We have developed *Rev1* transgenic mice to evaluate whether overexpression of Rev1 has some function on tumorigenesis. Tritium β-rays induce a variety of DNA lesions including 8-oxoguanine and abasic sites as well as single- and double- strand breaks. Rev1 can bypass these DNA lesions, as well bypass ^{6}O-methylguanine, which is the major DNA adduct formed by alkylating agents such as azoxymethane (AOM). While the DNA lesions induced by AOM are not as complicated as those induced by tritium β-rays, AOM-induced tumorigenesis provides a short-term assay for Rev1-dependent effects as compared with long-term tritium-induced cancer assays. Based on this rationale, in this study we explored whether *Rev1* transgenic mice display increased sensitivity for AOM-induced tumorigenesis. Our data indicate that overexpression of Rev1 accelerates chemical-induced tumorigenesis, and therefore may represent a sensitive model system for evaluating the effects of tritium in carcinogenesis.

II. MATERIALS AND METHODS

II.A. Animals

Female C57BL/6N mice were purchased from Charles River (Kanagawa, Japan). The development of *Rev1* Tg mice was described the submitted manuscript. Tail DNA was used for genomic PCR to determine the genotype of each mouse. The handling and sacrificing of all animals were carried out in accordance with the guidelines of the Institute of Laboratory animal Science, Hiroshima University.

II.B. Tumorigenesis experiment

Both *Rev1* Tg and normal C57BL/6N female mice were treated with 25 mM $ZnSO_4$ in drinking water beginning at 3 wk of age. Starting at 7 weeks of age, they were given weekly subcutaneous injections of AOM (15mg/ kg body weight) for 3 weeks. The mice were examined at 518 days after birth to identify any developing tumors. During the experiments, moribund mice were killed by euthanasia. Tumor tissue obtained from AOM-treated animals was collected for weight measurements and histopathology. The tumors were identified by both macroscopic and microscopic analyses.

II.C. Statistical Analyses

CoxPHFit was used to analyze the difference between two groups in tumorigenesis. StatMateIII was used to calculate the P value in tumor incidence between two groups. $P < 0.05$ was considered statistically significant.

III. RESULTS

AOM is known to form DNA adducts and has been recognized as a colon and liver carcinogen in rats and mice. To examine whether *Rev1* over-expression accelerates AOM-induced tumor development in mammals, we investigated a large cohort of wild-type and *Rev1* Tg female mice over an extended period to identify differences in tumor development. Wild-type and *Rev1* Tg mice were given weekly subcutaneous injections of AOM (15mg/ kg body weight) for 3 weeks and followed up for 518 days. We observed that AOM treatment increased mortality in both *Rev1* Tg and wild-type mice during this period, but the mortality rate was significantly different between the two groups (Fig. 1.). The median time of tumor-free survival was 511d for wild-type and 485d for *Rev1* Tg mice, that suggested the value in *Rev1* Tg mice was significantly shorter than that in wild-type mice.

AOM treatment induced a variety of tumors in both *Rev1* Tg and wild-type mice group. Among AOM-induced tumors, hepatocellular carcinoma was the predominate tumor type observed in both groups (Fig. 2.). Whereas the wild-type mice injected with AOM

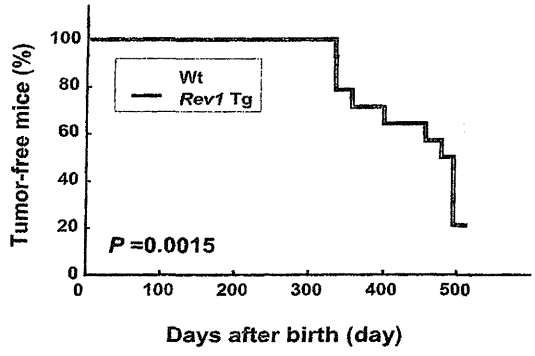

Fig. 1. Overexpressed *Rev1* accelerates AOM-induced tumorigenesis
Kaplan-Meier tumor-free survival curves of wild-type and *Rev1* Tg mice. CoxPHFit analysis showed significant difference between the two groups (P =0.0015)

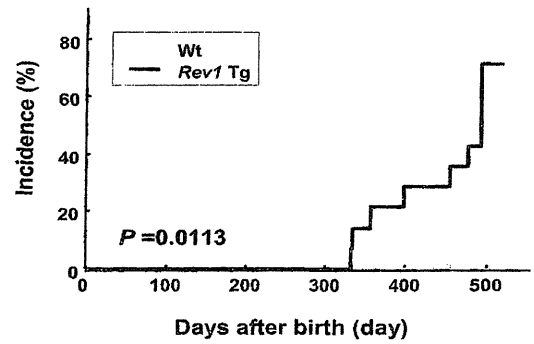

Fig. 2. *Rev1* Tg mice developed hepatocellular carcinoma at a higher incidence and a shorter latency than those of wild-type . CoxPHFit analysis showed significant difference between the two groups (P =0.0113)

developed hepatocellular carcinoma at the age of 455days, *Rev1* Tg mice injected with AOM began to develop hepatocellular carcinoma at as early as 333 days of age, 4 months earlier than wild-type mice. These data provide evidence that overexpression of Rev1 increases both the incidence of tumors and accelerates hepatic tumorigenesis induced by AOM.

Large intestinal adenomas were not detected in both groups during the time period we investigated, in spite of the fact that AOM is also known as a colon carcinogen in rodents. Aberrant crypt foci (ACF), which are putative preneoplastic lesions of colon cancer, and small intestinal adenomas were found in the large intestine of wild-type and *Rev1* Tg mice (Table 1). In wild-type mice, two adenomas were observed in the small intestines, and 13 ACF were found in the colons. On the other hand, four adenomas were found in the small intestines, and 22 ACF in the colon of *Rev1* Tg mice. There is no difference between the average number of mice with small intestinal adenoma and colonic ACF in the two groups.

The spectrum of tumor types observed in *Rev1* Tg mice was slightly different from that seen in the wild type. The difference was an increased incidence in lymphoma in the *Rev1* Tg mice. Seven of the 14 *Rev1* Tg mice developed lymphoma, whereas only two of the 9 wild-type mice did so (Table 1).

Our present data clearly indicate that overexpression of *Rev1* is in itself sufficient to predispose mice to develop other types of tumors in addition to hepatocellular carcinoma.

IV. DISCUSSION

In our previous study, we have developed *Rev1* transgenic mice and found that overexpression of *Rev1* enhanced MNU-induced tumor development.
In this study, it was explored whether *Rev1* transgenic mice are useful animal model as a high sensitive assay system for evaluating cancer risk of tritium. *Rev1* Tg mice and wild-type mice were treated with AOM as a model chemical carcinogen, as AOM is a known liver and colon carcinogen. *Rev1* Tg mice developed various types of tumors, including hepatocellular carcinoma, small intestinal adenoma, lymphoma, sarcoma and hardarian grand tumor in response to AOM exposure. Furthermore, *Rev1* Tg mice developed hepatocellular carcinoma at a higher incidence and a shorter latency than those of wild-type after AOM treatment. The incidence of AOM-induced lymphoma in *Rev1* Tg mice was higher than that in wild-type mice. From our data, overexpression of *Rev1* is sufficient to predispose mice to AOM-induced tumor with higher and shorter incidence compared with wild-type mice. Our present data clearly indicates that tumor-accelerating action of *Rev1* overexpression in mice induced by chemical carcinogens.

Although AOM induces colon cancers, there was no difference in small intestinal adenomas or colonic ACF in both wild-type and *Rev1* Tg mice groups. Development of small intestinal and colonic tumors has much longer term than that of hepatic tumor. In this study, the mice were examined at 518 days after birth to identify any developing tumors. It would be required for long period to detect the difference in the small intestinal adenomas or colonic ACF. The spectrum of tumor types observed in *Rev1* Tg mice was slightly different from that seen in the wild type. The difference was an increased incidence in lymphoma in the *Rev1* Tg mice. Expression level of Rev1 may be different in the tissue. In *Rev1* Tg mice, expression level of Rev1 in the hematopoietic tissue may be higher than that in the liver.

We have found that overexpression of Rev1 reduced cellular sensitivity to the cytotoxic effect of MNU and increased the mutagenicity of MNU, which was analyzed by measuring the frequency of HPRT mutations using human cell lines (manuscript in preparation). Furthermore, over-expressed Rev1 had the same function for radiation induced cytotoxicity and mutagenicity, respectively. Hence, as for chemical carcinogens, overexpression of Rev1 may accelerate radiation-induced carcinogenesis. It was reported that hypomorphic Rev1 accelerated UV-

TABLE 1. Tumor spectra

Organ	Wild-type (n=9)		*Rev1* Tg (n=14)	
	No. of tumors	No. of mouse with tumors (%)	No. of tumors	No. of mouse with tumors (%)
Hepatocellular carcinoma	7	7 (78 %)	11	11 (79 %)
Small intestinal adenoma	2	1 (11 %)	4	2 (14 %)
Aberrant crypt foci (ACF) in large intestine	13	5 (56 %)	22	6 (43 %)
Lymphoma	2	2 (22 %)	7	7 (50 %)
Sarcoma/ hemangiosarcoma	2	2 (22 %)	2	2 (14 %)
Hardarian grand tumor	0	0 (0 %)	2	2 (14 %)
Total	19	8 (89 %)	37	13 (93 %)

NOTE: The numbers of each tumor type and the number of animals with tumors in the indicated organs are presented. Parentheses denote tha percentage of animals with tumors in the respective organs. Total wild-type mice = 9; total *Rev1* Tg mice = 14

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Molecular nature of radiation injury and DNA repair disorders associated with radiosensitivity

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Abstract Ionizing radiation (IR), as well as a wide variety of chemicals and reactive oxygen species, can cause insults in DNA integrity. However, IR is distinct from other agents in that produces clustered DNA damage, particularly double-strand DNA breaks (DSBs). The discovery of radiosensitive human diseases has revealed that the molecular mechanisms underlying the biological effects of IR impact cellular responses to and repair of DSBs. One class of diseases, including ataxia-telangiectasia, displays a defect in checkpoint response to DSBs. Another class of diseases exhibits severe combined immunodeficiency and defects in DSB repair. Importantly, radiosensitive human diseases are also associated with increased risks of leukemia/lymphoma. In this review, we summarize the molecular nature of IR-induced DNA damage, and provide an overview of the molecular mechanisms of checkpoint response to and repair of DSBs. Lastly, we discuss the roles of these mechanisms in the development of the immune system and the suppression of lymphoma/leukemia, based on the clinical features and experiments with model mice.

Keywords Ionizing radiation · Double-stranded DNA breaks · Ataxia-telangiectasia · Severe combined immunodeficiency · Non-homologous end-joining

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Introduction

Ionizing radiation (IR) produces a wide variety of oxidative DNA damage. Cells recognize the damage and activate signal transduction pathways and repair systems to protect cells from such injury. The discovery of a radiosensitive human disease [1] has stimulated studies on the molecular mechanisms underlying the biological effects of IR. Now we recognize that the mechanisms; namely, checkpoint response to and repair of double-stranded DNA breaks (DSBs), have very important roles not only in the protection of cells from IR, but also in the development, aging, immune system and tumor suppression. In this review, we introduce the molecular nature of IR-induced DNA damage in comparison with chemicals damage. Then, we discuss roles of the checkpoint response and the repair mechanism in the development of the immune system and the suppression of lymphoma/leukemia, based on clinical features and experiments with model mice.

Damage to DNA by endogenous and environmental insults

DNA is a stable biomacromolecule. Even though *N*-glycosyl bonds with purine bases are relatively unstable, the frequency of hydrolysis of the *N*-glycosyl bonds in DNA molecules, producing abasic sites, is once per 1,000 years. Deamination of cytosine, producing uracil, occurs once per 70,000 years [2]. However, such rare events are significant in terms of maintaining genetic information. Because the genome size of humans is 3×10^9 base pairs, approximately 300 abasic sites and 4 uracils are estimated to be produced in 1 h in one human cell. Furthermore, targets of such hydrolytic and oxidative damage are naturally present

in DNA, i.e. carbons and nitrogens of bases and deoxyribose, and phosphodiester bonds [3]. The primary oxidative compounds are further decayed to many different chemical species because of their instability. Thus, many kinds of oxidative damage can be produced, and nearly 100 different types of oxidative damage have been identified [4, 5]. It has been estimated that oxidative damage causes the turnover of 2,000–10,000 purine bases per day in one human cell [6]. In addition to oxidative DNA damage, nucleophilic centers of bases, almost all oxygens, nitrogens, except for *N*-glycosyl bonds, as well as phosphates are targets of alkylation [7]. Many metabolic intermediates including *S*-adenosylmethionine (SAM) can be endogenous alkylating agents. Thus, many kinds of alkylating damage are also produced in cells. Reaction mechanisms of oxidation and alkylation of DNA are reviewed in detail [4, 7].

IR and environmental chemicals insult DNA as endogenous reactive species, producing oxidative DNA damage and introducing adducts to DNA, respectively. For example, benzo[*a*]pyrene, a carcinogen from cigarette smoking, and aflatoxin, a carcinogen produced by fungi, make adducts to the 2-amino position and N7 position on guanine, respectively [7, 8] and such DNA damage is specific to environmental chemicals. In contrast, oxidative DNA damage by IR can be also produced by endogenous reactive oxygens, already present in un-irradiated cells. The total amount of oxidative DNA damage produced in un-irradiated human cells in 1 day is estimated to correspond to that produced by 10 Gy of γ-ray irradiation with a dose rate of 7 mGy/min, which is roughly comparable to 7 mSv/min [9]. The amount of damage from environmental exposure of IR (1 mSv/year) is far less than the endogenous damage level.

The estimation of the amount of oxidative damage as above-mentioned seems to be somehow curious, because we are quite healthy even with the presence of similar amounts of oxidative damage to that produced by chronic irradiation of 7 mGy/min. A specific property distinguishing IR from endogenous oxygen species must be present. The most important such property of IR is the production of clustered DNA damage, especially DSBs. Because oxidation of deoxyribose causes strand breaks, damage to two sugars in opposite strands produce DSBs. One Gy of γ-ray irradiation to human cells produces 16–40 DSBs in addition to 600–1,000 single-stranded breaks (SSBs) [9, 10]. As a consequence, the lethal effect of IR is attributed to DSBs. This notion is supported by defective DNA repair mechanisms in cells from patients with radiosensitive diseases (see below).

Chemical structures of strand breaks in DNA

Strand breaks arise by abstraction of hydrogen atom from deoxyribose (Fig. 1a). Abstraction of H can occur

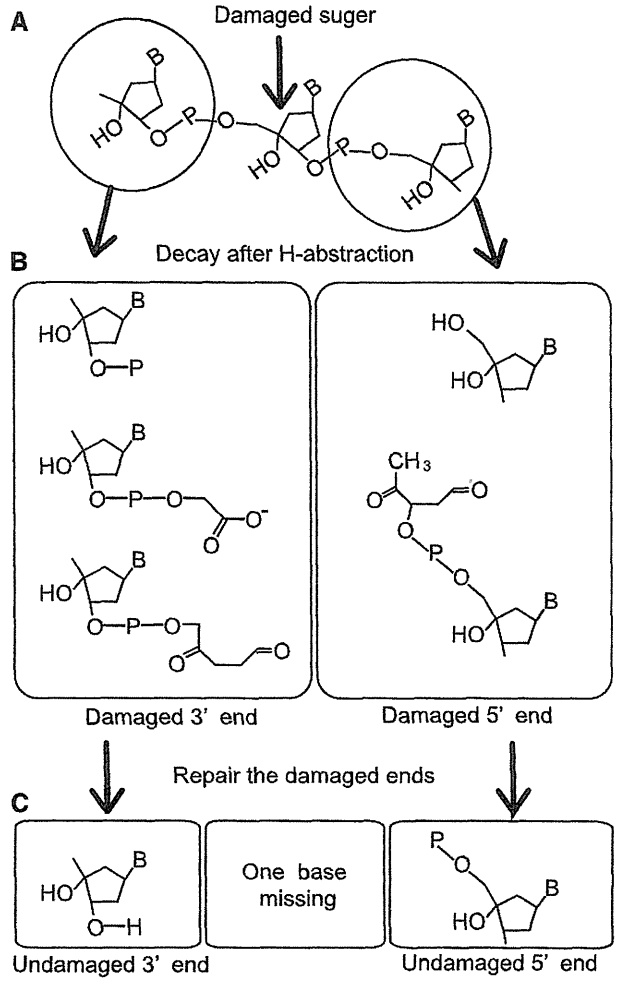

Fig. 1 Schematic representation of sugar damage in DNA. **a** Strand breaks are initiated by abstraction of hydrogen atom from deoxyribose. **b** Examples of chemical structures of the break ends. **c** Undamaged break ends after repair processing

at C2′, C3′, C4′ and C5′ positions [4]. The base attached to the damaged sugar is released in all cases, and some fragment of the deoxyribose moiety remains at the 5′ or 3′ break end (Fig. 1b) [4]. This indicates that the template strand for the missing base is required for accurate repair synthesis after trimming of break ends to produce 3′-OH for primer and 5′-phosphate for ligation (Fig. 1c) [11]. In the case of SSBs, the damage is repaired easily because of the presence of the opposite template strand. Contrarily, in the case of DSBs, it is easy to imagine that accurate repair synthesis is impossible because the two proximate bases located in the opposite strands are missing. Thus, direct re-joining of the DSB has the probability of loss of genetic information. If the DSB is repaired by homologous recombination, such risk could be negligible.

Radiosensitive diseases

A disorder characteristic of radiosensitive disease has been discovered in the course of treatment for lymphosarcoma that developed in a patient with ataxia-telangiectasia (AT) [1]. After irradiation to the tumor site, the patient developed severe dermatitis and deep tissue necrosis resulting in death in 8 months [1]. The clinically observed radiosensitivity of AT patients [1, 12] was demonstrated to be a defect at the cellular level [13]. Subsequently, many syndromes associated with radiosensitivity, the Nijmegen breakage syndrome (NBS) [14], ataxia-telangiectasia-like disorder (ATLD) and Nijmegen breakage syndrome-like disorder (NBSLD) have been reported [15–18]. These syndromes have phenotypic characteristics similar to AT at the cellular level. To date, genes responsible for the disorders have been identified as *ATM* for AT, *NBS1* for NBS, *MRE11A* for ATLD and *RAD50* for NBSLD [18–22]. Importantly, these gene products are all required for checkpoint response, a signal transduction pathway that senses DSBs (see below).

In addition, another class of disorders with severe combined immunodeficiency (SCID) is also associated with radiosensitivity, and the responsible genes have been identified as *DNA-PKcs*, *Artemis* and *LIG4* (DNA ligase IV) [23–26]. Notably, these gene products are all involved in the repair process, non-homologous end-joining (NHEJ), of DSBs induced by RAG proteins as well as IR (see below).

Cell biological properties

The cell biological characteristics of AT, NBS, ATLD and NBSLD are all similar. In addition to radiosensitivity, they are sensitive to radiomimetics like bleomycin and a wide variety of DNA damaging agents. In the early days, it had been generally considered that the primary defect in AT is a DNA repair defect. However, an obvious DNA repair defect has not been observed [13]. Rather, AT cells exhibit characteristics of radioresistant DNA synthesis (RDS) so that RDS has been used as a diagnostic marker for AT [27, 28]. This property is also observed in NBS [29–32], ATLD [22] and NBSLD cells [18]. In normal cells, replicative DNA synthesis is inhibited by IR as a consequence of checkpoint response. This property of RDS strongly suggests a defect of the checkpoint function in AT, NBS, ATLD and NBSLD cells. However, several reports have suggested that the sensitivity of those cells is not explained by only a defect of checkpoint response [33]. More recently, it has been recognized that ATM has a function to enhance homologous recombination at the damage sites by recruitment of repair enzymes (see below).

Cells deficient in *DNA-PKcs*, *Artemis* and *LIG4* all do show normal radiation induced cell cycle checkpoint response, and thus the radiosensitivity can be attributed to impaired NHEJ [34]. Patients with a defect in NHEJ are rather characteristic of SCID (see below).

Molecular functions

DNA damage response is initiated from recognition of specific DNA damage by sensor proteins (Fig. 2). For DSBs, MRE11A–RAD50–NBS1 (MRN) complex initially recognizes the DSB end, and recruits and activates ATM via specific interaction with NBS1 [19, 35, 36]. ATM is a member of the phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family of serine/threonine protein kinases [20]. ATM phosphorylates more than 700 proteins containing the phosphorylation motif (SQ/TQ) in response to IR [37], including MRN complex, a histone variant, H2AX, a checkpoint mediator, MDC1, a checkpoint kinase, CHK2 and p53. Phosphorylations of MRN complex, H2AX and MDC1 initiate recruitment of many factors required for signal transduction and homologous recombination, enhancing the repair process [38–42]. It has been considered that a marginal repair defect observed in AT cells [33] might reflect the reduced efficiency of homologous recombination. The cell cycle is arrested at the G1 phase through phosphorylation of CHK2 and activation of p53 (Fig. 2) [39, 43, 44].

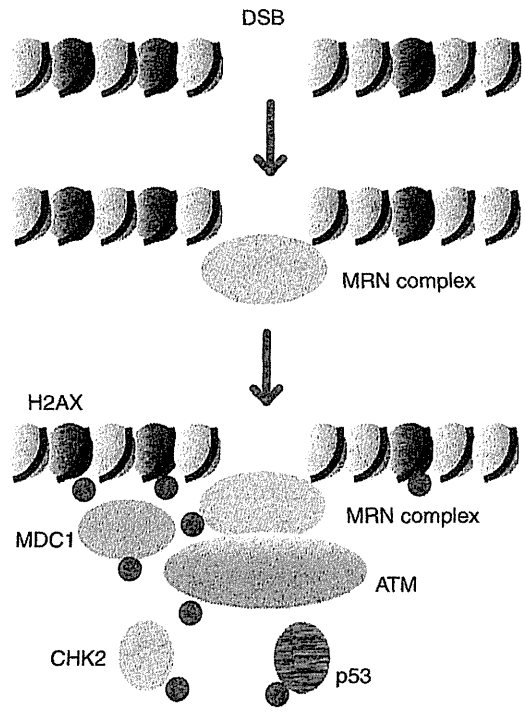

Fig. 2 DNA damage response to DSBs

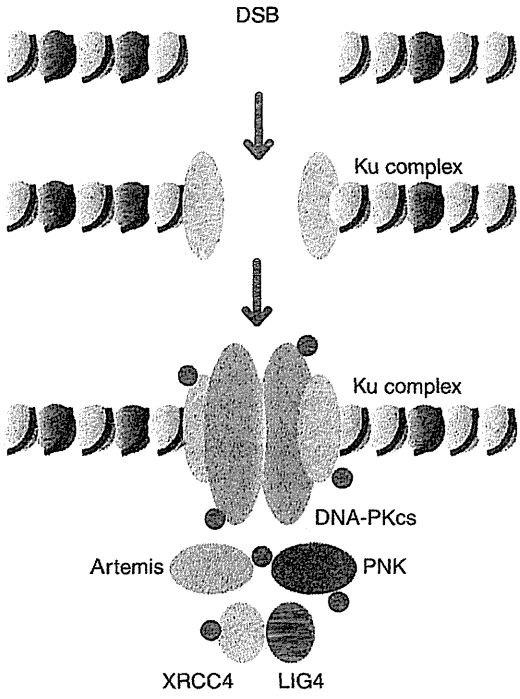

Fig. 3 NHEJ of DSBs

DSB repair through NHEJ is initiated by binding Ku70–Ku80 complex to the break ends (Fig. 3). The Ku complex recruits and activates DNA-PKcs via specific interaction with Ku80 [35]. DNA-PKcs is also a member of PIKK family [45], but its contribution to checkpoint response is insignificant. Rather, it phosphorylates multiple proteins involved in NHEJ [46]. *Artemis*, a nuclease, and PNK, a kinase/phosphatase, process the ends [11, 47, 48], and DNA ligase IV, a complex with XRCC4, ligates two DSB ends (Fig. 3) [49, 50].

Immunological disturbances and etiological aspects

In patients with AT, NBS and ATLD, immune deficiency is observed in the humoral immune system. IgA and IgG deficiency can be found [51–57]. B cells from patients have an intrinsic defect in maturation from IgM to other classes, as evidenced by normal or raised IgM. This suggests a defect in class switch recombination (CSR). This assumption has been documented at the molecular level using genomic DNA purified from peripheral blood cells from patients with AT, NBS and ATLD [58, 59]. Furthermore, the gene functions of *Atm* [60, 61] and *Nbs1* [62, 63] for CSR are confirmed by genetically engineered model mice. However, the molecular link between CSR and ATM pathway is currently unknown.

Patients with mutations in *DNA-PKcs*, *Artemis* and *LIG4* all show $T^-B^-NK^+$ SCID phenotype [23, 24, 64, 65]. Absence of mature B and T cells, but the presence of NK cells is characteristic of defects in V(D)J recombination. Mutations in RAG1 or RAG2 genes, encoding a lymphoid-specific endonuclease, also give rise to $T^-B^-NK^+$ SCID. The involvement in V(D)J recombination has been also demonstrated by model animals for *DNA-PKcs* [66–68], *Artemis* [69] and *LIG4* [70]. Therefore, SCID phenotype is attributed to the defect in the end-joining process after break induction by RAG proteins.

Increased risks of leukemia/lymphoma and etiological aspects

AT patients have a high risk of developing leukemia/lymphoma, which includes both B and T cell tumors, but not myeloid tumors. Incidence of childhood T cell lymphoma and T-ALL is greatly increased, although no differences between tumors in non-AT children are observed. In young adult AT patients, susceptibility to T cell prolymphocytic leukemia (T-PLL) is obvious. It is quite a contrast to non-AT patients who develop T-PLL with a median age of 69 years. However, no differences between AT and non-AT patients have appeared in clinical settings. Analysis of chromosome translocation associated with T-ALL and T-PLL reveals an involvement of a TCR gene. The translocation is attributed to irregular rejoining after processing with RAG proteins. It is characteristic to T-ALL and T-PLL in non-AT patients (reviewed in [71]). These clinical features imply two possibilities to explain the high incidence of lymphoma/leukemia in AT patients: early onset of tumorigenesis could be associated with an increased number of abnormal re-joining at V(D)J recombination sites and/or increased genetic instability accelerates tumorigenesis of the particular cell which has this rearrangement. The two possibilities are mutually not exclusive. In addition, there is no evidence that the susceptibility is attributed to immunodeficiency in AT patients. Importantly, evidence from model mice is striking [72]. $Atm^{-/-}$ mice are highly susceptible to thymic lymphoma. However, it is largely suppressed in $Atm^{-/-}$ and $Rag1^{-/-}$ double knock out mice. This is consistent with the idea that RAG-induced DSBs are the primary factor for the higher incidence of lymphoma/leukemia, even though any contribution of genetic instability to accelerate tumorigenesis could not be formally excluded in AT patients.

NBS patients are susceptible to lymphoma. In most cases, non-Hodgkin's lymphomas (NHL) develop [73]. Because the risk for NHL is increased by immunosuppressive therapy [74], the high incidence of NHL in NBS patients could be attributed to their immunodeficiency.

Although only a limited number of patients with a defect in NHEJ have been reported, susceptibility to leukemia/

lymphoma has been suggested [75–78]. Strikingly, in many cases, EBV-associated B cell lymphoma develops, suggesting that severe immunodeficiency fails to counteract EBV infection. Experiments with mouse models provide an important insight. SCID mice have an increased incidence of lymphoma. Notably, the risk in SCID mice is significantly suppressed by *Rag2* mutation [79, 80] just like the case of $Atm^{-/-}$ mice.

Conclusion

Radiosensitive diseases are associated with immunodeficiency, which are classified into two groups. One group includes AT, NBS and ATLD with a defect in cell cycle checkpoint. The other group shows a striking phenotype of SCID with a defect in NHEJ. The two groups are distinguishable in the clinical aspects of immunological disturbances and cell biological properties, and these properties are very consistent with defects in the biochemical pathway at molecular levels. Accumulating evidence indicates the immunodeficiency is attributed to defects in damage response to and repair mechanisms of DNA damage produced during development of the immune system. In the absence of checkpoint response, the risk of abnormal re-joining appears to be increased. Without NHEJ, the DSBs must be repaired by other mechanisms for survival of the cells. Consequently, an accumulated number of cells with the abnormal rearrangement could increase the risk of development of lymphoma/leukemia. A link between immunodeficiency and radiosensitivity shows a common molecular mechanism underlying both situations and the critical roles of checkpoint response and NHEJ to protect the cell from radiation injury.

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Associations of Ionizing Radiation and Breast Cancer-Related Serum Hormone and Growth Factor Levels in Cancer-Free Female A-Bomb Survivors

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Levels of exposure to ionizing radiation are increasing for women worldwide due to the widespread use of CT and other radiologic diagnostic modalities. Exposure to ionizing radiation as well as increased levels of estradiol and other sex hormones are acknowledged breast cancer risk factors, but the effects of whole-body radiation on serum hormone levels in cancer-free women are unknown. This study examined whether ionizing radiation exposure is associated with levels of serum hormones and other markers that may mediate radiation-associated breast cancer risk. Serum samples were measured from cancer-free women who attended biennial health examinations with a wide range of past radiation exposure levels ($N = 412$, ages 26–79). The women were selected as controls for separate case-control studies from a cohort of A-bomb survivors. Outcome measures included serum levels of total estradiol, bioavailable estradiol, testosterone, progesterone, prolactin, insulin-like growth factor-1 (IGF1), insulin-like growth factor-binding protein 3 (IGFBP-3), and ferritin. Relationships were assessed using repeated-measures regression models fitted with generalized estimating equations. Geometric mean serum levels of total estradiol and bioavailable estradiol increased with 1 Gy of radiation dose among samples collected from postmenopausal women (17%$_{1Gy}$, 95% CI: 1%–36% and 21%$_{1Gy}$, 95% CI: 4%–40%, respectively), while they decreased in samples collected from premenopausal women (−11%$_{1Gy}$, 95% CI: −20%–1% and −12%$_{1Gy}$, 95% CI: −20%– −2%, respectively). Interactions by menopausal status were significant ($P = 0.003$ and $P < 0.001$, respectively). Testosterone levels increased with radiation dose in postmenopausal samples (30.0%$_{1Gy}$, 95% CI: 13%–49%) while they marginally decreased in premenopausal samples (−10%$_{1Gy}$, 95% CI: −19%–0%) and the interaction by menopausal status was significant ($P < 0.001$). Serum levels of IGF1 increased linearly with radiation dose (11%$_{1Gy}$, 95% CI: 2%–18%) and there was a significant interaction by menopausal status ($P = 0.014$). Radiation-associated changes in serum levels of estradiol, bioavailable estradiol, testosterone and IGF1 were modified by menopausal status at the time of collection. No associations with radiation were observed in serum levels of progesterone, prolactin, IGFBP-3 or ferritin. © 2011 by Radiation Research Society

INTRODUCTION

Exposure to ionizing radiation via diagnostic imaging, particularly CT scans, continues to increase worldwide (*1*). Numerous studies have proven the association between exposure to ionizing radiation and breast cancer (*2*). Moreover, data from the A-bomb survivors indicate that the breast is particularly susceptible to ionizing radiation exposure, with an attributable fraction for breast cancer of 27% for all women exposed to more than 5 mGy (*3*) [a radiation dose roughly twice the yearly background dose (*4*)]. Breast cancer has also been associated with levels of endogenous hormones, particularly estradiol (E_2) (*5–7*). Estrogens stimulate cell proliferation (*8*) and possibly exert a direct genotoxic effect on breast epithelial cells (*9*).

Other circulating hormones and proteins, including non-sex hormone-binding globulin (SHBG)-bound

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estradiol, testosterone (*7*) and insulin-like growth factor 1 (IGF1), have been associated with breast cancer (*10*). Additional serum markers have been hypothesized to be associated with breast cancer, including IGFBP-3 (*11*), progesterone (*12*), prolactin (*13*) and ferritin. Ferritin levels and ionizing radiation are associated with increased levels of free radicals, and high ferritin levels have been reported to result in cell transformation and immortalization in mammary glands (*14*).

The question of whether ionizing radiation exposure modifies the underlying levels of risk-related circulating markers has not been addressed, however, and could be important in mechanistic studies of breast cancer or other hormone-related cancers after ionizing radiation exposure. The primary purpose of this project is to examine whether serum levels of risk-related markers vary with past radiation exposure in a representative sample of cancer-free female A-bomb survivors.

MATERIALS AND METHODS

Subjects

Study subjects were selected from the Adult Health Study (AHS), a longitudinal cohort study of A-bomb survivors initiated in 1958 (*15*). The AHS features biennial clinical examinations with serum collection and well-characterized radiation doses for numerous body organs (*16*). Serum samples were selected in the context of concurrent case-control studies wherein controls were selected by incidence density sampling from all subjects who met eligibility requirements and were at risk for a first primary cancer of any type at the time an index case was diagnosed. Eligibility was defined as women who were free from various medical conditions (oophorectomy, current exogenous hormone treatment, prior malignancies, pregnant), were cancer-free for at least 2 years after serum collection, and had estimated radiation organ doses. Hormone-related diseases/conditions were based on self-reports or, in the case of extreme measured values, a medical chart review. Samples were matched to an index case on age at collection (±2 years), sample collection year (same decade), city (Hiroshima or Nagasaki), and counter-matched using breast radiation dose strata (low, medium and high with cut-points at 5 mGy and 790 mGy) (*17*). Counter-matching assures a wide exposure range while still providing an appropriate sample for inference of the exposure effects within the underlying cancer-free cohort (*18*). Because it is not known which organ system might be responsible for changes to serum markers, we used doses to the colon for purposes of analyses. The colon is a centrally located organ, and colon doses have been used as representative doses in other studies (e.g. for overall solid cancer risk estimates), although the results should be relatively insensitive to the organ chosen since the doses to all organs are highly correlated. Radiation doses to the colon were adjusted to account for random errors in the individual radiation dose estimates (*19*). Menopausal status at the time of sample collection was assigned using a two-step process. First, we used a conservative age range to assign menopausal status. If a woman was less than 43 years of age at the time of sample collection, she was designated premenopausal (N = 218); if she was older than 55 years, she was designated postmenopausal (N = 139). For women in the intermediate age group (43–55 years old), FSH (follicular stimulating hormone) levels were measured and used to assign menopausal status primarily based upon the manufacturer's test guidelines (premenopausal cutoff: FSH <=15 mIU/ml, postmenopausal cutoff: FSH >=23 mIU/ml) (*20*). Of those in the intermediate age range and assigned by FSH values (N = 152), 75 were designated premenopausal, 60 were postmenopausal, and the others were in between the two cutoff values and were designated perimenopausal (N = 17). Due to the paucity of samples collected from perimenopausal women, they were excluded. The final data set included 492 samples collected from 412 women (293 premenopausal and 199 postmenopausal samples). Of the 80 subjects with two separate samples, 63 had samples that were both collected prior to menopause, 6 subjects had samples that were both collected after menopause, and 11 had one sample collected before and one after menopause.

All blood samples were obtained with informed consent, and the Human Investigation Committee of the Radiation Effects Research Foundation (RERF) approved the study protocol.

Sample Storage and Serum Assays

Sera were collected in the Hiroshima and Nagasaki laboratories between 1969 and 1992 and stored in vials at −80°C. Samples for this study were pulled from the freezers simultaneously and assayed for total E_2, bioavailable E_2, testosterone, progesterone, prolactin, IGF-1, IGFBP-3, ferritin, and total protein and protein fractions (albumin and globulin). All assays were performed blinded to age, city of collection and radiation exposure but were not performed in duplicate due to the limited serum amounts available. Commercial laboratories with internal quality assurance systems performed all assays. Supplementary Table 1 (http:dx.doi.org/10.1667/RR2631.1.S1) lists the assays and their precision as reported by the kits' manufacturers.

Serum levels of IGF-1, IGFBP-3, FSH, prolactin, testosterone, progesterone and ferritin were measured at a single laboratory (SRL Laboratory, Hachioji, Japan). Total protein concentration and protein fractions were measured by SRL using the Biuret method and cellulose acetate membrane electrophoresis, respectively. Serum levels of total E_2 and percentage bioavailable E_2 were measured at a single laboratory (Mitsubishi Kagaku Bio-clinical Laboratories, Tokyo, Japan). Percentage bioavailable E_2 (the fraction of serum E_2 that is free or loosely bound to albumin) was determined by ammonium sulfate precipitation of SHBG-bound E_2 (*21*). Total E_2 was multiplied by this percentage to obtain the concentration of bioavailable E_2. Serum ferritin was measured by the conventional chemiluminescence enzyme immunoassay (CLEIA). When the aliquots of samples were transported to outside laboratories for testing, they were packed in appropriate protective shipping boxes with sufficient amounts of dry ice and checked at the time of receipt by the measuring laboratories.

Methods of Analysis

Due to the skewed distributions of the markers, analyses were performed using log-transformed values for all of the markers except IGF1 and IGFBP-3, which did not require transformation. Effects of radiation exposure were estimated with adjustment only for other factors that were significantly related to marker level for statistical efficiency. Explanatory factors considered were: age at time of sample collection, year of birth, menopausal status (pre or post), total protein, body mass index [weight (kg)/height (m^2)] and its square, history of exogenous hormone use (yes or no), age at time of radiation exposure (including dichotomy at 15 years as a surrogate for pre- or postmenarche at time of exposure), number of full-term pregnancies, age at first full-term delivery, and radiation dose. Decisions for inclusion of factors other than radiation dose were based on a backward selection process where the least significant explanatory factor was removed until all factors were significant at a level of $P \leq 0.05$. The variable selection process was conducted separately for each marker. Analyses were performed using a regression method for correlated (repeated) measurements (generalized estimating equations; GEE) (*22*). P values and 95% confidence intervals were based on the Wald statistic.

TABLE 1
Characteristics of Study Cohort and Collected Samples by Menopausal Status

Variable	Premenopausal samples (N = 293)	Postmenopausal samples (N = 199)	P value[a]
City (Hiroshima/Nagasaki)[b]	187/106	145/54	0.036
	Mean (range)	Mean (range)	
Age ATB (years)	13.4 (1.0–24.0)	28.2 (12.0–49.0)	<0.001
Age at collection (years)	40.3 (26.3–51.9)	57.7 (43.0–78.9)	<0.001
Radiation dose (weighted gray)	0.54 (0.0–3.2)	0.49 (0.0–2.8)	0.472
BMI (kg/m^2)	22.4 (15.6–32.4)	22.8 (14.6–39.3)	0.267
Number of full-term pregnancies	2.7 (0–11)	3.6 (0–11)	<0.001
Year of sample collection	1971.9 (1969–1991)	1975.4 (1970–1992)	<0.001
IGF-1 (ng/ml)	116.0 (0.0–320.0)	94.6 (62–130)	<0.001
IGFBP-3 (μg/ml)	2.53 (0.4–4.3)	2.37 (2.1–2.8)	0.002
	Median (interquartile range[c])	Median (interquartile range[c])	
Total E_2 (pg/ml)	86.65 (59.2–116.0)	18.50 (13.1–25.2)	<0.001
Bioavailable E_2 (pg/ml)	39.48 (27.3–53.7)	9.19 (5.8–13.2)	<0.001
Testosterone (ng/dl)	15.7 (10.4–23.3)	10.5 (6.7–18.3)	<0.001
Progesterone (ng/ml)	1.60 (0.40–10.0)	0.25 (0.19–0.30)	<0.001
Prolactin (ng/ml)	6.20 (4.0–9.2)	3.60 (2.4–5.1)	<0.001
Ferritin (ng/ml)	21.50 (7.5–41.0)	50.00 (29–94)	<0.001

Notes. The upper section is the count of samples by city. The middle section shows the averages and ranges of non-transformed variables. The bottom section shows the median and interquartile ranges of natural log-transformed variables.

[a] Counts tested with χ^2 test for independence, means tested with t test for equality, medians tested with the Wilcoxon rank-sum test for independent samples.

[b] 412 subjects (Hiroshima, N = 267/Nagasaki, N = 145). 80 subjects had multiple samples (63 subjects with two premenopausal samples, 6 subjects with two postmenopausal samples, 11 subjects with one premenopausal sample and one postmenopausal sample).

[c] 25th percentile to the 75th percentile.

Radiation effects were assessed using linear dose–response functions. Dose responses were estimated with and without regard to effect modification by menopausal status at the time of blood collection and ionizing radiation exposure before or after age 15. For markers that were transformed prior to analyses, the radiation effects were reported on the untransformed scale by exponentiating the regression coefficient obtained using the log-transformed data and represent the association between 1 Gy radiation dose and the percentage change in geometric mean of the marker level. IGF1 and IGFBP-3, which were not transformed, were also reported on a relative scale by dividing the dose coefficient by the regression constant. To test the strength of the observed radiation effects, we identified and removed the most influential observations using the size-adjusted criterion 2/sqrt(n) for the t-statistic-like standardized deletion diagnostic DFBETAS of Belsley *et al.* (*23*). To alleviate concerns about misclassification of menopausal status at the time of sample collection due to possible nonspecificity of the FSH results, we conducted a sub-analysis after restricting samples to those collected from women at ages less than 43 or greater than 55 (135 samples omitted) and compared results to the full analyses.

Statistical comparisons between samples collected pre- and postmenopause were performed using χ^2 tests (for counts), t tests for equality (for means) and Wilcoxon rank-sum tests for independent samples (for medians). Interactions of radiation effects by menopausal status or exposure before or after age 15 were tested using the likelihood ratio. To compare fits of statistically significant non-nested interaction models, the quasi-likelihood/independence criterion (QIC) was used (*24*). All results were obtained using Stata (*25*).

RESULTS

Table 1 shows the demographics of the study cohort and marker values for samples separated by menopausal status at the time of collection. The distribution of the radiation dose is broader than what would be expected from a random sampling of all cancer-free women since they were counter-matched (low, medium, high) against the dose status of the case to ensure a broad representation of doses. The average year of sample collection is somewhat later for postmenopausal samples because the AHS study is a fixed cohort; more women were postmenopausal in later calendar years.

Table 2 shows the agreement of assays for the 63 pairs of samples collected from women who were premenopausal at each collection. Comparisons of multiple samples collected from the same women while postmenopausal were not reported (N = 6 pairs). The earlier sample was collected at average age 39.7 years and the average interval between sample collections was 2.8 years. Correlation coefficients ranged from 0.11 for progesterone to 0.75 for ferritin. A paired t test comparing the difference of the paired values with zero was not significant for any marker except for serum prolactin, where the earlier value proved to be significantly higher than the second value.

The first column of Table 3 shows the associations of radiation with marker levels with no allowance for effect modification but with adjustment for other significant factors. No significant radiation associations were observed in any of the markers when ignoring menopausal status. Columns two through five in Table 3 show the radiation associations separately by menopausal status at the time of sample collection or by age

TABLE 2
Comparison of Assay Values from Women Donating Multiple Samples

Assayed biomarker	*N*	Sample 1 average value (SD)	Sample 2 average value (SD)	Correlation	Sample 1 − Sample 2 average value (SD)	*P* value
Log total E_2 (pg/ml)	63	4.47 (1.02)	4.34 (0.54)	0.14	0.13 (1.10)	0.10
Log bioavailable E_2 (pg/ml)	63	3.65 (0.94)	3.64 (0.54)	0.16	0.014 (1.03)	0.42
Log testosterone (ng/dl)	57	2.74 (0.59)	2.74 (0.59)	0.52	0.0066 (0.54)	0.55
Log progesterone (ng/ml)	54	0.49 (1.71)	0.53 (1.64)	0.11	−0.042 (2.3)	0.54
Log prolactin (ng/ml)	57	1.86 (0.85)	1.47 (0.61)	0.28	0.39 (0.91)	0.001
Log ferritin (ng/ml)	56	3.12 (1.30)	3.11 (1.35)	0.75	0.0082 (0.83)	0.93
IGF-1 (ng/ml)	57	129.0 (69.7)	149 (67.9)	0.16	−20.3 (88.1)	0.19
IGFBP-3 (μg/ml)	57	2.51 (0.55)	2.54 (0.43)	0.36	−0.029 (0.60)	0.96

Notes. Samples are confined to multiple premenopausal samples (there were only six persons who donated multiple postmenopausal samples). The average age at the time of Sample 1 collection was 37.9 years and the average year of collection was 1970.9. The average age at the time of collection for the second sample was 40.8 years and the average year of collection was 1973.7. Values in parentheses (SD) after averages are standard deviations. The *P* value in the last column is a paired *t* test (H_o: Sample 1 − Sample 2 = 0).

at time of bombing (≤15 or >15 years; ATB15). Because menopausal status at blood donation was highly correlated with ATB15 in this study sample (particularly for a woman's first/only serum sample), the latter two columns largely mirror the results displayed in the former two. Total and bioavailable E_2, testosterone and IGF-1 demonstrated statistically significant associations with radiation dose in one or both periods.

Total E_2 (Fig. 1) decreased with radiation dose in the premenopausal period (relative change 0.89 at 1 Gy; 95% CI: 0.80, 0.99; $P = 0.027$), or in other words, the level of total E_2 changed as $k*0.89^{dose}$, where k is the level of total E_2 when the dose was zero (results from other log-transformed variables are interpreted in the same way). The level of total E_2 increased with dose in the postmenopausal period (relative change 1.17 at 1 Gy; 95% CI: 1.01, 1.36; $P = 0.037$). Effect modification by menopausal status was highly significant ($P = 0.003$) and remained significant after removing the most influential values ($P = 0.038$). The association of total E_2 with radiation dose was similar when categorizing subjects based on ATB15 (rather than menopausal status at the time of collection); relative change for a 1-Gy dose at age less than 15 years was 0.86 (95% CI: 0.76, 0.96; $P = 0.010$) and relative change for exposure after age 15 was 1.12 (95% CI: 0.99, 1.26; $P = 0.063$). Models used to estimate the radiation effects on total E_2 included menopausal status, age at sample collection, total protein, BMI and a BMI*postmenopause interaction. The QIC values for the two models were very similar, but the value for the ATB15 interaction model was slightly lower (i.e. better fit) than for the model with interaction by menopausal status.

Bioavailable E_2 (Fig. 2) showed a significant decrease with radiation dose in the premenopausal period, with a relative change of 0.88 at 1 Gy (95% CI: 0.80, 0.98; $P = 0.022$) and a significant increase with radiation dose in the postmenopausal period with a relative change of 1.21 at 1 Gy (95% CI: 1.04, 1.40; $P = 0.011$). Effect modification by menopausal status was highly significant ($P < 0.001$) and remained so after exclusion of influential observations ($P = 0.003$). As with total E_2, bioavailable E_2 also demonstrated similar radiation effects according to whether exposure was before or after age 15 (0.87 at 1 Gy; 95% CI: 0.77, 0.98; $P = 0.019$ and a relative increase of 1.13 at 1 Gy; 95% CI: 1.00, 1.27; $P = 0.045$, respectively). The model used to estimate the radiation effects on bioavailable E_2 included menopause status, age at sample collection and total protein. Again the QIC value was slightly lower for the model with ATB15 interaction.

Testosterone (Fig. 3) exhibited an increase with radiation dose in the postmenopausal period (relative change 1.30 at 1 Gy; 95% CI: 1.13, 1.49; $P < 0.001$) and a marginally significant decrease with radiation dose in the premenopausal period (0.90 at 1 Gy; 95% CI: 0.81, 1.00; $P = 0.049$). Estimates of effect modification by menopausal status were highly significant when estimates were derived using all observations ($P < 0.001$) and after excluding outliers ($P = 0.003$), whereas no effect modification was evident with ATB15. The testosterone model included menopause status, age at sample collection, total protein and a total protein*postmenopause interaction, and BMI.

IGF-1 (Fig. 4) displayed a significant increase with radiation dose in the premenopausal period (relative change 1.11 per Gy; 95% CI: 1.02, 1.18; $P = 0.024$). Since the values from this assay were not log-transformed, the results are interpreted as the percentage change in marker levels per gray. No significant change with dose was observed in the postmenopausal period (relative change 0.91 per Gy; 95% CI: 0.75, 1.04; $P = 0.18$). Similar effects were seen when categorizing the women by age at exposure. Effect modification by menopausal status was significant ($P = 0.014$) and remained so after removing the most influential observations ($P = 0.017$). Comparing QIC values indicated that the model with menopausal interaction model was preferable to the one with ATB15 interaction. Estimates of the radiation effects were adjusted for age at sample collection, year of birth, total protein*premenopause interaction, and number of full-term pregnancies.

TABLE 3
Relative Radiation Associations with Adjusted Mean Marker Values and Effect Modification By Menopausal Status and Age at Radiation Dose

Marker	Relative radiation association at 1 Gy no effect modification (95% confidence intervals), *P* value	Relative radiation association at 1 Gy Separately by effect-modifier category[a] (95% confidence intervals), *P* value			
		Menopause status at time of sample collection		Age at time of the bomb	
		Pre (N = 293)	Post (N = 199)	≤15 (N = 190)	>15 (N = 302)
Total E_2[b,d]	0.97	0.89	1.17	0.86	1.12
	(0.89, 1.06)	(0.80, 0.99)	(1.01, 1.36)	(0.76, 0.96)	(0.99, 1.26)
	P = 0.52	**P = 0.027**	**P = 0.037**	**P = 0.010**	P = 0.063
		Interaction **P = 0.003**		Interaction **P = 0.002***	
Bioavailable E_2[b,e]	0.98	0.88	1.21	0.87	1.13
	(0.93, 1.06)	(0.80, 0.98)	(1.04, 1.40)	(0.77, 0.98)	(1.00, 1.27)
	P = 0.84	**P = 0.022**	**P = 0.011**	**P = 0.019**	**P = 0.045**
		Interaction **P <0.001**		Interaction **P = 0.002***	
Testosterone[b,f]	1.02	0.90	1.30	0.97	1.06
	(0.93, 1.12)	(0.81, 1.00)	(1.13, 1.49)	(0.84, 1.10)	(0.93, 1.20)
	P = 0.70	**P = 0.049**	**P < 0.001**	P = 0.617	P = 0.387
		Interaction **P < 0.001**		Interaction P = 0.337	
Progesterone[b,g]	1.08	1.08	1.10	1.00	1.21
	(0.90, 1.30)	(0.86, 1.35)	(0.81, 1.50)	(0.77, 1.29)	(0.93, 1.58)
	P = 0.39	P = 0.52	P = 0.54	P = 0.992	P = 0.16
		Interaction P = 0.905		Interaction P = 0.313	
Prolactin[b,h]	1.01	0.97	1.07	0.94	1.08
	(0.91, 1.11)	(0.86, 1.09)	(0.91, 1.26)	(0.82, 1.08)	(0.95, 1.24)
	P = 0.905	P = 0.641	P = 0.384	P = 0.413	P = 0.230
		Interaction P = 0.313		Interaction P = 0.161	
IGF-1[c,i]	1.04	1.11	0.91	1.11	0.98
	(0.96, 1.10)	(1.02, 1.18)	(0.75, 1.04)	(0.99, 1.20)	(0.85, 1.07)
	P = 0.29	**P = 0.024**	P = 0.18	P = 0.061	P = 0.850
		Interaction **P = 0.014***		Interaction **P = 0.091**	
IGFBP-3[c,j]	1	1.01	0.98	1.00	0.93
	(0.97, 1.03)	(0.97, 1.05)	(0.92, 1.05)	(0.97, 1.04)	(0.97, 1.03)
	P = 0.89	P = 0.48	P = 0.44	P = 0.81	P = 0.82
		Interaction P = 0.28		Interaction P = 0.98	
Ferritin[b,k]	0.97	0.98	0.96	0.87	1.07
	(0.83, 1.13)	(0.81, 1.18)	(0.73, 1.27)	(0.70, 1.09)	(0.86, 1.32)
	P = 0.72	P = 0.81	P = 0.78	P = 0.22	P = 0.55
		Interaction P = 0.92		Interaction P = 0.19	

Notes. Numbers in parentheses are 95% confidence intervals. The first column shows the radiation associations without adjustment for menopause status at the time of sample collection or age at the time of the exposure from the atomic bombings. The right two sets of columns show the radiation associations by menopause status (columns 2, 3) and age less than or greater than 15 at the time of the bombing (columns 4, 5). The "Interaction P" values test whether the results differ significantly by menopausal status or age at the time of the bombing. Note that there were no detected effects when effect modification was ignored. The models were developed using a backwards elimination process for statistical efficiency as described in the text. Covariates for each model are listed in the footnotes. All effect modification models included the main effect variable regardless of whether it was selected in the elimination process. *QIC value indicates that this interaction model was better than the alternative interaction model. This score was compared only between the two interaction models and only when both models were significant.

[a] Effects of radiation are displayed separately for each of two categories of either menopausal status at time of serum collection (pre- or postmenopause) or age at time of exposure (up to age 15 or after age 15). The main effect was always included when testing the interaction.

[b] Log-transformed variable (relative change is calculated using X^{dose}, where X is the reported coefficient).

[c] Non-transformed variable (relative change is calculated using X * dose, where X is the reported coefficient).

[d] Adjusted for age and menopause status at sample collection, total protein, BMI and BMI*postmenopause interaction.

[e] Adjusted for age and menopause status at sample collection, total protein.

[f] Adjusted for age and menopause status at sample collection, total protein, total protein*postmenopause interaction, BMI.

[g] Adjusted for menopause status at sample collection, total protein.

[h] Adjusted for age and menopause status at sample collection.

[i] Adjusted for age at sample collection, year of birth, total protein*premenopause interaction, number of full-term pregnancies.

[j] Adjusted for year of birth, total protein*premenopause interaction, BMI, BMI^2.

[k] Adjusted for age and menopausal status at sample collection, BMI, number of full-term pregnancies.

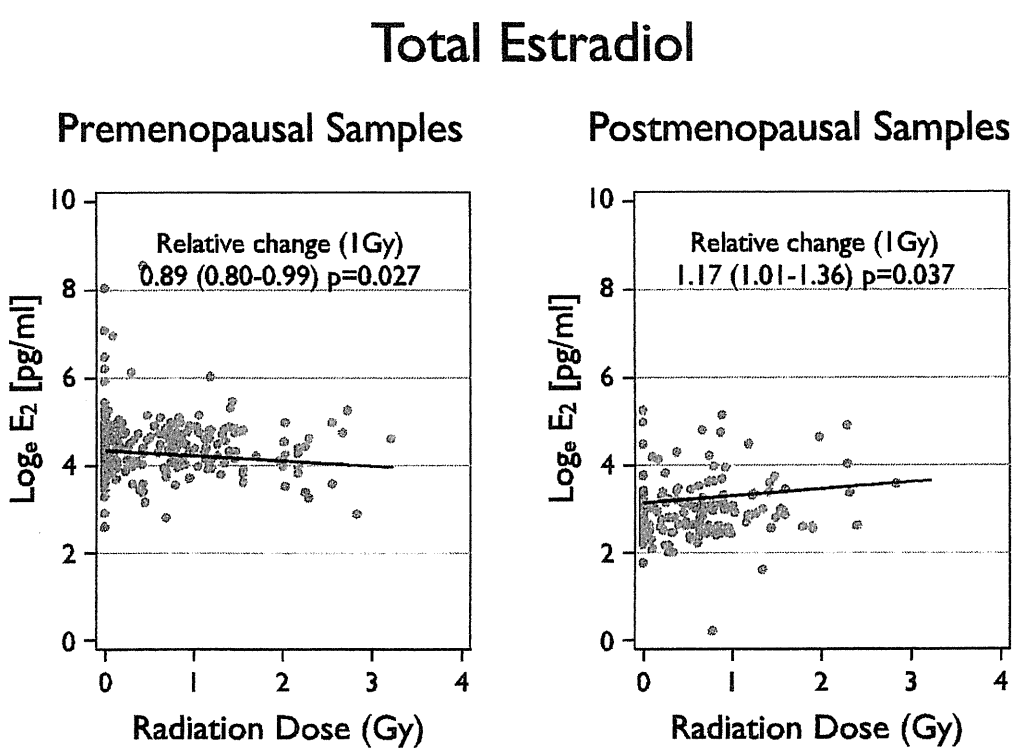

FIG. 1. Log total estradiol (E_2) by menopausal status and radiation dose. The relative change is the percentage change at a dose of 1 Gy. The regression lines and *P* values are based on the final models obtained for the markers (Table 3). The slopes of the two regression lines by menopausal status are significantly different ($P = 0.003$).

The other markers (IGFBP-3, progesterone, prolactin and ferritin) demonstrated no associations with radiation dose, either with or without effect modification. No effect modification of the radiation effects was observed in relation to the sample storage period, time since exposure, or the number of full-term pregnancies, although E_2 levels did increase at a marginally significant level for each additional full-term pregnancy (results not shown). Model fits generally improved when including the number of full-term pregnancies (as either an effect modifier or a main effect), but the estimated coefficients for the number of pregnancies were never statistically significant and therefore the results were not reported. No improvements in model fit were detected by the addition of a quadratic dose term. Point estimates were generally unchanged by the removal of samples retrieved when women were aged 43 to 55.

DISCUSSION

The present study was designed to investigate the associations of ionizing radiation exposure with sex and growth hormones in cancer-free A-bomb survivors using stored sera. Increasing levels of ionizing radiation exposure led to disparate changes by menopausal status in a number of serum markers. Levels of E_2 and bioavailable E_2 increased in postmenopausal samples while they decreased in premenopausal samples. Caution must be used when interpreting the results in premenopausal samples, especially E_2 levels, which are highly variable in premenopausal women due to the menstrual cycle. Table 2 illustrates the high variability of serum marker levels among premenopausal women. Controlling for the day of the menstrual cycle was not possible, because the clinical examination protocol did not attempt to record these values. It should be noted, however, that the point estimates for the radiation findings were largely unchanged after separately removing the most influential measurements (e.g., those associated with the E_2 peak at ovulation) and excluding those in the perimenopausal age range 43–55. Also, it is unlikely that there is any association with the timing of blood sample collection and radiation dose, eliminating most concerns of confounding. Nevertheless, chance findings for the premenopausal hormone associations cannot be completely ruled out.

The magnitude of change in total E_2 levels was about 10% at 1 Gy of radiation (negative in premenopausal women and positive in postmenopausal women). It is not clear that either of these are clinically significant changes, particularly among premenopausal women where there is so much variation. However, if E_2 levels were assigned to quartiles in samples taken from postmenopausal women, a 10% change in any particular result would generally be sufficient to increase its quartile assignment by one

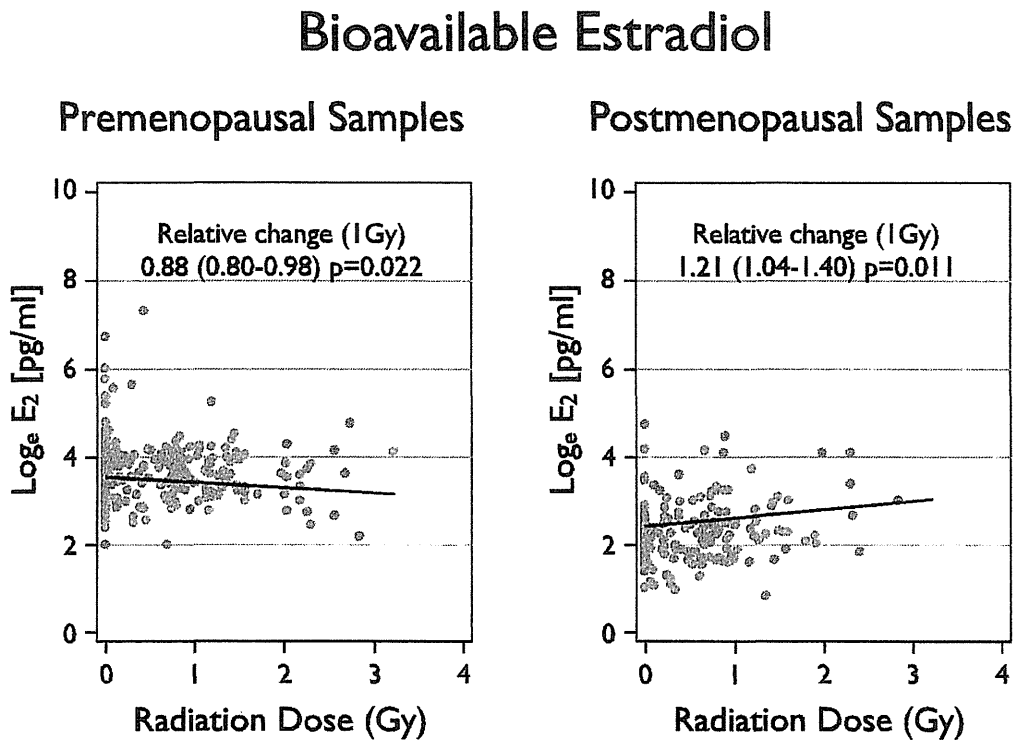

FIG. 2. Log bioavailable estradiol (E_2) concentration by menopausal status and radiation dose. The relative change is the percentage change at a dose of 1 Gy. The regression lines and *P* values are based on the final models obtained for the markers (Table 3). The slopes of the two regression lines by menopausal status are significantly different ($P < 0.001$).

category. Interpolating the results from Fig. 1 of the meta-analysis performed by Key *et al.* (*7*), this increase in total E_2 would roughly correspond to a 25% increase in breast cancer risk.

The significance of IGF-1 and IGFBP-3 levels in pre- or postmenopausal breast cancer has been under debate. The most recent evidence indicates that high levels of IGF-1 confer an increased risk of breast cancer (*10*). In our study, radiation exposure appeared to increase the level of IGF-1 in premenopausal women but had no effect on levels of IGF-1 in postmenopausal women. Again, however, large variations were observed in the premenopausal IGF-1 levels. IGFBP-3 was not associated with radiation exposure.

If the results are correct, questions arise regarding the radiation-related biological pathways that may affect sex hormone levels, particularly the most potent hormone E_2. In premenopausal women, E_2 is produced primarily by the ovaries whereas after ovarian involution, estrogen is primarily produced in adipose tissue by converting androgen to estrogen via aromatase. A number of speculative mechanisms for an association between radiation and sex steroid alterations can be suggested, including damage to primordial ovarian follicles leading to decreased ovarian aromatase and depressed E_2 production as observed in the samples taken from women who were exposed at less than 15 years of age (*26, 27*). Ataya *et al.* reported similar findings with decreased ovarian size and long-term decreased serum E_2 levels in rhesus monkeys with ovarian exposure to ionizing radiation (*28*). Larsen *et al.* reported decreased ovarian volume in spontaneously ovulating women who had received chemotherapy and radiotherapy for cancers diagnosed at mean age 5.4 years; however, E_2 levels were reported to be higher in those receiving (any type of) therapy compared to a control group of similar age (*29*).

For the results seen among older birth cohorts (i.e. postmenopausal samples), radiation-induced upregulation of inflammatory cytokines, specifically TNFα, can lead to increased estrogen biosynthesis (*30–33*); radiation can also alter lipid metabolism (*34–36*), possibly leading to hyperandrogenism through hypothalamus-pituitary-adrenal axis dysregulation (*37*). Either of these latter mechanisms would be consistent with the observed increased E_2 and testosterone levels among the postmenopausal women.

No effect modification by the number of full-term pregnancies was evident in the radiation effects on any of the markers. The number of women reporting any history of exogenous hormone intake was less than 10%, and that variable was not a significant risk factor in any of the tested models. Therefore, we do not believe that hormone treatment had a meaningful impact on our

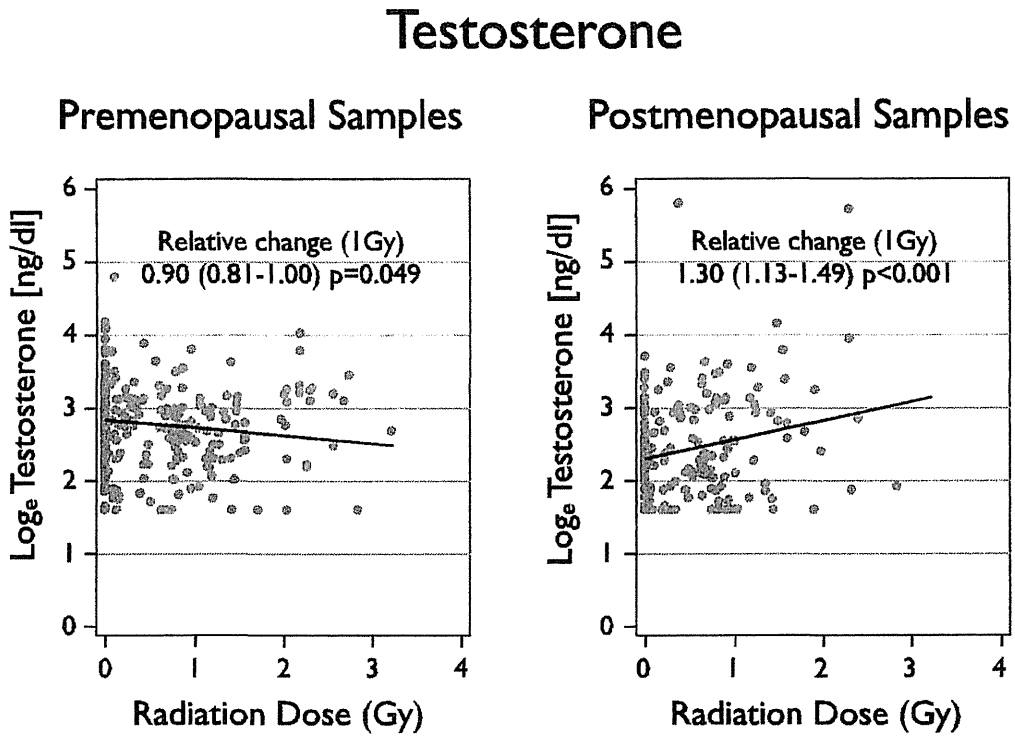

FIG. 3. Log testosterone by menopausal status and radiation dose. The relative change is the percentage change at a dose of 1 Gy. The regression lines and *P* values are based on the final models obtained for the markers (Table 3). The slopes of the two regression lines by menopausal status are significantly different ($P < 0.001$).

findings. Interaction models categorizing exposures before or after age 15 more often had a better fit than models categorized by menopausal status at the time of serum collection. However, this may be due to misclassification of menopausal status as opposed to an important biological effect involving menarche status at the time of radiation exposure. McDougall *et al.* found no association of breast cancer risk and menarche status at the time of radiation exposure but did not consider serum marker levels (*38*).

Associations of radiation and markers modified by menopausal status were tested in the results of eight bioassays (the two parallel analyses of modification by either menopause status or ATB15 are effectively the same), which may raise concerns of finding associations due to multiple testing. Using the conservative Bonferroni correction for multiple testing ($\alpha = 0.05/8 = 0.00625$), all of the tests for interaction remain significant with the exception of IGF-1 ($P = 0.014$).

The study boasts a number of strengths, including well-characterized radiation doses to a representative population of women, and data collected prospectively from cancer-free women as part of a long-term follow-up regimen, including medical history data on hormone-related conditions. However, there are also several weaknesses. The day of blood collection in relation to the menstrual cycle was not available, which is problematic for premenopausal estradiol measurements in particular. The use of FSH to assign menopausal status has been shown to be not fully specific (*39*). It is also likely that misclassification occurred when assigning menarche status by age. Due to the limited number of samples, duplicate assays for quality control were not attempted. A limited number of women did have multiple samples collected while premenopausal, for which the correlation between marker assays was generally poor, probably due to different times in the menstrual cycle. Despite these limitations, no major changes in the interactions between the measured radiation associations and menopausal status were detected after separately removing the most influential observations and those observations drawn from women in the perimenopausal age range, which helps confirm the robustness of the associations.

Conclusions

Previous studies have proven the potency of ionizing radiation as a carcinogen via direct damage effects to DNA while separate lines of research have established serum levels of E_2, testosterone and IGF1 as risk factors for breast cancer. The results of this study suggest that ionizing radiation may lead to changes in serum levels of cancer-related hormones and proteins in cancer-free women and that these changes are dependent upon

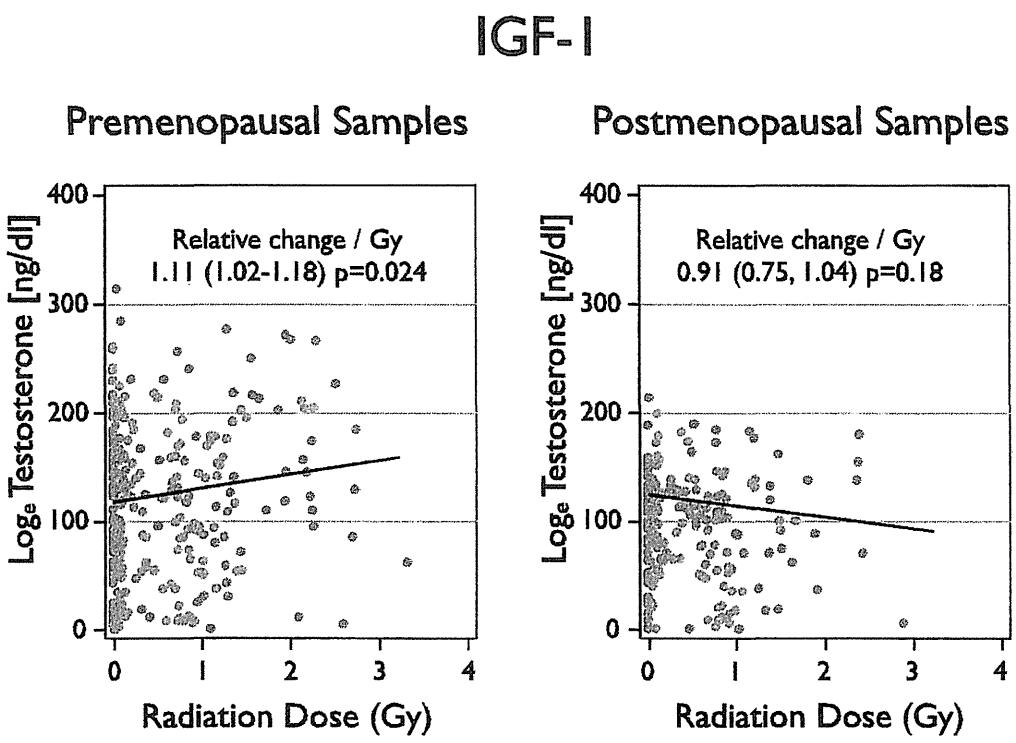

FIG. 4. IGF-1 by menopausal status and radiation dose. The relative change is the percentage change at a dose of 1 Gy. The regression lines and *P* values are based on the final models obtained for the markers (Table 3). The slopes of the two regression lines by menopausal status are significantly different ($P = 0.014$).

either the menopausal status at the time of collection or the menarche status at the time of exposure. Several mechanisms for these changes are suggested, but they are all speculative. Researchers of cancer etiology after whole-body ionizing radiation exposure should be aware of possible underlying changes in the levels of serum markers, which may confer independent carcinogenic risks.

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Lymphocyte subset characterization associated with persistent hepatitis C virus infection and subsequent progression of liver fibrosis

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ABSTRACT

This study aims to deepen the understanding of lymphocyte phenotypes related to the course of hepatitis C virus (HCV) infection and progression of liver fibrosis in a cohort of atomic bomb survivors. The study subjects comprise 3 groups: 162 HCV persistently infected, 145 spontaneously cleared, and 3,511 uninfected individuals. We observed increased percentages of peripheral blood T_H1 and total CD8 T cells and decreased percentages of natural killer (NK) cells in the HCV persistence group compared with the other 2 groups after adjustment for age, gender, and radiation exposure dose. Subsequently, we determined that increased T_H1 cell percentages in the HCV persistence group were significantly associated with an accelerated time-course reduction in platelet counts—accelerated progression of liver fibrosis—whereas T_C1 and NK cell percentages were inversely associated with progression. This study suggests that T_H1 immunity is enhanced by persistent HCV infection and that percentages of peripheral T_H1, T_C1, and NK cells may help predict progression of liver fibrosis.

1. Introduction

Hepatitis C virus (HCV) infects some 120 to 170 million people worldwide, and persistent HCV infection is a major cause of liver diseases, including chronic hepatitis, cirrhosis, and hepatocellular carcinomas [1,2]. Both innate and adaptive arms of the host immune system are closely involved in persistent infection, liver injury, and virus clearance [3,4]. For instance, cytotoxic granule release and cytokine production of natural killer (NK) cells are inhibited by direct binding of HCV envelope protein E2 to CD81 on NK cells or stabilizing the human leukocyte antigen (HLA)-E expressions on hepatocytes in HCV-infected patients [5,6]. However, the comprehensive understanding of interactions between HCV and the immune system remains incomplete [3,4]. Moreover, aging, gender, and several environmental factors, such as alcohol drinking, smoking, and ionizing radiation, have been reported to influence host immune functions as well as HCV spontaneous clearance [7–9], which may increase the complexity of virus–host interactions. Therefore, a comprehensive characterization of host immunologic phenotypes in HCV infection is needed, especially with a cohort-based study design without conceivable selection bias [10]. Nevertheless, few studies along those lines have been carried out. One prospective cohort study (the Adult Health Study [AHS]) of atomic bomb survivors—a longevity cohort with biennial health examinations—has been conducted at the Radiation Effects Research Foundation (RERF) and provides clinicoepidemiological data related to HCV infection and immunologic status [11,12]. Within the cohort study, we conducted a cross-sectional analysis for peripheral blood lymphocyte subsets among HCV persistently infected, spontaneously cleared, and uninfected groups, aiming to delineate immunologic distinctions among these 3 groups. We also aimed to identify the lymphocyte subsets that can predict hepatitis progression in HCV-persistent individuals on the basis of a longitudinal analysis of time-course changes of platelet counts.

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