitor cells, iPS cells were less sensitive to radiation. To examine the effect of ionizing radiation on the early differentiation pathway of iPS cells, we assessed embryoid body (EB) formation. Although EB formation was observed at all radiation doses, EB diameter decreased in a radiation dose-dependent manner. At the same time, we analyzed the expression of genes specific to differentiation in the initial iPS cells and cells of EB. The expression of the endoderm marker *Afp* increased remarkably in cells of EB derived from non-irradiated iPS cells; however, in irradiated cells, this expression significantly decreased in a radiation dose-dependent manner. Further, the expressions of the pluripotent stem cell markers *Nanog* and *Oct-4* and the early mesoderm marker *Brachyury* significantly decreased. The results of the present study suggest that radiosensitivity with regard to gene expression differs at various stages in the early differentiation pathways of iPS cells that lead to the formation of the 3 germ layers; the sensitivity is the highest in the genes expressed during the differentiation pathways of iPS cells, leading to the formation of the endoderm.

1. **Introduction**

Induced pluripotent stem (iPS) cells were first generated from mouse adult fibroblasts through reprogramming by transduction of 4 defined transcription factors (Oct3/4, Sox2, Klf4, and c-Myc)[1)]; thereafter, human iPS cells were generated from human adult fibroblasts using the same transcription factors[2)]. These cells show pluripotency by expressing embryonic stem (ES) cell-marker genes, such as *Oct3/4* and *Nanog*, by expressing ES cell-specific surface antigens, such as CD15; and by showing teratoma formation after subcutaneous injection into immunodeficient mice[1,3)]. Because iPS cells can differentiate into various types of functional cells such as hematopoietic cells[4-9)], neuronal cells[10,11)], and cardiovascular cells[12,13)], many researchers are investigating the application of iPS cells in regenerative medicine.

Generally, immature cells are thought to be more radiosensitive than mature cells; however, it spears that rare stem cells are more resistant than their daughter cells, the progenitors[14,15)]. In our previous studies on radiosensitivity of human hematopoietic stem/progenitor cells (HSPCs), we showed that HSPCs in human peripheral blood, which are relatively mature compared to those in bone marrow and placental/umbilical cord blood, are more radiosensitive than HSPCs in cord blood[16,17)]. However, little is known about the influence of ionizing radiation on differentiation and proliferation of stem cells, especially iPS cells. Therefore, investigating whether iPS cells are sensitive to ionizing radiation and the mechanism will add vital information to the field of regenerative medicine and embryology. In the present study, the clonogenic and differentiation potential of induced pluripotent stem (iPS) cells exposed to ionizing radiation were analyzed for determing the effect of ionizing radiation on proliferation and differentiation of mouse induced pluripotent stem cells.

2. Materials and Methods

Reagents and growth factors

Recombinant human interleukin-3 (IL-3) and stem cell factor (SCF) were purchased from BioSource (Tokyo, Japan). Recombinant human erythropoietin (EPO) was purchased from Sankyo Co. Ltd. (Tokyo, Japan). Recombinant human granulocyte colony-stimulating factor (G-CSF) was purchased from Kirin Brewery Co. Ltd. (Tokyo, Japan). Recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) was purchased from Pepro Tech Inc. (NJ, USA). These factors were administered at the following concentrations: EPO, 4 U/mL; G-CSF and GM-CSF, 10 ng/mL; and SCF and IL-3, 100 ng/mL each.

Mouse iPS cell culture

The germline-competent mouse iPS cell line 20D17 carrying the Nanog promoter-driven green fluorescent protein (GFP)/internal ribosome entry site/puromycin-resistant gene was generated[3]. The iPS cells used in this study were obtained from RIKEN BioResource Center (Tsukuba, Japan). These cells were maintained in an undifferentiated state on 50 Gy-irradiated primary mouse embryo fibroblasts (MEFs; Oriental Yeast Co. Ltd., Tokyo, Japan) in knockout Dulbecco's modified Eagle's medium (DMEM, Nakarai Tesque, Kyoto, Japan) supplemented with 15% ES cell-qualified fetal bovine serum (ES-FBS; Invitrogen USDA, Tokyo, Japan), 0.1 mM nonessential amino acids (NEAA; Sigma, St. Louis, USA), 0.1 mM 2-mercaptoethanol (2-ME; Sigma), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco, NY, USA), and 1000 U/mL leukemia inhibitory factor (LIF; Invitrogen, Tokyo, Japan), which have been reported to play an important role in maintaining the iPS cell line an undifferentiated state at 37°C in a humidified atmosphere containing 95% air/5% CO_2[18]. All of the cells were passaged every 2 days by using 0.25%

trypsin-ethylenediaminetetraacetic acid. The culture medium used for iPS cells was changed every day.

Colony assay for mouse iPS cells

Colony-forming cells were assayed by culturing the cells with methylcellulose by using MethoCult® (StemCell Tech- nologies, Vancouver, Canada). The non-irradiated and 1, 2, 4, or 7.5 Gy-irradiated iPS cells were plated onto separate wells of a 24-well plate (0.3 mL/well) in culture medium containing 10% ES-FBS. Each plate was incubated at 37°C in a humidified atmosphere containing 95% air/5% CO_2 for 6 days. Colonies expressing GFP were counted under an inverted microscope (Olympus, Tokyo, Japan).

Embryoid body formation of iPS cells

The culture for embryoid body (EB) formation was performed in a 1.5-mL round-bottomed conical polypropylene tube with a screw cap (Assist, Tokyo, Japan), as previously reported[19]. Briefly, the non-irradiated and 1, 2, 4, or 7.5 Gy-irradiated iPS cells were suspended at 2×10^4 cells/mL in α-modified Eagle's medium (α-MEM) (Gibco) supplemented with 10% ES-FBS, 0.1 mM NEAA, 0.1 mM 2-ME, 100 U/mL penicillin, and 100 μg/mL streptomycin, without LIF. One milliliter of each iPS cell suspension was placed in a conical tube, and the screw cap was placed loosely to allow oxygen supply. Ten conical tubes were used in each experiment. After 5 days of culture, EBs were transferred to a dish of diameter 60 mm (BD Falcon, NJ, USA), and the diameter of EB was measured by DP2-BSW. These EBs were then transferred to conical tubes. After 7 days of culture, each EB was harvested. Total RNA in each EB was extracted using the RNeasy® micro kit (QIAGEN, Valencia, CA, USA), and quantified using a NANODROP 1000 spectrophotometer (Thermo, Yokohama, Japan) according to the manufacturer's instructions.

Colony assay for mouse HSPCs

Colony-forming cells were assayed by culturing them with methylcellulose by using MethoCult®. Mouse HSPCs were plated onto separate wells of a 24-well plate (0.3 mL/well) in culture medium containing EPO, SCF, IL-3, G-CSF, and GM-CSF as colony-stimulating factors. Each plate was incubated at 37°C in a humidified atmosphere containing 95% air/5% CO_2 for 7 days. Colonies containing more than 50 cells were counted under an inverted microscope (Olympus).

Isolation of HSPCs from mouse bone marrow cells

BALB/c mice aged 7–8 weeks were used as the source of mouse bone marrow cells. These mice were maintained in a specific pathogen-free facility at Hirosaki University. The experiment was approved by the Animal Research Committee of Hirosaki University and performed in accordance with the Guidelines for Animal Experimentation, Hirosaki University (Hirosaki, Japan). Bone marrow cells were flushed out from the femur by using phosphate-buffered saline supplemented with 0.5% bovine serum albumin (Boehringer Mannheim GmbH, Germany). Clumps were dispersed by gently passing the cell suspension through a syringe several times. The remaining clumps of cells and debris were removed by passing the cell suspension through a 70-μm mesh nylon strainer. In order to isolate the HSPCs from bone marrow cells, these cells were then processed using the EasySep® mouse hematopoietic progenitor enrichment kit (StemCell Technologies), and HSPCs were isolated according to the manufacturer's instructions.

In vitro *irradiation*

For the removal of MEFs, MEF-dependent iPS cells (passages 9–12) were initially trypsinized and plated for 2 × 30 min on a 0.1% gelatin (Sigma)-coated dish at 37°C in a humidified atmosphere containing 95% air/5% CO_2. The number of iPS cells was counted, and the cells were suspended at 1.0×10^5 cells/mL in α-MEM (Gibco) supplemented with 10% ES-FBS, 0.1 mM NEAA, 0.1 mM 2-ME, 100 U/mL penicillin,

and 100 μg/mL streptomycin, without LIF. The iPS cells were then irradiated at a dose of 1, 2, 4, or 7.5 Gy with X-rays (150 kVp, 20 mA), using 0.2-mm copper and 0.5-mm aluminum filters from a distance of 30 cm from the focal point at a dose rate of 3.3–3.4 Gy/min.

The mouse HSPCs prepared from mouse bone marrow cells were irradiated at a dose of 0.5, 1, 2, or 4 Gy with X-rays (150 kVp, 20 mA), using 0.2-mm copper and 0.5-mm aluminum filters from a distance of 30 cm from the focal point at a dose rate of 3.3–3.4 Gy/min.

Real-time reverse transcription-polymerase chain reaction

Purified total RNA was quantified using a NANODROP 1000 spectrophotometer (Thermo). First-strand cDNA was synthesized using the iScript cDNA Synthesis kit (Bio-Rad Lab. Inc., CA, USA) according to the manufacturer's instructions. Gene expression was assessed by real-time reverse transcription-polymerase chain reaction (RT-PCR) performed using Power SYBR® green PCR master mix (Applied Biosystems, Tokyo, Japan) with typical amplification parameters (95°C for 10 min, followed by 50 cycles of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s). Fold differences were determined by ΔCt values, and the expression levels of genes in EB cells were compared with those in iPS cells; the expression levels of genes in irradiated EB cells were compared with those in non-irradiated EB cells after normalization with *GAPDH*, a housekeeping gene. The oligonucleotide primer sets used in real-time RT-PCR were purchased from TAKARA Bio Inc. (Shiga, Japan) (Table 1).

Table 1. Origins, National Center for Biotechnology Information gene accession numbers and sequences of synthetic oligonucleotide PCR primers for stem cell genes, differentiation genes and housekeeping gene.

Gene	Accession number	Primer sequence	Mainly expressing cells
Nanog	NM_028016.2	F: 5'-GAATTCTGGGAACGCCTCATC-3' R: 5'-CCTTGTCAGCCTCAGGACTTG-3'	Pluripotent stem cells
Pou5f1 (Oct-4)	NM_013633.2	F: 5'-CAGACCACCATCTGTCGCTTC-3' R: 5'-AGACTCCACCTCACACGGTTCTC-3'	Pluripotent stem cells
Afp	NM_007423.4	F: 5'-AATGGTCCGGCTGTGGTGA-3' R: 5'-CAGCAGTGGCTGATACCAGAGTTC-3'	Endoderm cells
Nes (Nestin)	NM_016701.3	F: 5'-GGGCCAGCACTCTTAGCTTTGATA-3' R: 5'-TGAGCCTTCAGGGTGATCCAG-3'	Ectoderm cells
T (Brachyury)	NM_009309.2	F: 5'-CCATGCTGCAGTCCCATGA-3' R: 5'-GCTCACAGACCAGAGACTGGGATAC-3'	Mesoderm cells
Gapdh	NM_008084.2	F: 5'-TGTGTCCGTCGTGGATCTGA-3' R: 5'-TTGCTGTTGAAGTCGCAGGAG-3'	

Statistical analysis

Differences between the 2 groups were analyzed with the Student *t*-test or Mann-Whitney *U* test. A *P* value less than 0.05 was considered statistically significant. Statistical analyses were performed using the Excel 2007 software program (Microsoft, USA) with the add-in software program Statcel 2 (OMS, Tokyo, Japan).[20)]

3. Results

Radiosensitivity of iPS cells

iPS cells and mouse HSPCs exposed to X-rays at 1–7.5 Gy were assayed in the methylcellulose culture for clonogenic potential. Figure 1 shows the radiation survival curve of each type of cell. The survival fraction (S) was calculated using the following fomula:

$$S = 1 - (1 - e^{-D/D0\ n})$$

The D, D_0 and n values mean radiation dose, the dose of 37% surviving dose and the number of targets, respectively. The D_0 values for iPS cells and HSPCs were 1.85 and 1.11, respectively; and the n value for both iPS cells and HSPCs was 1.00, indicating that iPS cells were more resistant to radiation than were HSPCs.

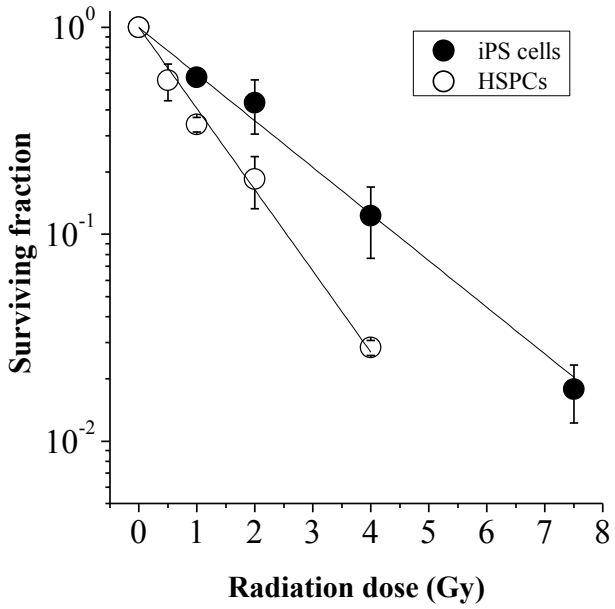


Fig. 1. Survival curves of iPS cells and mouse HSPCs. iPS cells and HSPCs exposed to X-rays at 1-7.5 Gy were assayed in the methylcellulose culture for clonogenic potential. The D_0 values for iPS cells and HSPCs were 1.85 ± 0.18 and 1.11 ± 0.05, respectively; and the n values for iPS cells and HSPCs were 1.00 ± 0.13 and 1.00 ± 0.13, respectively. The values are mean ± standard deviation (S.D.) from 4 separate experiments.

Embryoid body formation of iPS cells

To determine the differentiation potential of iPS cells, non-irradiated and irradiated iPS cells were assayed for EB formation. The plating efficiency (the ratio of growing clonal cells to seeding cells) of EB formation was 100% for the non-irradiated iPS cells. The colony morphology of EB resembled a highly dense mass as shown in Fig. 2. The diameter of the colonies derived from the 0-Gy control culture was approximately 670 ± 76 μm. When iPS cells were exposed to X-rays at 1–7.5 Gy, EB formation was observed. However, the diameter of EB showed a radiation dose- dependent decrease: 650 ± 100 μm at 1 Gy, 620 ± 96 μm at 2Gy, 550 ± 100 μm at 4 Gy, and 410 ± 63 μm at 7.5 Gy (Table 2).

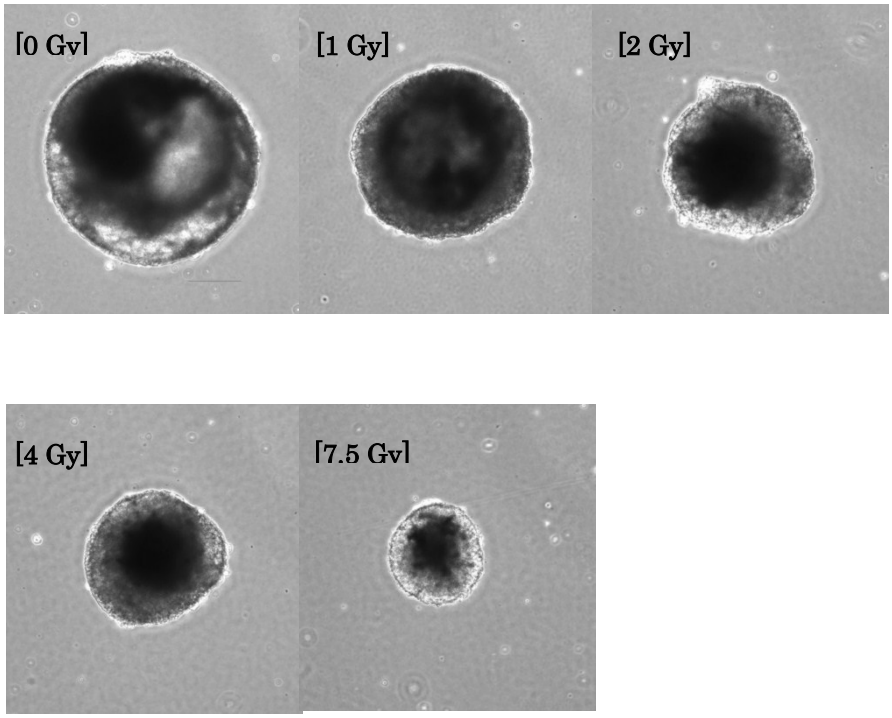


Fig. 2. Morphology of EB derived from iPS cells. Each EB was formed by iPS cells exposed to X-rays at 1-7.5 Gy.

Table 2. Plating efficiency of EB derived from iPS cells and their diameter

	Radiation dose				
	0 Gy	1 Gy	2 Gy	4 Gy	7.5 Gy
Number of iPS cells plated	40	40	40	40	40
Number of EB formation	40	40	40	40	40
Plating efficiency (%)	100	100	100	100	100
Diameter of EB (μm)	672.4 ± 75.7	651.6 ± 102.4	616.3 ± 96.1*	545.0 ± 101.3*	412.7 ± 62.5*

*P < 0.05; values compared with those of 0-Gy EB.

Effect of ionizing radiation on gene expression

To investigate the effect of ionizing radiation on gene expression, the expression of differentiation-related specific genes were examined in the initial iPS cells and EB cells. The expression of the endoderm marker *Afp* in non-irradiated EB cells markedly increased to approximately 3×10^5-fold compared to that in the initial iPS cells (Fig. 3). In contrast, a significant decrease was observed in the expression levels of the pluripotent stem cell markers *Nanog* and *Oct-4* and the mesoderm marker *Brachyury* (0.09-, 0.06-, and 0.12-fold, respectively) compared to the corresponding expression levels in the initial iPS cells. In addition, no significant changes were observed in the expression of the ectoderm marker *Nestin*.

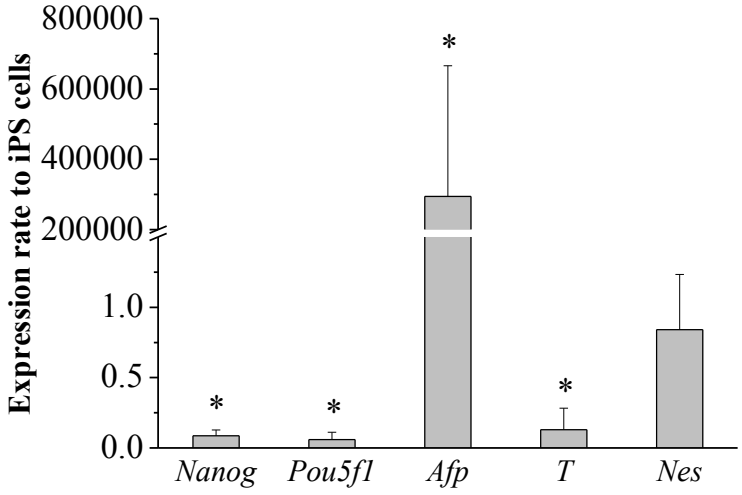


Fig. 3. Differentiation-related specific gene expression detected in EB cells. Total RNA was obtained from EB cells. mRNA expression was analyzed by real-time RT-PCR. mRNA expression levels in each cell were evaluated by the ratio of the level of mRNA expressed in the initial iPS cells to that of mRNA expressed in EB cells (baseline 1.0). The values are mean ± S.D. from 4 separate experiments. *P < 0.05.

Next, the expression of these genes was analyzed in cells of EB derived from irradiated iPS cells (Fig. 4). *Afp* expression significantly decreased in a radiation dose-dependent manner, showing a 0.19-fold decrease at 4 Gy and a 0.04-fold decrease at 7.5 Gy as compared to EB cells derived from non-irradiated iPS cells. In contrast, no significant differences were observed in the expression of other genes in almost all cases.

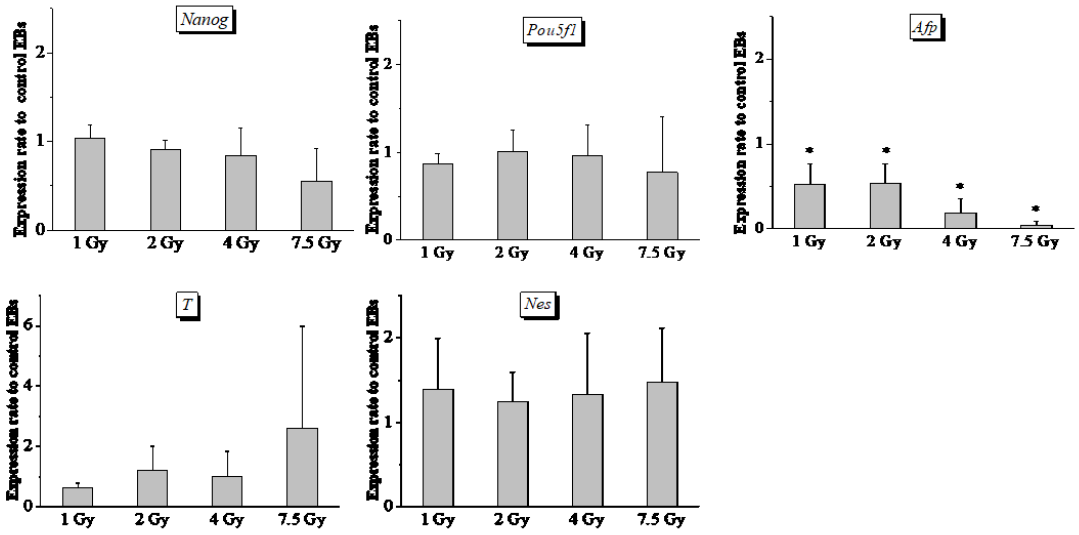


Fig. 4. Behavior of differentiation-related specific gene expression detected in EB cells derived from non-irradiated and irradiated iPS cells. mRNA expression levels in each cell were evaluated by the ratio of the level of mRNA expressed in the control EB cells derived from non-irradiated iPS cells to that of mRNA expressed in EB cells (baseline 1.0). The values are mean ± S.D. from 4 separate experiments. *P < 0.05.

4. Discussion

In this study, we investigated the effect of ionizing radiation on differentiation and proliferation of mouse iPS cells. The D_0 values of iPS cells and HSPCs were 1.85 and 1.11 (Fig. 1), respectively. These results suggest that iPS cells are more resistant to radiation than are HSPCs. Bañuelos *et al*. reported a similar radiation survival curve obtained in the study of mouse ES cells[21]. In addition, we reported that HSPCs in human peripheral blood, which are relatively mature compared with those in the bone marrow and placental/umbilical cord blood, are more radiosensitive[15,16]. In other words, the radiosensitivity of stem cells is possibly different from that of other types of functional cells. Moreover, molecular mechanisms related to radiosensitivity have been investigated by several researchers[22-25]. Wang *et al*. reported that notch signaling reduces radiosensitivity in glioma stem cells through activation of the PI3K/Akt pathway[22]. We reported that tyrosine kinase with immunoglobulin and the epidermal growth factor homology domain 2 may be implicated in the radiosensitivity of individual HSPCs[23]. Furthermore, cancer stem cells have been reported to be radioresistant, because they can enter a quiescent phase such as G_0 and possess signaling pathways such as the Wnt/b-catenin pathway for survival[24,25]. In addition, pluripotent stem cells have an antioxidative system comprising *Hspb1* and *Sod2*[26]. *Hspb1* positively regulates the expression levels of *Sod2*; *Sod2* is downregulated to varying degrees upon differentiation of ES cells until the day-4 EB stage. Therefore, pluripotent stem cells can protect themselves from various extracellular oxidative stresses owing to the presence of the antioxidative system.

In this study, to observe the effects of ionizing radiation on differentiation of iPS cells into the 3 germ layers, we assessed EB formation. Although EB formation was observed at all radiation doses (Figs. 2; Table 2), EB diameter showed a radiation dose-dependent decrease suggesting a harmful effect of ionizing radiation on differentiation and proliferation of iPS cells. The mRNA levels of each lineage marker, including *Nanog*,

Oct-4, *Afp*, *Brachyury*, and *Nestin*, were analyzed in the initial iPS cells and EB cells (Fig. 3). In the control culture performed with non-irradiated iPS cells, the expression of *Afp* during EB formation increased remarkably compared to that in the initial iPS cells. The expression levels of *Nanog*, *Oct-4*, and *Brachyury* decreased, whereas those of *Nestin* showed no significant changes. The expression pattern of *Afp* and *Nestin* in this study is consistent with the findings of a previous study on mouse ES cell-derived pluripotent EBs[27]. On the other hand, the downregulation of *Brachyury*, an early mesoderm marker, showed a pattern different from that observed in a previous study[4]. In addition, Morizane *et al*. reported that the expression of *Brachyury* increased remarkably on day 4, after which it was downregulated[28]. The difference in the behavior of *Brachyury* expression in this study could possibly be attributed to the different culture conditions.

With respect to the effects of ionizing radiation on the gene expression levels of each lineage marker, *Afp* expression significantly decreased in a radiation dose-dependent manner despite its increased mRNA levels in EB derived from non-irradiated iPS cells. In contrast, no significant effect was observed in the expression levels of *Nanog*, *Oct-4*, *Brachyury*, and *Nestin* (Fig. 4). Because the expression of *Afp* in cells of EB derived from irradiated iPS cells decreased in a radiation dose-dependent manner, ionizing radiation possibly suppresses differentiation and proliferation of iPS cells to the endoderm lineage, thereby leading to a decrease in EB diameter and gene expression levels. Perhaps, this could be attributable to a delay in differentiation, and cells being arrested in the G2 phase[29]. Subsequent analyses are underway for determining the mechanism(s) underlying these results.

In conclusion, these results suggest that the radiosensitivity of iPS cells is low compared with other stem/progenitor cells, especially HSPCs. In addition, radiosensitivity with regard to gene expression differs at various stages in the early differentiation pathways of iPS cells that lead to the formation of the 3 germ layers; the sensitivity is the highest in the genes expressed during the differentiation of iPS cells, leading to the formation of the endoderm. It is necessary to assess the function of mature

cells derived from irradiated iPS cells through differentiation into the 3 germ layers. Future studies need to assay intracellular protein and cell surface antigen expression in iPS cells to investigate the effects of ionizing radiation on them in detail.

Chapter 2

Placental/umbilical cord blood-derived mesenchymal stem cell-like stromal cells support hematopoietic recovery of X-irradiated human $CD34^+$ cells

Abstract

Aims: The potential of human mesenchymal stem cell-like stroma prepared from placental/umbilical cord blood for hematopoietic regeneration by X-irradiated hematopoietic stem cells is herein assessed.

Main methods: Placental/umbilical cord blood-derived mesenchymal stem cell-like stromal cells were applied to a regenerative ex vivo expansion of X-irradiated human $CD34^+$ cells in a serum-free liquid culture supplemented with a combination of interleukine-3 plus stem cell factor plus thrombopoietin.

Key findings: The total number of cells and of lineage-committed myeloid hematopoietic progenitor cells generated in the co-culture of both non-irradiated and X-irradiated cells with stromal cells was significantly higher than those in the stroma-free culture. In addition, the number of $CD34^+$ cells and $CD34^+/CD38^-$ cells, immature hematopoietic stem/progenitor cells also increased more than the stroma-free culture. The stromal cells produced various types of cytokines, although there was little difference between the co-cultures of non-irradiated and X-irradiated cells with stromal cells. Furthermore, when X-irradiated cells came in contact with stromal cells for 16 h before cytokine stimulation, a similar degree of hematopoiesis was observed, thus suggesting the critical role of cell-to-cell interaction. Significance: The present results showed the potential efficacy of human mesenchymal stem cell-like stroma for hematopoietic regeneration from irradiated hematopoietic stem/progenitor cells.

1. Introduction

Since the hematopoietic system is a radiosensitive tissue, the exposure to high dose radiation leads to myelosuppression and increases the possibility of an individual's death[30,31]. Under those conditions, hematopoietic stem cell transplantation using bone marrow (BM) transplantation or cord blood (CB) transplantation is usually used for the regeneration of hematopoiesis/immunity[32,33]. However, there are some specific issues regarding the number of donor in human BM transplantation and the number of mononuclear cells in CB transplantation[34-36]. In addition, long periods of severe neutropenia or thrombocytopenia have led to problems in many patients who have undergone these types of transplantation[35]. Since the source of hematopoietic stem cells for transplantation is from a different individual, the regulation of graft versus host disease (GVHD) after hematopoietic stem cell transplantation causes serious problems in many cases[32,37]. One possible approach to solve this problem is to transplant hematopoietic stem/progenitor cells (HSPC) expanded ex vivo from autologous stem cells[38]. These have been many investigations addressing the ex vivo expansion of HSPC[33,39]. These studies can be divided based on; liquid culture with a combination cytokines alone and the combination of the liquid culture with a stroma layer. The majority of the previous studies have been performed in breast cancer and multiple myeloma patients after high-dose chemotherapy, showing moderate outcomes[39-46]. In the case of an individual exposed to irradiation, the ex vivo expansion of hematopoietic stem cells has been demonstrated to be a useful tool in animal studies[33,47,48]. However, there are still many questions concerning this type of therapy. In particular, since the use of a combination of cytokines alone has little potential to maintain and expand immature hematopoietic stem cells ex vivo, the effectiveness of a stroma layer has been demonstrated in many cases. However, there are unresolved questions, particularly in terms of the establishment of a safety protocol for human-derived stroma layers, since almost previous reports are derived from nonhuman primates[49].

In the present study, the potential of human mesenchymal stem cell-like stromal cells prepared from placental/umbilical cord blood for hematopoietic regeneration by X-irradiated hematopoietic stem cells was studied.

2. Materials and Methods

Growth Factors and Reagents

Recombinant human interleukin-3 (IL-3), stem cell factor (SCF) and thrombopoietin (TPO) were purchased from Bio Source (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 Pepro Tech Inc. (NJ, USA). Recombinant human fibroblast growth factor-2 (FGF-2) was provided Kaken Pharmaceutical Co. Ltd. (Tokyo, Japan). These factors were administered at the following concentrations: IL-3 and SCF, 100 ng/ml; TPO, 50 ng/ml; EPO, 4 U/ml; G-CSF, GM-CSF and FGF-2, 10 ng/ml medium. The fluorescence-labeled monoclonal antibodies (mAbs) fluorescein isothiocyanate (FITC)-conjugated anti-human CD13 (CD13), FITC-conjugated anti-human CD34 (CD34), FITC-conjugated anti-human CD41 (gpIIbIIIa, CD41), FITC-conjugated anti-human CD235a (CD235a), FITC-conjugated anti-human HLA-ABC (HLA-ABC), phycoerythrin (PE)-conjugated anti-human CD14 (CD14), PE-conjugated anti-human CD38 (CD38), PE-CD41, PE-conjugated anti-human CD90 (CD90), PE-conjugated anti-human CD105 (CD105), PE-conjugated anti-human HLA-DR (HLA-DR) and PE-cyanin-5-forochrome tandem (PC5)-conjugated anti-human CD15 were purchased from Beckman Coulter Immunotech (Marseille, France) and PE-conjugated anti-human CD73 (CD73) was purchased from Becton Dickinson Biosciences (San Jose, CA). Mouse IgG_1-FITC, mouse IgG_1-PE, mouse IgG_1-PC5 and mouse IgG_{2a}-FITC (Becton Dickinson Biosciences) were used as isotype controls. 7-Aminoactinomycin D (7-AAD)

was purchased from Beckman Coulter Immunotech.

Collection and Purification of Cord Blood CD34$^+$ Cells

This study was approved by The Committee of Medical Ethics of Hirosaki University Graduate School of Medicine (Hirosaki, Japan). After informed consent was obtained from the mother, cord blood for use in this study was collected after full-term deliveries using a sterile collection bag containing the anticoagulant, citrate-phosphate dextrose, according to the guidelines of the Tokyo Cord Blood Bank. These samples were isolated separately and used for each experiment. Within 24 h after the collection of cord blood, light-density mononuclear cord blood cells were separated by centrifugation on a Lymphosepar I (1.077 g/ml, Immuno-Biological Laboratories, Takasaki, Japan) for 30 min at 300 ×*g* and washed three times with phosphate-buffered saline (PBS, Sigma St. Louis, USA) containing 5 mM ethylenediaminetetraacetic acid (EDTA). These cells were then processed for CD34$^+$ cell enrichment according to the manufacturer's instructions. An Easy Sep® human CD34 selection kit (StemCell Technologies, Vancouver, Canada) was used for the positive selection of CD34$^+$ cells. At the end of the procedure, the CD34$^+$ cell purity as measured by a fluorescence cell analyzer (EPICS® XL, Beckman-Coulter, Fullerton, CA) was 72-98%. Freshly prepared CD34$^+$ cells obtained from the above protocol were used in this study.

Culture of Placental/Umbilical Cord Blood-derived Mesenchymal Stem Cell-like Stromal Cells

Mesenchymal stem cell-like stromal cells were pre-established by culturing excluded cells from positive selection of CD34$^+$ cells in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml) and FGF-2 at 37°C in a humidified atmosphere containing 95% air/5% CO_2. The culture medium was exchanged twice per week. The adherent and expanded cells were considered to be the mesenchymal stem cell-like stromal cells,

which were used to perform the allogenic co-culture. The confirmation that the harvested cells were mesenchymal stem cell-like stromal cells was determined by their expression of a characteristic antigen for mesenchymal stem cells, such as CD73 (95-99%), CD90 (Thy-1, 40-54%), CD105 (93-95%) and HLA-ABC (66-80%) using flow cytometry (n=3, data not shown) according to the findings of previous reports[50-52]. At the same time, the expressions of CD15 (SSEA-1), CD34, CD45 and HLA-DR were also analyzed, but no positive expression was observed (data not shown). These results were therefore consistent with those from previous reports.

In vitro Irradiation of CD34$^+$ Cells

The CD34$^+$ cells were irradiated a dose of 2 Gy with X-rays (200 kVp, 15 mA) using 0.3 mm copper and 0.5 mm aluminum filters at a distance of 45 cm from the focus at a dose rate of 0.87-0.89 Gy/min.

Co-culture of CD34$^+$ Cells with Stromal Cells

Mesenchymal stem cell-like stromal cells were initially cultured in DMEM containing 10% FBS and FGF-2 in a 24-well plate at a concentration of 3×10^4 cells/0.5 ml/well. The next day, the stromal cells were irradiated a dose of 20 Gy with X-rays (200 kVp, 15 mA) using a 2.0 mm aluminum filter at a distance of 35 cm from the focus at a dose rate of 4.0-4.2 Gy/min to repress a growth. One to three days after irradiation, non-irradiated and irradiated CD34$^+$ cells (2.5×10^2 cells/0.5 ml/well for non-irradiated and 2.5×10^3 cells/0.5 ml/well for irradiated) were suspended in Isocave's modified Dulbocco's medium (IMDM, Gibco BRL) supplemented with BIT9500 (a serum substitute for serum-free culture, StemCell technologies), were cultured with or without stromal cells at 37˚C in a humidified atmosphere containing 95% air/5% CO_2. The cultures were treated with SCF + TPO + IL-3, and fresh medium with cytokines was exchanged in the cultures at day 7 and 10-11. On day 14, the floating cells were harvested and the number of viable cells was determined by Trypan blue exclusion.

Flow Cytometric Analysis

The expression of specific cell surface antigens was analyzed by direct immuno-fluorescence flow cytometry using single to triple staining combinations of mAbs, including FITC-CD13, FITC-CD34, FITC-CD235a, PE-CD14, PE-CD38, PE-CD41 and PC5-CD15. Briefly, the cells were incubated with saturated concentrations of the relevant mAbs for 20 min at room temperature. After labeling, dead cells were discriminated by 7-AAD, and the remaining cells were washed and analyzed by flow cytometry. For each experiment, an isotype-matched irrelevant mAb was used as a negative control.

Methylcellulose cultures

The colony-forming cells including colony-forming unit-granulocyte-macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E) and colony-forming unit-granulocyte-erythroid-macrophage-megakaryocyte (CFU-Mix) were assayed by methylcellulose culturing using MethoCult® (StemCell technologies). The expanded cells were plated onto each well of a 24-well plate (0.3 ml/well) in culture medium containing EPO, SCF, IL-3, G-CSF and GM-CSF as colony-stimulating factors. Each plate was incubated at 37˚C in a humidified atmosphere containing 95% air/5% CO_2 for 14 days. The colonies containing more than 50 cells were counted with an inverted microscope (Olympus, Tokyo, Japan).

Plasma Clot Culture

CFU-Meg was assayed by the plasma clot technique using platelet-poor human plasma. Ten percent human platelet-poor AB plasma, SCF, TPO, penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mM), MEM vitamin (1%), MEM non-essential amino acids (1%; GIBCO® Invitrogen), thioglycerol (1×10^{-5} M; Sigma), L-asparagine (2 μg/ml), $CaCl_2$ (74 μg/ml; Wako Pure Chemicals, Tokyo, Japan) and 0.2% bovine serum albumin (BSA, Boehringer Mannheim GmbH) were added to the

culture medium based on IMDM. Harvested cells were added to the culture medium at a concentration of 2.5×10^3 cells/ml. This baseline culture was plated onto a 24-well plate at 0.3 ml/well and then was incubated at 37˚C for 14 days in a humidified atmosphere containing 95% air/5% CO_2.

Identification of Megakaryocyte Colonies by Immunofluorescence

The plasma clot cell cultures were fixed by 15 and 10 min incubation in acetone: methanol (2:1). The plates were dried in an air flow overnight and stored at -20˚C. Prior to staining, the plates were returned to room temperature and then PBS containing 0.5% BSA (PBS-B) was added to soften the clot. The plates were incubated at room temperature with FITC-CD41 mAb diluted 1:100 in PBS-B for 1 h followed by a single wash with PBS-B. The nuclei were then counterstained with propidium iodine (PI, 0.3 ng/ml, Sigma). After a final wash, the colonies containing of more than 3 cells were counted with an inverted microscope.

Cytokine assay

The cytokines released from the cultures in the conditioned media with or without stromal cells were measured using the Bio-Plex protein array system (Bio-Rad Laboratories, Hercules, CA, USA). The Bio-Plex cytokine assay is designed for the multiplexed quantitative measurement of multiple cytokines in a single well using as little as 50 μl of sample. For the cytokine assay, premixed multiplex beads of the Bio-Plex human Th1/Th2 panel (Bio-Rad Laboratories), which included 27 cytokines, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, Eotaxin, FGF-2, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF were used. The data obtained from this system were analyzed using the Bio-Plex Manager 4.0 software program (Bio-Rad Laboratories). The detectable concentration of each cytokine ranged from 30 fg/ml to 42 ng/ml.

Statistical analysis

The differences between two groups were analyzed by Student's *t*-test. A p value less than 0.05 was considered to be statistically significant.

3. Results

The potential of stromal cells for the expansion of HSPC from X-irradiated CD34$^+$ cells

To evaluate the potential of stromal cells for the expansion of HSPC from X-irradiated CD34$^+$ cells, freshly prepared non-irradiated and X-irradiated CD34$^+$ cells were cultured with stromal cells in the presence of multiple cytokines, TPO plus SCF plus IL-3. At the same time, in order to determine the effect of cell-to-cell interactions between the CD34$^+$ cells and stromal cells, the cytokines were added in two different ways, addition of cytokines at the same time as the co-culture and delayed addition 16 h after co-culture, respectively. In the cultures of non-irradiated cells with or without stromal cells, the total number of cells significantly increased to 1.2×10^6 cells (approximately 4800-fold) and 4.1×10^5 cells (approximately 1600-fold), respectively, from the initial number of cells (2.5×10^2 cells/culture; Fig. 5). When the cytokines were added 16 h later, the total number of cells detected in the co-culture increased 3.7-fold higher than the stroma-free culture (Fig. 5). In the culture of 2-Gy irradiated CD34$^+$ cells, the co-culture led to a 2.1- and 8.9-fold greater increase in total cell numbers in comparison to stroma-free culture, respectively.

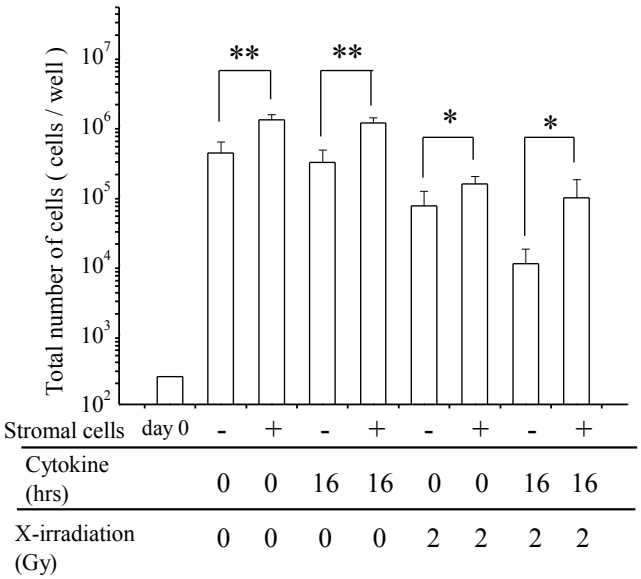


Fig. 5. The total number of cells generated in the liquid culture with or without stromal cells. Freshly prepared CB-$CD34^+$ cells were cultured with a combination of TPO + IL-3 + SCF in either stroma-free cultures or with stromal cells for 14 days. Values represent the means ± S.D. of four to five separate experiments. * $P < 0.05$. ** $P < 0.01$.

The number of $CD34^+$ cells and $CD34^+/CD38^-$ cells, relatively more immature cells in hematopoiesis than $CD34^+$ cells, contained in the expanded cells was evaluated using flow cytometry (Fig. 6). In the culture of non-irradiated cells with or without stromal cells, the numbers of $CD34^+$ cells increased to 1.7×10^5 cells (approximately 800-fold) and 2.3×10^4 cells (approximately 100-fold) from the initial input (approximately 2.2×10^2 cells), showing a 7.3-fold greater increase in the stromal culture. Similarly, the number of $CD34^+/CD38^-$ cells also increased to 1.4×10^5 cells (approximately 15,000-fold) and 1.8×10^4 cells (approximately 2000-fold) from the initial culture (11 cells), respectively (Fig. 7). In the case of 2 Gy-irradiated cells, the number of $CD34^+$ cells and $CD34^+/CD38^-$ cells generated in the co-culture resulted in a 6.9- and 6.5-fold increase in comparison to the stroma-free control, respectively. Furthermore, a significant increase was observed in both cultures with the delayed addition of cytokines.

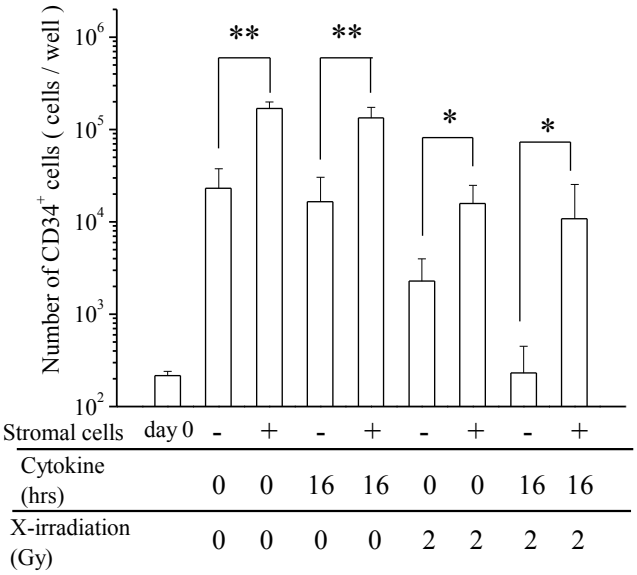


Fig. 6. The number of CD34$^+$ cells generated in a liquid culture with or without stromal cells. Values represent the means ± S.D. of three to four separate experiments. * $P < 0.05$. ** $P < 0.01$.

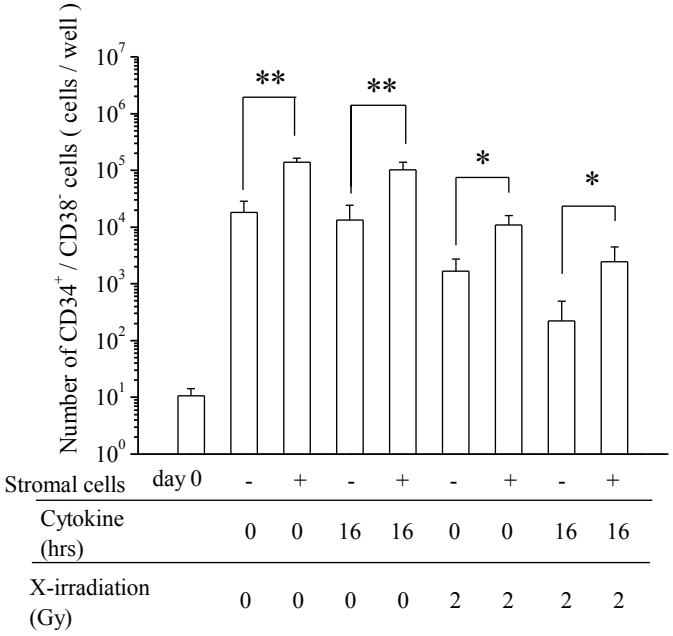


Fig. 7. The number of CD34$^+$ / CD38$^-$ cells generated in a liquid culture with or without stromal cells. Values represent the means ± S.D. of three to four separate experiments. * $P < 0.05$. ** $P < 0.01$.

In order to determine the proportion of mature cells contained in the harvested cells, the expression of CD13, CD14, CD15, CD34, CD38, CD41 and CD235a was analyzed using flow cytometry (Table 3). The expression of cells expressing CD13, a marker antigen of the myeloid lineage, was in the range of 44%-54% in the culture without stromal cells; however, a significant decrease was observed in the cultures with stromal cells. The expression of CD14 and CD15, marker antigens of the leukocyte lineage, was 4%-10% and 18%-28% in the culture without stromal cells, respectively. At this time, the expression of CD14 detected in the harvested cells from the co-culture of non-irradiated cells showed no significant difference in comparison to the stroma-free culture, while a significant increase was observed in the culture of X-irradiated cells. On the other hand, the expression of CD15 detected in the cells from the co-culture of non-irradiated cells showed a significant decrease in comparison to the stroma-free culture, while no significant difference was observed in the culture of X-irradiated cells. The $CD41^{+}$ cells, mature megakaryocytes, were 26%-31% in the both non-irradiated and X-irradiated cells, representing 1/4-1/3 of the generated cells. The expression of CD235a, a marker antigen of the erythroblast lineage, was 4.2% - 4.9% in the harvested cells. However, no significant difference was observed in the cells cultured with or without stromal cells.

Table 3. Phenotypic analysis of generated cells in the culture with or without stromal cells

Antigens	Stromal cells	0 Gy (%)		2 Gy (%)	
		0 h	16 h	0 h	16 h
$CD13^+$	-	46.9 ± 5.7	44.7 ± 4.5	48.9 ± 3.4	54.3 ± 2.9
	+	29.8 ± 3.6*	27.9 ± 5.7*	33.5 ± 5.5*	32.3 ± 4.7*
$CD14^+$	-	7.6 ± 2.8	9.8 ± 7.2	6.2 ± 2.4	4.0 ± 1.2
	+	11.7 ± 3.9	12.5 ± 4.2	13.2 ± 4.9*	10.2 ± 3.8*
$CD15^+$	-	26.2 ± 6.2	27.6 ± 6.1	19.0 ± 8.7	18.3 ± 13.9
	+	10.8 ± 2.4*	13.6 ± 6.2*	11.0 ± 2.4	13.9 ± 10.6
$CD41^+$	-	25.7 ± 10.9	30.8 ± 10.1	31.4 ± 11.2	25.7 ± 10.4
	+	9.6 ± 5.6	4.7 ± 1.7*	12.2 ± 6.2*	14.9 ± 6.4
$CD235a^+$	-	5.4 ± 1.9	5.7 ± 2.0	7.0 ± 3.2	5.1 ± 1.9
	+	4.7 ± 1.4	4.2 ± 1.4	4.9 ± 3.3	4.5 ± 3.1

* $P < 0.05$ compared to stromal cells (-) cultured cells by t test.

Generation of hematopoietic progenitor cells

After ex vivo expansion, the total number of lineage-committed myeloid hematopoietic progenitor cells including CFU-GM which are progenitors for granulocyte-macrophages, BFU-E which differentiate into erythroblasts and erythrocytes and CFU-Mix which is a pluripotent hematopoietic progenitor, thus giving rise to erythroid, granulocyte, macrophage and megakaryocytic cells and CFU-Meg which is a progenitor for megakaryocyte and platelet were assayed, respectively. The total number of hematopoietic progenitor cells, colony-forming cells (CFC), was obtained from the sum of each value of CFU-GM, BFU-E and CFU-Mix. In the culture of non-irradiated cells with or without stromal cells, the total number of CFC increased to 1.3×10^5 cells (approximately 2600-fold) and 3.7×10^4 cells (approximately 800-fold),

respectively, from the initial numbers (48 cells/culture; Fig. 8). At this time, the total number of CFC detected in the culture with the both methods of cytokine addition resulted in a 3.3- and 4.8-fold greater increase, respectively, in comparison to the stroma-free culture (Fig. 8). In the co-culture of X-irradiated cells, the total number of CFCs was also significantly increased, increasing 2.5-fold with the same time addition of cytokines and 60-fold by the delayed addition, respectively.

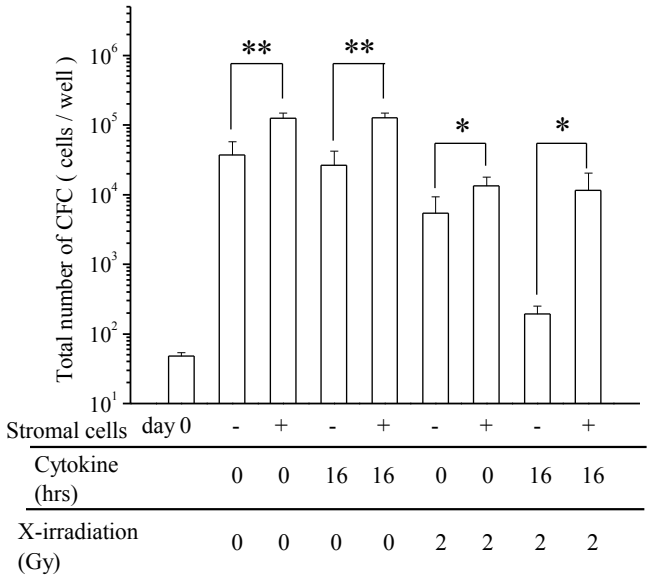


Fig. 8. The number of total CFC generated in a liquid culture with or without stromal cells. The cells harvested from the culture were assayed for the number of CFU-GM, BFU-E and CFU-Mix using a methylcellulose culture. Values represent the means ± S.D. of four to five separate experiments. * $P < 0.05$. ** $P < 0.01$.

The number of CFU-GM, BFU-E and CFU-Mix detected in the culture of non-irradiated cells with or without stromal cells increased by approximately 600- to 3500-fold and approximately 200- to 1000-fold, respectively (Fig. 9). All of the individual progenitors, CFU-GM, BFU-E and CFU-Mix, detected in the co-culture of non-irradiated cells showed a significantly higher increase than in the stroma-free cultures. In contrast, no significant difference was observed in the number of CFU-Meg detected in the non-irradiated culture (Fig. 10). However, the total number of CFU-Meg

significantly increased in both cultures of X-irradiated cells (4- and 25-fold higher, respectively).

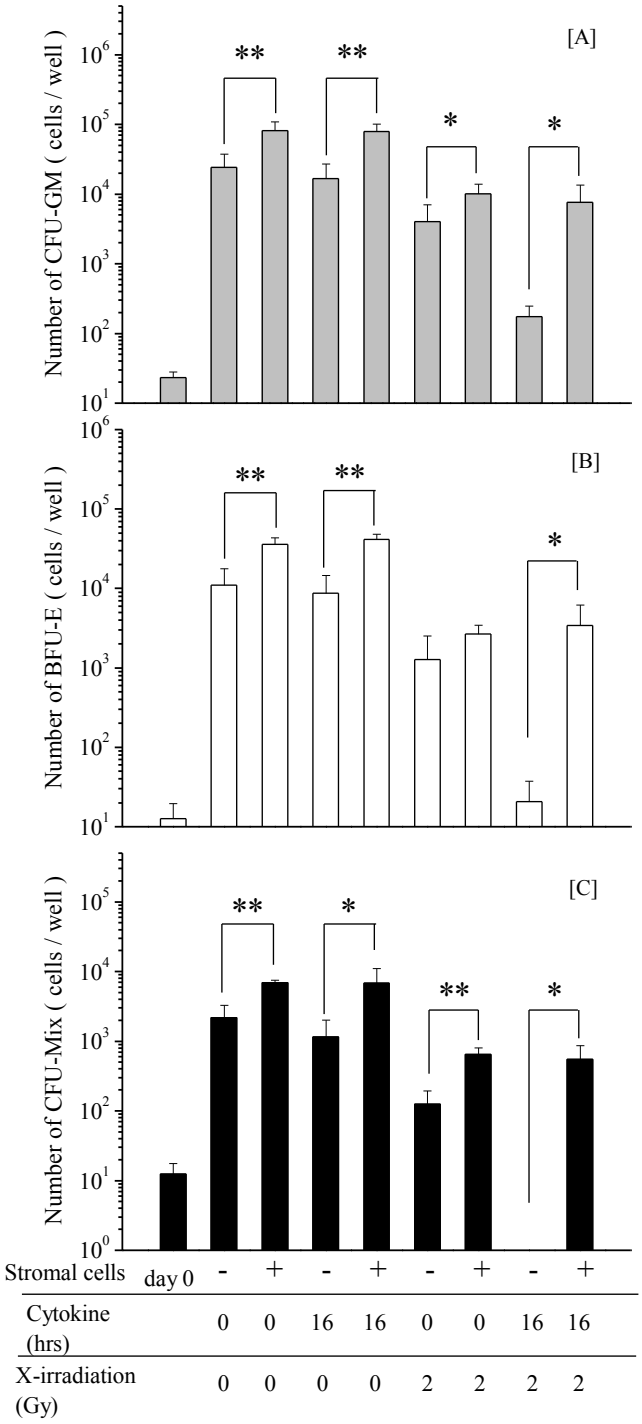


Fig. 9. The number of each progenitor cell generated in a liquid culture with or without stromal cells. Values represent the means ± S.D. of four to five separate experiments. * $P < 0.05$. ** $P < 0.01$.

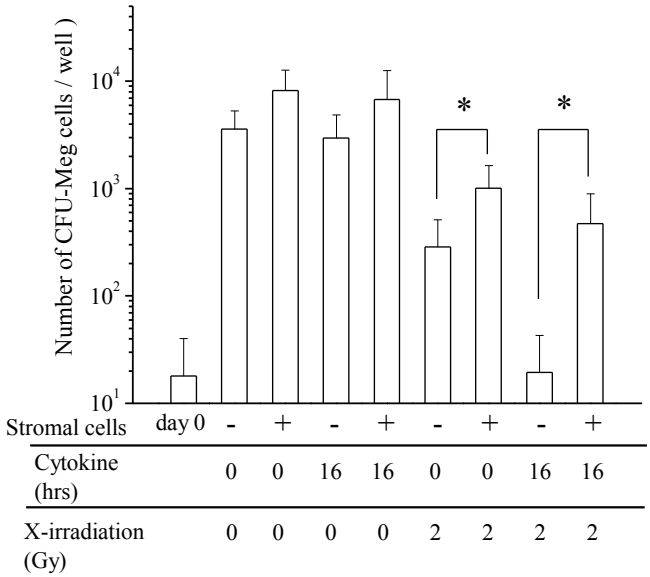


Fig. 10. The number of CFU-Meg generated in a liquid culture with or without stromal cells. The cells harvested from the culture were assayed for the number of CFU-Meg using the plasma clot technique. Values represent the means ± S.D. of four to five separate experiments. * $P < 0.05$.

Cytokine production in the media conditioned with or without stromal cells

To determine the content of the cytokines released by expanded cells and/or stromal cells, each conditioned medium harvested from the cultures was analyzed for the measurement of cytokines using a multi-protein array system. Fig.11 shows that the conditioned media from the stroma-free culture contained IL-1α, IL-6, IL-8, Eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, RANTES and VEGF. In the case of co-culture, there was an increase of IL-1α, IL-6, IL-13, IL-17, Eotaxin, G-CSF, IFN-γ, IP-10, PDGF BB, RANTES and VEGF. However, little difference was observed between the cultures of non-irradiated and X-irradiated cells except for PDGF BB and RANTES. Interestingly, 2 Gy irradiated cells alone produced IL-10, IL-13 and GM-CSF, in comparison to the culture of non-irradiated cells without stromal cells, although the concentration of each was very low.

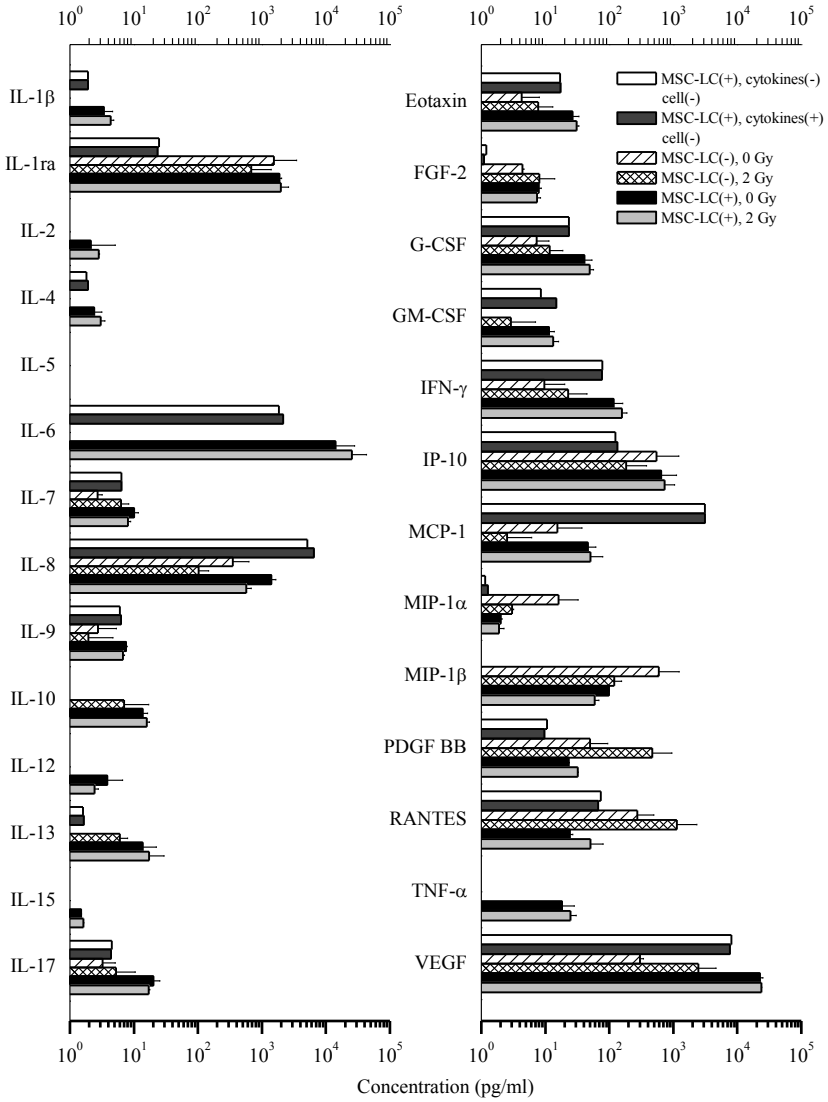


Fig. 11. Cytokine concentration detected in the medium conditioned by the cultures with or without stromal cells. The foot note including stromal cells (+), cytokines (-), $CD34^+$ cells (-) and stromal cells (+), cytokines (+), $CD34^+$ cells (-) indicates the control culture of this culture system. Each cytokine was measured in duplicate using the Bio-Plex cytokine protein array system. The data are represented as the mean ± S.D. of two different cultures.

4. Discussion

In the present study, $CD34^+$ cells were irradiated a radiation dose of 2 Gy that was previously reported to decrease the survival rate of the progenitor cells to about 30 %[16]. A combination of IL-3 plus SCF plus TPO was used as an effective cytokine mixture for hematopoietic regeneration of X-irradiated HSPC based on previous findings[53,54]. The total number of cells generated in the co-culture of both non-irradiated and X-irradiated cells with stromal cells was significantly higher than those in the stroma-free cultures (Fig. 5). For hematopoietic progenitors, co-culture of non-irradiated and irradiated $CD34^+$ cells led to an increase of CFC in comparison to stroma-free cultures (Fig. 8). These results are consistent with the previous findings observed in animal models[47,55], suggesting that this co-culture system has the potential for ex vivo expansion of irradiated human $CD34^+$ cells.

Stromal cells have been isolated from many tissues, including bone marrow, adipose tissue, fetal liver, peripheral blood and cord blood[34,47,55-59]. These mesenchymal stem cells have been known to release cytokines[57,58,60] and to maintain stem cells in a primitive, quiescent state, by playing a significant role in the bone marrow microenvironment[47,57]. In the present study, various myeloid hematopoiesis-related cytokines, such as IL-6, IL-9, G-CSF and GM-CSF, were detected in the conditioned media harvested from the co-cultures (Fig. 11). Although the precise action of all of the cytokines detected in the co-culture is not clear, they may make some contribution to hematopoiesis. In addition, the number of $CD34^+$ cells and $CD34^+/CD38^-$ cells, immature hematopoietic stem cells[61,62], was also increased more than in the stroma-free culture (Fig. 6, 7). The number of $CD34^+$ cells and $CD34^+/CD38^-$ cells generated in the co-culture of irradiated $CD34^+$ cells was almost the same as in stroma-free culture of non-irradiated cells. These findings demonstrated the characteristics of stromal cells, to maintain stem cells in a primitive state, as shown in the previous studies[47,57]. The proportion of $CD13^+$, $CD15^+$ and $CD41^+$ cells detected in all or some cultures showed a

significant decrease (Table 3). Stromal cells counteract the differentiation events triggered by cytokines and thus allow cells to retain their undifferentiated properties[63]. For example, stromal cells express Notch ligands and $CD34^+$ cells express Notch receptors and it has been shown that the ligand-receptor interaction prevents differentiation with little change in proliferation. Therefore, it is possible that Notch signaling derived from stromal cells regulates differentiation of HSPC[64]. However, a significantly increase of CD14 expressing monocyte/macrophages was observed in the co-culture of X-irradiated cells in comparison to the stroma-free culture. These results may be due to the large increase of CFU-GM, monocyte/macrophage progenitors, in the culture[65,66].

In order to determine the effect of cell-to-cell interactions between $CD34^+$ cells and stromal cells, the cytokines were added in two different ways. In these experiments, similar hematopoiesis was observed in both cultures of non-irradiated cells with or without stromal cells (Fig. 5 - 8). A decrease of hematopoiesis was observed in the stroma-free culture of 2 Gy-irradiated cells with cytokine delayed addition in comparison to the control culture. When X-irradiated cells came in contact with stromal cells before cytokine addition, a similar rate of hematopoiesis was observed in the culture with the same time addition, suggesting the importance of cell-to-cell interactions. The interaction between hematopoietic stem cells and stromal cells in the co-culture has been noted in previous studies[47,55,67,68]. Although they demonstrated a greater increase in the total number of cells and HSPC, the non-contact system of irradiated $CD34^+$ and mesenchymal stem cells showed a significant decrease of the expansion rate of the total number of cells in comparison to the contact system[47]. In contrast, Belkacémi et al. investigated the possibility of the ex vivo expansion of the residual progenitors exposed to different dose levels of total body irradiation[69]. However, they were concerned with the sustained defect in the stroma which limits the functionality of HSPC, because immature progenitors, long-term culture initiating cells, were not detectable even after a dose >1.2 Gy. Therefore, a more detailed study is

required including an improvement of the culture system, such as the use of more suitable cytokine combinations, an analysis of cell components in generated cells and the exposure to a >2 Gy dose.

Since previous studies have primarily used animal models, little research has been reported regarding a human co-culture system for regeneration of human HSPC, with either human hematopoietic stem cells or stromal cells[33,47,48]. Hérodin et al. suggested that autologous stem cell transplantation might be applied to those exposed to radiation, provided a sufficient amount of residual HSPC is collected after irradiation and these cells are then efficiently expanded ex vivo[32]. The establishment of an ex vivo expansion system from residual hematopoietic stem cells of irradiated victims with a normal human feeder layer could be required. The present results showed the potential efficacy of human CB-derived mesenchymal stem cell-like stromal cells for hematopoietic regeneration from irradiated HSPC. However, since there are various problems regarding this ex vivo expansion system, such as the optimal protocol for the establishment of CB-derived stromal cells further research is required to establish a more effective ex vivo expansion system.

Chapter 3

The effects of X-irradiation on *ex vivo* expansion of cryopreserved human hematopoietic stem/progenitor cells

Abstract

In our previous study (Life Sciences **84**: 598-605, 2009), we demonstrated that placental/umbilical cord blood-derived mesenchymal stem cell-like stromal cells have the effect to support the regeration of freshly prepared X-irradiated hematopoietic stem/progenitor cells (HSPCs). Generally, HSPCs are supplied from companies, institutions, and cell banks that cryopreserve them for clinical and experimental use. In this study, the influence of cryopreservation on the responses of HSPCs to irradiation and co-culture with stromal cells is assessed. After cryopreservation with the optimal procedure, 2 Gy-irradiated HSPCs were cultured with or without stromal cells supplemented with combination of interleukin-3, stem cell factor, and thrombopoietin. The population of relatively immature $CD34^+/CD38^-$ cells in cryopreserved cells was significantly higher than in fresh cells prior to cryopreservation; furthermore, the hematopoietic progenitor populations of $CD34^+/CD45RA^+$ cells and $CD34^+/CD117^+$ cells in cryopreserved cells were significantly lower than that in fresh cells. However, the rate of expansion in the cryopreserved HSPCs was lower than in the fresh HSPCs. In the culture of cryopreserved cells irradiated with 2 Gy, the growth rates of $CD34^+$ cells, $CD34^+/CD38^-$ cells, and hematopoietic progenitors were greater than growth rates of their counterparts in the culture of fresh cells. Surprisingly, the effect to support the hematopoiesis in co-culture with stromal cells was never observed in the X-irradiated HSPCs after cryopreservation. The present results demonstrated that cryopreserving process increased the rate of immature and radio-resistant HSPCs but decreased the

effects to support the hematopoiesis by stromal cells, thus suggesting that cryopreservation changes the character of HSPCs.

1. Introduction

The hematopoietic stem cell (HSC) has the potential to produce all the functional hematopoietic cells[70]. By tapping this potential, HSC transplantation has been utilized worldwide[71-83]. In particular, allogeneic HSC transplantation using the bone marrow or placental/umbilical cord blood (CB) is achieving effective results in the therapy of various hematopoietic diseases such as leukemia, lymphoma, myelodysplasia, aplastic anemia, hemoglobinopathies, metabolic storage diseases, and immunodeficiencies[82,83]. In addition, this medical treatment has also been used in victims of accidental irradiation for the reconstitution of their hematopoietic function[30]. In the case of a lethal neutron-irradiation nuclear accident that occurred in Japan in 1999, one patient received a bone marrow transplantation while another received a CB transplantation[84-86].

We have reported the potential efficacy of human mesenchymal stem cell-like stromal cells prepared from CB for hematopoietic regeneration from irradiated hematopoietic stem/progenitor cells (HSPCs)[87]. Although the fresh HSPCs were often used in our study as well as other studies because of the close connection to a hospital, the general supply of HSPCs depends on various companies, institutions, and cell banks that cryopreserve them for clinical and experimental use. On the other hand, the risk estimation for radio-sensitive hematopoietic systems and the emergent treatment of radiogenic sickness during space flight have been necessary for the development of space programs[88]. The use of cryopreserved human HSPCs is one of the methods utilized in these therapy. However, the proliferating capacities of cryopreserved HSPCs are generally quite lower in comparison to those of freshly prepared HSPCs[89,90]. Furthermore, a precise investigation regarding the *ex vivo* co-culture system for expansion of cryopreserved HSPCs is much needed.

In order to examine these issues, we cryopreserved and thawed human $CD34^+$ cells prepared from CB, and then evaluated the proliferating capacities of these cells in our

co-culture system of mesenchymal stem cells. In addition, responses of cryopreserved cells to high dose radiation were compared with those of fresh cells.

2. Materials and Methods

Growth factors and reagents

Recombinant human interleukin-3 (IL-3), stem cell factor (SCF) and thrombopoietin (TPO) were purchased from Bio Source (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 Pepro Tech Inc. (NJ, USA). Recombinant human fibroblast growth factor-2 (FGF-2) was provided by Kaken Pharmaceutical Co. Ltd. (Tokyo, Japan). These factors were added at the following concentrations: IL-3 and SCF, 100 ng/ml; TPO, 50 ng/ml; EPO, 4 U/ml; G-CSF, GM-CSF and FGF-2, 10 ng/ml medium. The fluorescence-labeled monoclonal antibodies (mAbs), fluorescein isothiocyanate (FITC)-conjugated anti-human CD13 (CD13), FITC-conjugated anti-human CD34 (CD34), FITC-conjugated anti-human CD41 (gpIIbIIIa, CD41), FITC-conjugated anti-human CD235a (CD235a), phycoerythrin (PE)-conjugated anti-human CD14 (CD14), PE-conjugated anti-human CD38 (CD38), PE-CD41, PE-conjugated anti-human CD105 (CD105), PE-cyanin-5-forochrome tandem (PC5)-conjugated anti-human CD15, PC5-conjugated anti-human CD117 (CD117), and PC5-conjugated anti-human CD123 (CD123) were purchased from Beckman Coulter Immunotech (Marseille, France). PE-conjugated anti-human CD45RA (CD45RA), PE-conjugated anti-human CD73 (CD73), and PE-conjugated anti-human CD110 (CD110) were purchased from Becton Dickinson Biosciences (San Jose, CA). Mouse IgG_1-FITC, mouse IgG_1-PE, mouse IgG_1-PC5, and mouse IgG_{2a}-FITC (Beckman Coulter Immunotech) were used for

isotype controls.

Collection and purification of CBCD34$^+$ cells

This study was approved by The Committee of Medical Ethics of Hirosaki University Graduate School of Medicine (Hirosaki, Japan). After informed consent was obtained from all the mothers, CB for use in this study was collected after full-term deliveries using a sterile collection bag containing the anticoagulant citrate-phosphate dextrose, according to the guidelines of the Tokyo Cord Blood Bank. These samples were separately isolated and used for each experiment. Within 24 hours after the collection of the CB, light-density mononuclear CB cells were separated by centrifugation on a Lymphosepar I (1.077 g/ml, Immuno-Biological Laboratories, Takasaki, Japan) for 30 minutes at 300 ×*g* and washed three times with phosphate-buffered saline (PBS, St. Louis Sigma, USA) containing 5 mM ethylenediaminetetraacetic acid (EDTA). These cells were then processed for enrichment of CD34$^+$ cells according to the manufacturer's instructions. An Easy Sep® human CD34 selection kit (StemCell Technologies, Vancouver, Canada) was used for the positive selection of CD34$^+$ cells. At the end of the procedure, the purity of CD34$^+$ cells as measured by a direct immune-fluorescence flow cytometry (EPICS® XL, Beckman-Coulter, Fullerton, CA) was 63-93%.

Cryopreservation and thawing of purified CD34$^+$ cells

The expression of CD34, CD38, CD45RA, CD110 (c-mpl), CD117 (c-kit), and CD123 (IL-3 receptor) in the purified CD34$^+$ cells was measured by flow cytometry. The purified CD34$^+$ cells were suspended in the freezing medium, containing 60% Isocave's modified Dulbocco's medium (IMDM, Gibco BRL) plus 30% FBS plus 10% dimethyl sulfoxide (Sigma), and then were subsequently cryopreserved at lower than -80ºC using BICELL (Nihon Freezer, Tokyo, Japan) as the cryoprotectant[91-93]. The purified CD34$^+$ cells were thawed and cultured with or without stromal cells after cryopreservation (1 week-10 months). For rapid thawing after cryopreservation, frozen cryogenic vials with

Pink Cap (Becton Dickinson and Company, NJ, USA) were taken from the freezer and immediately placed in a 37ºC water bath until ice crystals disappeared with shaking[93,94]. Thawed $CD34^+$ cells were measured by flow cytometry for the antigen expression as mentioned above.

Culture of placental/umbilical CB-derived mesenchymal stem cell-like stromal cells

Mesenchymal stem cell-like stromal cells were previously established by culturing the mononuclear cells excluded from the positive selection of $CD34^+$ cells in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml) and FGF-2 at 37˚C in a humidified atmosphere with 5% CO_2 in air. The culture medium was exchanged twice per week. The adherent and expanded cells were considered to be the mesenchymal stem cell-like stromal cells, which were used to perform the co-culture with allogeneic HSPCs. The confirmation that the harvested cells were mesenchymal stem cell-like stromal cells was determined by their expression of antigens characteristic for mesenchymal stem cells, such as CD73, CD90, CD105 and HLA-ABC, using flow cytometry[87].

In vitro irradiation of cryopreserved $CD34^+$ cells

The $CD34^+$ cells were irradiated with a dose of 2 Gy at a dose rate of 0.8 - 0.9 Gy/min of X-rays (200 kVp, 15 mA) with 0.3 mm copper and 0.5 mm aluminum filters at a distance of 45 cm from the focus.

Co-culture of cryopreserved $CD34^+$ cells with stromal cells

Mesenchymal stem cell-like stromal cells were initially cultured in DMEM containing 10% FBS and FGF-2 in a 24-well plate (Becton Dickinson and Company) at a concentration of 3×10^4 cells/0.5 ml/well. The next day, the stromal cells were irradiated with a dose of 20 Gy at a dose rate of 3.9 - 4.2 Gy/min of X-rays (200 kVp, 15

mA) with a 2.0 mm aluminum filter at a distance of 35 cm from the focus to suppress growth. On day 3 after irradiation, the non-irradiated or $CD34^+$ cells irradiated with 2 Gy (2.5×10^2 cells/0.5 ml/well for non-irradiated and 2.5×10^3 cells/0.5 ml/well for irradiated) were suspended in serum-free IMDM supplemented with BIT9500 (a serum substitute for serum-free culture, StemCell technologies) and low-density lipoproteins (CALBIOCHEM, Darmstadt Germany), and then cultured with or without stromal cells at 37˚C in a humidified atmosphere with 5% CO_2 in air. All the cultures were incubated in the presence of SCF + TPO + IL-3. On day 14, the suspended cells were harvested and the number of viable cells was determined by Trypan blue exclusion.

Flow cytometric analysis

The expression of specific cell-surface antigens was analyzed by direct immuno-fluorescence flow cytometry using single to triple staining combinations of mAbs, including FITC-CD13, FITC-CD34, FITC-CD235a, PE-CD14, PE-CD38, PE-CD41 and PC5-CD15. Briefly, the cells were incubated with the saturated concentrations of the relevant mAbs for 30 minutes at room temperature. After labeling, the cells were washed with PBS and analyzed by flow cytometry. For each experiment, an isotype-matched irrelevant mAb was used as a negative control.

Methylcellulose cultures

The colony-forming cells, including granulocyte-macrophage colony-forming unit (CFU-GM), erythroid burst-forming unit (BFU-E) and granulocyte-erythroid-macrophage-megakaryocyte colony-forming unit (CFU-Mix), were assayed by methylcellulose culturing using MethoCult® (StemCell technologies). The expanded cells were plated onto each well of a 24-well plate (0.3 ml/well) in the culture medium containing EPO, SCF, IL-3, G-CSF and GM-CSF as colony-stimulating factors. Each plate was incubated at 37˚C in a humidified atmosphere with 5% CO_2 in air for 14 days. The colonies containing more than 50 cells were counted under an inverted

microscope (Olympus, Tokyo, Japan).

Plasma clot culture

The megakaryocyte colony-forming unit (CFU-Meg) was assayed by the plasma clot technique using platelet-poor human plasma. Ten percent human platelet-poor AB plasma, SCF, TPO, penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mM), MEM vitamin (1%), MEM non-essential amino acids (1%; GIBCO® Invitrogen), thioglycerol (1×10^{-5} M; Sigma), L-asparagine (2 μg/ml), $CaCl_2$ (74 μg/ml; Wako Pure Chemicals, Tokyo, Japan) and 0.2% bovine serum albumin (BSA, Boehringer Mannheim GmbH) were added to the culture medium based on IMDM. Harvested cells were added to the culture medium at a concentration of 2.5×10^3 cells/ml. This baseline culture was plated onto a 24-well plate at 0.3 ml/well and was subsequently incubated at 37˚C for 14 days in a humidified atmosphere with 5% CO_2 in air.

Identification of megakaryocyte colonies by immunofluorescence

The plasma clot cell cultures were fixed by 15 and 10 minute incubation in acetone: methanol (2:1). The plates were dried in an air flow overnight and stored at -20˚C. Prior to staining, the plates were placed at room temperature, and PBS containing 0.5% BSA (PBS-B) was added to soften the clot. The plates were incubated at room temperature with FITC-CD41 mAb diluted 1:100 in PBS-B for 1 hour followed by a single wash with PBS-B. The nuclei were then counterstained with propidium iodide (0.3 ng/ml, Sigma). After a final wash, the colonies containing more than 3 cells were then counted under an inverted microscope.

Statistical analysis

The data are expressed as the mean ± S.D. The comparisons of two different cultures were performed with Student's *t*-test, Welch's *t*-test or Mann-Whitney's *U*-test. The comparisons before and after cryopreservation were performed by a paired *t*-test or

Friedman test. The *p*-value less than 0.05 was considered to be statistically significant. The statistical analysis was performed using the Excel 2007 software program (Microsoft, USA) with the add-in software program Statcel 2[20].

3. Results

The expression of cell-surface antigens on CD34$^+$ cells before and after cryopreservation.

The expression of cell-surface antigens on fresh and cryopreserved CD34$^+$ cells was analyzed by flow cytometry (**Fig. 12**). After cryopreservation, the population of CD34$^+$/CD38$^-$ cells, which are more immature HSPCs as compared with CD34$^+$/CD38$^+$ cells[61,62], showed a significant increase in comparison to that of fresh cells before cryopreservation (approximately 3.4 ± 1.1% for fresh cells and 5.9 ± 1.3% for cryopreserved cells), although no significant difference was observed in the expression of CD34. On the other hand, the subpopulation of CD34$^+$/CD45RA$^+$ cells, which are progenitors committed to granulocyte and macrophage lineages[95], and the subpopulation of CD34$^+$/CD117$^+$ cells, which are relatively immature HSPCs, showed a significant decrease after cryopreservation (approximately 16.0 ± 5.2% and 14.4 ± 9.2% for fresh cells and 9.3 ± 3.3% and 6.9 ± 5.1% for cryopreserved cells, respectively). No significant difference in the population of CD34$^+$/CD38$^+$, CD34$^+$/CD110$^+$ or CD34$^+$/CD123$^+$ cells before and after the cryopreservation was observed.

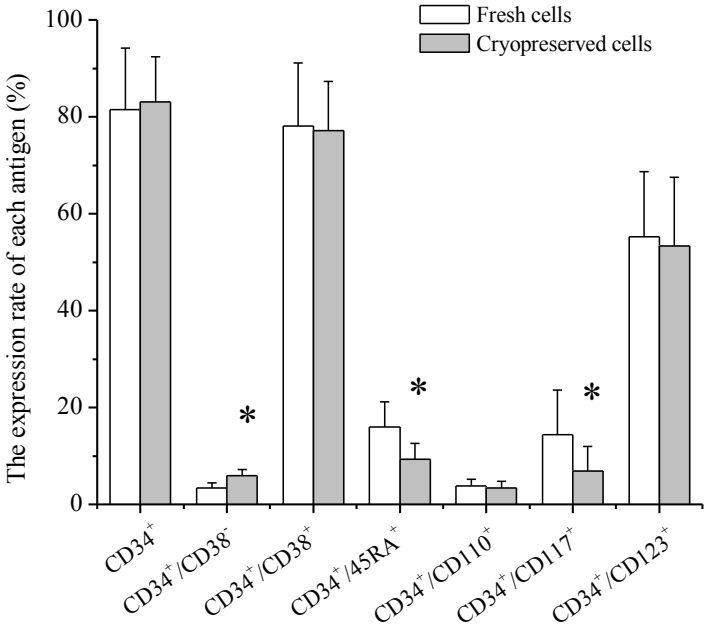


Fig. 12. Changes in surface antigens of purified CD34+ cells before and after cryopreservation and thawing. Each surface antigen of the freshly purified and thawed CD34+ cells was analyzed by flow cytometory. The rate of expression for each antigen is shown. Values represent the mean ± S.D. of six separate experiments. The statistical analysis was performed by a paired t-test. *P<0.05.

Ex vivo expansion of non-irradiated or X-irradiated CD34$^+$ cells after the cryopreservation.

In the culture of non-irradiated cells, the numbers of total and CD34$^+$ cells in the co-culture with stroma cells (approximately 7.9×10^5 and 6.5×10^4 cells/well) significantly increased in comparison to cultures without stromal cells (approximately 3.4×10^5 and 2.5×10^4 cells/well) (**Fig. 13 and 14**). However, no significant difference between the numbers of CD34$^+$/CD38$^-$ cells in the cultures with or without stroma cells was observed (**Fig. 15**).

However, the numbers of total harvested cells, CD34$^+$ cells, and CD34$^+$/CD38$^-$ cells decreased in X-irradiated cells in comparison to the non-irradiated control. No beneficial effect of stromal cells was observed in X-irradiated cells (**Fig. 13, 14 and 15**).

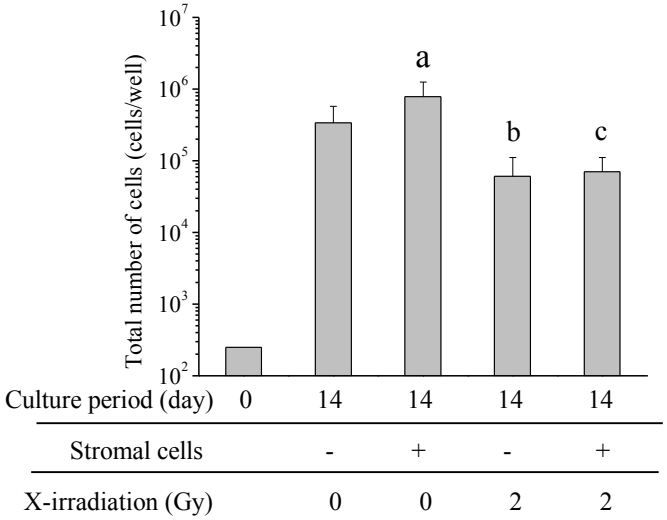


Fig. 13. The total number of cells generated in the liquid culture with or without stromal cells. The cryopreserved CD34+ cells were cultured in the presence of TPO + IL-3 + SCF with or without stromal cells for 14 days. Values represent the mean ± S.D. of ten separate experiments. a $P<0.05$ compared to the culture without stromal cells. b $P<0.05$ compared to the non-irradiated cells in the culture without stromal cells. c $P<0.05$ compared to the non-irradiated cells in the culture with stromal cells.

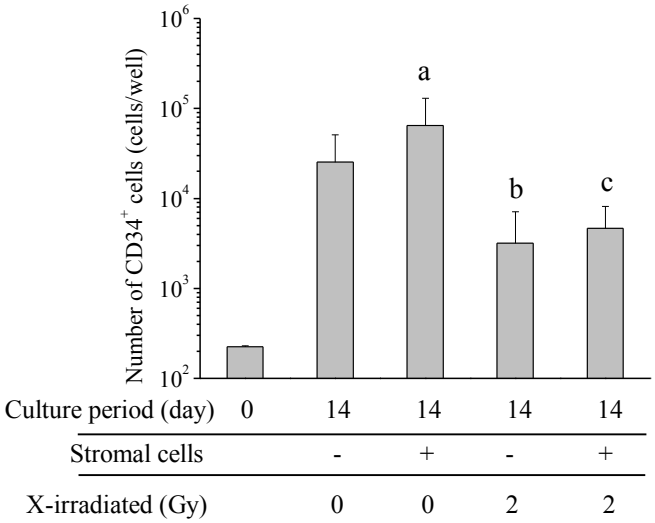


Fig. 14. The number of CD34+ cells generated in the liquid culture with or without stromal cells. Values represent the mean ± S.D. of ten separate experiments. a $P<0.05$ compared to the culture without stromal cells. b $P<0.05$ compared to the non-irradiated cells in the culture without stromal cells. c $P<0.05$ compared to the non-irradiated cells in the culture with stromal cells.

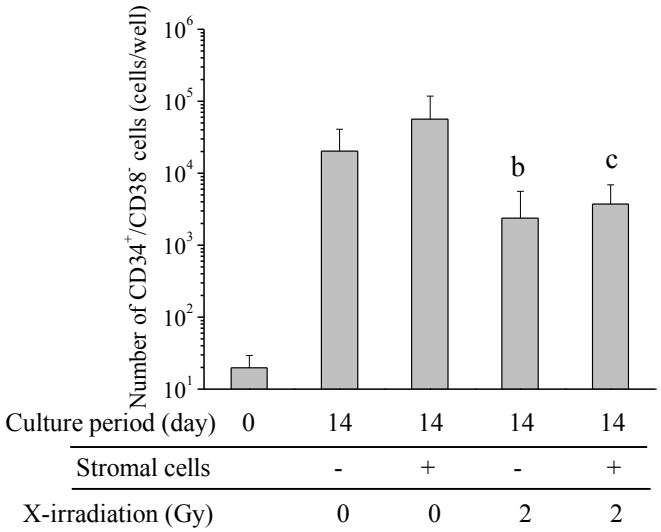


Fig. 15. The number of CD34+/D38- cells generated in the liquid culture with or without stromal cells. Values represent the mean ± S.D. of nine separate experiments. b $P<0.05$ compared to the non-irradiated cells in the culture without stromal cells. c $P<0.05$ compared to the non-irradiated cells in the culture with stromal cells.

In order to determine the proportions of hematopoietic lineage cells contained in the expanded cells, the expression of immature markers (CD34 and CD38), myeloid lineage markers (CD13, CD14 and CD15), megakaryocytic markers (CD41), and erythrocytic marker (CD235a) was analyzed using flow cytometry (**Table 4**). No significant difference in the proportion of each hemopoietic cell lineage was observed between the non-irradiated and X-irradiated cells. On the other hand, the expression of CD41 was significantly decreased by co-culture with stromal cells in comparison to stroma-free culture in both non-irradiated and X-irradiated conditions. Moreover, the culture with stromal cells increased the expression of CD15 of X-irradiated cells in comparison to the stroma-free culture.

Table 4. Phenotypic analysis of the generated cells in the culture with or without stromal cells

Antigen	Stromal cells	0 Gy (%)	2 Gy (%)
$CD13^+$	-	50.7 ± 5.8	53.9 ± 6.4
	+	39.2 ± 8.0	45.4 ± 14.5
$CD14^+$	-	8.9 ± 1.5	10.1 ± 2.1
	+	14.5 ± 5.3	19.1 ± 7.0
$CD15^+$	-	35.8 ± 14.3	31.1 ± 10.6
	+	54.9 ± 9.7	52.9 ± 7.0*
$CD41^+$	-	32.0 ± 11.1	31.9 ± 7.2
	+	7.4 ± 5.3*	11.7 ± 6.2*
$CD235a^+$	-	8.4 ± 3.3	6.7 ± 2.2
	+	6.2 ± 3.3	6.5 ± 3.4

*$P < 0.05$ compared to stromal cells (-) cultured cells by the *t*-test.

Ex vivo expansion of hematopoietic progenitor cells in non-irradiated or X-irradiated $CD34^+$ cells after cryopreservation.

After *ex vivo* expansion, the number of lineage-committed hematopoietic progenitor cells including CFU-GM, which are progenitors for granulocyte-macrophages, BFU-E, which differentiate into erythroblasts and erythrocytes, and CFU-Mix, containing pluripotent hematopoietic progenitors differentiating into erythroid, granulocyte, macrophage and megakaryocytic cells, were assayed using methylcellulose cultures. The total number of hematopoietic progenitor cells, which are colony-forming cells (CFC), was obtained from the sum of each value of CFU-GM, BFU-E, and CFU-Mix (**Fig. 16**). In the culture of non-irradiated cells, there was a significant difference

between the CFC numbers in the culture with and without stromal cells (5.8×10^4 cells and 2.1×10^4 cells, respectively); however, the co-culture with stromal cells did not effect the number of CFU-GM, BFU-E and CFU-Mix (**Fig. 16 and 17**). The co-culture with stromal cells increased the mean values of the number of CFC, CFU-GM, BFU-E and CFU-Mix in comparison to cultures without stromal cells after X-irradiation, although no significant difference was observed. The number of CFU-Meg, which is a progenitor for megakaryocytes and platelets, was assayed using a plasma clot culture. This number tended to decrease after the co-culture with stromal cells in comparison to the cultures without stromal cells in both the non-irradiated and X-irradiated cells (**Fig. 18**).

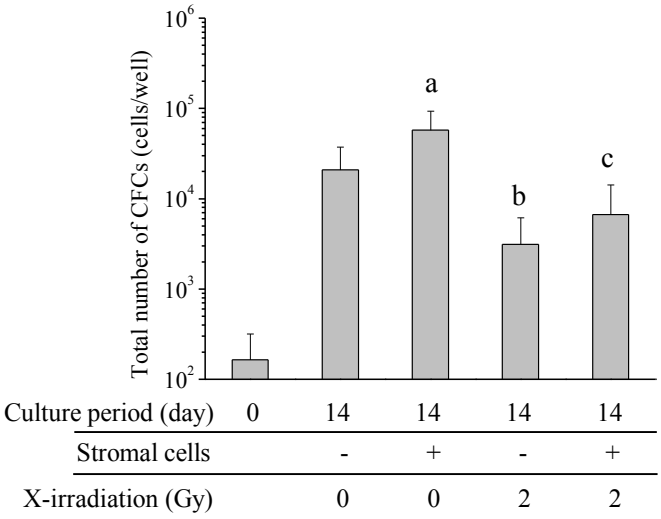


Fig. 16. Total number of CFC generated in the liquid culture with or without stromal cells. The cells harvested from the culture were assayed for the number of CFU-GM, BFU-E, and CFU-Mix using a methylcellulose culture. Values represent the mean ± S.D. of ten separate experiments. a $P<0.05$ compared to the culture without stromal cells. b $P<0.05$ compared to non-irradiated cells in the culture without stromal cells. c $P<0.05$ compared to the non-irradiated cells in the culture with stromal cells.

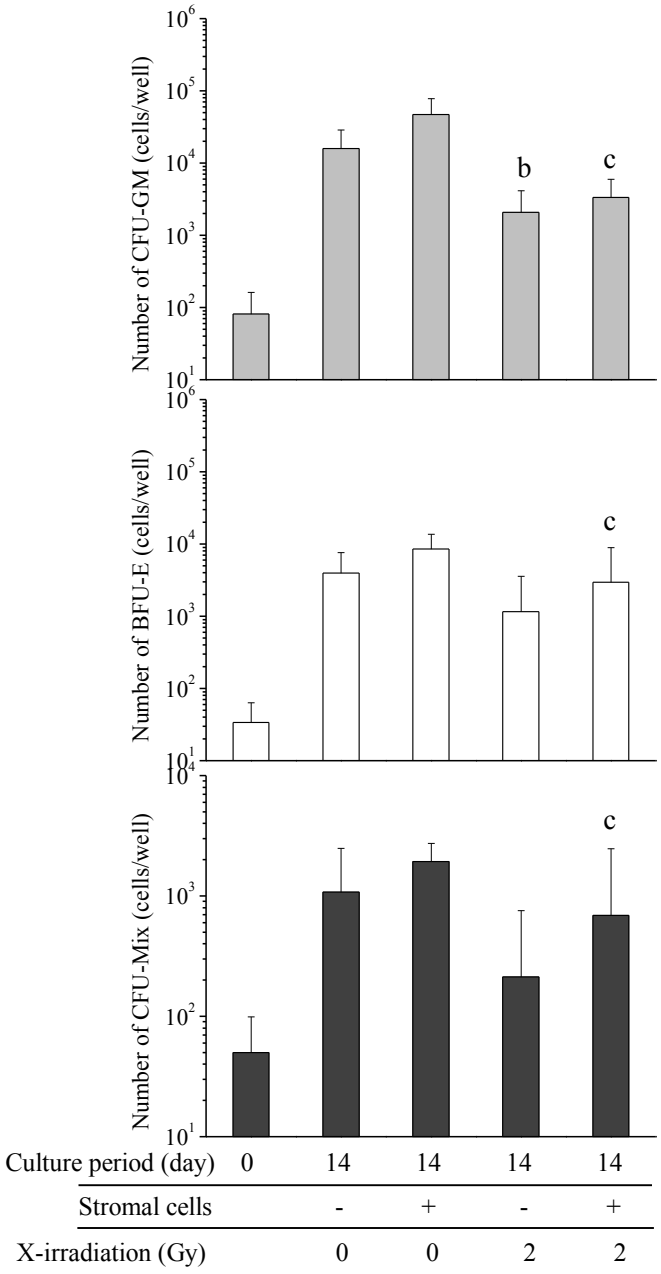


Fig. 17. The number of each progenitor cells generated in the liquid culture with or without stromal cells. A. CFU-GM, B. BFU-E and C. CFU-Mix. Values represent the mean ± S.D. of nine or ten separate experiments. b $P<0.05$ compared to non-irradiated cells in the culture without stromal cells. c $P<0.05$ compared to the non-irradiated cells in the culture with stromal cells.

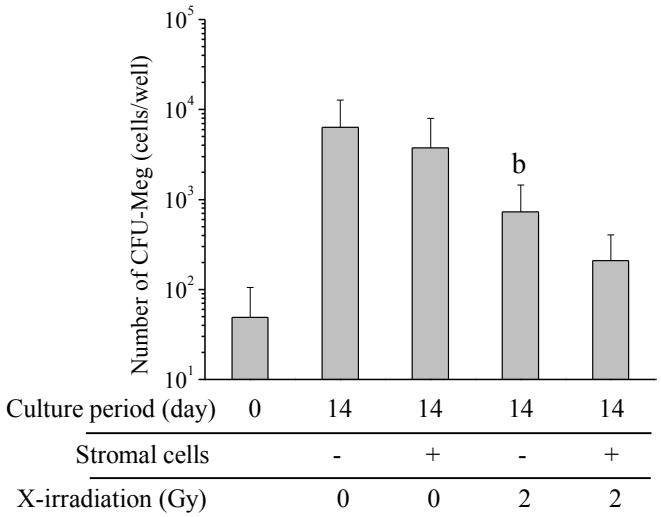


Fig. 18. The number of CFU-Meg generated in the liquid culture with or without stromal cells. The cells harvested from the culture were assayed for the number of CFU-Meg using the plasma clot technique. Values represent the mean ± S.D. of five or six separate experiments. b $P<0.05$ compared to the non-irradiated cells in the culture without stromal cells.

4. Discussion

Previous reports demonstrated that immature HSPCs, such as $CD34^+/CD38^-$ cells, have a capacity to survive environmental stress such as extremely low temperature[30,96,97]. Meanwhile, Broxmeyer *et al.* have reported that CB can be stored frozen for at least 15 years with highly viable and functional hematopoietic stem/progenitor cells for successful CB transplantation[98]. The present study also demonstrated an increase in the population of $CD34^+/CD38^-$ cells after cryopreservation, although no changes were seen in expression rate of CD34. However, the growth rate of $CD34^+/CD38^-$ cells in the culture of non-irradiated cryopreserved cells was lower than that in the culture of the fresh cells shown in our previous study, although the growth rates of total cells and $CD34^+$ cells were equivalent to that in the fresh cells (approximately 1600- and 110-fold increase for fresh cells and 1300- and 110-fold increase for cryopreserved cells, respectively)[87]. Consistent with these results, the number of each progenitor cell, CFU-GM, BFU-E and CFU-Mix, after the cryopreservation was smaller than that obtained in the fresh cells, but the number of CFU-Meg was not decreased (**Figs. 17 and 18**). These results were consistent with the previous reports that the proliferating capacities of cryopreserved HSPCs are generally quite lower in comparison to the freshly prepared HSPCs[89,90]. However, the final constitution of hematopoietic cells from cryopreserved HSPCs may be normal because of the existence of cryo-resistant immature cells after thawing. A slow proliferation rate in cryopreserved HSPCs may result from the loss of cryo-sensitive hematopoietic progenitor cells such as $CD34^+/CD45RA^+$ and $CD34^+/CD117^+$ cells. On the other hand, the capacity to amplify $CD34^+$ cells and $CD34^+/CD38^-$ cells from cryopreserved cells in the co-culture with stromal cells was lower than that of the fresh cells as shown in our previous study (approximately 6.4- and 6.8-fold increase for fresh cells and 2.6- and 2.8-fold increase for cryopreserved cells compared to that of cultures without stromal cells, respectively), although the capacity to amplify total cells and CFC from

cryopreserved cells in the co-culture with stromal cells was equivalent with that of the fresh cells. These results suggest that the interaction of cryo-resistant cells and stromal cells for self-renewal is lower or that the stress of freezing and thawing may induce damage to cell-to-cell interaction process, including Notch, Frizzled, or Wnt receptors. Notch and Wnt signaling have been known to maintain, proliferate and control differentiation in the HSPCs[57].

In the culture of X-irradiated cells, the growth rates of $CD34^+$ cells and $CD34^+/CD38^-$ cells were slightly higher than in the culture of the fresh cells as shown in our previous study (approximately 13% and 12% [**Figs 14 and 15**] and 10% and 9% [**Fig. 6 and 7**], respectively). The generation of BFU-E, CFU-Mix and CFU-Meg was also greater than in the culture of the fresh cells (**Figs. 9, 10, 17 and 18**). These results suggest that cryo-resistant cells might possibly contain radio-resistant cells. Xiao and Dooley reported that higher levels of anti-apoptotic proteins Bcl-2 and Bcl-x were observed in $CD34^+$ cells as compared with the mononuclear cells, containing some hematopoietic lineage[99]. These findings raise the possibility that immature cells, such as $CD34^+/CD38^-$ cells, but not $CD34^+/CD45RA^+$ nor $CD34^+/CD117^+$ cells are radio-resistant cells with a higher level of intracellular Bcl-2. On the other hand, the capacity of stromal cells to support expansion of all the types of cells in cryopreserved cells after X-irradiation was not strong (**Figs. 13 – 18**). In particular, the capacities of stromal cells to support expansion of the $CD34^+$ cells and $CD34^+/CD38^-$ cells in X-irradiated cryopreserved cells were much lower than that obtained by the fresh cells (approximately 6.9- and 6.5-fold increase for fresh cells and 1.5- and 1.6-fold increase for cryopreserved cells as compared with cultures without stromal cells, respectively). These results suggest that the process of freezing and thawing largely interferes with HSPCs receiving the beneficial effect of stromal cells and that the interaction between HSPCs and stromal cells is important to protect the cells from radiation damage.

In order to evaluate the effects of cryopreservation on *ex vivo* hematopoiesis, the specific cell-surface antigens were analyzed (**Table 4**). Zweegman *et al.* suggested that

stromal sulfated glycoconjugates contained in human bone marrow stroma inhibited a megakaryocytic differentiation step[100]. In the present study, a decrease in CD41 expression in the co-culture with stromal cells was observed similar to our previous data on using fresh cells. However, in contrast to fresh cells, co-cultures of cryopreserved cells with stromal cells increased the mean rate of CD15-positive cells and irradiated cells significantly showed increased expression of CD15 in comparison to control cells without stromal cells (**Table 3 and 4**). These results show that the responses of HSPCs to stromal cells may change after cryopresevation, thereby promoting the differentiation into the myeloid cell lineage.

In conclusion, the present data demonstrate that the stress of cryopreservation influences the proliferative capacity of HSPCs, suggesting that consideration of these influences is necessary in experiments using cryopreserved HSPCs. In particular, the present results indicate that cryo-resistant cells might possibly include radio-resistant cells. However, the placental/umbilical CB-derived mesenchymal stem cell-like stromal cells did not affect the capacities of proliferation and sustaining of immature cells in the culture of irradiated cells after the cryopreservation/thawing process. The treatment of emergent radiogenic sickness has been developed for long stays at the International Space Station and/or a trip to Mars in the future[101]. Langell et al. noted that galactic cosmic radiation and solar energetic particles are known to represent two types of ionizing deep-space radiation. Especially, the largest of solar particle events produce solar particles with a lethal dose[102]. The findings of this report support treatment with high-dose radioprotectant amifostine 30 min prior to undergoing radiation exposure as a pharmacological countermeasure. On the other hand, hematopoietic stem cells must be transplanted to individuals exposed with such a lethal dose immediately after irradiation. At this time, cryopreserved HSPCs, such as a backup of HSPCs, are now scheduled to be taken up into space and they will receive combined damage due to both cryopreservation and irradiation. In space, the cryopreserved HSPCs will receive not only low-linear energy transfer (LET) radiation, such as X-ray and γ-ray, but also high-LET radiation,

such as proton and α-particle[103]. Therefore, further investigation of the effect of high-LET radiation on the cryopreserved HSPCs will be needed for in the future.

Conclusions

All the results Chapter 1 to Chapter 3 indicate as follows:

1. The radiosensitivity of iPS cells is low compared with other stem/progenitor cells, especially HSPCs. In addition, radiosensitivity with regard to gene expression differs at varias stages in the early differentiation pathways of iPS cells that lead to the formation of the 3 germ layers; the sensitivity is the highest in the genes expressed during the differentiation of iPS cells, leading to the formation of the endoderm.
2. The potential efficacy of human CB-derived mesenchymal stem cell-like stromal cells for hematopoietic regeneration from irradiated HSPC.
3. The stress of cryopreservation influences the proliferative capacity of HSPCs. In particular, cryo-resistant cells might possibly include radio-resistant cells. However, the placental/umbilical CB-derived mesenchymal stem cell-like stromal cells did not affect the capacities of proliferation and sustaining of immature cells in the culture of irradiated cells after the cryopreservation/thawing process.

Taken together, all the results from Chapter 1 to Chapter 3 above-mentioned may contribute as useful information when knowing the influence of radiation on the each stem cells proliferation and differentiation from the irradiated stem cells. It is necessary to assess the function of mature cells derived from irradiated iPS cells through differentiation into the 3 germ layers, in the study on irradiated iPS cells. In the study for irradiated HSPCs, the optimal protocol for the establishment of CB-derived stromal cells further research is required to establish a more effective ex vivo expansion system. In addition, further investigation of the effect of high-LET radiation on the cryopreserved HSPCs will be needed for in the future.

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要旨

生体幹細胞の分化・増殖に対する放射線の影響

林　直樹

弘前大学大学院保健学研究科保健学専攻　放射線技術科学領域

幹細胞は自己複製能と多分化能を有する細胞である．その幹細胞には大きく分けてES細胞やiPS細胞のような多能性幹細胞と，造血幹細胞や間葉系幹細胞のような体性幹細胞に分けられる．それら幹細胞が多量の放射線に曝露されると増殖能を失い個体の発生阻害もしくは個体の死を招く．しかしながら，多能性幹細胞，特にiPS細胞の放射線への影響についてはほとんど検討されていない．一方，造血組織は放射線感受性の高い組織として知られている．放射線曝露後の造血維持には，造血幹細胞の増殖及び未分化維持能が必要となってくるが，サイトカイン刺激のみでは幹細胞の未分化維持が困難であることが多い．本研究では多能性幹細胞及び造血幹/前駆細胞への放射線の影響を明らかにし，さらに造血幹/前駆細胞と間葉系幹細胞を共培養することによる増殖及び未分化維持能への効果について検討した．

先ず，マウス多能性幹細胞の増殖及び分化に及ぼす電離放射線の影響について検討した．iPS細胞に放射線曝露したところ，造血幹/前駆細胞よりも放射線感受性が低いことが確認された．また，放射線曝露の有無によって胚様体の形成に影響しなかったが，そのサイズは線量依存的に低下した．一方，分化能は放射線非曝露iPS細胞由来では内胚葉系への誘導が促進されていたが，放射線曝露iPS細胞由来ではその分化誘導が抑制された．次に，臍帯血由来間葉系幹細胞の放射線曝露ヒト$CD34^{+}$細胞への造血支持能力を検討した．臍帯血由来間葉系幹細胞を樹立し，放射線非曝露及び曝露造血幹/前駆細胞を共培養した結果，放射線曝露の有無に関わらず間葉系幹細胞の造血支持能が示された．特に，細胞間接触が重要な役割を果たしていることが示唆された．一方，一度凍結され

た造血幹/前駆細胞では，間葉系幹細胞との共培養における造血支持能が確認できなかった．本研究の結果，iPS 細胞は放射線感受性が低く，放射線に対する防御機構が備わっている可能性が示唆された．また，分化能に対する放射線の影響は一様ではなく，内胚葉系組織への分化誘導は抑制されるが，その他の細胞への分化には影響しないことが示唆された．一方，造血幹細胞は放射線曝露された場合も，存在する環境においてその影響が異なり，間葉系幹細胞と一緒に置かれることにより，増殖及び未分化維持能に影響することが示唆された．しかしながら，造血幹細胞を凍結すると，間葉系幹細胞の効果が低下することが示唆された．

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