hand, FGM1 was abundant in the stomach, in which the concentration of FGM1 was similar with that of FGA1, and fucosylation of GM1 was almost complete like that of GA1, suggesting that the fucosyltransferase equally acts on GA1 and GM1 in the stomach (Fig. 1) (*5*).

Ceramide monohexosides (CMH) were the most abundant neutral glycolipids in all regions, amounting to 1.7–4.1 μg/mg dry weight. As shown in Fig. 1, gastric CMH migrated to a similar position on a TLC plate to GalCer containing 2-hydroxy fatty acyl sphingosine, but to a higher position than intestinal CMH, which is composed of 2-hydroxy fatty acyl phytosphingosine, as reported in the literature (*2*).

As to the acidic lipids, the distribution of sulphatides was restricted to the stomach and cecum, and the amount in the cecum was significantly higher than those in the other regions. Also, CS and GM3 were uniformly distributed in the tract, their highest amounts being observed in the ileum and colon, respectively.

Then the molar ratios of individual lipids to CMH were calculated using their mean molecular weights [behenic acid (22:0)-containing ones], that is, 783 for CMH, 1,310 for Gb_4Cer, 1,513 for Forssman glycolipid, 1,310 for GA1, 1,456 for FGA1, 466 for CS and 1,236 for GM3, the CMH:Gb_4Cer:Forssman glycolipid:GA1:FGA1:CS:GM3 ratio in the jejunum being found to be 1.00:0.05:0.04:0.73:0.002:0.17:0.05, which resembled the reported ratio (*2*), showing that the jejunum as well as the duodenum and ileum contain GA1 in relatively high molar proportions.

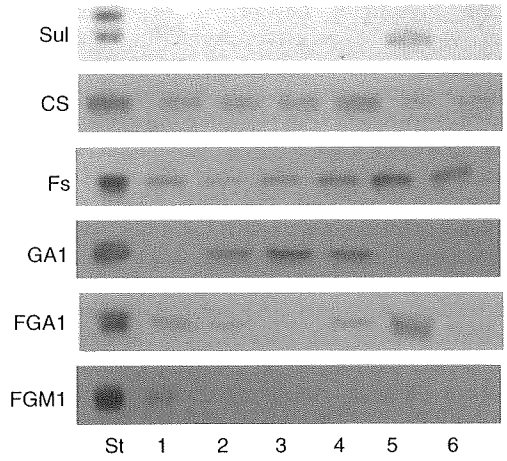

Fig. 2. **TLC- and TLC-immunostaining of lipids from different regions of the murine digestive tract.** For CS and sulphatides (Sul), acidic lipids, corresponding to 0.5 mg dry weight, were developed on TLC plates with chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1, v/v/v), and were detected with cupric acetate–phosphoric and orcinol–sulphuric acid reagents, respectively. For TLC-immunostaining of glycolipids, total lipid extracts, corresponding to 0.1 mg dry weight, were developed on TLC plates with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, v/v/v), followed by detection with anti-Forssman (Fs), anti-GA1, anti-FGA1 and anti-FGM1 antibodies. 1, Stomach; 2, duodenum; 3, jejunum; 4, ileum; 5, cecum; 6, colon.

Thus, the following glycolipids were abundant in the murine digestive tract in region-specific manners, FGA1 and FGM1 in the stomach, GA1 in the small intestine, FGA1 and sulphatides in the cecum. Accordingly, the small intestine seemed to be the site for colonization by *Lactobacilli*, because GA1 was characterized as the receptor with strongest affinity toward *L. johnsonni, L. casei* and *L. reuteri* (*13*).

Bacterial lipids—Extensive studies on the structures of bacterial lipids including those of *Lactobacillus* species have appeared in the literature (*28, 29–33*). In accord with previous reports, CL and PG were the major phospholipids in *L. johnsonii* and *L. intestinalis*, amounting to 0.12–0.65 μg of dry weight (Table 2) (*33*). Also, the major glycolipid, whose mobility on a TLC plate was similar with that of Galα1–2Glcα1–3DG from *L. casei*, was present in both bacteria, amounting to 1.03 μg/mg in *L. johnsonii* and 0.41 μg/mg in *L. intestinalis*, as determined by TLC-densitometry with *N*-stearoyl LacCer as the standard for quantitation. The other glycolipids in *L. johnsonii* were supposed to be Glcα1–3DG, Glcβ1–6Galα1–2Glcα1–3DG and Glcβ1–6Glcβ1–6Galα1–2Glcα1–3DG on the bases of their mobilities on a TLC plate,

Table 2. **Amounts of lipids in *Lactobacilli*.**

	L. johnsonii (μg/mg dry weight)	*L. intestinalis* (μg/mg dry weight)
CL	0.13	0.65
PG	0.12	0.21
Monohexaosyl DG (Glcα1–3DG)	0.15	tr
Dihexaosyl DG (Galα1–2Glcα1–3DG)	1.03	0.41
Trihexaosyl DG (Glcβ1–6Galα1–2Glcα1–3DG)	0.39	0.03
Tetrahexaosyl DG (Glcβ1–6Glcβ1–6Galα1–2Glcα1–3DG)	0.61	–

Quantitative determination of lipids was performed by TLC-densitometry with the following standards, CL from bovine heart, dioleoyl PG and N-stearoyl derivatives of GalCer, LacCer, Gb_3Cer and Gb_4Cer. tr, trace amount. Values are the means for three different experiments.

Table 1. **Amounts of lipids in the murine digestive tract (microgram/milligram dry weight).**

	Cho	CL	PE	PG	PC/PS	SM	CMH	Gb_4Cer	Fs	GA1	FGA1	FGM1	Sul	CS	GM3
Stomach	6.5	0.5	5.9	1.5	7.3	1.6	3.5	0.37	0.25	–	0.87	0.79	0.20	0.19	0.40
Duodenum	7.4	0.7	6.1	1.6	11.1	1.3	1.7	0.21	0.12	1.6	0.17	–	–	0.16	0.25
Jejunum	5.5	0.8	4.7	1.2	8.1	1.1	2.3	0.21	0.16	2.8	0.01	–	–	0.23	0.20
Ileum	6.1	0.2	4.0	1.0	6.6	0.9	2.4	0.69	0.33	2.2	0.28	–	tr	0.42	0.39
Cecum	6.3	0.3	5.3	1.2	7.5	1.2	4.1	0.53	1.11	tr	1.56	–	0.88	0.11	0.90
Colon	7.1	0.3	5.3	0.8	7.1	1.2	2.4	0.82	0.57	–	0.08	–	–	0.11	1.20

Cho, cholesterol; Fs, Forssman glycolipid; Sul, sulphatides; tr, trace amount. Values are the means for three different experiments.

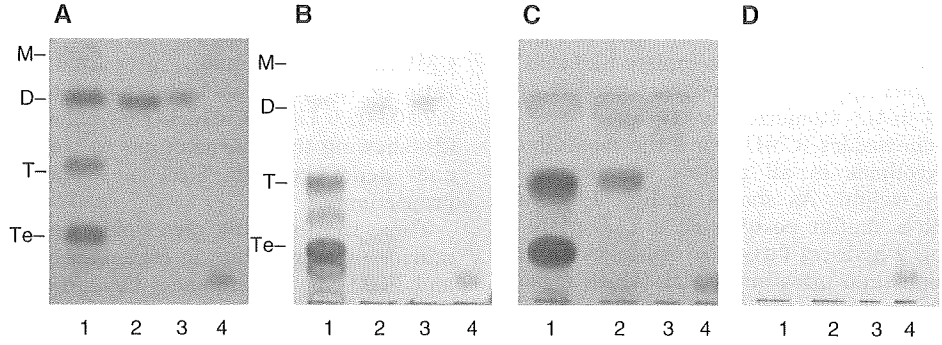

Fig. 3. **TLC-immunostaining of lipids from *Lactobacilli*.** Lipid extracts of *L. johnsonii* (*1*) and *L. intestinalis* (*2*), corresponding to 1 mg dry weight for A, and 0.1 mg dry weight for B–D, were developed on TLC plates with chloroform/methanol/water (65:35:8, v/v/v), and the spots were visualized with cupric acetate–phosphoric acid (A), anti-*L. johnsonii* (B), anti-*L. intestinalis* (C), and anti-GA1 (D) antisera. 3, Galα1–2Glcα1–3DG from *L. casei*; 4, GA1. M, D, T and Te indicate the positions of mono-, di-, tri- and tetrahexaosyl DGs, respectively.

as described in the literature (*28*, *29–32*). Tri- and tetraglycolipids were present in *L. johnsonii* at significantly high concentrations, but were only present in trace amounts in *L. intestinalis*.

Bacterial antigens reacting with anti-L. johnsonii and anti-L. intestinalis antisera—Immunization of rabbits with *L. johnsonii* and *L. intestinalis* yielded antisera with ELISA titres of more than 1:12,800. Both the anti-*L. johnsonii* and anti-*L. intestinalis* antisera reacted with Galα1–2Glcα1–3DG in *L. casei*, as well as that in *L. johnsonii* and *L. intestinalis*, but not with monohexaosyl DG (Glcα1–3DG). Although tri- and tetrahexaosyl DGs were not detectable in *L. intestinalis*, even on spotting of lipids corresponding to 5 mg dried bacteria, those in *L. johnsonii* were intensively stained with anti-*L. intestinalis* antisera to a similar levels to in the case of anti-*L. johnsonni* antisera, indicating their strong antigenicities (Fig. 3). In fact, the relative densities of spots/μg of Galα1–2Glcα1–3DG were significantly lower than those of tri and tetrahexaosyl DGs, *i.e.* Glcβ1–6Galα1–2Glcα1–3DG and Glcβ1–6Glcβ1–6Galα1–2Glcα1–3DG, respectively (*28*,*32*). However, since Galα1–2Glcα1–3DG was widely distributed in *Lactobacillus* species in relatively higher amounts than those of tri- and tetrahexaosyl DGs, it was revealed to contribute to a *Lactobacillus* antigen, as already reported by others (*30*, *34*).

Similarly, the strong antigenicity of diglucosyl DGs, *i.e.* kojibiosyl (Glcα1–2Glc) DG in *Acholeplasma laidlawi* and gentibiosyl (Glcβ1–6Glc) DG in *Mycoplasma neurolyticum*, has been well studied (*34*), and antibodies towards digalactosyl DG have been reported not to cross-react with GalCer or gangliosides (*35*, *36*), but to be involved in the production of natural antibodies in patients suffering from multiple sclerosis (*37*). Accordingly, one can suggest that *Lactobacillus* antigens including glycolipids are also involved in the production of natural antibodies.

Lipid antigens reacting with anti-lactobacillus antisera in murine tissues—Anti-*L. johnsonii* and anti-*L. intestinalis* antisera contained antibodies that reacted with GA1, but glycolipids that reacted with anti-GA1 antibodies were not present in the lipids from either bacterium (Fig. 3). Therefore, antigens reactive with antisera were explored by TLC-immunostaining with lipids from the murine digestive tract and several standard glycolipids. Among the glycolipids examined, GalCer and GA1 were reactive with antisera, but structurally related glycolipids such as GlcCer, LacCer, Gb_3Cer, Gg_3Cer, Gb_4Cer, Forssman glycolipid, Lc_4Cer, nLc_4Cer and $IV^3Gal\alpha$-nLc_4Cer were not reactive, indicating that terminal galactose moieties are not always included in the epitope and that gangliotetraose is a preferable antigen for anti-lactobacillus antisera (Fig. 4). On comparison of the densities of spots per microgram of glycolipids stained with antisera, the intensities of Galα1–2Glcα1–3DG and GA1 were found to be similar, but that of GalCer was lower than those of Galα1–2Glcα1–3DG and GA1 (Fig. 4). Thus, symbiotic *Lactobacilli* were shown to generate antibodies toward receptor glycolipid GA1.

To determine why anti-GA1 antibodies were yielded on immunization with *Lactobacilli*, the antigens reacting with anti-GA1 antibodies in *Lactobacilli* were explored by Western blotting. As shown in Fig. 5, although the protein-profiles on SDS–PAGE differed between the two bacteria, anti-lactobacillus antisera reacted with the same antigenic proteins with molecular weights of 43, 50, 55 and 75 kDa. In addition, a protein with a molecular weight of 26 kDa in both bacteria exhibited a positive reaction with anti-GA1 antibodies, indicating the presence of gangliotetraose-like glycans in their protein fractions. If the molecules mimicking GA1 in *Lactobacilli* to generate anti-GA1 antibodies exhibit receptor activity for a ligand, binding of bacteria to GA1 in the small intestine and bacterial aggregation through the ligand might allow effective colonization on the surface of the small intestine. In this connection, modification of GA1 through fucosylation might regulate the number of colonies of *Lactobacilli*.

The production of antibodies to GA1 and gangliosides in human autoimmune diseases such as Guilain–Barré syndrome has been reported to be due to infection by *Campylobacter jejuni*, which causes gastroenteritis (*38*). In fact, an oligosaccharide mimicking GA1, that is, GalGalNAc-Gal-(Glc)HepII-(Glc)HepI, was detected in the lipooligosaccharides (LOS) of a gram-negative bacterium, *C. Jejuni* (*39*), and an immune response to the bacterial LOS was thought to result in autoimmune diseases through a reaction with gangliosides in neural tissues,

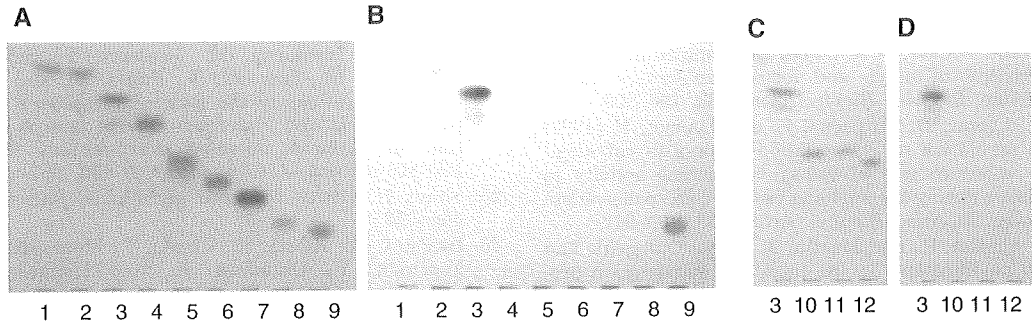

Fig. 4. **TLC and TLC-immunostaining of standard glycolipids.** Glycolipids (0.5–1.5 μg) were developed on TLC plates with chloroform/methanol/water (65:35:8, v/v/v) for A and B, and with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, v/v/v) for C and D, and the spots were visualized with orcinol–sulphuric acid for A and C, and with anti-*L. johnsonii* antisera for B and D. 1, GlcCer; 2, GalCer NFA; 3, Galα1–2Glcα1–3DG from *L. casei*; 4, LacCer; 5, Gb_3Cer; 6, Gg_3Cer; 7, Gb_4Cer; 8, Forssman antigen; 9, GA1; 10, Lc_4Cer; 11, nLc_4Cer; 12, $IV^3Gal\alpha$-nLc_4Cer.

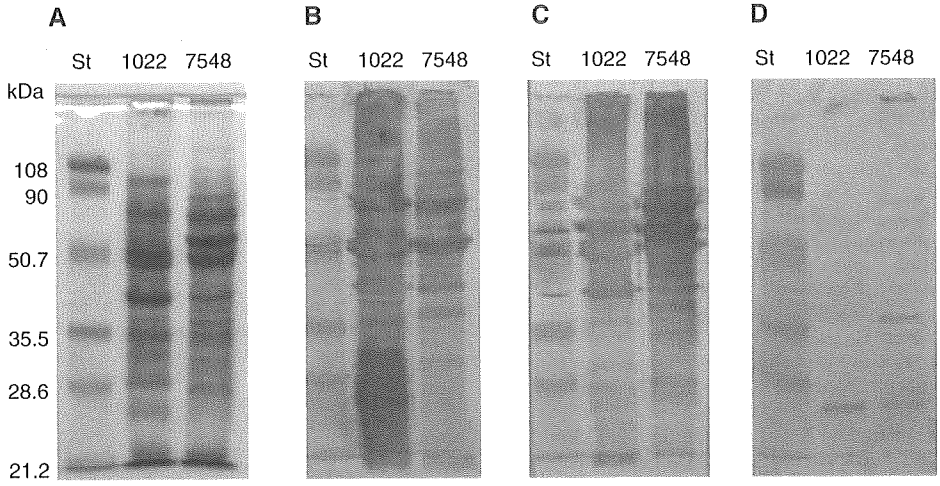

Fig. 5. **SDS-PAGE and Western blotting of proteins from *Lactobacilli*.** *L. johnsonii* (1,022) and *L. intestinalis* (7,548) suspended in PBS by sonication were denatured by heating with the sample buffer, and the resultant solutions were analysed by SDS–PAGE with CBB-staining (A), and by Western blotting with anti-*L. johnsonii* (B), anti-*L. intestinalis* (C), and anti-GA1 (D) antibodies. No band was obtained by staining with normal rabbit serum (1:500). St, protein molecular markers.

playing a crucial role in the pathogeneses of the diseases. In this connection, *Lactobacilli*, gram-positive bacteria, also carry glycans mimicking GA1 in the digestive tract of mice, and the resemblance in the epitope structure between bacteria and the host might be essentially related with a mechanism for evading immune responses to establish symbiosis with *Lactobacilli* in the digestive tract.

CONFLICT OF INTEREST

None declared.

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RADIATION RESEARCH 173, 205–213 (2010)
0033-7587/10 $15.00

DOI: 10.1667/RR1991.1

No Evidence of Increased Mutation Rates at Microsatellite Loci in Offspring of A-Bomb Survivors

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Kodaira, M., Ryo, H., Kamada, N., Furukawa, K., Takahashi, N., Nakajima, H., Nomura, T. and Nakumura, M. No Evidence of Increased Mutation Rates at Microsatellite Loci in Offspring of A-Bomb Survivors. *Radiat. Res.* 173, 205–213 (2010).

To evaluate the genetic effects of A-bomb radiation, we examined mutations at 40 microsatellite loci in exposed families (father-mother-offspring, mostly uni-parental exposures), which consisted of 66 offspring having a mean paternal dose of 1.87 Gy and a mean maternal dose of 1.27 Gy. The control families consisted of 63 offspring whose parents either were exposed to low doses of radiation (< 0.01 Gy) or were not in the cities of Hiroshima or Nagasaki at the time of the bombs. We found seven mutations in the exposed alleles (7/2,789; mutation rate 0.25 × 10^{-2}/locus/generation) and 26 in the unexposed alleles (26/7,465; 0.35 × 10^{-2}/locus/generation), which does not indicate an effect from parental exposure to radiation. Although we could not assign the parental origins of four mutations, the conclusion may hold since even if we assume that these four mutations had occurred in the exposed alleles, the estimated mean mutation rate would be 0.39 × 10^{-2} in the exposed group [(7 + 4)/2,789)], which is slightly higher than 0.35 × 10^{-2} in the control group, but the difference is not statistically significant.

INTRODUCTION

Studies on the genetic effects of radiation have been pursued for many years at the Atomic Bomb Casualty Commission (ABCC) and its successor, the Radiation Effects Research Foundation (RERF). While somatic mutations have been documented to occur in a dose-related manner, as seen by chromosome aberrations in blood lymphocytes (*1*), no indication of genetic (trans-generational) effects of radiation has been seen in several end points such as malformation, stillbirth or chromosome aberration (*2–5*). Currently, in addition to epidemiological (*6, 7*) and clinical studies (*8*), laboratory studies to determine whether A-bomb radiation caused genetic effects at the DNA level, that is, whether a significant increase in mutation rate is observed among the children of A-bomb survivors, are also being conducted using several molecular biology techniques (*9–15*). One of those molecular studies relates to the instability of repeated DNA sequences. Polymorphic tandem repeat sequences, such as minisatellites and microsatellites, are known to exhibit high spontaneous mutation rates (i.e., alterations in the number of repeats) in germ cells, and it has been suggested that these may serve as versatile tools for genetic studies because it might be possible to draw conclusions by studying a relatively small number of the offspring.

Mini- and microsatellites are conventionally classified based on the number of nucleotides that comprise the unit of repeat sequence. Minisatellites are composed of repeat-sequence units of 10 to 100 bp, and the number of repeats may exceed 500. They are located at around 1,000 sites in the human genome, some of which are known as hypervariable minisatellites in humans due to their high spontaneous mutation rates in germ cells (0.5 × 10^{-2} to 10 × 10^{-2}/locus/generation) (*16*). The mechanisms of mutagenesis acting at these hypervariable loci are complex, but they appear to involve gene conversion in meiosis (*17, 18*). Several studies measured the mutation induction rates at hypervariable minisatellite loci in humans, with results that sometimes appeared to be discordant. For example, increased mutation rates were reported in residents of contaminated areas after the Chernobyl accident (mean estimated dose of around 0.03 Sv), in areas exposed to atmospheric nuclear tests at Semipalatinsk (mean estimated dose may be over 1 Sv), and in areas along the Techa River exposed to the release of radionuclides (mean dose of about 0.1 Sv) (*19–22*). In contrast, no significant increase in mutation rates was observed in

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studies of A-bomb survivors who suffered from a one-time acute exposure at a mean dose of 1.9 Sv or in the Chernobyl cleanup workers, who were also exposed to relatively acute radiation at doses of up to over 1 Sv (*9–12, 23, 24*). A study using DNA from sperm to evaluate mutation rates in three patients before and after radiotherapy for seminoma (estimated doses of 0.38 to 0.82 Gy) reported no significant increase in the mutation rates at two hypervariable minisatellite loci examined (*25*).

On the other hand, microsatellites comprise shorter repeat units of 1 to 6 bp, with repeat numbers of 100 copies or less. Their entire length can range up to several hundred bases, and they are dispersed and found at over 100,000 sites in the human genome. Spontaneous mutation rates at microsatellite loci in germ cells range from 10^{-2} to 10^{-4}/locus/generation which are much higher than those at unique protein-coding loci (10^{-5} to 10^{-6}/locus/generation) but are considerably lower than those seen at hypervariable minisatellite loci (*26–28*). In contrast to mutations in minisatellites, microsatellite mutations are thought to derive from "slippage" during DNA replication that results in gains or losses of repeat units (*29*). Therefore, mini- and microsatellites may differ in mutation behavior after exposure to radiation. Several studies examined the genetic effects of radiation and found significantly increased mutation rates at microsatellite loci; one study examined barn swallows and wheat grown in highly contaminated sites after the Chernobyl accident (*30, 31*), and another examined Japanese medaka fish that were irradiated chronically at low dose rates (*32*). However, it is not clear whether microsatellite mutations are induced in mammals after radiation exposure. In humans, only three small-scale studies have been reported; in studies of the survivors of A-bombs and the Chernobyl cleanup workers, there was no clear evidence of increased mutation rates (*11, 33*). The third study reported an elevated mutation rate in offspring born to parents who were exposed to ^{137}Cs γ rays at Goiania, Brazil, but these results do not appear to be conclusive, because only 12 microsatellite loci were examined for 10 exposed families (*34*).

As a part of the effort to take advantage of the relatively low spontaneous mutation rates at microsatellite loci compared with the mutation rate at hypervariable minisatellite loci to increase the statistical power of mutation studies, Furitsu *et al.* (*35*) developed assays to examine as many as 72 microsatellite loci and studied 64 families of cleanup workers and 66 controls after the Chernobyl accident. Those results again indicated no genetic effects of radiation, although the possibility remained that the radiation effects were too low to detect because the mean radiation dose was only 0.039 Sv.

In view of the results of the past studies in humans, it is important to examine not only as many loci as possible but also as many people as possible who were exposed to large doses. In the present study, the offspring of A-bomb survivors (doses to the exposed parents are mainly 1 Gy or larger) were screened for mutations at 40 microsatellite loci that are known to exhibit relatively high mutation rates.

MATERIALS AND METHODS

Subject Families and Ethical Considerations

Blood samples were collected from A-bomb survivors who participated in the Adult Health Study (AHS) and from their family members (spouses and children). They provided written informed consent for examining DNA following the form approved by the Ethics Committee for Genome Research in RERF established in accordance with the "Guidelines from Three Ministries" in Japan (*36*). Subsequently, mononuclear cells were isolated and a fraction of the cells were infected with Epstein-Barr virus (EBV) to establish lymphoblastoid cell lines, while the remaining cells were stored in liquid nitrogen without culture. White blood cells were also isolated and stored at −80°C.

DNA from family members (father-mother-children) was examined in 49 families in the exposed group (highest-dose cases were selected) and 51 families in the control group. The protocol for this study was reviewed and approved by the Institutional Review Board of both RERF and Osaka University.

Radiation Dose

The dose information for the parents (gonadal dose) is summarized in Table 1. The doses were estimated using the Dose System 2002 (DS02) with a relative biological effectiveness (RBE) of 10 for the neutron component (*37*). Of 129 offspring examined, 66 were in the exposed group and 63 were in the control group, and 115 of these subjects were the same ones examined in our previous minisatellite studies (*9–12*). The exposed families consisted mostly of uni-parental exposures but also included two families (a single offspring in one family and three siblings in the other) that had high-dose-exposed fathers (>2 Gy) and low-dose-exposed mothers (<0.1 Gy). The total number of offspring derived from irradiated germ cells was 70 (mean dose 1.56 Gy); of these, 34 were derived from exposed fathers (mean dose 1.87 Gy, range 0.78–3.09 Gy) to 23 daughters and 11 sons and 36 from exposed mothers (mean dose of 1.27 Gy, range 0.02–2.51 Gy) to 16 daughters and 20 sons. The total number of offspring derived from unexposed germ cells was 188 (95 from fathers to 40 daughters and 55 sons and 93 from mothers to 47 daughters and 46 sons).

The mean parental age at the time of exposure was 15.3 ± 5.1 years (mean ± SD) in the exposed group and 14.2 ± 5.7 years in the control group. The mean interval between radiation exposure and the birth of the offspring was 14.5 years (range 1.5–28.9 years) in the exposed group and 15.4 years (range 3.1–32.0 years) in the control group. The mean age of the fathers at the time of having the offspring was 30.1 ± 4.3 years (range 20.5–39.9 years) in the exposed group and 31.3 ± 5.2 years (range 21.3–51.6 years) in the control group. As for the mothers, the mean age at the time of delivery was 28.8 ± 5.3 years (range 20.2–41.8 years) in the exposed group and 27.4 ± 4.2 years (range 19.4–39.4 years) in the control group. There was no significant difference in the age-related parameters between the exposed and the control group.

Microsatellite Analysis

1. DNA samples

For screening candidate mutations at microsatellite loci, DNA from later passages of lymphoblastoid cells was first used. Mutations

TABLE 1
Parental Doses in the 129 Study Subjects

Testis doses of fathers in Gy (mean)	Ovary doses of mothers in Gy (mean)					Total
	< 0.01 (0[a])	0.01–0.10 (0.05)	0. 50–0.99 (0.87)	1.00–1.99 (1.42)	> 2.00 (2.38)	
<0.01 (0[a])	63	0	5	24	3	95
0.50–0.99 (0.85)	2	0	0	0	0	2
1.00–1.99 (1.43)	18	0	0	0	0	18
>2.00 (2.63)	10	4	0	0	0	14
Total	93	4	5	24	3	129

[a] Zero dose includes those parents who were not in the cities of Hiroshima or Nagasaki at the time of the bombs (classified as not in city, NIC).

at microsatellites occur somatically in the process of EBV transformation and/or during culture, and cells having such mutations sometimes proliferate clonally during culture. The errors in mistyping of microsatellites may be due to clonal expansion of cells having those somatic mutations (*38*). To avoid this possibility, in this study, we did not use DNA samples from cell lines with high clonal expansion.

When putative mutations were detected, DNA from another independent lymphoblastoid cell line from the same donor was used for confirmation. We ultimately confirmed *de novo* mutations by using DNA from uncultured cells.

2. Microsatellite loci

We selected 40 microsatellite loci that were previously documented to show relatively high mutation rates ($\geq 0.5 \times 10^{-3}$/locus/generation) or high heterozygosity rates in a population (>70%). The genomic location and size of the repeat unit of each locus are shown in Table 2.

3. Detection of microsatellite mutations

Microsatellite sequences were amplified with PCR using fluorescence-labeled primers flanking the repeat sequence, and the amplicon sizes in offspring were compared with those of both parents. Most of the primer sequences and PCR conditions used in this study are described in Furitsu *et al.* (*35*). We examined ten loci for which PCR conditions were not described or that were modified from the descriptions of Furitsu *et al.* The primer sequences and annealing temperatures for PCR are summarized in Supplementary Table 1. Five microsatellites in that table (D2S1338, D3S1358, D5S818, D13S317, D16S539) were amplified by multiplex PCR in the first screening.

Fluorescence-labeled PCR products from offspring and their parents were subjected to capillary electrophoresis with size markers and were analyzed with software (GeneScan or GeneMapper Analysis) provided by Applied Biosystems.

When the allele size for an offspring was found to differ from the sizes of the parental alleles, the new allele was considered as a putative mutation. Since it was reported that more than 85% of microsatellite mutations result from addition or deletion of a single repeat unit (*39*), we assumed that the mutant allele was derived from the parental allele of the closest size (*26–28*).

4. Exceptional cases

Occasional failures of PCR amplification were encountered and were apparently caused by sequence variations in regions that corresponded to the primer sequences. In such cases, one parent might show only one band, and thus appear to be homozygous, although he or she could actually be heterozygous bearing one "non-amplifying" (apparently null) allele (*28, 40*). Consequently, the offspring would appear to have inherited no allele from one parent and two copies of one of the two alleles from the other, a condition analogous to uni-parental disomy. Three such cases were discovered: one at DS19S47 and two at D10S1214 (in siblings). However, an alternative possibility also exists but is less likely to happen. That is, a mutation occurred at the mother's allele to give rise to the same size of the allele that was transmitted from the father. To discriminate the two possibilities, we examined the existence of hidden non-amplifying alleles by PCR amplification using new primer sets that were specific to sequences outside of the original primer regions. We found that none of the cases were caused by new mutations but rather were caused by the non-amplifying allele.

Mosaicism was also encountered; namely, individuals can show three bands as the result of a somatic mutation that occurred at one allele soon after fertilization. Two such cases were encountered, one at D21S1245 and the other at ACTBP2. Those were not considered to be germ-cell mutations. In the present study, both mosaic offspring were encountered only in the control group, thus we do not discuss the effect of a parental exposure in the post-zygotic mutation rate in offspring.

5. Mutation rates

The mutation rate is defined as the number of mutations per allele per generation.

6. Statistical analysis

Statistical analyses were conducted using Fisher's exact test. To evaluate the possibility that A-bomb radiation exposure increases the germline mutation rate, P values were obtained from one-sided likelihood-ratio tests, with values <0.05 indicating statistical significance.

RESULTS

Characteristics of the Mutations

In 70 exposed haploid sets (2,789 alleles) and 188 unexposed haploid sets (7,465 alleles) (Supplementary Table 2), 58 putative mutations were found in the first screening. Subsequently, second cell lines from 17 families were used to confirm the putative mutations but 9 out of 23 were not detected, indicating that these mutations arose during *in vitro* cell culture, while 14 were detected, counting results of the first screening. Next, we examined 49 putative mutations (i.e., 58 – 9) by using stored, uncultured whole blood samples; we found that 12 had occurred during *in vitro* culture of the cells and 37 were true *de novo* mutations (Table 3). The number of mutations detected at each locus is presented in Table 2. Possible parental origins were successfully assigned in the majority of the cases except for four

TABLE 2
Microsatellite Loci Screened in this Study with Mutations Detected at each Locus

Microsatellites	Type of repeat unit	Chromosome no.	Gene name[a]	Number of mutations derived from		
				Exposed allele	Unexposed allele	Undetermined allele
D9S58	2	9			2	
D9S63	2	9	FNBP1 (intron)			
D13S120	2	13	KIAA0774 (intron)*			
D13S128	2	13	FARP1 (intron)		2	1
D19S47	2	19				
D1S389	4	1				
D1S1612[b]	4	1	RPL7AP18*			
D2S1338[b]	4	2		1	1	
D3S1358[b]	4	3	LARS2 (intron)			
D3S1359[b]	4	3	MON1A (intron)			1
D3S1744	4	3			1	
D4S2431	4	4				
FGA	4	4	FGA (intron)			
D5S2501	4	5	FLJ43080 (intron)*		1	
D5S818[b]	4	5				
CSF1R (TAGA)	4	5	CSF1R (intron)		1	
CSF1R (CCTT/CTTT)	4	5	CSF1R (intron)		1	
ACTBP2	4	6	ACTBP2	2		
D7S1517	4	7	HYAL4		1	
D7S1482[b]	4	7		2	4	
D8S1179	4	8			1	
D9S748	4	9				
D10S1214	4	10		1	4	1
D10S1237	4	10	AFAP1L2 (intron)		1	
D12S66	4	12		1		
D12S67	4	12	MGAT4C (intron)*			
D12S391	4	12			1	
D12S1090	4	12			1	
VWA	4	12	VWA			
D13S317[b]	4	13				
D15S657	4	15	LOC100132798 (intron)*			
D16S539[b]	4	16				
D18S51	4	18	BCL2 (intron)			
D18S1270	4	18				
D19S244	4	19	CYP4F3 (intron)			
D19S245	4	19			1	1
D19S247	4	19	GNA15 (intron)			
D21S11	4	21			1	
D21S1245	4	21	TTC3 (intron)			
DXS981	4	X			2	
Total number of mutations				7	26	4

[a] Microsatellite loci that are mapped in coding (transcribed) regions are denoted by the appropriate gene name, but these with an asterisk are not registered in the Online Mendelian Inheritance in Man (OMIM). Those mapped in non-coding regions are shown as blanks.
[b] These eight microsatellite loci were added to the 32 loci described by Furitsu *et al.* (*34*).

TABLE 3
Mutations in the Alleles from Exposed and Unexposed Parents

	Exposed group		Control group	Statistical test (one-sided)
	Exposed alleles	Unexposed alleles	Unexposed alleles	
Number of alleles examined	2,789	2,462	5,003	
Mutations of parental origin determined	7	9	13	
Mutations of parental origin undetermined	4		4	
Total number of mutations (mean mutation rate: %)	11[a]/2,789 (0.39%)	26[a]/7,465 (0.35%)		$P = 0.43$[a]
	7[b]/2,789 (0.25%)	30[b]/7,465 (0.40%)		$P = 0.91$[b]

[a] All four mutations with unassigned parental origin in the exposed group were assumed to be radiogenic in origin.
[b] Four mutations with unassigned parental origins in the exposed group were assumed to be spontaneous.

TABLE 4
Gain or Loss of Repeat Units at Mutated Microsatellite Loci

	Exposed alleles	Unexposed alleles
Expansion (gain of repeat units)	6	12
Contraction (loss of repeat units)	1	11
Undetermined	0	3
Total number of mutations	7	26

mutations in the exposed group and four in the control group.

Mutations at di- or tetranucleotide repeats. Among the 37 mutations, five occurred at dinucleotide repeat loci (5 mutations/5 loci) and 32 at tetranucleotide repeat loci (32 mutations/35 loci) (Table 2). Thus there appears to be no preferential induction of mutations at di- and tetranucleotide repeat loci.

Mutations showing gain or loss of the repeat units. The direction (expansion or contraction) of the 33 mutations was examined (four mutations whose parental origin could not be assessed in the exposed families are not included here) (Table 4). Among the mutations that occurred at unexposed alleles, gains and losses seemed to occur equally (12 gains and 11 losses), but mutations at exposed alleles appeared to consist of more gains than losses (6 and 1, respectively). However, the latter difference was not statistically significant ($P = 0.14$, two-sided test).

Sex differences in mutation rates. It was possible to determine the parental origins of 22 mutations in the unexposed alleles, and the spontaneous mean mutation rates were 0.45×10^{-2}/locus/generation (17/3,745, Table 5) for the paternal alleles and 0.13×10^{-2}/locus/generation (5/3,720, Table 6) for the maternal alleles. The paternal mean mutation rate was about three times higher than that in the maternal alleles ($P < 0.05$), which is consistent with the data of other researchers showing that microsatellite mutation rates in humans are three to six times higher in males than in females (*27, 28, 39*). Among the exposed alleles, the mean mutation rates were 0.30×10^{-2}/locus/generation (4/1,349, Table 5) for the paternal alleles and 0.21×10^{-2}/locus/generation (3/1,440, Table 6) for the maternal alleles, but this difference was not statistically significant.

Effects of parental age. The mean parental ages at the birth of their offspring were compared between couples whose offspring exhibited or did not exhibit microsatellite mutations; they were 30.1 and 31.3 years old for the fathers and 27.9 and 27.8 years for the mothers, respectively. This difference was not statistically significant for either parent.

Effects of Parental Exposure to Radiation

1. Mutation rates in the exposed and unexposed alleles

In offspring from the exposed and the control families, 20 and 17 mutations were found, respectively (Table 3). Among those, we could not determine the parental origins of four mutations in the exposed group and four mutations in the control group. Those non-assigned mutations created a problem in estimating radiation-induced mutation rates, because only one parent was exposed in most of the exposed families (this is not a problem in the control families because neither parent was exposed). Thus two extreme cases were assumed: All non-assigned mutations in the exposed group occurred either in the exposed alleles or in the unexposed alleles. In the former case, the mean mutation rate in the exposed alleles was 0.39×10^{-2}/locus/ generation [(4 + 7)/2,789], which was slightly higher than 0.35×10^{-2}/locus/generation (26/7,465) in the control group (Table 3), but this difference is not statistically significant ($P = 0.43$). In the latter case, the mean mutation rate was 0.25×10^{-2}/locus/generation (7/2,789) in the exposed group, which is lower than the value of 0.40×10^{-2}/locus/generation [(4 + 26)/7,465] (Table 3) in the control group.

TABLE 5
Mutations in Paternal Alleles

	Exposed group		Control group	
	Exposed paternal alleles	Unexposed paternal alleles	Unexposed paternal alleles	Statistical test (one-sided)
Number of alleles examined	1,349	1,262	2,483	
Number of mutations of paternal origin	4	6	11	
Number of mutations of undetermined parental origin	4 (1 in a family of father's exposure and 3 in families of mother's exposure)		4	
Total number of mutations (mean mutation rate: %)	5[a]/1,349 (0.37%) 4[b]/1,349 (0.30%)	17[c]/3,745 (0.45%)		$P = 0.73$[d]

[a] It was assumed that one unassigned mutation in a family with father's exposure was derived from the exposed paternal allele.
[b] It was assumed that one unassigned mutation in a family with father's exposure was derived from the unexposed maternal allele.
[c] It was assumed that all of the seven unassigned mutations (three in the exposed and four in the control group) were derived from maternal alleles to estimate the smallest spontaneous mutation rate in paternal alleles of the control group.
[d] Statistical test of the differences between *a* and *c*.

TABLE 6
Mutations in Maternal Alleles

	Exposed group		Control group	
	Exposed maternal alleles	Unexposed maternal alleles	Unexposed maternal alleles	Statistical test (one-sided)
Number of alleles examined	1,440	1,200	2,520	
Number of mutations of maternal origin	3	3	2	
Number of mutations of parental origin undetermined	4 (3 in families of mother's exposure and 1 in a family of father's exposure)		4	
Total number of mutations (mean mutation rate: %)	6[a]/1,440 (0.42%)	5[c]/3,720 (0.13%)		$P = 0.06$[e]
	3.8[b]/1,440 (0.26%)	6.3[d]/3,720 (0.17%)		$P = 0.30$[f]

[a] It was assumed that three unassigned mutations in families with mother's exposure were derived from the exposed maternal alleles.
[b] It was assumed that only 1/4 of three unassigned mutations in families with mother's exposure were derived from exposed maternal alleles.
[c] It was assumed that all five unassigned mutations (one in a family with father's exposure and four in the control group) were derived from paternal alleles and were excluded from calculating the spontaneous mutation rate in females.
[d] It was assumed that only 1/4 of five unassigned mutations were derived from maternal alleles.
[e] Statistical test of differences between cases *a* and *c*.
[f] Statistical test of differences between cases *b* and *d*.

2. Mutation rates in paternal and maternal alleles

Since the mutation rate in paternal alleles was about three times higher than that in the maternal alleles in the control group, radiation effects on paternal and maternal germ cells were assessed separately. Tables 5 and 6 summarize the possible mutations detected in paternal and maternal alleles, respectively.

Mutations in paternal alleles. In the exposed group, 14 mutations were detected. Four occurred in the exposed alleles, six occurred in the unexposed alleles, and four could not be assigned a parental origin (one in a family with father's exposure and three in families with mother's exposure, Table 5). In the control group, 15 mutations were detected but for four of them, the parental origin could not be determined. Therefore, if we restrict the confirmed cases regarding the parental origin, the mean mutation rate is 0.30×10^{-2}/locus/generation in the exposed group (4/1,349, Table 5), which is not higher than 0.45×10^{-2}/locus/generation in the control group (17/3,745, Table 5). Even if we consider that the one unassigned mutation in a family with father's exposure occurred in the paternal allele, and the remaining unassigned mutations occurred in the maternal alleles (i.e., three mutations in the exposed group but in families with mother's exposure and four mutations in the control group), which gives the lowest estimation of the spontaneous mutation rate in the paternal alleles, the difference is not statistically significant: 0.37×10^{-2}/locus/generation [(4 + 1) = 5/1,349] in the exposed paternal alleles compared to 0.45×10^{-2}/locus/generation (17/3,745) in the unexposed paternal alleles ($P = 0.73$, Table 5).

Mutations in maternal alleles. In the exposed group, 10 mutations were found. Three occurred in the exposed alleles, three occurred in the unexposed alleles, and four could not be assigned a parental origin (three in families with mother's exposure and one in a family with father's exposure, Table 6). In the control group, six mutations were detected, but the parental origin could not be determined in four of them.

Mutation rates were estimated under two different sets of hypothetical conditions. Under the first set of conditions, the minimum estimate of the spontaneous mutation rate was made by assuming that all five unassigned mutations (one in a family with father's exposure and four in the control group) occurred in paternal alleles, which gave an estimate of 0.13×10^{-2}/locus/generation (5/3,720, Table 6). The corresponding maximum rate of mutation induction after radiation exposure was estimated by assuming that all three unassigned mutations in families with maternal exposure occurred in the exposed maternal alleles, which gave an estimate of 0.42×10^{-2}/locus/generation [(3 + 3) = 6/1,440, Table 6]. That rate was nearly three times higher than the spontaneous rate of 0.13×10^{-2} but the difference was only marginally significant due to the small number of the mutations ($P = 0.06$, Table 6). Under the second set of conditions, unassigned mutations were allocated to paternal and maternal origins in proportion to the spontaneous rate, namely, 3 to 1. In that situation, the mutation rate in the exposed alleles was 0.26×10^{-2}: [3 + (3/4)]/1,440 in the exposed alleles and 0.17×10^{-2}; [5 + (1/4) + (4/4)]/3,720 in the unexposed alleles (Table 6). The difference was not statistically significant ($P = 0.30$, Table 6).

DISCUSSION

Different Radiation Response in Mice and Humans

The present results indicated no mutagenic effect of parental exposure to ionizing radiation at microsatellite loci in the offspring of A-bomb survivors. The results are at variance with the results of mutagenesis studies obtained at hypervariable tandem repeat sequences in male mice (*41*).

Mouse hypervariable loci consist of arrays of 4 to 6 base repeats as do human microsatellites but contain a much larger number of repeats which often exceed 1,000 (>several kb in allele length) and are thus referred to as expanded simple tandem repeats (m-ESTRs) (*41–45*). Although mutations at both m-ESTRs and human microsatellites (h-microsatellites) are thought to occur via replication slippage, spontaneous mutation rates differ considerably between the two species, e.g., as high as 10×10^{-2}/locus/generation in the ESTRs compared to less than 1×10^{-2}/locus/generation in h-microsatellites (*26–28, 42–45*). Since the mutation induction rate at m-ESTR loci may reach as high as a few percent per Gy, which is too high to be explained by radiation-induced damage to the target loci themselves, it is generally believed that the ESTR mutations are induced through genomic instability (*41, 46–48*). After irradiation of mouse spermatogonia cells, the mutation rate increased in a dose-dependent manner at the m-ESTR loci, and the estimated doubling dose (the dose required to double the spontaneous level of mutations) ranged from 0.4 to 1.1 Gy in most studies (*41, 47, 48*). Thus we expected that if the radiosensitivity for mutagenesis at m-ESTR and h-microsatellite loci were not largely different, genetic effects of parental exposure to radiation would have been detected in the present study. However, in contrast to high radiosensitivity at m-ESTR, no radiation-associated mutation induction at h-microsatellites was observed. It is noted that with our sample size, 2,789 alleles (mean dose 1.56 Gy) in the exposed group and 7,465 alleles in the control group, we could have detected the radiation effect with a 70% probability if the mean mutation rate had doubled in the exposed group compared with that in the control group (0.35%; 26/7465, Table 3).

In the mouse specific locus test, which is believed to detect mutations via direct damage of the genes by irradiation, the X- or γ-ray-induced mutation rate in the offspring mated at 6 to 7 weeks after irradiation did not differ from that in the offspring mated 8 to 40 weeks after irradiation (*49*). In the m-ESTR mutation studies, however, the radiation effect has not been studied beyond 15 weeks after the irradiation. In this regard, it should be noted that most of the A-bomb survivors had their children more than 15 years after their radiation exposure. Thus it is possible that either the instability is less pronounced in humans than in mice and/or unstable conditions that might have been induced in human germ cells immediately after radiation exposure did not persist beyond 15 years after the exposure.

Last, ionizing radiation can induce mutations in somatic and germ cells of various species tested so far, and hence it is unlikely that humans are an exception. Radiation doses in animal studies are considerably higher than those received by the survivors of the bombs. For example, specific locus tests in mice mainly used acute doses of 3 to 5 Gy whereas only a small fraction of A-bomb survivors have estimated doses of 2 Gy or larger. The mean parental dose of the survivors (exposed group) in the F_1 epidemiological cohort is about 0.4 Gy (*50*). Nonetheless, a clear dose response has been observed in m-ESTR studies for a dose range of 0.5 to 1 Gy (*41, 47, 48*), which corresponds to the dose range of the A-bomb survivors.

Feasibility of Detecting Radiation Effects in Females

Although no discernible effect from radiation exposure was observed, the observed mutation rates after maternal exposures appeared to be elevated compared with spontaneous rates ($0.26–0.42 \times 10^{-2}$/locus/generation and $0.13–0.17 \times 10^{-2}$/locus/generation, respectively, Table 6) although the difference was not significant. If we take the mutation rates at face value, then we may estimate the sample size to detect the possible difference with a probability of 0.8, i.e., 48,000 unexposed alleles and 16,000 exposed alleles, about 10 times larger than the present study. Unfortunately, obtaining such a large number of samples is not realistic at this time.

Biological Implications of New Mutations in Human Genome

Recent reports on genome sequencing revealed that even apparently healthy individuals carry thousands of nonsynonymous single nucleotide polymorphisms that cause amino acid substitutions and nearly 1000 insertions or deletions that involve protein-coding sequences in their genome (*51–55*). Some of those variations may be old and are either neutral or even advantageous, whereas others may be rather new and may cause a genetic burden to their hosts. In any event, when we present the data on the genetic effects of radiation, the risk would be better understood if we were able to know the number of pre-existing mutations in our genome and the ones that were added as a consequence of radiation exposure. Three different approaches, epidemiological, clinical and molecular studies, are indispensable for an unbiased understanding of the genetic effects of A-bomb radiation.

SUPPLEMENTARY INFORMATION

Supplementary Table 1. Primer sequences and annealing temperature for ten loci that were not described previously. **Supplementary Table 2**. Number of alleles examined. http://dx.doi.org/10.1667/RR1991.1.S1

ACKNOWLEDGMENTS

The authors thank Dr. E. Nakashima for valuable suggestions for statistical analysis, J. Kaneko, M. Imanaka, A. Miura and E. Nishikori at RERF, and L. T. T. Thuy at Osaka University for their excellent technical assistance. The Radiation Effects Research

Foundation (RERF), Hiroshima and Nagasaki, Japan is a private, non-profit foundation funded by the Japanese Ministry of Health, Labour and Welfare (MHLW) and the U.S. Department of Energy (DOE), the latter in part through the National Academy of Sciences. This publication was supported by RERF Research Protocols RP7-85, RP5-85, RP1-01, B40-05 and B46-07 and by grants from MEXT Japan (16201013, 20241014), the Heiwa Nakajima Foundation, and the Japan Space Forum to TN.

Received: August 28, 2009; accepted: October 9, 2009

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Mutation Research 696 (2010) 107–113

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

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Community address: www.elsevier.com/locate/mutres

Effects of fission neutrons on human thyroid tissues maintained in SCID mice

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ARTICLE INFO

Article history:
Received 18 September 2009
Received in revised form 8 December 2009
Accepted 28 December 2009
Available online 12 January 2010

Keywords:
Human thyroid tissue
Fission neutrons
SCID mice
Gene expression
Mutation
RBE

ABSTRACT

Morphology and function (secretion of thyroid hormone) of human thyroid tissues from Graves' disease patients are well maintained in C57BL/6J-*scid* mice. Serum level of thyroid hormone was reduced by fission neutrons from the nuclear reactor UTR-KINKI, and changes in thyroid hormone by fission neutrons were bigger than those by low LET radiations, X-rays and ^{137}Cs γ-rays, suggesting high relative biological effectiveness (RBE; 6.5) of fission neutrons. Microarray analyses revealed that about 3% of genes showed more than 4-fold change in gene expression in the unexposed thyroid tissues against surgically resected thyroid tissues from the same patient, probably due to the difficult oxygen and nutrient supply shortly after transplantation. Dose-dependent changes in gene expression against unexposed concurrent controls were observed with increasing doses of fission neutrons (0.2–0.6 Gy) and ^{137}Cs γ-rays (1.0–3.0 Gy) and showed high RBE (4.2). Furthermore, there were some specific genes which showed more than 4-fold change in gene expression in all the thyroid tissues exposed to higher doses of radiation, especially neutrons (0.4 and 0.6 Gy), but none at lower doses (0.2 Gy of neutrons and 1.0 and 2.0 Gy of γ-rays). These genes related to degeneration, regeneration, apoptosis, and transcription, respond specifically and very sensitively to neutron injury in human thyroid tissues. This is the first experimental report that fission neutrons can induce some morphological and functional disorders in human tissues, showing high RBE against γ-ray exposure. These results are useful to evaluate the risks of fission neutrons and cosmic rays to humans.

1. Introduction

Radiation induces various types of damage in human and animals. Among various radiation sources, neutrons are several times more effective than X- and γ-radiation in inducing neoplastic cell transformation, mutation *in vitro*, germ-cell mutation *in vivo*, chromosomal aberrations *in vivo* and *in vitro* and cancer in experimental animals [1]. In spite of the evidence in experimental animals, there is a scarcity of evidence in humans; epidemiological study of A-bomb survivors on the difference between Hiroshima and Nagasaki and a few accidents at nuclear sites [1]. In humans, exposure to neutrons can occur from the nuclear fission reactions usually associated with the production of nuclear energy and from cosmic radiation (in the flying body) in the natural environment [2–4]. Consequently, it is of utmost importance to study the direct effects of fission neutrons on human organs and tissues for investigating the precise risk.

In the improved severe combined immunodeficient (super-SCID) mice, 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 [5–10]. 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 [10]. Furthermore, transplanted human thyroid tissues secreted thyroid hormone (T3), and T3 secretion was stimulated by the injection of human thyroid stimulating hormone (TSH)

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

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doi:10.1016/j.mrgentox.2009.12.017

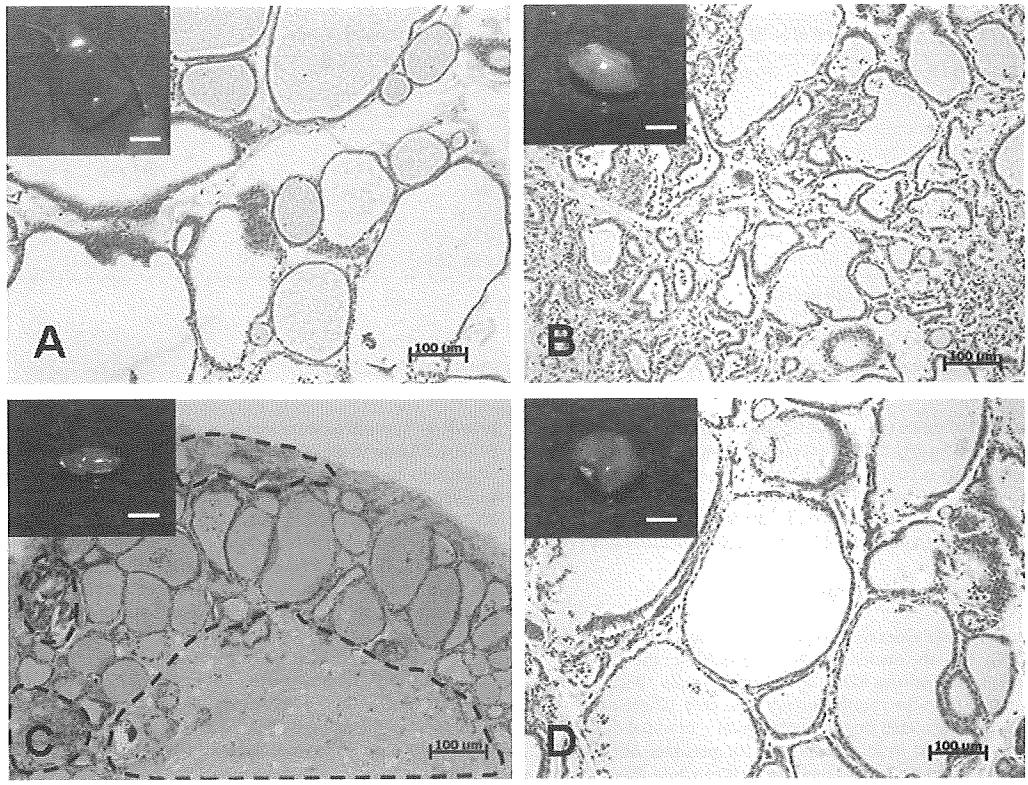

Fig. 1. Macroscopic and microscopic views of transplanted human thyroid tissues with or without radiation exposures. (A) Surgically resected human thyroid tissue from Graves' disease patient (20 years, female). (B) Human thyroid tissues from a Graves' disease patient (20 years, female) exposed to neutrons (0.2 Gy × 4 times at 1 week interval) and exposed tissue was removed 5 weeks after transplantation, (C and D) transplanted thyroid tissues exposed to neutrons (0.2 Gy × 6 times) and removed 7 months after transplantation (C) and concurrent unexposed controls (D), respectively. Histologically, follicles became small, some disappeared and were replaced to connective tissues (C) (marked by broken line). Scale bars in gross features: 3 mm. Microscopic views; haematoxylin and eosin staining. Scale bars: 100 μm.

[10]. Expression analysis by microarray indicated that gene expression was also well maintained in the transplanted human thyroid tissues [11]. The expression of only 3% of genes showed more than 4-fold change in gene expression 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 slightly [11].

The thyroid gland is one of the most important endocrine organs for the development and growth, and one of the most sensitive organs to radiation. Radiation exposure, therefore, causes general disorder in human beings [12]. In fact, consecutive irradiation with X-rays or ^{137}Cs γ-rays for approximately 2 years resulted in the disappearance of follicles and significant decrease of thyroid hormone secretion [11]. Mutations in *p53* and *c-kit* genes were induced significantly by high dose and high dose rate of X-rays and γ-rays in human thyroid tissues from old head and neck cancer patients and a Graves' disease patient, while mutations were not detected by low dose rate exposure [11]. Furthermore, lower doses (1–3 Gy) of ^{137}Cs γ-rays can induce changes in gene expression in the transplanted human thyroid tissues.

In the present study, human thyroid tissues from Graves' disease patients were transplanted into the improved SCID mice, and exposed to fission neutrons or ^{137}Cs γ-rays to examine the induced changes in morphology, function, cancer-related genes, and changes in gene expression in the transplanted human thyroid tissues to confirm the direct link between radiation sources.

2. Materials and methods

2.1. Human thyroid tissues

Thyroid tissues resected from two Graves' disease patients (20 and 23 years, females) were used for heterotransplantation to the SCID mice. Goiter was resected because of cosmetic problem, and blood level of thyroid hormone of the patients before the surgical resection was within normal range. Only the human tissues free of mycoplasma, human hepatitis B and C antigens/antibodies, adult T cell leukemia, human immunodeficiency virus, and Wassermann reaction were accepted into the SPF room of the barrier section of the Institute of Experimental Animal Sciences, Osaka University. Use of human tissues were permitted by the ethics committees of Osaka University, Graduate School of Medicine, Kuma Hospital and National Institute of Biomedical Innovation, 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

C57BL/6J-*scid*/*scid* mice ($N_{12}F_{22-24}$) were used for the experiment. C.B17-*scid*/+ male and female mice were provided by Dr. M.J. Bosma [13], Institute of Cancer Research, Philadelphia, in 1986, and then C.B17-*scid*/*scid* mice were maintained by selective sister–brother inbreeding of C.B17-*scid*/*scid* homozygote showing undetectable serum IgG and IgM (<1 μg/ml) by T. Nomura to diminish the leaky and leukemic mice [5,6,14]. C.B17*scid*/*scid* male (N_1F_3) was mated with C57BL/6J female (F_{153}) (provided by E.S. Russell, Jackson Laboratory at F_{129} in 1976 and inbred by sister–brother mating for further generations). Progeny was crossed and *scid* homozygote mouse was repeatedly back-crossed to C57BL/6J mouse to make congenic strain of C57BL/6J-*scid*/*scid* ($N_{12}F_{20}$) by T. Nomura [15]. 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 [5,6], and 2 months old C57BL/6J-*scid*/*scid* mice showing undetectable serum IgG and IgM (<1 μg/ml) were used for the heterotransplantation of human thyroid tissues. 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 [5–11]. Briefly, resected human thyroid gland was cut into 5–6 mm cubic masses in a 0.9% NaCl solution contain-

Table 1
Human thyroid hormone (T3) in peripheral blood of SCID mice with human thyroid tissues after neutron exposure.

Weeks after transplantation (first exposure)	Exposed			Unexposed[a]		
	Dose (Gy)	No.	Thyroid hormone (pg/ml)	No.	Thyroid hormone (pg/ml)	p^b
1	0.2 × 1	2	481, 409	1	445	1.000
2	0.2 × 2	2	509, 468	1	494	0.902
3	0.2 × 3	2	458, 385	1	461	0.644
4	0.2 × 4	2	385, 388[c]	2	443, 438	0.007
20	0.2 × 6	3	331, 275, 375[c]	2	391, 394	0.152
24	0.2 × 6	3	295, 334, 236[c]	2	328, 354	0.202

Thyroid tissues from Graves' disease patient (20 years, female) were used.
[a] Unexposed concurrent controls to the exposed groups.
[b] *t*-Test was applied after testing quality of variance by SPSS Statistics System.
[c] $p < 0.01$ vs. the mean of unexposed groups (5 samples from 1 to 4 weeks after transplantation).

ing high concentrations of antibiotics (penicillin G, 50,000 units/ml; panipenem, 25 mg/ml; 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. Thyroid stimulating hormone (TSH) was not given to the SCID mice with thyroid tissues of Graves' disease which is characterized by stimulating autoantibody [16].

2.4. Neutron and γ-ray exposure of human thyroid tissues

SCID mice with human thyroid tissues were exposed to fission neutrons at about 0.2 Gy/h and γ-rays at 0.2 Gy/h by the nuclear reactor, UTR-KINKI in the Kinki University Atomic Energy Research Institute. UTR-KINKI has an ample space for the irradiation of biological materials in the central portion of its core, where neutrons and γ-rays of about 0.2 Gy/h is available during operation at a nominal output of 1 W. Neutron and γ-ray doses at the irradiation port in the presence of C57BL/6J mice were measured using the paired chamber method. Neutron dose was reduced with increasing numbers of mice; *i.e.* total weight (g) of mice [17]. Actual dose rate, when four C57BL/6J mice (25 g each) were placed, was 0.197 Gy/h of neutrons and 0.198 Gy/h of γ-rays. Human thyroid tissues from Graves' disease patients were also exposed to reference doses of ^{137}Cs γ- rays by Gammacell 40 Exactor (Nordion International Inc., Canada) at a dose rate of 1.19 Gy/min. Dosimetry of ^{137}Cs γ-rays 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.

Human thyroid tissues from Graves' disease patient (20 years, female) were transplanted s.c. and then a half of SCID mice with human thyroid tissues were exposed to 0.2 Gy of fission neutrons by UTR-KINKI 1–4 times (2 samples in each group) at 1, 8, 15 and 22 days after transplantation. The other half were not exposed to neutrons and used for concurrent unexposed controls. Six days after exposure, blood was taken from each SCID mouse with thyroid tissue transplantation for the measurement of human thyroid hormone in the blood of SCID mice [18]. Blood was also taken from unexposed SCID mice with thyroid tissues 7, 14, 21 and 28 days after transplantation. Eleven thyroid tissues were also exposed to 0.2 Gy of fission neutrons 6 times at 1, 8, 15, 22, 29 and 36 days after transplantation, and then thyroid tissues were removed 5–13 months after transplantation (3–11 months after neutron exposure) for the examination of mutations. Peripheral blood was also taken from these exposed and unexposed SCID mice with thyroid tissue transplantation for the examination of human thyroid hormone secretion.

2.5. Measurement of thyroid hormone (T3)

Peripheral blood was taken from the SCID mice to which human thyroid tissue had been transplanted. Human thyroid hormone T3 was measured by the radioimmunoassay (T-3 RIABEAD, Dinabot, Tokyo, Japan) as reported previously [10,11,18].

2.6. Analysis of gene expression in the transplanted human thyroid tissue

Twenty two human thyroid tissues from the other Graves disease patient (23 years, female) were exposed to 0.2 Gy (4 thyroid tissues), 0.4 Gy (4 tissues) and 0.6 Gy (3 tissues) of fission neutrons and 1.0 Gy (4), 2.0 Gy (4) and 3.0 Gy (3) of ^{137}Cs γ-rays 1 day after transplantation to SCID mice. Eight thyroid tissues were not exposed to radiations and used for concurrent controls. Two weeks after radiation exposures, thyroid tissues were removed from SCID mice for the analysis of gene expression by microarray [11]. Human thyroid tissues removed from the SCID mice were kept in the liquid nitrogen.

Level of gene expression in transplanted thyroid tissue was compared with original (surgically resected) thyroid tissue to observe spontaneous changes after transplantation [11]. Radiation exposed thyroid tissues were also compared with concurrent unexposed controls by GeneChip (HG-Focus Array; 8500 genes, Affymetrix, Inc., Santa Clara, CA, USA) to examine the changes in gene expression by radiation exposure.

To extract RNA in high efficiency, thyroid tissues were crushed by pressing in frozen metal blocks (Cryopress, Microtec Co., Ltd., Funabashi, Japan) and then the powdered specimens were homogenized by microtube homogenizer (Homogenizer PT-101, PA Cosmo Bio., Tokyo, Japan) in Trizol reagent solution as described by manufacturer's protocol (Invitrogen, Japan K.K., Tokyo, Japan). 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. Total RNA (1.18–2 μg) was used to synthesize cDNA and biotinylated cRNA, and fragmentation of the cRNA. Those procedures were performed according to the manufacture's protocol (Affymetrix, http://www.affymetrix.com). 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 using the Microarray Suite 5 (MAS5) algorithm. The data of gene description were derived from NetAffx™ Analysis Center (Affmetrix Inc.) on July 4, 2009, and Entrez Gene (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, U.S.A.) in September, 2009.

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 neutrons

There were no substantial differences in gross and histological features in the unexposed thyroid tissues after transplantation, as it was in previous reports [10,11]. However, macroscopic and his-

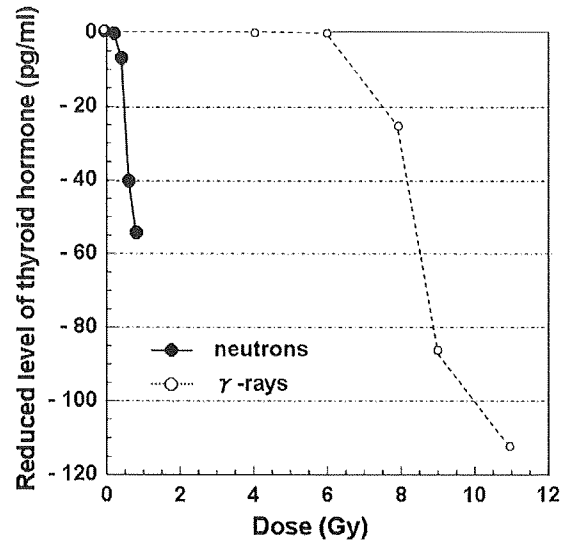

Fig. 2. Reduced concentration of human thyroid hormone (T3) in the peripheral blood of SCID mice with human thyroid tissues after neutron and γ-ray exposures. Thyroid tissues from a Graves' disease patient (20 years, female) were transplanted s.c. to the SCID mice and exposed to fission neutrons. Thyroid hormone (T3) in the blood of SCID mice with human thyroid tissue transplantation was measured by radioimmunoassay. Reduced level of serum thyroid hormone (T3) in exposed SCID mice from those of concurrent unexposed controls (Table 1) were plotted at each dose (closed circles, solid line). Similarly, reduced level of T3 after ^{137}Cs γ-ray exposure (open circles, broken line) were also plotted (from Table 2 of Ref. [11]) in the same figure in comparison with neutron exposed groups.

tological changes were observed in the thyroid tissues exposed to 0.8 Gy (0.2 Gy, 4 times) (Fig. 1B) and 1.2 Gy (0.2 Gy, 6 times) of fission neutrons and removed 7 months after transplantation (Fig. 1C), while such damage was not observed in concurrent unexposed controls (Fig. 1D). Histologically, follicles became small (Fig. 1B and C), some disappeared and were replaced by connective tissues (Fig. 1C, marked by broken line).

3.2. Functional changes in human thyroid tissues by neutrons

Table 1 shows the serum level of human thyroid hormone (T3) detected in the blood of the SCID mice to which human thyroid tissues had been transplanted. Serum level of T3 in unexposed control groups showed no decreases up to 4 weeks after transplantation, but slight decreases were observed at 20 weeks after transplantation. There were no differences in the serum level of T3 between exposed and concurrent unexposed groups at 0.2 Gy of neutrons. However, serum level of T3 decreased slightly and dose-dependently at doses from 0.4 to 0.8 Gy in comparison with unexposed concurrent controls (Table 1 and Fig. 2). Decrease of T3 was also observed 20 and 24 weeks after 1.2 Gy (0.2 Gy, 6 times) of neutron exposure. Results showed large differences from those by γ-rays which showed apparent threshold dose (~6 Gy) [11] (Fig. 2). Reduced serum levels of thyroid hormone by 0.4, 0.6 and 0.8 Gy of fission neutron exposure were nearly equivalent to those by approximately 6.5, 8.2, and 8.5 Gy of ^{137}Cs γ-rays (Fig. 2). This suggests high relative biological effectiveness (RBE) of fission neutrons from UTR-KINKI, the values estimated from the slopes from 0.2 to 0.8 Gy of neutrons and from 6 to 11 Gy of γ-rays (Fig. 2) being approximately 6.5 after the reduction of γ-ray component of the nuclear reactor UTR-KINKI.

3.3. Changes of gene expression in human thyroid tissues by fission neutrons

Levels of gene expression in transplanted thyroid tissues were compared with those of the original tissues; *i.e.* surgically resected thyroid tissues. In the previous report [11], about 3% genes showed more than 4-fold change in gene expression in the first week after transplantation, probably due to the difficulty of oxygen and nutrient supply within 7 days after surgical extirpation and transplantation, but further changes were not observed 2–4 weeks after transplantation. In the present study, consequently, levels of gene expression were compared 2 weeks after transplantation and radiation exposure, and similar results were observed in the thyroid tissues from Graves' disease patient (23 years, female). About 3% of genes showed more than 4-fold change (both increase and decrease) in gene expression in 8 unexposed thyroid tissues [increases (>4); 201.8 ± 12.5 (mean ± SE, $n = 8$), decreases (<1/4); 69.8 ± 13.5 against surgically resected original thyroid tissue from the same patient]. Neutron exposed thyroid tissues from the Graves' disease patient (23 years, female) were also compared with unexposed concurrent control thyroid tissues from the same patient. Numbers of genes which showed more than 4-fold change in gene expression against concurrent unexposed thyroid tissues increased dose-dependently at dose ranges from 0.2 to 0.6 Gy of fission neutrons and from 1.0 to 3.0 Gy of ^{137}Cs γ-rays (Fig. 3). RBE was estimated as 4.2 after the reduction of γ-ray component of UTR-KINKI.

There were many genes which showed more than 4 folds change in gene expression among all the unexposed transplanted thyroid tissues against surgically resected original thyroid tissue (Fig. 4, Table 2), suggesting that these genes are sensitive to the lack of oxygen and nutrients just after surgical extirpation and trans-

Table 2
Genes showing more than 4-fold change in all the transplanted unexposed thyroid tissues.

	Gene name	Gene symbol
(1) Increase	Matrix metallopeptidase 12 (macrophage elastase)	MMP12
	Secreted phosphoprotein 1	SPP1
	Chemokine ligand 10	CXCL10
	Collagen, type I	COL1A1
	Perilipin 2	PLIN2
	Matrix metallopeptidase 9 (gelatinase B)	MMP9
	Chemokine ligand 9	CXCL9
	Guanylate binding protein 2	GBP2
	Tenascin C	TNC
	TIMP metallopeptidase inhibitor 1	TIMP1
	Rho family GTPase 3	RND3
	Versican	VCAN
	Collagen, type VI	COL6A3
	Collagen, type I	COL1A2
	Complement component 1, r subcomponent	C1R
	Collagen, type III	COL3A1
	Lysyl oxidase-like 2	LOXL2
	Cathepsin K	CTSK
	Transforming growth factor, beta-induced	TGFBI
	Chemokine ligand 11[a]	CXCL11
	Surfactant protein B[a]	SFTPB
	Plexin domain containing 1[a]	PLXDC1
(2) Decrease	Murine osteosarcoma viral oncogene homolog	FOS
	Murine osteosarcoma viral oncogene homolog B	FOSB
	Carbonic anhydrase IV	CA4
	Thyroid peroxidase	TPO
	Nebulin	NEB
	Solute carrier organic anion transporter family	SLCO2A1
	Deiodinase, iodothyronine, type I[a]	DIO1
	Chloride intracellular channel 3[a]	CLIC3
	Fc fragment of IgG binding protein[a]	FCGBP
	Solute carrier family 25[a]	SLC25A15
	Dual specificity phosphatase 1[a]	DUSP1
	Latent transforming growth factor beta binding protein 4[a]	LTBP4

Eight thyroid tissues from Grave's disease patient (23 years, female) were transplanted to SCID mice and removed 2 weeks after transplantation for gene expression analysis. Levels of gene expression were compared with surgically resected original thyroid tissue from the same patient.

[a] Seven of 8 thyroid tissues showed more than 4-fold change in gene expression.

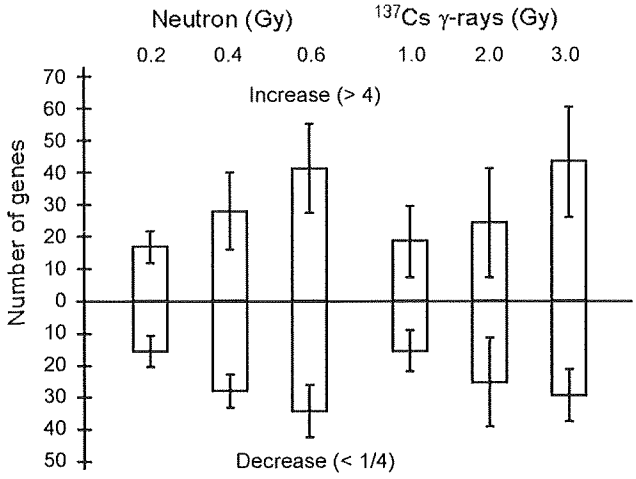

Fig. 3. Dose-dependent changes in gene expression in the transplanted human thyroid tissues after neutron and γ-ray exposures. Human thyroid tissues from Graves' disease patient (23 years, female) in SCID mice were exposed to 0.2, 0.4 and 0.6 Gy of fission neutrons and 1.0, 2.0 and 3.0 Gy of ^{137}Cs γ-rays, and compared with concurrent unexposed thyroid tissue from the same patient. Numbers of genes which showed more than 4-fold change in gene expression against unexposed concurrent controls were plotted on the ordinate. Histograms and horizontal bars show the mean and standard error of the mean. p values are 0.077 for neutrons and 0.157 for γ-rays by Kruskal Wallis test (SPSS statistics).

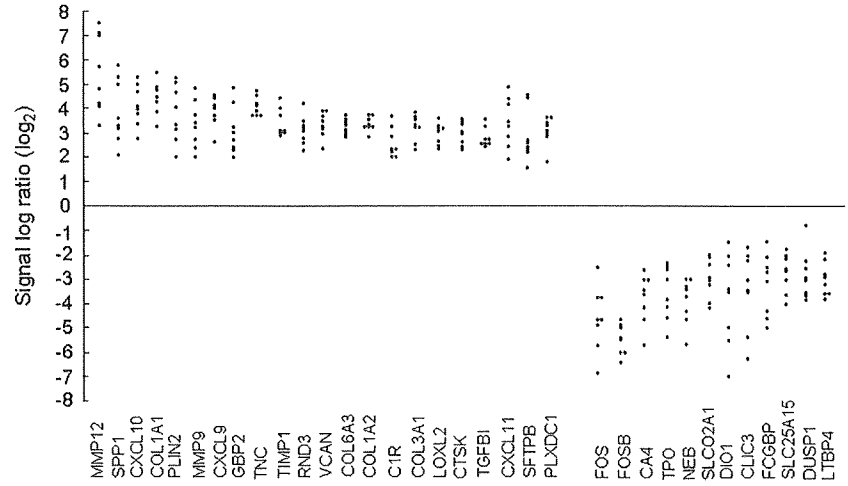

Fig. 4. Individual variation in gene expression of each unexposed thyroid tissue. Level of gene expression in 8 thyroid tissues from Graves' disease patient (23 years, female) was compared with that of surgically resected original thyroid tissue from the same patient. Genes showing signal log ratio (log_2) more than 2 (4-fold change) in gene expression in all the thyroid tissues were given on the abscissa and individual value was plotted on the ordinate.

plantation. Such genes are shown in Table 2. These spontaneous changes are related to degeneration and regeneration processes of transplanted thyroid tissues. In contrast, these findings were not observed in radiation exposed thyroid tissues at low doses of radiation. There were no common genes which showed more than 4-fold changes in gene expression in thyroid tissues exposed to 0.2 Gy of neutrons and 1.0 and 2.0 Gy of ^{137}Cs γ-rays, against unexposed concurrent controls, suggesting that radiation injury may be random phenomenon, while 4 and 14 genes for 0.4 and 0.6 Gy of neutron exposure and 5 genes for 3.0 Gy of γ-ray exposure showed more than 4-fold change (Table 3). Among 13 of 14 genes which showed more than 4-fold change in all the thyroid tissues exposed to higher doses of fission neutrons and ^{137}Cs γ-rays (Fig. 5, Table 3), eight were related to genes responding to injury or stress (C7orf68, ADM, SERPINEI, ENO2, ANGPTL4, PLIN2, COL9A3, and DIO1), 3 were related to apoptosis (BNIP3, HK2, and TFF3), and 2 were related to transcription (CEBRD and BHLHE40). This indicates that some genes are specifically sensitive to higher doses of radiation, especially to neutrons. Two genes, perilipin 2 and deiodinase showed more than 4-fold change in both unexposed and neutron exposed thyroid tissues. There were no changes in gene expression of *RET*, *BRAF* and Thyroxine (T3) related genes in all the thyroid tissues which were exposed to fission neutrons (0.2 Gy, 1–4 times at 1 week interval or single exposure of 0.2–0.6 Gy) and ^{137}Cs γ-rays (1.0–3.0 Gy).

4. Discussion

Human thyroid tissues were well maintained for a long period in C.B17-*scid* mice, showing no substantial differences in the histology, secretion of human thyroid hormone, and response to thyroid stimulating hormone [10,11]. Level of gene expression was also well maintained in the transplanted thyroid tissues for several weeks [11]. 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 not observed by the low dose rate exposure to the same dose of ^{137}Cs γ-rays, indicating significant dose rate effects in human thyroid tissues.

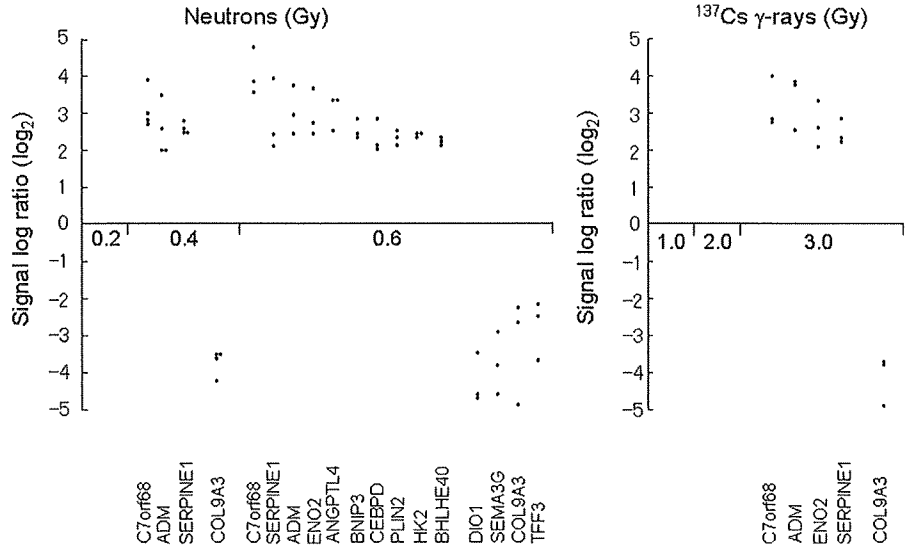

Fig. 5. Individual variation in gene expression of neutron and ^{137}Cs γ-ray exposed thyroid tissues. Level of gene expression in each thyroid tissue from Graves' disease patient (23 years, female) was compared with that of unexposed concurrent control thyroid tissue of the same patient. Genes showing signal log ratio (log_2) more than 2 (4-fold change) in gene expression in all the thyroid tissues were given on the abscissa and individual value was plotted on the ordinate.

Table 3
Genes showing more than 4-fold change in gene expression in all the thyroid tissues exposed to fission neutrons and ^{137}Cs γ-rays in comparison with unexposed concurrent controls.

Radiations	Dose (Gy)	Gene name	Gene Symbol
(A) Neutrons			
(1) Increase	0.2	None	
	0.4	Chromosome 7 open reading frame 68	C7orf68
	0.4	Adrenomedullin	ADM
	0.4	Serpin peptidase inhibitor, clade E (nexin)	SERPINE1
	0.6	Chromosome 7 open reading frame 68	C7orf68
	0.6	Serpin peptidase inhibitor, clade E (nexin)	SERPINE1
	0.6	Adrenomedullin	ADM
	0.6	Enolase 2	ENO2
	0.6	Angiopoietin-like 4	ANGPTL4
	0.6	BCL2/adenovirus interacting protein	BNIP3
	0.6	CCAAT/enhancer binding protein	CEBPD
	0.6	Perilipin 2	PLIN2
	0.6	Hexokinase 2	HK2
	0.6	Basic helix-loop-helix family	BHLHE40
(2) Decrease	0.2	None	
	0.4	Collagen, type IX	COL9A3
	0.6	Deiodinase, iodothyronine	DIO1
	0.6	Sema domain, immunoglobulin domain	SEMA3G
	0.6	Collagen, type IX	COL9A3
	0.6	Trefoil factor 3	TFF3
(B) ^{137}Cs γ-rays			
(1) Increase	1.0	None	
	2.0	None	
	3.0	Chromosome 7 open reading frame 68	C7orf68
	3.0	Adrenomedullin	ADM
	3.0	Enolase 2	ENO2
	3.0	Serpin peptidase inhibitor, clade E (nexin)	SERPINE1
(2) Decrease	1.0	None	
	2.0	None	
	3.0	Collagen, type IX, alpha 3	COL9A3

Twenty-two thyroid tissues from the Graves' disease patient (23 years, female) were transplanted to SCID mice and exposed to 0.2 Gy (4 tissues), 0.4 Gy (4) and 0.6 Gy (3) of fission neutrons or 1.0 Gy (4), 2.0 Gy (4) and 3.0 Gy (3) of ^{137}Cs γ-rays. Radiation exposed thyroid tissues were removed 2 weeks after transplantation for gene expression analysis. Levels of gene expression were compared with unexposed concurrent control thyroid tissue from the same patient.

In the present study, morphology and function (secretion of thyroid hormone) of human thyroid tissues are well maintained in congenic C57BL/6J-*scid* mice. Neutron exposure reduced thyroid hormone secretion and changes in serum level of thyroid hormone by fission neutrons were larger than those of low LET radiations, X-rays and γ-rays (Fig. 2) [11], suggesting high relative biological effectiveness (RBE; 6.5) of fission neutrons.

In the previous study, large doses of γ-ray exposure (11–33 Gy) at high dose rate for long period (~3 years) could induce mutations in *c-kit* and *p53* genes in the transplanted human thyroid tissues [11,19–21]. In the present study, however, no mutations of cancer-related genes, *p53*, *K-ras*, *c-kit*, *β-catenin*, *RET*, *BAK* and *BRAF* nor tumors have been detected in these eleven thyroid tissues exposed to 1.2 Gy of fission neutrons (0.2 Gy, 6 times at 7-day intervals) and in the unexposed concurrent control tissues which were observed for 5–13 months after transplantation (data not shown). Longer term exposure of the thyroid tissue to larger doses of fission neutrons may be necessary to induce specific mutations and cancer in human thyroid tissues. We succeeded to induce mutation and human skin cancer in normal human skin maintained on SCID mice only after the continuous (~2 years) ultraviolet light B (UVB) irradiation [9]. Topical exposures, in contrast to whole body exposure to neutrons, may be less hazardous to critical internal organs of SCID mice and can produce more changes to the transplanted human tissues, resulting in the induction of mutation and cancer in human tissues.

In contrast to gene mutation, changes in gene expression were detectable in the human thyroid tissues shortly after transplantation to SCID mice [11]. Spontaneous changes in gene expression were observed after surgical extirpation and transplantation of thyroid tissues to SCID mice, and many genes showed more than 4-fold changes in gene expression in all the unexposed thyroid tissues, suggesting these genes related to degeneration and regeneration processes are very sensitive to the lack of oxygen and nutrients (Table 2, Fig. 4). In the radiation exposed thyroid tissues, numbers of genes which showed more than 4-fold change in gene expression increased with increasing doses of γ-rays and neutrons (Fig. 3) and showed high RBE (4.2). Furthermore, there were several genes which showed more than 4-fold change in gene expression in all the thyroid tissues exposed to higher doses of radiation, especially neutrons (Fig. 5 and Table 3). Some genes related to degeneration, regeneration, apoptosis, and transcription, responded specifically and very sensitively to radiation injury. Mechanism on radiation effects will be elucidated.

This is the first experimental report that fission neutrons can induce morphological and functional disorders to human tissues, showing high RBE against γ-ray exposure. These results are essentially useful to evaluate the risks of fission neutrons and various environmental factors to humans, and also useful for preclinical studies to examine efficacy and safety of new drugs.

Conflicts of interest

None.

Acknowledgements

This work was supported by Research for the Future, MEXT Japan, the Japan Space Forum, Takeda Sci. Foundation, and Japan Health Sci. Foundation. We thank R. Orihara, E. Hatanaka, K. Yasuda, and K. Enomoto for their assistances.

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