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Differential radiation sensitivity to morphological, functional and molecular changes of human thyroid tissues and bone marrow cells maintained in SCID mice

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ABSTRACT

Morphology and function of human organs and tissues are well maintained in the improved SCID (severe combined immunodeficient) mice for a long period (~3 years). To study the radiation-induced damage on human thyroid gland, human thyroid tissues transplanted to SCID mice were consecutively exposed to X-rays or ^{137}Cs γ-rays at high and low dose rates for approximately 2 years. Consecutive irradiation resulted in the disappearance of follicles and significant decrease of thyroid hormone secretion. Mutations in *p53* and *c-kit* genes were induced significantly in human thyroid tissues from old head and neck cancer patients (av. 56.8 years, 4 males) and a Graves' disease patient (20 years, male) over the dose of 24 Gy (44.7 ± 5.9 Gy, mean ± S.E) and 11 Gy (20.2 ± 7.8 Gy), respectively, while mutations were not detected at lower doses nor in unexposed matched controls ($p < 0.01$). There were significant differences in mutation frequency in the transplanted human thyroid tissues (31 years, female) between high dose rate (1.19 Gy/min; 8 in 20 tissues) and low dose rate (0.00023 Gy/min; 0 in 14 tissues) exposures ($p < 0.01$). Mutations were not detected in *RET*, K-*ras* and *β-catenin* genes. Expression analysis by GeneChip indicated that gene expression was also well maintained in the transplanted human thyroid tissues. However, lower doses (1 or 3 Gy) of ^{137}Cs γ-rays can induce changes in gene expression in the transplanted human thyroid tissues. Furthermore, fatally irradiated SCID mice could survive with human bone marrow cell transplantation. When about half of mouse bone marrows were replaced by human bone marrow cells, the human bone marrow cells showed high sensitivity to γ-irradiation; 28.0% and 0.45% survival after 0.5 and 2.0 Gy exposures, respectively.

1. Introduction

Radiation induces various types of damage in human and animals. Abundant experimental studies, both *in vivo* and *in vitro*, have been carried out for the estimation of radiation risk to humans [1]. However, it is of utmost importance to study the direct effects of radiation on human organs and tissues for investigating the mechanism and precise risk.

In the improved severe combined immunodeficient (super-SCID) mice, not only malignant and benign human tumors, but also normal human organs and tissues, are well maintained in morphology and function for a long period (~3 years) by the consecutive transplantation of these tissues, although it has not been possible to maintain normal human organs and tissues in athymic nude mice [2–7]. For example, no substantial histological changes were observed in the human thyroid tissues maintained in SCID mice for 18 months, and rapid and high uptake of radioiodine into the transplanted human thyroid tissue was observed [7]. Furthermore, transplanted human thyroid tissues secreted thyroid hormone (T3), and T3 secretion was stimulated by the injection of human thyroid stimulating hormone (TSH) [7]. Human bone marrow cells

Abbreviations: SCID, severe combined immunodeficiency; SPF, specific pathogen free; SSCP, single strand conformational polymorphism.

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injected *i. v.* into SCID mice remained in the SCID mice, and human immunoglobulins were detectable in the peripheral blood of the SCID mice [8,9]. These findings suggest that the improved SCID mice will provide an invaluable experimental system for investigating the mechanism of radiation effects on human organs and tissues.

The thyroid gland is one of the most important endocrine organs for the development and growth of man and animals, and one of the most sensitive organs to radiation and endocrine disrupters. Radiation exposure, therefore, causes general disorder in human beings by inflicting serious damage to the thyroid gland itself, though radiation is used beneficially for therapeutic purposes to the organs [1]. Bone marrow is also one of the most sensitive organs to ionizing radiation. These two organs are organs of high sensitivity in humans for radiation carcinogenesis, showing the higher risk of thyroid cancer and leukemia in the populations exposed to X-rays and nuclear radiations.

In the present study, human thyroid gland was transplanted into the improved SCID mice, and consecutively exposed to X-rays or ^{137}Cs γ-rays for approximately 2 years to confirm the direct link between radiation exposure at low and high dose rates and the induced changes in morphology, function, and cancer-related genes, *p53*, K-*ras*, *c-kit*, *β-catenin* and *RET* [10–12]. Changes in gene expression in the transplanted human thyroid tissues after lower doses of radiation for a short period were examined to detect early changes. Furthermore, effectiveness of human bone marrow transplantation into the fatally irradiated SCID mice and radiation sensitivity of these human bone marrow cells were examined.

2. Materials and methods

2.1. Human thyroid tissues and bone marrow cells

Normal thyroid tissues were obtained with permission from 4 patients with hypopharyngeal cancer (2 cases; 56 years, males), esophageal cancer at neck region (one case; 70 years, male) and laryngeal cancer (one case; 45 years, male). In all cases, it was mandatory to resect the thyroid gland with cancer, because cancer had invaded into the surrounding tissues of the thyroid gland. Histologically, none of the cases showed any invasion into the thyroid gland. Thyroid tissues resected from 3 Graves' disease patients (20 years male and female, and 31 years female) were also used with consent for heterotransplantation to SCID mice. Goiter was resected because of cosmetic problems, and blood level of thyroid hormone of the patients before the surgical resection was within normal range.

Frozen human bone marrow aspirates were used for bone marrow transplantation with the consent of the donor. Human bone marrow cells were prepared just before *i. v.* or *i. p.* injection into SCID mice.

Only the human tissues and cells free of mycoplasma, human hepatitis B and C antigens/antibodies, adult T cell leukemia, human immunodeficiency virus, and Wasserman reaction were accepted into the SPF room of the barrier section of the Institute of Experimental Animal Sciences, Osaka University. Use of human tissues and cells was permitted by the ethics committees of Osaka University, Graduate School of Medicine and Kuma Hospital, and all experiments were performed following the guidelines of the Ministry of Education, Science and Culture and the Ministry of Health and Labor for the use of human tissues.

2.2. SCID mice

Inbred C.B17-*scid*/*scid* mice (F_{43-52}) were used for the experiments. C.B17-*scid*/+ male and female mice were provided by Bosma [13], Institute of Cancer Research, Philadelphia, in 1986, and then C.B17-*scid*/*scid* mice were maintained by selective brother × sister inbreeding of C.B17-*scid*/*scid* homozygotes showing undetectable serum IgG and IgM (<1 μg/ml) by T. Nomura to diminish the leaky and leukemic mice [2,3,14]. Mice were maintained in the complete barrier condition, lit from 4:00 to 18:00, at 23 ± 1 °C and 50–70% humidity with autoclaved mouse diet CRF-1 (Charles River Japan, Kanagawa, Japan) and acidified, chlorinated, and filtrated (by MILLIPORE) water. Serum IgG and IgM were examined at 4–6 weeks after birth by enzyme-linked immunosorbent assay [2,3], and 2 months old C.B17-*scid*/*scid* mice showing undetectable serum IgG and IgM (<1 μg/ml) were used for the heterotransplantation of human thyroid tissues and bone marrow cells. All animal experiments were carried out in the barrier section of the Institute of Experimental Animal Sciences following the Osaka University Guidelines for Animal Experimentation.

2.3. Maintenance of human thyroid tissues in SCID mice

Procedures for the heterotransplantation of human organs and tissues into the SCID mice were reported previously [2–7]. Briefly, a partially resected human thyroid gland was cut into 5–6 mm cubic masses in a 0.9% NaCl solution containing high concentrations of antibiotics (penicillin G; 50,000 units/ml, panipenem; 25 mg/ml, and streptomycin sulfate; 50 mg/ml). Mice were anesthetized with 0.77% tribromoethanol (Aldrich Chemical Co. Ltd., Milwaukee, WI, USA), and human thyroid tissues were implanted *s. c.* into the back of SCID mice by the surgical operation. To the SCID mice transplanted with normal thyroid tissues from cancer patients, human thyroid stimulating hormone (TSH, 20 μU/g body weight) was injected *i. p.* three times per week to maintain the function of the transplanted normal thyroid tissue [7]. TSH was not given to the SCID mice with thyroid tissues of Graves' disease which is characterized by stimulating autoantibodies to the TSH-receptor [15]. When mice died or were dying, transplanted human thyroid tissues were removed and transplanted to other SCID mice by the same procedure (Fig. 1).

2.4. Radiation exposure of human thyroid tissue

About three quarters of normal human thyroid tissues from each cancer patient were exposed to 5 Gy of X-rays at a dose rate of 1.13 ± 0.016 Gy/min (mean ± 95% CL) by X-ray apparatus Shimazu SHT 250M-3 (Shimazu Co., Kyoto, Japan) at 34 cm distance, 180 kVp and 20 mA with a filter of 0.5 mm Cu and 1.0 mm Al. Dosimetry was made each time by Condenser R-meter 500 Radcon with 550-3 probe (Victoreen Instr. Div. Cleveland, OH) adjusted to the standard ^{60}Co γ-ray source. The irradiated thyroid tissues were transplanted *s. c.* to the SCID mice. After transplantation, SCID mice with thyroid tissue xenografts were exposed monthly to 2 Gy of X-rays. When SCID mice died or became moribund, human thyroid tissues were removed, irradiated with 5 Gy of X-rays, and then transplanted to other SCID mice. The other one-quarter thyroid tissues from each cancer patient were unirradiated and served as matched controls.

Human thyroid tissues from Graves' disease patients were exposed to ^{137}Cs γ-rays at high and low dose rates. The high dose rate exposure was given by Gammacell 40 Exactor (Nordion International Inc., Canada) at a dose rate of 1.19 Gy/min. The low dose rate exposure was given at 0.00023 Gy/min (1 Gy/3 days) by Computed ^{137}Cs γ-ray Exposure Instrument SK-951 (Sangyo Kagaku, Osaka, Japan). About a half of human thyroid tissues from a Graves' disease patient (20 years, male) were exposed to 5 Gy of ^{137}Cs γ-rays at high dose rate, and then transplanted *s. c.* to the SCID mice. SCID mice with human thyroid tissue xenografts were exposed biweekly to whole body dose of 1 Gy. The other half of thyroid tissues from the same patient were similarly transplanted, but unirradiated as matched controls. To examine the dose rate effect of radiation, human thyroid tissues from another Graves' disease patient (31 years, female) were exposed weekly to 1 Gy of ^{137}Cs γ-rays at high and low dose rates after transplantation. For gene expression analysis, thyroid tissues from a third Graves' disease patient (20 years, female) were used.

2.5. Measurement of thyroid hormone (T3)

Peripheral blood samples were taken from the SCID mice to which human thyroid tissues had been transplanted. Human thyroid hormone T3 was measured by the radioimmunoassay (T-3·RIABEAD, Dinabot, Tokyo, Japan) as reported previously [7,16].

2.6. Examination of p53, K-ras, c-kit, β-catenin, and RET mutations

PCR and single strand conformational polymorphism without radioisotopes (Cold SSCP) analysis followed by direct sequencing were carried out to examine mutations in *p53*, K-*ras*, *c-kit*, *β-catenin*, and *RET* genes for the original and transplanted human thyroid tissues as described previously [16–19]. Briefly, DNA was extracted from the frozen specimens using QIAmp DNA Mini Kit (QIAGEN Inc., Valencia, CA, USA), and amplified by PCR (GeneAmp PCR System 9700, PerkinElmer, Norwalk, CT, USA). PCR products were subjected to Cold-SSCP. The SSCP bands which showed altered mobility were extracted from the gel and re-amplified by 25 cycles of PCR to enrich the altered bands. The mutated alleles were purified using the QIAquick PCR purification kit (QIAGEN). Direct sequencing was performed from both directions by the dideoxy chain termination method using the Big Dye Terminator cycle sequencing kit (PerkinElmer, Foster City, CA, USA) and automated DNA sequencer (ABI PRISM 310 Genetic Analyzer, PerkinElmer). PCR-SSCP analysis and sequencing results of each sample were confirmed more than twice from different PCR products of the same thyroid tissues. The PCR primers and sequencing condition are listed in Table 1. Exons 4 and 5 of *p53* gene were divided to two regions.

2.7. Analysis of gene expression in the human thyroid tissue after irradiation

The other line of investigation concerns early changes in gene expression of the human thyroid tissues exposed to lower doses of radiation. Human thyroid tissues from another Graves' disease patient (20 years, female) were transplanted *s. c.* to the SCID mice. In the first experiment, transplanted thyroid tissues were not irradiated

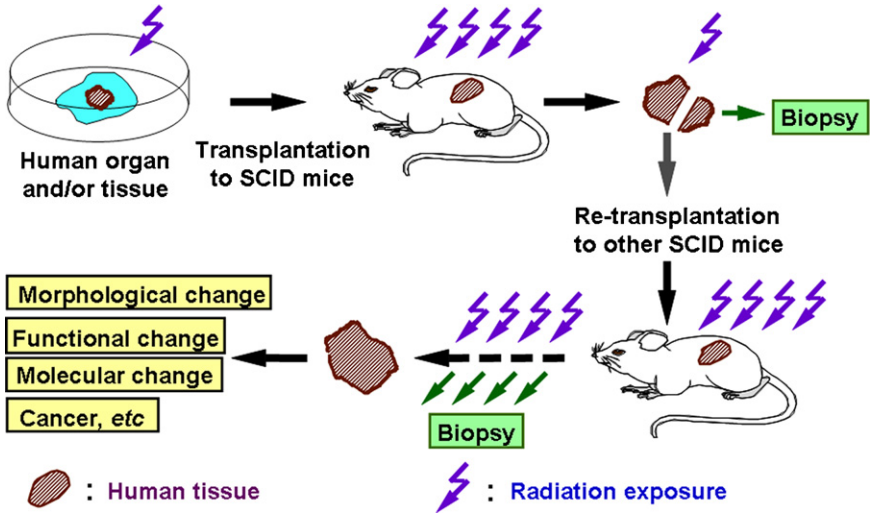

Fig. 1. Experimental procedure to maintain human organs and tissues for long period in the improved SCID mice. See details in Section 2.

and removed 1, 2, 3 and 4 weeks after transplantation, and the level of gene expression was compared with original (surgically resected) thyroid tissues by GeneChip (HG Focus Array, Affymetrix, Inc., Santa Clara, CA, USA). In the second experiment, human thyroid tissues transplanted *s. c.* to the SCID mice were unexposed or exposed once to 1 or 3 Gy of ^{137}Cs γ-rays at high dose rate (1.07 Gy/min) 4 days after transplantation. Two weeks after irradiation, thyroid tissues were removed, and changes in gene expression were compared between irradiated thyroid tissues and unexposed concurrent control tissues from the same donor. Transplanted human thyroid tissues removed from the SCID mice were kept in liquid nitrogen.

To extract RNA in high efficiency, thyroid tissues were crushed by pressing in frozen metal blocks (Cryopress, Microtech-Nichion, Funahashi, Japan) and then the powdered specimens were homogenized by microtube homogenizer (Homogenizer

Table 1
The PCR primer pairs for the amplification of *p53*, K-*ras*, *c-kit*, *β-catenin* and *RET* genes

Genes	Oligonucleotide	Temperature (°C)	
		Annealing	SSCP
p53			
exon 4a	5′-TTTTCACCCATCTACAGTCC-3′ upstream 5′-CAAGAAGCCCAGACGGAAAC-3′ downstream	58	20
exon 4b	5′-CCTGGCCCCTGTCATCTTCT-3′ upstream 5′-AAGAAATGCAGGGGGATACG-3′ downstream	58	20
exon 5a	5′-TCTGTCTCCTTCCTCTTCCTA-3′ upstream 5′-CATGTGCTGTGACTGCTTGT-3′ downstream	57	35
exon 5b	5′-TGTGCAGCTGTGGGTTGATTC-3′ upstream 5′-CAGCCCTGTCGTCTCTCCAG-3′ downstream	62	25
exon 6	5′-TTGCTCTTAGGTCTGGCCCCT-3′ upstream 5′-TAGGGAGGTCAAATAAGCAG-3′ downstream	60	35
exon 7	5′-TGCCACAGGTCTCCCCAAGG-3′ upstream 5′-AGGGGTCAGCGGCAAGCAGA-3′ downstream	60	25
exon 8	5′-TCTTGCTTCTCTTTTCCTAT-3′ upstream 5′-CGCTTCTTGTCCTGCTTGCT-3′ downstream	56	10
K-*ras*			
exon 1	5′-CATGTTCTAATATAGTCACA-3′ upstream 5′-CTCTATTGTTGGATCATATTCGTCC-3′ downstream	48	25
exon 2	5′-ACTGTGTTTCTCCCTTCTCA-3′ upstream 5′-CACAAAGAAAGCCCTCCCCA-3′ downstream	48	5
c-kit			
exon 11	5′-TGATCTATTTTTCCCTTTCTC-3′ upstream 5′-AGCCCCTGTTTCATACTGAC-3′ downstream	56	20
exon 17	5′-CATGGTCGGATCACAAAGAT-3′ upstream 5′-ATTATGAAAGTCACGGAAAC-3′ downstream	58	15
β-catenin			
exon 3	5′-GCTGATTTGATGGAGTTGGA-3′ upstream 5′-GCTACTTGTTGTTCTTGAGTGAA-3′ downstream	56	25
RET			
exon 10	5′-ATTAAAGCTGGCTATGGCAC-3′ upstream 5′-CACTCACCCTGGATGTCTTC-3′ downstream	58	25
exon 11	5′-ATCCACTGTGCGACGAGCTG-3′ upstream 5′-GAAGGTCATCTCAGCTGA GG-3′ downstream	60	25
exon 16	5′-TCTCTTTAGGGTCGGATTCC-3′ upstream 5′-CACACTTACACATCACTTTG-3′ downstream	55	20

Exons 4 and 5 of *p53* were divided into two regions, exon 4a (262 bp), exon 4b.(173 bp), exon 5a (155 bp) and exon 5b (167 bp) for PCR.

PT-101, PA Cosmo Bio., Tokyo, Japan) in TRizol reagent (Invitrogen, Tokyo, Japan). Total RNA was extracted from the homogenate in the TRizol reagent solution as described by manufacturer's protocol. The extracted RNA was purified by RNeasy Mini Kit (Qiagen, Tokyo, Japan). Quantity and quality of the RNA was characterized by spectrophotometer and gel electrophoresis. Two micrograms of total RNA was used to synthesize cDNA and biotinylated cRNA, and for fragmentation of the cRNA. Those procedures were performed according to the manufacturer's protocol (Affymetrix, http//www.affymetrix). The procedures for hybridization, staining and washing, and scanning were carried out as described by Affymetrix protocol. Expression analysis was made by the GeneChip operating software GCOS. The data of gene description were derived from NetAffx Analysis Center (Affymetrix Inc.) on 13 November 2007.

2.8. Radiation exposure of SCID mice and human bone marrow cells

C.B17-*scid* mice were exposed to ^{137}Cs γ-rays at doses 2.0 and 4.0 Gy at high dose rate, and then 0.5 ml of human bone marrow cells (4.7×10^7 ml^{-1}) were injected *i. v.* into SCID mice. The same volume of saline was injected to irradiated mice as concurrent controls. Four months later, bone marrow cells were washed out from the femur of the surviving SCID mice, and stained following the fluorescence *in situ* hybridization protocol for fluorescein isothyanate (F1TC) labeled human chromosome 1-specific paints to identify how many human bone marrow cells survived in the mouse bone marrow.

C.B17-*scid* mice were pre-irradiated with 2 Gy of ^{137}Cs γ-rays at high dose rate, and 24 h later human bone marrow cells (1.2×10^7 cells/mouse) were injected *i. p.* Seven weeks after human bone marrow transplantation, these mice were exposed again to 0, 0.5, 1.0 and 2.0 Gy of ^{137}Cs γ-rays. Bone marrow cells were washed out from the femur of mice 7 days after ^{137}Cs γ-ray exposure, and 10^4 cells were stained with human FITC-labeled anti-human CD3, CD20 and CD33 antibodies. Flow cytometric immunophenotyping was performed on a FACScan spectrofluorometer (Becton Dickinson Immunocytometry System, San Jose, CA, USA). Total numbers of human bone marrow cells in the mouse femur were calculated by multiplying the percent of CD3, CD20 and CD33 positive cells to total washed out cells from mouse femur.

Statistical analyses were carried out by SPSS Statistics System (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Morphological changes in human thyroid tissues by radiation

Gross and histological features of human tissues were well maintained in the improved SCID mice for a long period and over several mouse generations [7]. In the present study, similar results were obtained in human thyroid tissues from head and neck cancer patients and Graves' disease patients (Fig. 2A and D). There was no substantial difference from the surgically resected original human thyroid tissues from the patients. After the consecutive irradiation, however, transplanted human thyroid tissues became small and pale (Fig. 2B and C), and histologically, follicles disappeared with increasing doses of radiation and were replaced by necrotic and connective tissues (Fig. 2E and F).

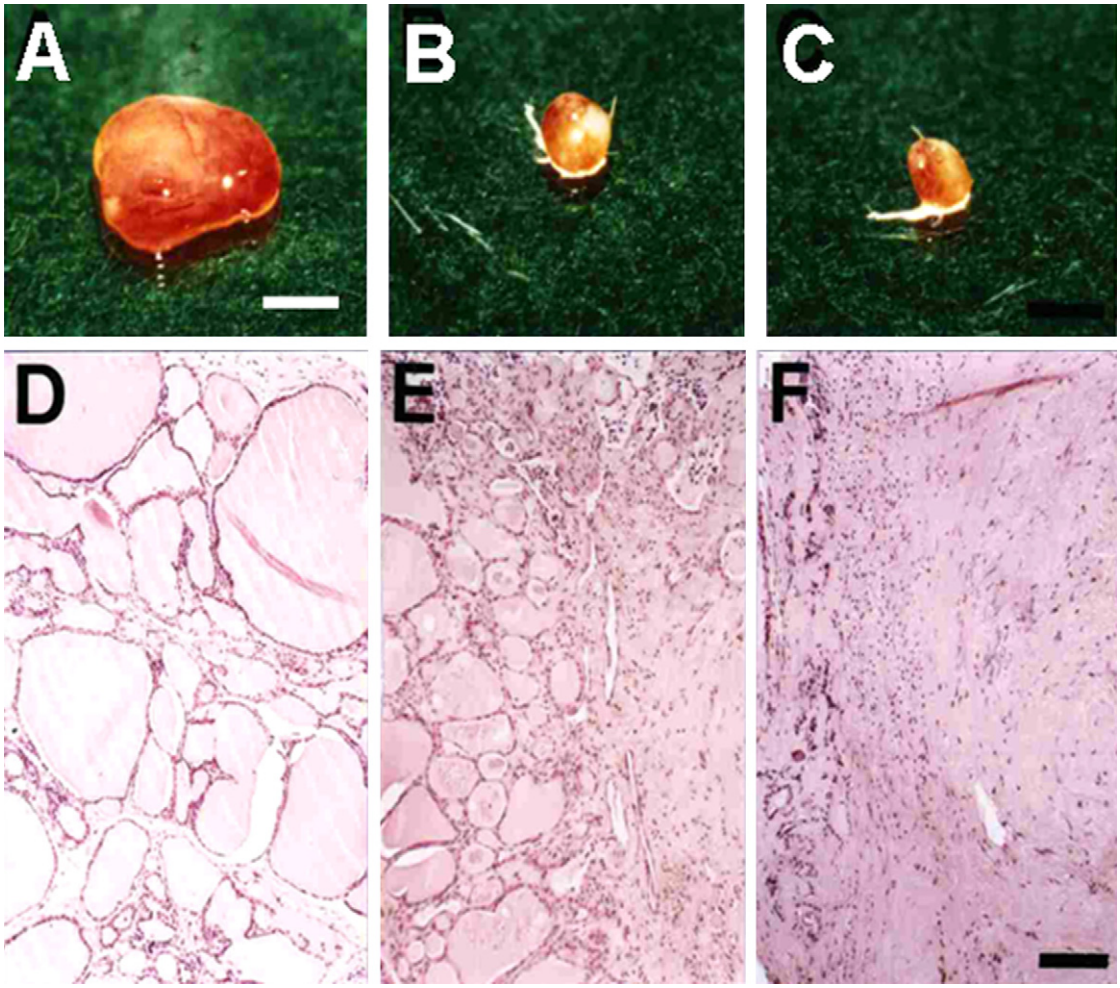

Fig. 2. Macroscopic and microscopic views of transplanted human thyroid tissues with or without irradiation. (A–C) Gross features of human thyroid tissues from a Graves' disease patient (20 years, male) exposed to 0 (A), 18 (B) and 23 (C) Gy of ^{137}Cs γ-rays; photographs of thyroid tissues were taken 30 (A), 26 (B), 36 (C) weeks after transplantation. Similar results were obtained after X-ray exposure. Scale bar: 3 mm. (D–F) Microscopic views of human thyroid tissues from the same patient exposed to 0 (D), 11 (E) and 18 (F) Gy of ^{137}Cs γ-rays, *i.e.*, 30 (D), 13 (E), and 26 (F) weeks after transplantation. Haematoxylin and eosin staining. Scale bar: 100 μm.

Table 2
Human thyroid hormone (T3) in peripheral blood of SCID mice with human thyroid tissues after ^{137}Cs γ-ray exposure

Weeks after transplantation	Exposed			Unexposed[a]		p[b]
	Dose (Gy)	No.	Thyroid hormone (pg/ml)	No.	Thyroid hormone (pg/ml)	
3	6	10	441 ± 28[c]	7	433 ± 14	0.83
9	9	11	334 ± 18	10	420 ± 32	<0.05
13	11	7	331 ± 31	3	443 ± 68	0.12
26	18	7	287 ± 26	4	367 ± 16	<0.05
38	24	4	193 ± 12	9[d]	366 ± 36	<0.01

Thyroid tissues from a Graves' disease patient (20 years, male) were transplanted *s. c.* to the SCID mice and thyroid hormone (T3) of exposed and unexposed groups was measured by radioimmunoassay (see Section 2).
[a] Unexposed matched controls to the exposed groups.
[b] *t*-Test was applied between exposed and unexposed groups after testing quality of variance by SPSS Statistics System.
[c] Mean ± S.E.
[d] 38–80 weeks.

3.2. Functional changes in human thyroid tissues by radiation

Table 2 shows the serum level of human thyroid hormone (T3) detected in the blood of SCID mice which had been transplanted with human thyroid tissues of Graves' disease. T3 was not detectable in the SCID mice without human thyroid tissue transplantation. After the consecutive γ-ray exposure, serum level of T3 decreased gradually and significantly. The results were compatible to the morphological changes. No significant reduction in the level of serum T3 was observed following low dose rate (0.00023 Gy/min) exposure to 8 Gy of ^{137}Cs γ-rays (440 ± 5 pg/ml, n = 4 at low dose rate exposure *vs.* 446 ± 19, n = 4 in unexposed matched controls, mean ± S.E.), while significant reduction was observed by high dose rate (1.19 Gy/min) exposure to 8 Gy (303 ± 69, n = 5, p < 0.05).

3.3. Mutations of p53, K-ras, c-kit, β-catenin and RET genes

Since the involvement of the *p53* tumor suppressor gene and other oncogenes has been reported in thyroid tumors in humans [10–12] and also in radiation-induced thyroid cancer in rats [20], X-irradiated and unirradiated human thyroid tissues (see Section 2) were examined for *p53*, K-*ras*, *c-kit*, *β-catenin* and *RET* mutations. There were no mutations in these loci in any of the original thyroid tissues of 7 donors. However, a variety of mutations were detected in exons 4, 5, and 6 of the *p53* gene and exons 11 and 17 of the *c-kit* gene in the transplanted thyroid tissues following consecutive radiation exposures, though mutations of K-*ras*, *β-catenin* and *RET* genes were not detected (Table 3). Among 11 normal thyroid tissues which received more than 24 Gy of X-rays, six developed mutations in *p53* and *c-kit* genes, while no mutations were detected in 6 thyroid tissues exposed to X-ray doses lower than 20 Gy and in 6 unirradiated matched controls (Table 3). The difference was statistically significant (p < 0.05 by Fisher's exact test). Cumulative incidence of mutations also increased significantly with increasing doses of X-rays (Fig. 3A).

Fig. 3B shows the cumulative incidence of mutations in human thyroid tissues of Graves' disease (20 years, male) which had been exposed to 5 Gy of ^{137}Cs γ-rays before transplantation and exposed biweekly to 1 Gy at high dose rate. Though no mutations were detected in the human thyroid tissues exposed to the doses lower than 10 Gy and in unexposed matched controls, significant increases of *p53* and *c-kit* mutations were observed in the thyroid tissues exposed to doses higher than 11 Gy (p < 0.01, Table 3) and the incidence increased significantly with increasing doses (Fig. 3B). The average age of the donors (cancer patients) for the X-ray experiment was 56.8 years, while the age of Graves' disease patient was 20 years. The average dose for the induction of mutations in the normal thyroid tissue of older donors was 44.7 ± 5.9 Gy (mean ± S.E., n = 6), whereas it was 20.2 ± 7.8 Gy (n = 4) in the case of younger donor (p < 0.05 by *t*-test) (see legend to Table 3 for details). The thyroid tissue of younger age seems more sensitive to radiation-induced mutations than that of older age, as it was the case in radiation induced thyroid cancer in man and rats [1].

Weekly exposures of 20 human thyroid tissues from another Graves' disease patient (31 years, female) to ^{137}Cs γ-rays at high dose rate also induced 8 mutations. However, low dose rate exposure did not induce any mutations in 14 thyroid tissues exposed

Table 3
Radiation-induced mutations in *p53*, *c-kit*, K-*ras*, *β-catenin*, and *RET* genes in human thyroid tissues

Dose (Gy)	Dose rate (Gy/min)	No. of thyroid tissues			Mutations					
		Examined	With mutation	p[a]	No.	*p53*	*c-kit*	K-*ras*	*β-catenin*	*RET*
X-rays										
24–65	1.13	11	6	<0.05	6	2[b]	4[c]	0	0	0
–20	1.13	6	0	–	0	0	0	0	0	0
Control	–	6	0	–	0	0	0	0	0	0
^{137}Cs γ-rays										
11–59	1.19	11	4[d]	<0.01	6	3[e]	3[f]	0	0	0
–10	1.19	13	0	–	0	0	0	0	0	0
Control	–	26	0	–	0	0	0	0	0	0

Human thyroid tissues from laryngo-pharyngeal cancer and esophageal cancer patients and from a Graves' disease patient (20 years, male) were exposed to X-rays and ^{137}Cs γ-rays, respectively. Experimental procedures are described in Section 2.
[a] Fisher's exact test was carried out against control.
[b] Exon 6 codon 209 (*AGA* → *TGA*: Arg → STOP) (24 and 42 Gy).
[c] Exon 17 codon 825 (G*TT* → GCT: Val → Ala) (38, 42, 57, and 65 Gy).
[d] One specimen developed 1 *p53* and 2 *c-kit* mutation.
[e] Exon 4 codon 61 (G*AT* → G*GT*: Asp → Gly) (59 Gy), exon 5 codon 152 (CC*G* → CC*A*: Pro → Pro) (17 Gy), exon 5 codon 175 (CG*C* → CG*T*: Arg → Arg) (11 Gy).
[f] Exon 11 codon 557 (*TGG* → *CGG*: Trp → Arg) (11 Gy), exon 17 codon 825 (G*TT* → GCT: Val → Ala) (11 and 12 Gy).

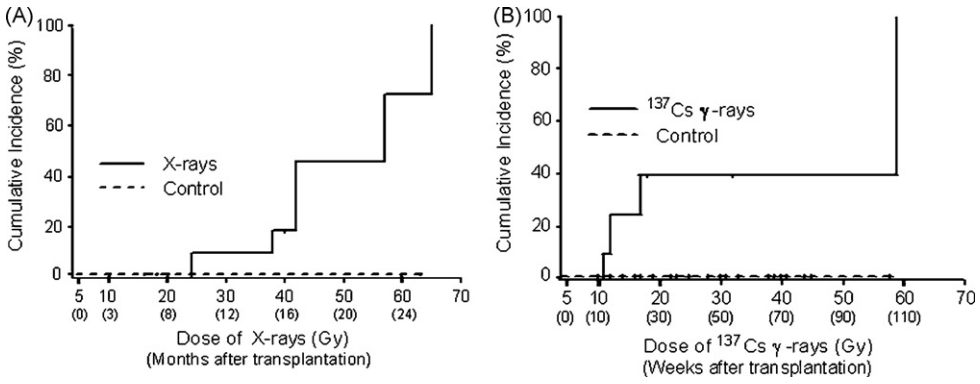

Fig. 3. Cumulative incidence of mutations in human thyroid tissues maintained in SCID mice after X-ray (A) and ^{137}Cs γ-ray (B) exposure. Cumulative incidence of mutation (%) against radiation doses in the remaining human thyroid tissues were analyzed and drawn by SPSS Statistics System (SPSS Inc., Chicago, IL, USA). Figures in parentheses on the abscissa indicate approximate months (A) or weeks (B) after heterotransplantation of human thyroid tissues. Five Gy was given before transplantation. Solid lines show irradiated groups and broken lines show unirradiated matched controls. There were significant differences ($p < 0.05$ in A, $p < 0.01$ in B) between the irradiated group and matched control group by Log Rank, Breslow and Tarone–Ware statistic tests (SPSS).

Table 4
Radiation-induced mutations in *p53*, *c-kit*, K-*ras*, *β-catenin*, and *RET* genes in human thyroid tissues after high and low dose rate exposures

Dose (Gy)	Dose rate (Gy/min)	No. of thyroid tissues			Mutations					
		Examined	With mutation	*P*[a]	No.	*p53*	*c-kit*	K-*ras*	*β-catenin*	*RET*
11–33	1.19	20	8	<0.01	8	5[b]	3[c]	0	0	0
11–33	0.00023	14	0	–	0	0	0	0	0	0

Human thyroid tissues from a Graves disease patient (31 years, female) were exposed to ^{137}Cs γ-rays at high and low dose rates. Details are described in Section 2.

[a] Fisher's exact test was carried out between high and low dose rate exposures.

[b] Exon 5 codon 142 (CCT → C*T*T: Pro → Leu) (12 and 16 Gy), exon 5 codon 148 (GA*T* → GAC: Asp → Asp) (14 Gy), exon 5 codon 152 (CC*G* → CC*A*: Pro → Pro) (14 Gy), exon 5 codon 180 (GAG → G: GA or AG deletion) (12 Gy).

[c] Exon 11 codon 557 (*T*GG → *C*GG: Trp → Arg) (12 Gy), exon 17 codon 825 (G*T*T → G*C*T: Ala) (11 and 11 Gy).

up to 33 Gy (Table 4). Differences in the incidence of mutation between high and low dose rate exposures were highly significant ($p < 0.01$ by Fisher's exact test and by Log Rank, Breslow and Tarone–Ware statistic tests, SPSS Statistics System). The results clearly show that there exists a significant and apparent dose rate effect in radiation-induced mutations in cancer-related genes in human thyroid tissues. In the present study, specific mutations have not been induced in the human thyroid tissue by radiation.

3.4. Changes in gene expression in transplanted human thyroid tissues

Table 5 shows that the expression of only 1% of genes (mostly related to degeneration and degradation) increased and the expression of 2% of genes (mostly related to the cell function) decreased during the first week after transplantation of surgically resected original tissues. However, further changes were not observed 2–4 weeks after transplantation, but instead recovered. More than 70% of genes which showed changes in gene expression were same in all the transplanted thyroid tissues, irrespective of the period (1–4 weeks) maintained in the SCID mice after transplantation. These results suggest that functions of transplanted human thyroid tissues are well maintained at molecular level. All the changes were observed in the first week after transplantation, probably due to the lack of blood flow, *i.e.* lack of oxygen supply and nutrients. Following radiation exposure, considerable numbers of genes showed further changes in gene expression dose-dependently against concurrent unexposed controls (Table 6). However, changes in expression level of *RET* gene were not observed in the human thyroid tissues after a single dose of ^{137}Cs γ-rays.

3.5. Radiation effects on transplanted human bone marrow cells

Fatally irradiated SCID mice can survive by human bone marrow transplantation (Table 7). SCID mice died of intestinal disorder 5–8 days after ^{137}Cs γ-ray exposure (4 Gy), irrespective of human bone marrow transplantation. SCID mice without human bone marrow transplantation died 3–4 weeks after radiation exposure to 2 Gy, because of the repair deficiency of radiation-induced double strand breaks [2,13]. However, all of 2 Gy irradiated SCID mice with human

Table 5
Changes in gene expression in the transplanted human thyroid tissues—comparison with surgically resected original thyroid tissue

Weeks after transplantation	No. of genes examined	Change in gene expression	
		Increase (> ×4)	Decrease (< ×(1/4))
1	8500	92	195
2	8500	88	138
3	8500	95	129
4	8500	87	127

Human thyroid tissues from a Graves' disease patient (20 years, female) were transplanted *s. c.* to SCID mice, and changes in gene expression (4-fold differences) were analyzed by GeneChip (Affymetrix HG Focus Array; 8500 genes).

Table 6
Changes in gene expression in human thyroid tissues after ^{137}Cs γ-ray exposure at a high dose rate

Dose (Gy)	No. of genes examined	Changes in gene expression	
		Increase (> ×4)	Decrease (< ×(1/4))
1.0	8500	11	11
3.0	8500	33	30

Human thyroid tissues from a Graves disease patient (20 years, female) were removed from SCID mice 2 weeks after ^{137}Cs γ-ray exposure at a high dose rate (1.19 Gy/min) and submitted to GeneChip analysis (HG Focus Array, Affymetrix).

Table 7
Survival of ^{137}Cs γ-ray-irradiated SCID mice by human bone marrow transplantation

Dose (Gy)	Transplantation of human bone marrow	No. of mice	Survival at 4 weeks after irradiation
4.0	+	4	0
4.0	–	4	0
2.0	+	4	4[a]
2.0	–	4	0
0	+	2	2[a]

[a] Survived until these mice were euthanized at 4 months after irradiation.

bone marrow transplantation survived more than 4 weeks and until these mice were euthanized at 4 months after radiation exposure. In the surviving SCID mice, about a half of bone marrow cells in the femur were stained by FITC-labeled human chromosome 1-specific painting (Fig. 4), *i.e.*, a half of mouse bone marrow were replaced by human bone marrow cells.

Human bone marrow cells maintained in the mouse bone marrow were very sensitive to radiation (Fig. 5). Total numbers of cells from femur were 2.2, 0.8, 0.98, 0.13×10^7 by 0, 0.5, 1.0 and 2.0 Gy of γ-ray exposure, and 10^4 cells were scanned by FACScan. Percentages of CD3 positive cells were 38.0, 32.3, 25.4 and 2.7, those of CD20 positive cells were 5.8, 4.5, 3.2, 0.45 and those of CD33 positive cells were 6.1, 4.2, 2.9 and 0.53 at doses of 0, 0.5, 1.0 and 2.0 Gy, respectively. Numbers of human-CD3, CD20 and CD33 positive cells decreased with doses of γ-rays, and 28.0 and 0.45% survived after 0.5 and 2.0 Gy exposures, respectively (Fig. 5).

4. Discussion

In the highly inbred non-leaky C.B17-*scid* mice, human thyroid tissues (and also bone marrow cells) are well maintained for a long period, showing no substantial differences in the histology, secretion of human thyroid hormone, and response to thyroid stimulating hormone [7]. Although there were some changes in gene expression during the first week after transplantation, further changes were not observed after that. Instead, suppressed gene expression was recovered during the period of 2–4 weeks after transplantation, indicating that gene functions are also well maintained in the improved SCID mice.

Large doses of X-rays and γ-rays at high dose rate significantly induced degeneration of the human thyroid tissue, reduced thyroid hormone secretion, and induced mutations in cancer related genes. However, all of these adverse effects of radiation on human thyroid tissues were reduced by the low dose rate exposure to the same dose of ^{137}Cs γ-rays, indicating significant dose rate effects in human thyroid tissues. In fact, extremely large doses of radiation could induce mutations in cancer related genes in the human thyroid tissues, but there was no mutation by the same doses of γ-rays at very low dose rate. Thyroid tissues of the younger person seem

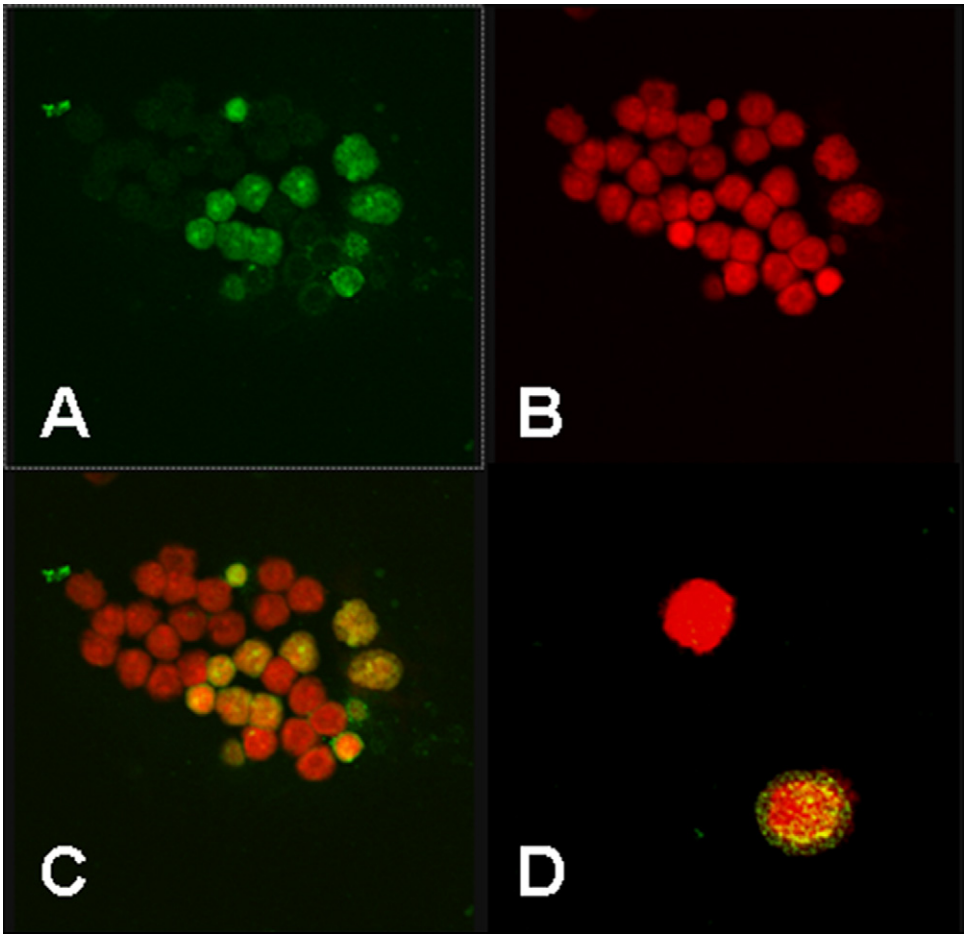

Fig. 4. Staining of human cells by FITC-labeled chromosome 1-specific paint. Human bone marrow cells were injected *i. v.* to SCID mice. Four months later, bone marrow cells were washed out from the femur of the SCID mice. Cells were stained by FITC-labeled human chromosome 1-specific paint, and images taken by the confocal laser microscope (TCS SP II, Leica Microsystems, Germany) were captured into a computer and processed using the Adobe Photoshop (Adobe System Inc., USA). (A) Human cells (green), (B) propidium iodide (PI) counter stained mice and human cells (red), and (C) overlay of both pictures. (D) Magnified picture of mouse and human bone marrow cells.

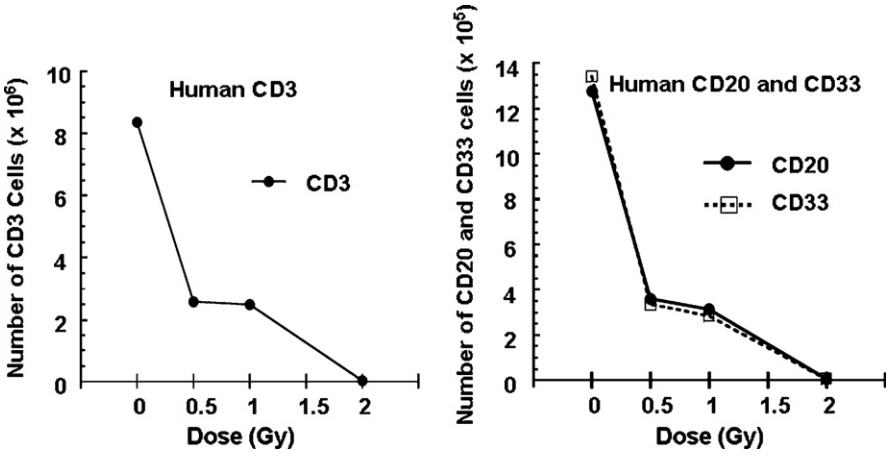

Fig. 5. Survival curve of human bone marrow cells maintained in the femur bone marrow of SCID mice after ^{137}Cs γ-ray exposure. SCID mice were pre-irradiated with 2 Gy of ^{137}Cs γ-rays and received *i. p.* injection of human bone marrow cells (1.2×10^7 cells/mouse). Seven weeks after transplantation of human bone marrow cells, recipient SCID mice were exposed to 0, 0.5, 1.0 and 2.0 Gy of ^{137}Cs γ-rays at high dose rate. Seven days after irradiation, mice were euthanized and cells were washed out from the femur of the SCID mice. Cells from the femur were counted and 10^4 cells were stained with FITC-labeled anti-human CD3, anti-human CD20, and anti-human CD33 antibodies. Flow cytometric immunophenotyping was performed on a FACScan (Becton-Dickenson Immunocytometry System, San Jose, CA, USA). Total numbers of CD3, CD20, and CD33 positive cells were plotted against ^{137}Cs γ-ray doses.

more sensitive to radiation, developing mutations earlier and at a higher rate than those of older persons (Fig. 3).

In radiation-related thyroid cancer in human, involvement of *p53* [10] and *RET* [21] mutations has been suggested, especially in the children after Chernobyl catastrophe, but *ras* mutation is uncommon [22]. Mutations of *c-kit* gene were commonly detected in the transplanted human thyroid tissue after radiation exposure. However, *c-kit* mutation has not been studied in both radiation-related and sporadic thyroid cancers. Triple mutations (one *p53* and two *c-kit* mutations) were detected in one irradiated thyroid tissue. However, cancer has not yet developed. Longer-term exposure of the thyroid tissue of children to radiation and radioiodine may be necessary to induce some specific mutations and cancer in human thyroid tissues. We succeeded to induce human skin cancer by the continuous (~2 years) ultraviolet light B (UVB) irradiation of normal human skin maintained on SCID mice [6]. The every day doses of UVB were equal to 1 h exposure on the beach in the mid-summer in Osaka. Topical exposures, in contrast to whole body exposure to γ-rays, may produce damage more effectively on the transplanted human skin and are not hazardous to critical internal organs, resulting in the induction of cancer in human skin.

In contrast to gene mutation, changes in gene expression were easily and rapidly detectable in various organs and tissues. This may give an invaluable methodology to evaluate irreversible or reversible changes of organ functions by the various environmental factors, and it is also useful for preclinical studies to examine efficacy and safety of new drugs. Human bone marrow cells survived and proliferated in the mouse bone marrow, replacing about a half of mouse bone marrow with human bone marrow cells. Since human precancerous and cancerous tissues are well maintained in the SCID mice [2–8], it will be possible with human bone marrow system. This model is of utmost importance to investigate human immune systems for immunotherapy, *etc*.

It will be easy to launch 5–10 mice, which were transplanted with radiation sensitive and critical human organs, thyroid tissues and human bone marrow, into space to know the risk of cosmic radiations to humans. Studies on the effects of γ-rays on these human tissues are now extended to those of neutrons, a major component in the flying body and space base, because in a preliminary study, it has been observed that neutrons have been found to induce 10-fold more changes than γ-rays in gene function in human thyroid tissues.

Conflict of interest

None.

Acknowledgements

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