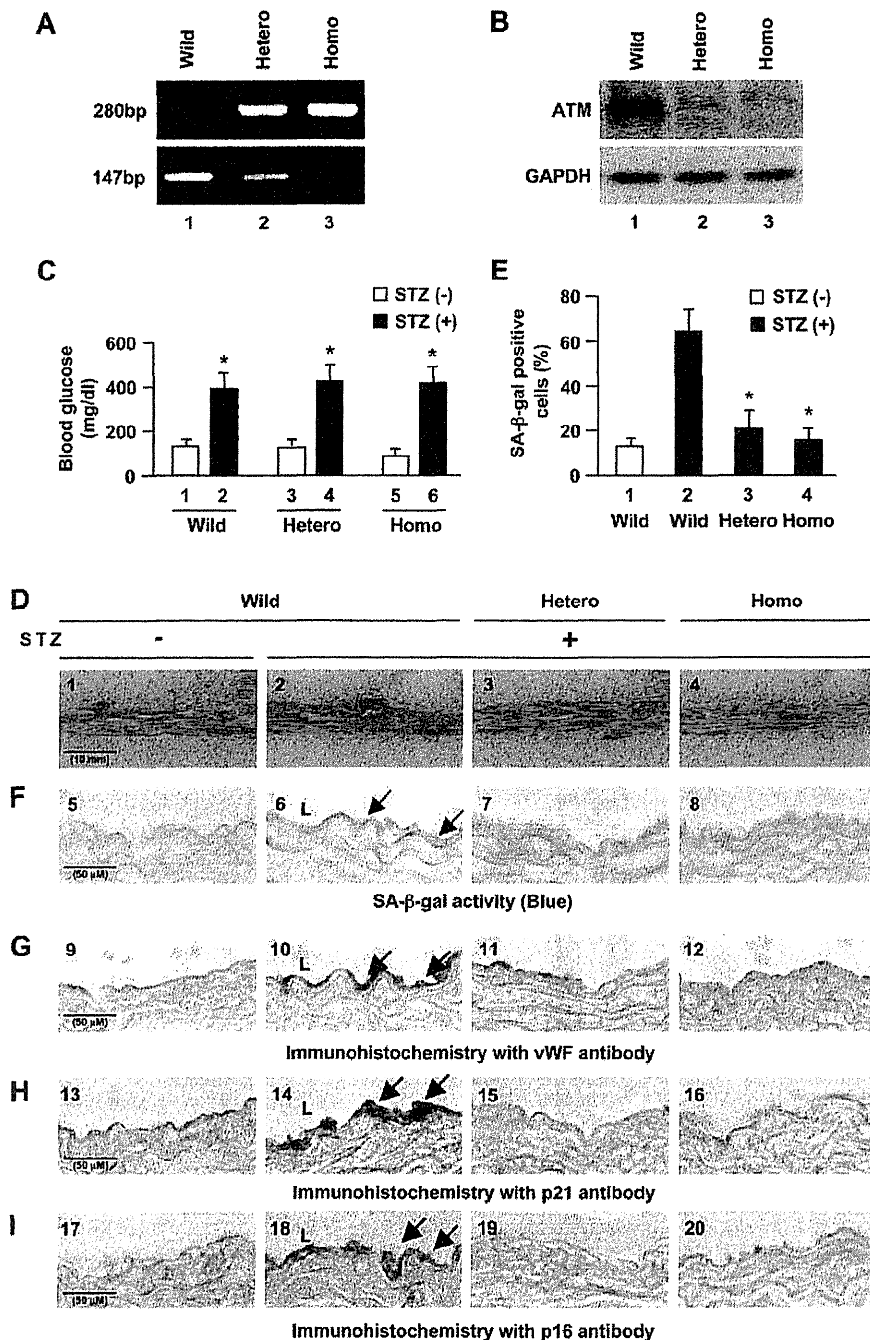

FIGURE 6. **Senescent endothelial cells in aortas of STZ-diabetic ATM knock-out mice.** Six respective $ATM^{+/+}$ (*Wild*), $ATM^{+/-}$ (*Hetero*), and $ATM^{-/-}$ (*Homo*) mice were used. *A*, genotyping analysis for ATM mice. *B*, Western blot analysis of ATM expression in the thoracic aortas. *C*, blood glucose levels measured before and after treatment with STZ. Values are mean ± S.E. (*error bars*). *, $p < 0.05$ *versus* STZ (−) mice (*lane 1*, *lane 3*, or *lane 5*, respectively). *D*, SA-β-gal activity (*blue*) in the thoracic aortas from mice at 10 days after treatment with STZ. *E*, quantitation of percentage of SA-β-gal-positive cells in the STZ-treated thoracic aortic samples. Values are mean ± S.E. *, $p < 0.05$ *versus* STZ (+) mice. *F*, sections of SA-β-gal stained thoracic aortas. *Arrows* indicate SA-β-gal-positive cells mostly localized to the luminal surface in the cross-section of the thoracic aortas. *G*, immunohistochemistry for von Willebrand factor, an endothelial cell marker, in the thoracic aortas (*brown*). *Arrows* indicate positive staining in the endothelium. *H* and *I*, immunohistochemical staining of p21 and p16 (*brown*). *Scale bar*, 50 μm and 10 mm, respectively.

Ser^{473} phosphorylation in response to insulin or ionizing radiation, which results in radiosensitivity or resistance to insulin in cell lines derived from A-T patients and ATM knock-out mice (15) or in muscle cells (29). However, in vascular endothelial cells stimulated by oxidative stress, we demonstrated for the first time to our knowledge that ATM regulates endothelial senescence through activation of Akt. Further, the pathway downstream of Akt through p53 and p21 has been well studied as a central pathophysiological signaling pathway in endothelial cells, especially in response to insulin (6, 7). However, whether the Akt/p53/p21 pathway is also activated by oxidative stress has not been clear. Our results thus indicate that this pathway is activated by oxidative damage and most importantly, regulated by ATM. γ-H2AX and 53BP1 foci formation was induced by oxidative stress, thus further providing supportive evidence that the oxidative stress-induced ATM-Akt/p53/p21 pathway is activated by DNA DSBs (supplemental Fig. 3).

Although the DNA damage response has been pursued mainly in oncogenesis and cancer-related fields and recently in regulation of senescence (1–3), as ATM has been shown to be activated by oxidative stress in hematopoietic cells (8), it is not beyond reason and in fact quite reasonable that ATM and the DNA damage response are involved in the vasculature as a response to oxidative stress albeit unexpected and unappreciated. Previous studies by ourselves initially suggested that the DNA damage response is activated in cardiovascular pathogenesis through the action of poly(ADP-ribose)polymerase-1 (PARP-1) (30), but identification and characterization of the actions of the central signaling molecule, ATM, in cardiovascular regulation provides further compelling evidence for the importance of this pathway in regulation of the vasculature and its diseases.

Our findings might explain in part the underlying mechanisms of the congenital disease condition of A-T in which the ATM gene is mutated. One of the hallmark characteristic phenotypes of A-T is vascular dysplasia and dysfunction, which pronounces as telangiectasia. Pathologically, vascular degeneration as characterized by loss of elastic fibers and proliferation of smooth muscle cells is seen. Patients that sur-

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vive to later years are also known to be prone to ischemic heart disease (31). Although the precise underlying mechanism for the latter is unknown, in general, progression of coronary artery disease involves accelerated atherosclerosis in which oxidative stress of the endothelium and related cells plays a major contributory role. Response to oxidative stress in the vasculature may be disrupted in A-T patients. Dysregulation of endothelial cell function might lead to pathophysiological states such as vascular dysfunction in developmental states and accelerated atherosclerosis in adults. Better understanding the cellular response to oxidative stress with a focus on ATM is expected to shed further light on the pathogenesis of A-T and to clarify alternative pathological pathways that are activated in response to oxidative stress when deficient for ATM as well as to provide new insight into the molecular mechanisms underlying age-related cardiovascular pathologies such as atherosclerosis in which ATM might pose a new therapeutic target for vascular pathologies involving oxidative stress.

In conclusion, we show that oxidative stress can induce cellular senescence in HUVECs as shown by staining for SA-β-gal, which was associated with an ATM-dependent Akt/p53/p21 signaling pathway. Our findings might in part explain underlying mechanisms of the pathogenesis of the disease ataxia telangiectasia in which vascular dysplasia and dysfunction are seen, as well as suggesting that ATM may be a new therapeutic target for cardiovascular pathologies.

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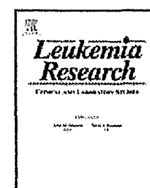

Letter to the Editor

Erdheim–Chester disease: Multisystem involvement and management with interferon-α

Erdheim–Chester disease (ECD) is a rare, non-Langerhans form of histiocytosis of unknown origin, with involvement of skeletal system and multiple organs [1]. Recently, interferon-α (IFN-α) has been reported as a valuable therapy for ECD [2,3]. We report another case of ECD treated with IFN-α and discuss the therapeutic efficacy.

A 47-year-old Japanese male presented to our hospital with 2-year history of multiple xanthogranulomas (xanthelasmas) on the both eyelids and face, periorbital congestion and exophthalmos. Thoracoabdominal CT scanning and magnetic resonance imaging (MRI) disclosed soft-tissue infiltration in the renal pelvis and uterus, bilateral hydronephrosis and periaortic fibrosis ("coated aorta" appearance) (Fig. 1A–D). A periorbital skin biopsy demonstrated diffuse infiltration with CD68+/S100−/CD1a− foamy histiocytes and Touton giant cells (Fig. 2), leading to the diagnosis of ECD. The long bone radiographs and bone scintigraphy showed a symmetric medullary sclerosis of the femur and tibia, which is a characteristic radiological feature of ECD (Fig. 1E and F). Furthermore, brain MRI revealed the absence of hyperintensity of the posterior pituitary lobe on T1-weighted images, without sellar mass lesions or stalk thickening, and hormonal examinations were compatible with diabetes insipidus (Fig. 1G). Initial complete blood counts showed a white blood cell count of 9.0×10^9/l with a normal differential count, a hemoglobin level of 9.5 g/dl, and a platelet count of 541×10^9/l.

The renal function gradually worsened, and required the insertion of ureteral stent on the right kidney. Renal scintigraphy showed a remarkable decrease in left kidney uptake, suggesting severe renal impairment due to chronic renal obstruction and xanthomatous invasion (Fig. 1H). Renovascular hypertension was also observed. Since the periaortic infiltration also extended to the aortic arch and pericardial region with the presence of pericardial effusion, the treatment with IFN-α was initiated (3×10^6 units, subcutaneously 3 times per week). Treatment was generally well tolerated, except for fever following injections. IFN-α treatment improved xanthelasmas after 1 month and attenuated the substantial progression of pericardial effusion. Renal function has been kept managable with ureteral stenting and diuretics. Thirteen months later, IFN-α was transiently discontinued due to progression of anemia caused by renal impairment, which recovered within 2 months. IFN-α was reintroduced, maintained at the dose of 3×10^6 units twice per week for 8 months, and then tapered to once per week. The patient's condition remained generally stable after 32 months of follow up with IFN-α therapy, except for the slow progression of the renal impairment.

Since the initial description in 1930, about 250 cases of ECD were reported in the literature. The most frequent clinical features of ECD are bone pain, exophthalmos and diabetes insipidus. Renal and retroperitoneal involvement frequently occurs in about 30% (17/59) of ECD patients in isolation or as a component of disseminated disease [1]. The "hairy kidney" appearance on CT scans is highly suggestive of the diagnosis [4]. This affectation is usually asymptomatic, but can present with dysuria, abdominal pain, enlarged palpable kidneys and ureteric obstruction, leading to hydroneprosis and acute or chronic renal failure. The prognosis of ECD is generally worse than that of Langerhans cell histiocytosis, and pulmonary involvement and cardiac failure are regarded as the most common causes of death [1,2,5]. Severe renal failure could also result in the emergent complication or the fatal outcome in some cases [6–8].

There is no standard treatment for ECD owing to its rarity, while several options including steroids, various cytotoxic agents and hematopoietic stem cell transplantation have been reported with variable effects [1,9]. IFN-α has been recently shown to be a promising treatment for ECD [2,3,10]. In the Braiteh's report, marked improvement was noted in three patients, with regression of retro-orbital disease within 1 month and gradual and durable improvement of bone lesions, pain and diabetes insipidus over the following month [2]. Otherwise, the efficacy of IFN-α varies among the sites of disease involvement, and especially cardiovascular and central nervous system involvement, and mesenteric lesions may resist to IFN-α treatment [3,11]. As for renal disease, IFN-α seems to effective for ureteral obstruction, allowing withdrawal of ureteral stents, in two patients in the Haroche's report describing eight patients' responses [3]. In our case, xanthelasmas responded well to IFN-α, and IFN-α treatment appears to be effective in preventing progression of cardiovascular and renal involvements. Thus, early therapeutic intervention by IFN-α may be important for stabilization of ECD, although the occurrence of life-threatening events such as myocardial infarction and acute renal failure should also be kept in mind during the IFN-α treatment, as in our case. Whereas IFN-α might be a valuable first-line therapy for ECD at the present time, the establishment of further effective therapeutic modality is warranted, considering the slow progression of renal impairment in our case. Failure of IFN therapy might lead to more aggressive or

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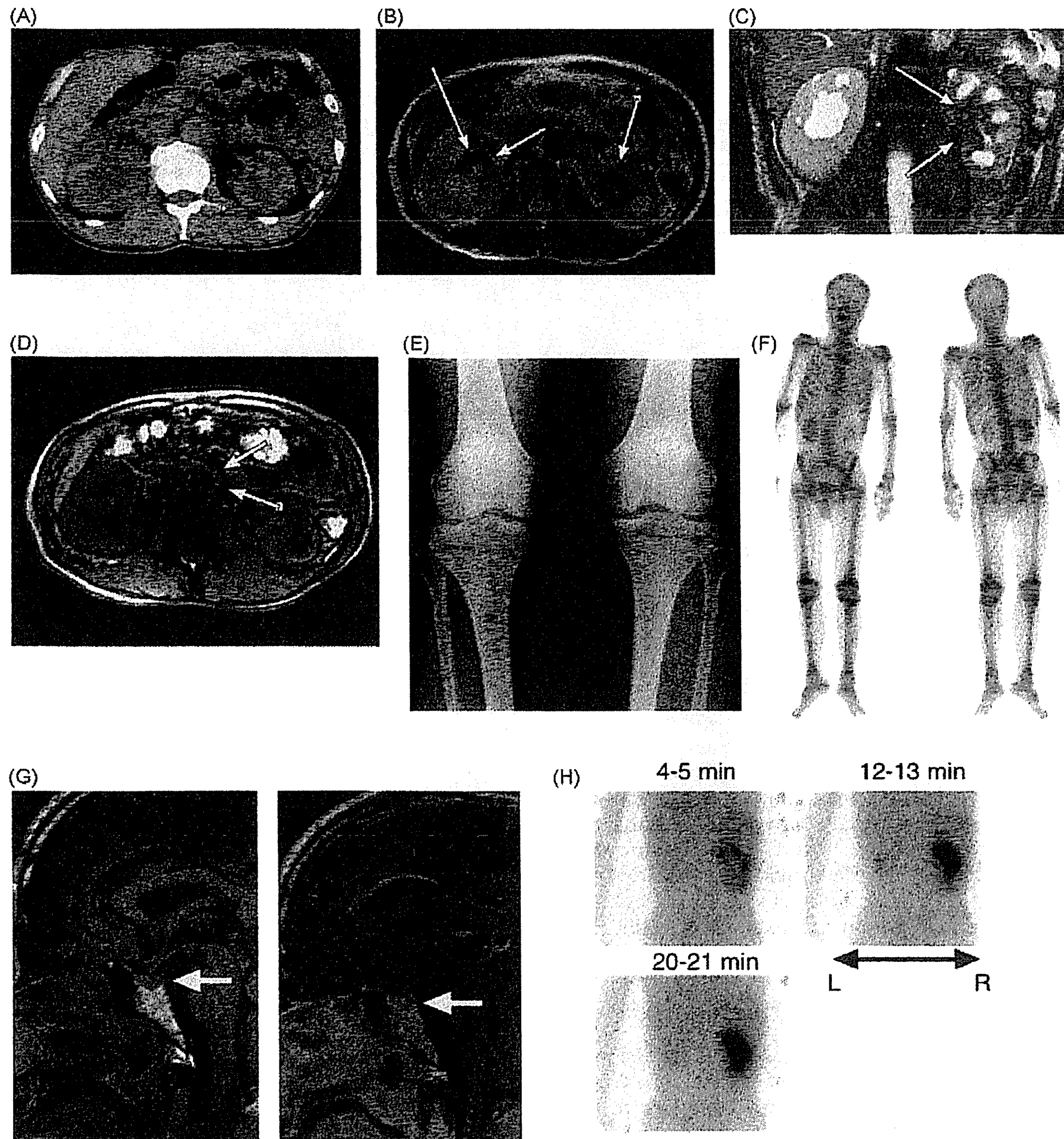

Fig. 1. Radiological findings. Abdominal CT (A) and MRI (B) show soft-tissue infiltration in the both renal pelvis (arrows) and associated hydronephrosis. Left renal parenchyma is thinned, suggesting chronic renal impairment. (C) A coronal reformatted enhanced image showing bilateral pelvocaliectasis (arrows). (D) Abdominal MRI scan with periaortic infiltration ("coated aorta") (arrows). Bone radiograph (E) and scintigraphy (F) showing a symmetric medullary sclerosis of the femur and tibia. (G) Brain MRI shows the absence of hyperintensity of the posterior pituitary lobe (arrows) on T1-weighted image, which is also evident after contrast enhancement (right). (H) Renal scintigraphy demonstrates the abrogation of left kidney uptake.

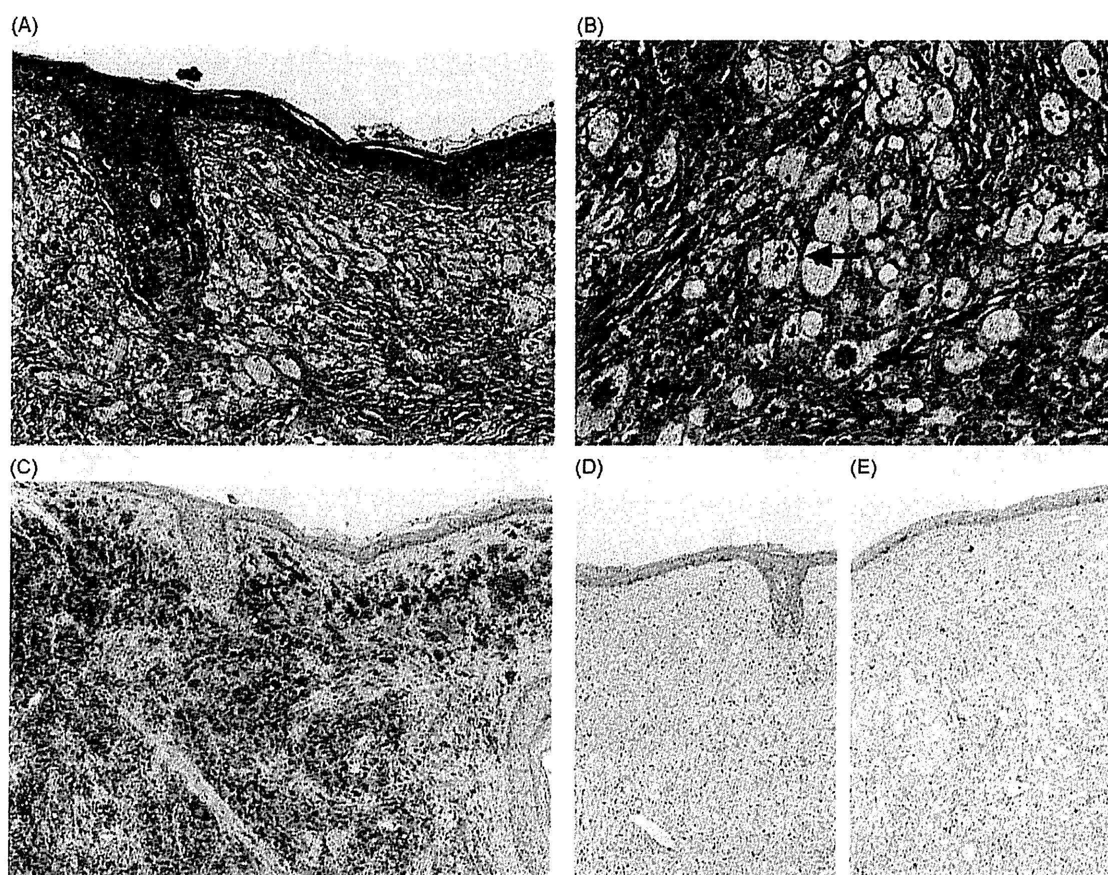

Fig. 2. Histological and immunohistochemial findings of the skin biopsy. (A) Hematoxylin-Eosin (H-E) stains, 100×. Skin biopsy shows dense infiltration of large foamy histiocytes. (B) High-power view of H-E staining. Arrows show Touton giant cells. Immunohistochemistry for CD68 (C), S100 (D) and CD1a (E) (brown), 100×. The foamy histiocytes are stained strongly positive for CD68 (C), but negative for S100 (D) and CD1a (E). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

experimental alternative therapeutic regimens including imatinib [12].

Conflict of interest statement

All authors declare that there are no competing financial interests regarding this article.

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T-Cell Immunosenescence and Inflammatory Response in Atomic Bomb Survivors

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Kusunoki, Y., Yamaoka, M., Kubo, Y., Hayashi, T., Kasagi, F., Douple, E. B. and Nakachi, K. T-Cell Immunosenescence and Inflammatory Response in Atomic Bomb Survivors. *Radiat. Res.* 174, 870–876 (2010).

In this paper we summarize the long-term effects of A-bomb radiation on the T-cell system and discuss the possible involvement of attenuated T-cell immunity in the disease development observed in A-bomb survivors. Our previous observations on such effects include impaired mitogen-dependent proliferation and IL-2 production, decreases in naive T-cell populations, and increased proportions of anergic and functionally weak memory CD4 T-cell subsets. In addition, we recently found a radiation dose-dependent increase in the percentages of $CD25^{+}/CD127^{-}$ regulatory T cells in the CD4 T-cell population of the survivors. All these effects of radiation on T-cell immunity resemble effects of aging on the immune system, suggesting that ionizing radiation might direct the T-cell system toward a compromised phenotype and thereby might contribute to an enhanced immunosenescence. Furthermore, there are inverse, significant associations between plasma levels of inflammatory cytokines and the relative number of naïve CD4 T cells, also suggesting that the elevated levels of inflammatory markers found in A-bomb survivors can be ascribed in part to T-cell immunosenescence. We suggest that radiation-induced T-cell immunosenescence may result in activation of inflammatory responses and may be partly involved in the development of aging-associated and inflammation-related diseases frequently observed in A-bomb survivors. © 2010 by Radiation Research Society

INTRODUCTION

Epidemiological studies of A-bomb survivors have suggested a relationship between radiation dose and the mortality or morbidity rates for various noncancer diseases (*1, 2*). Risk estimates made for the years from 1950 to 1997 were found to be elevated for death from all solid cancers combined and also for death from all noncancer diseases combined, with excess relative risks (ERRs) per Gy of 0.47 and 0.14, respectively (*1*). While the estimated ERR for noncancer disease death is clearly low, the death toll has risen to about 32,000, representing approximately 70% of the total recorded deaths in the cohort (*1*). Mechanisms for radiation-related cancer, although not totally understood, are much clearer than those for radiation-related noncancer diseases, about which almost nothing is known. An interesting hypothesis is that radiation effects on the immune system may be involved in part in radiation-related diseases, especially for noncancer diseases.

Advancing age is accompanied by a variety of alterations in the immune system, many of which will tend to increase the susceptibility of elderly people to a wide range of diseases. Thus, for example, age-dependent decreases in T-cell numbers and/or function are almost certain to lead to increases in vulnerability to disease-causing pathogens as well as to several adverse manifestations of chronic inflammation. In A-bomb survivors, dose-dependent increases in morbidity have been associated with a variety of inflammatory diseases, such as chronic liver diseases, thyroid diseases and heart diseases (*2*). To gain further insights into the mechanisms of radiation-related noncancer diseases, we have been focusing on aging- and radiation-related alterations in T-cell immunity. In this paper, we first summarize the long-term effects of A-bomb radiation on the T-cell system, including current study results on regulatory T cells, and then we discuss a possible involvement of attenuated T-cell immunity in the development of diseases frequently observed in A-bomb survivors.

MATERIALS AND METHODS

Blood Donors

A total of 1,035 study subjects were randomly selected from Hiroshima participants in the Adult Health Study (AHS) at the Radiation Effects Research Foundation (RERF) (*3*). For the present study, blood samples from the subjects were obtained with informed consent when they participated in the AHS health examination program between 2006 and 2008. The study protocol has been approved by the Human Investigation Committee of RERF (the RERF Institutional Review Board). Cancer prevalence within the study subjects by dose

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TABLE 1
Characterization of the Study Population

	Age (years)[b]						
	<70		70–79		80+		
Dose (Gy)	Male	Female	Male	Female	Male	Female	Total
<0.005[a]	29	26	73	102	14	97	341
0.005–0.5	11	34	62	86	22	133	348
0.5–1.0	18	23	19	41	19	48	168
1.0–4.0	28	32	29	40	17	32	178
Total	86	115	183	269	72	310	1,035

[a] Individuals in this dose category were exposed at distances in excess of 3 km from the hypocenter and hence received doses that are equivalent to zero.
[b] Age at the time of the examinations that were conducted between 2006 and 2008.

category was 14% at <0.005 Gy, 16% at 0.005–0.5 Gy, 22% at 0.5–1.0 Gy, and 24% at ≥1.0 Gy and tended to be higher in survivors exposed to higher doses, in accord with a recent observation in the AHS population (*4*). The age-, gender- and radiation dose-specific distributions of the 1,035 study subjects are listed in Table 1. Radiation doses are based on Dosimetry System 02 (DS02) estimates (*5*).

Flow Cytometry

Mononuclear cell fractions separated by the Ficoll-Hypaque gradient technique were analyzed by three-color flow cytometry using a FACScan flow cytometer (BD Biosciences, San Jose, CA) as described previously (*6*). For assessment of regulatory T (Treg) cells, approximately 100,000 mononuclear cells were stained with Alexa Fluor® 488-labeled CD25 mAb (Serotec Ltd, Oxford, UK), PE-labeled anti-CD127 mAb (BD Biosciences), and PerCP-labeled CD4 mAb (BD Biosciences). Analyses of naïve and effector/memory cell subsets in the CD4 T-cell population involved staining mononuclear cell fractions with FITC-labeled anti-CD45RA mAb (Beckman Coulter, Inc., Fullerton, CA), PE-labeled anti-CD62L mAb (BD Biosciences), and PerCP-labeled CD4 mAb.

TNF-α Measurement

Of the 1,035 subjects examined for their T-cell subsets, we selected 69 subjects whose DS02 doses exceeded 1 Gy as well as 86 age- and gender-matched subjects who were exposed to less than 5 mGy and analyzed their plasma TNF-α levels. Since the sample selection was originally made for the purpose of examining associations between inflammatory responses and mutant frequency at the glycophorin A locus in erythrocytes (*4*), all subjects were all heterozygous for blood-type MN at that locus. TNF-α levels were analyzed using a highly sensitive enzyme-linked immunosorbent assay kit (Quantikine HS, R&D systems, Minneapolis, MN). The minimum detectable concentration was 0.05 pg/ml.

Data Analysis

Associations of the percentage (Y) of each CD4 T-cell subpopulation with age at time of examination (*age*), gender (*gender*) and radiation dose (*dose*) were analyzed using a multiple regression model (*7*). This model assumes that the percentage of each T-cell subpopulation, or level of TNF-α, is related to each variable in an exponential manner with adjustment for age, gender and other parameters (V) measured for the same individuals:

$$\log Y = \alpha + \beta_1 \times age + \beta_2 \times gender + \beta_3 \times dose + \beta_4 \times V,$$

where *gender* is an indicator of female sex, i.e., *gender* = 0 for male and *gender* = 1 for female, and *dose* is radiation dose in grays. The α is a constant term, and β_1, β_2, β_3 and β_4 are regression coefficients for variables to be estimated. The age term was subtracted by 70 years so that α corresponds to log-transformed percentage of CD4 T-cell subset, i.e., the subset percentage is calculated to be e^{α} (= exponential [α]), for nonirradiated males at 70 years of age. The percentage change of subset percentage was estimated to be $100(e^{10\beta_1} - 1)$ per 10 years increment of age and $100(e^{\beta_3} - 1)$ per 1 Gy radiation dose. All statistical analyses were carried out using the SAS program (SAS Institute Inc., Cary, NC).

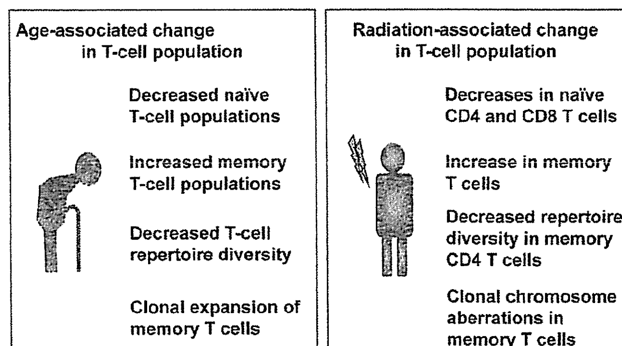

FIG. 1. Age- and radiation-associated alterations in the T-cell system in humans. Left panel: An outline of aging of the T-cell system from a review by Weng (*8*). Right panel: Alterations in the T-cell systems we have observed in association with radiation exposure among A-bomb survivors (*6, 9–12*).

RESULTS AND DISCUSSION

Evidence for T-Cell Immunosenescence from Previous Studies on A-Bomb Survivors

Age-associated changes in T-cell populations are primarily characterized as (1) decreases in cell numbers and functions such as proliferative responses to T-cell receptor (TCR) stimulation and T-helper function in naïve T-cell populations, (2) increases in memory T-cell populations, (3) decreased TCR repertoire diversity along with a reduced antigen recognition, and (4) frequent emergence of an oligoclonally expanded population of memory T cells (Fig. 1, left panel) (*8*). Radiation effects on the human immune system that are in accordance with the age-associated changes in T-cell populations are also depicted in Fig. 1 (right panel). Among A-bomb survivors, we have observed radiation-associated alterations such as (1) decreases in naïve CD4 and CD8 T cells (*6, 9–11*), (2) increases in memory CD4 and CD8 T cells (*6*), and (3) decreased repertoire diversity in memory CD4 T cells (*11*). We also observed (4) clonal chromosome aberrations of memory T-cell origin in heavily exposed survivors, suggesting clonal expansion of memory T cells (*12*). Thus the effects of radiation on T-cell immunity mostly resemble the effects of aging on the immune system.

Although other studies have reported that clonal expansion of a subset of memory T cells, $CD28^-$ or $CD57^+$, frequently occurred in older unirradiated

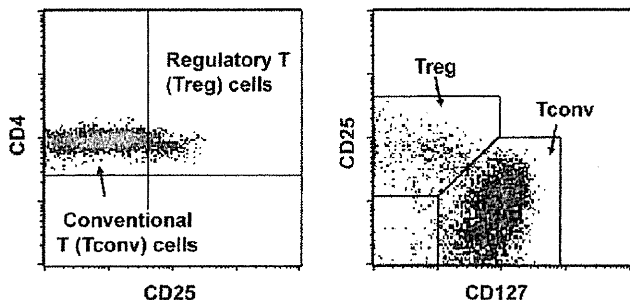

FIG. 2. Flow cytometry patterns of regulatory T (Treg) and conventional T (Tconv) cells that were discriminated with CD25 expression levels alone (left panel) or with both CD25 and CD127 levels (right panel) in the peripheral blood CD4 T-cell fractions from a typical A-bomb survivor.

individuals (*8, 13*), no significant radiation-associated change in percentages of such cells in either CD4 (*14*) or CD8 (*6*) T-cell subsets was observed among A-bomb survivors. To further evaluate the maintenance of T-cell memory in A-bomb survivors, we recently analyzed memory CD4 T-cell subsets using HSCA-2 (recognizing the low-molecular-mass glycoform of CD43), a monoclonal antibody that we established to classify human memory T cells (*15*). We found that functionally weak ($CD43^{middle}$) and anergic ($CD43^{low}$) memory CD4 T-cell subsets dose-dependently increased among A-bomb survivors, suggesting that attrition of the competent memory T-cell population is related to radiation exposure (*16*).

Immunosenescence in the T-cell system may be involved in long-lasting impairments in T-cell functions among A-bomb survivors. In fact, our previous observations indicated that previous radiation exposure dose-dependently induced deleterious effects on T-cell functions in A-bomb survivors. *In vitro* T-cell proliferative responses to PHA (*17*) and alloantigens (*18*) and the frequency of IL-2-producing T cells (*19*) were all found to decrease in association with radiation dose. Also noted were dose-dependent decreases in the proliferative responses of A-bomb survivors' T cells that were exposed to superantigen staphylococcal enterotoxins (*10*). The superantigen responses correlated well with the naïve CD4 T cell percentages, suggesting that the immunosenescence in naïve CD4 T cells that we consistently observed in A-bomb survivors might account in part for their deficits of T-cell proliferative function (*10*). In addition, the other T-cell functions, which were inversely related to radiation, also tended to decrease in association with aging. Taken together, there is accumulating evidence of T-cell immunosenescence being associated with aging and previous radiation exposure in A-bomb survivors and consequently leading to diminished functions in their adaptive immune systems.

Radiation Effects on Regulatory T Cells (Current Study)

$CD4^+$ regulatory T (Treg) cells express CD25 and suppress T-cell activation and function, and their number increases with age (*20*). Our previous measurement of Treg, which were identified by their high CD25 expression level, did not find any effect of age or radiation on these cells, probably due to the difficulty in accurately discriminating Treg from conventional T (Tconv) cells (data not shown). Recently, however, Treg cells were shown to be clearly identified by their $CD25^+$/$CD127^-$ phenotype (*21, 22*), allowing us to make more accurate measurements. Figure 2 shows representative flow cytometry patterns of Treg cells that were identified by the $CD25^+$ phenotype alone and by the $CD25^+$/$CD127^-$ phenotype in an A-bomb survivor. We could not find a significant effect of age on Treg cells (Table 2). This is probably due to the advanced ages of our study subjects; all subjects were 60 years old or older. As for radiation effects, there was a dose-dependent increase in the percentages of Treg cells in the CD4 T-cell population. The same trend was suggested for the ratio between Treg and Tconv cells. Those results suggest that A-bomb radiation might direct T-cell immunity toward suppressor phenotypes in relation to immunosenescence. It has been demonstrated that Treg cells have a strong influence in suppressing pathological immune responses in autoimmune diseases (*23*). Clinical studies in A-bomb survivors thus far have found no evidence that supports the idea that there is an increase in autoimmune diseases (*24*). Therefore, the current observation for Treg cell counts is not contradictory to the absence of an excess prevalence of autoimmune diseases in A-bomb survivors.

Radiation Effects on Naïve and Memory CD4 T-Cell Subsets (Current Study)

Naïve and memory T cells are mature T cells that have not previously experienced antigen encounter and that mediate recall responses to second and subsequent exposure to antigens, respectively. Memory T-cell populations are produced by antigen stimulation of naïve T cells and survive for many years and are classified based on the expression of other surface markers including CD62L into central and effector memory T cells and effector T cells with several differences in their functions, migration capacities and proliferation abilities. Our previous examinations of the peripheral blood lymphocyte populations of A-bomb survivors using CD45RO and CD62L as markers showed decreased percentages of naïve CD4 and CD8 T cells and increased percentages of memory CD8 T-cell subsets in a dose-dependent manner (*6*). In the present study, we discriminated naïve and memory CD4 T-cell subsets by using CD45RA and CD62L as markers and assessed the percentages of these subsets in the peripheral blood CD4 T-cell population among A-bomb survivors. We observed a dose-dependent decrease in the percentage of $CD45RA^+$/$CD62L^+$ (naïve, Tn) cells within the CD4 T-cell population, confirming

TABLE 2
Age, Gender and Radiation Effects on the Percentages of T-Cell Subpopulations in the Peripheral Blood CD4 T-Cell Populations among a Total of 1,035 A-Bomb Survivors[a]

T-cell subpopulation	Estimated percentage for nonirradiated males at 75 years of age	Percentage change of T-cell subpopulation percentage per unit		
		Age (10 years)[b]	Gender[c]	Dose (Gy)[d]
Regulatory T (Treg) and conventional T (Tconv) cells				
$CD25^+/CD127^-$ (Treg)	6.3	1.2 (−1.2, 3.5)[e] $P = 0.34$	−7.3 (−11.7, −5.3) $P = 0.0004$	2.7 (0.1, 5.3) $P = 0.044$
25.9$CD25^-/CD127^+$ (Tconv)	89.7	− 0.8 (−1.2, −0.5) $P = 0.0001$	0.9 (0.3, 1.5) $P = 0.0046$	− 0.1 (−0.5, 0.3) $P = 0.67$
Ratio (Treg/Tconv)	7.0	2.0 (−0.5, 4.6) $P = 0.13$	−8.2 (−12.4, −4.0) $P = 0.0002$	2.7 (−0.1, 5.5) $P = 0.052$
Naïve and memory				
$CD45RA^+/CD62L^+$ (Tn)	25.9	− 18.7 (−23.5, −13.9) $P = 0.0001$	5.6 (−2.2, 13.8) $P = 0.16$	− 5.6 (−10.9, −0.3) $P = 0.040$
$CD45RA^-/CD62L^+$ (Tcm)	44.5	0.4 (−1.6, 2.5) $P = 0.68$	0.3 (−3.0, 3.7) $P = 0.85$	4.5 (2.3, 6.8) $P = 0.0001$
$CD45RA^-/CD62L^-$ (Tem)	19.6	17.3 (13.4, 21.2) $P = 0.0001$	− 2.1 (−8.5, 4.3) $P = 0.51$	2.5 (−1.8, 6.7) $P = 0.26$
$CD45RA^+/CD62L^-$ (Teff)	0.4	8.6 (−3.0, 20.2) $P = 0.15$	− 22.3 (−42.1, −2.6) $P = 0.036$	− 8.0 (−21.1, 5.0) $P = 0.23$

Note. Treg, regulatory T cells; Tconv, conventional T cells; Tn, naïve T cells; Tcm, central memory T cells; Tem; effector memory T cells; Teff, effector T cells.

[a] Percentages of CD4 T-cell subpopulations for age, gender and dose were obtained by the multiple regression model, using the following formula: log (*percentage T cells*) = $\alpha + \beta_1 \times age + \beta_2 \times gender + \beta_3 \times dose$, where the value showing zero for the percentage of T cells was replaced with 0.01 when the value was transformed into natural log.

[b] Effects of age were estimated for 10 years.

[c] Gender = 0 for male and = 1 for female.

[d] Effects of dose were estimated for 1 Gy.

[e] 95% confidence interval.

our previous findings (Table 2). As for memory T-cell subsets, the percentage of $CD45RA^-/CD62L^+$ (central memory, Tcm) cells appeared to increase with radiation dose, whereas there was no significant radiation effect on $CD45RA^-/CD62L^-$ (effector memory, Tem) or $CD45RA^+/CD62L^-$ (effector, Teff) cells (Table 2). Those results clearly indicate that radiation exposure generated a reduction of Tn cell populations but an increase of Tcm cell populations among A-bomb survivors. Because Treg cells are known to suppress differentiation of resting naïve and memory T cells into effector cells (*24*), we analyzed the associations between the percentages of Treg cells with the $CD25^+/CD127^-$ phenotype and those of Teff cells with the $CD45RA^+/CD62L^-$ phenotype. A multiple regression analysis showed an inverse association between Treg and effector cell percentages ($r = 0.14$, $P = 0.0001$) after adjusting for age, gender and radiation dose. Although it needs to be determined whether the increase in the proportion of Treg cells is actually responsible for a suppression of the effector functions of T cells, elevated Treg cell levels in A-bomb survivors may be partly involved in the diminished T-cell responsiveness that we have observed among the survivors.

T-Cell Immunosenescence and Inflammation

A link between alterations in T-cell immunity and elevated inflammation among A-bomb survivors is suggested (*24*). We found that plasma levels of inflammatory cytokines such as IL-6, TNF-α and IFN-γ increased with radiation dose (*26*). Kim *et al.* (*27*) reported that treatment of T-cell-deficient nude and *Rag1*-knockout mice with poly I:C led to a lethal cytokine storm, whereas similar doses of poly I:C did not kill wild-type mice, which possess abundant T lymphocytes. They also showed that T cells, either Tn or Treg, were sufficient to control this cytokine response by the adoptive transfer of T cells prior to poly I:C treatment of *Rag1*-knockout mice, which resulted in decreased pro-inflammatory cytokine production. Those results suggest that T cells suppress the cytokine storm that occurs during the initial innate immune response. In A-bomb survivors, there were dose-dependent increases in plasma levels of inflammatory cytokines and CRP, and such enhanced inflammatory responses might be caused by alterations in the T-cell system of the survivors. For example, we found that plasma IL-6 and CRP levels were significantly elevated in the survivors with low percentages of peripheral blood CD4 T cells (*26*). A similar trend was also apparent in the survivors with low percentages of Tn (YK and TH, unpublished observation).

To seek a further link of T-cell immunosenescence to inflammatory responses among A-bomb survivors, in the present study we analyzed associations between percentages of T-cell subsets (that we currently examined) and plasma TNF-α levels. TNF-α concentration has already been measured among a large number of subjects for another study and purpose; 1.50 pg/ml was estimated for nonirradiated males at 75 years of age, and the effects (percentage increases) of age, gender and radiation dose

TABLE 3
Partial Correlation Coefficients between TNF-α Levels and Percentages of T-Cell Subsets in the Peripheral Blood CD4 T-Cell Population

	T-cell subset				
	Treg	Tn	Tcm	Tem	Teff
TNF-α	−0.08 (P = 0.36)	−0.20 (P = 0.014)	0.04 (P = 0.67)	0.23 (P = 0.005)	0.12 (P = 0.15)

Notes. Partial correlation coefficients adjusted for sex, age and radiation dose were calculated after TNF-α levels and percentages of T-cell subsets were naturally log-transformed. Each P value in parentheses is a significant level of partial correlation coefficient between natural log-transformed percentage of the T-cell subset and the log-transformed of TNF-α level. The value showing zero for the percentage of T cells was replaced with 0.01 when the value was transformed into natural log. Treg, regulatory T cells; Tn, naïve T cells; Tcm, central memory T cells; Tem; effector memory T cells; Teff, effector T cells.

on TNF-α concentration were 15% for 10 years, 15% for females relative to males, and 7% for 1 Gy, respectively (*28*). In this study, we remeasured the TNF-α concentration with a subset (155 survivors) of the current study subjects, whose T-cell data are available in Table 2, and we used these data (1.14 pg/ml being estimated for nonirradiated males at 75 years of age) for the calculation of partial correlation coefficients in Table 3. Plasma TNF-α levels were inversely associated with the percentages of Tn but not Treg cells, suggesting an involvement of Tn but not Treg cells in suppression of innate inflammatory responses manifested by increased TNF-α levels (Table 3). This may explain why we observed long-lasting inflammatory responses despite an increase in the relative number of immunosuppressive Treg cells in A-bomb survivors. It was also noted that plasma TNF-α levels were positively associated with the percentage of Tem cells (Table 3). There was a negative correlation between percentages of Tn and Tem cells ($r = -0.71$, $P = 0.0001$) but not between those of Treg and Tem cells ($r = 0.05$, $P = 0.13$). Thus T-cell immunosenescence manifested by a reduction in the relative number of Tn cells might be linked to the development and/or expansion of Tem cells, involving enhanced inflammatory responses.

Implications of T-Cell Immunosenescence in Disease Development among A-Bomb Survivors

We found previously that the CD4 T-cell percentages were significantly lower in survivors with a history of myocardial infarction (MI) than in survivors with no such history (*28*). We also noted that the T cells of survivors with a history of MI tended to be poor responders to several superantigens of *S. aureus* toxins and that these same individuals had proportionally fewer naïve CD4 T cells than survivors with no MI history (*10*). As suggested for a link between alterations in T-cell immunity and inflammation among A-bomb survivors, both IL-6 and CRP levels were significantly higher in survivors with a history of MI than in those without such a history (*26*). We thus believe that the T-cell immunosenescence associated with inflammatory reactions will prove to be a cause of increased risk of cardiovascular disease among A-bomb survivors, in much the same way as seen in other epidemiological studies of unirradiated individuals (*29–31*). However, because it is still possible that the disease itself or some medication might be responsible for such immunosenescence, prospective studies are needed to provide definitive information on the causal link between T-cell immunosenescence and inflammatory diseases.

Noncancer diseases frequently observed in A-bomb survivors include circulatory, respiratory and digestive diseases, especially cardiovascular disease, pneumonia and liver disease (*1, 2*). One possible explanation involves the failure of aging immune systems to control microbial infections, since infections might lead to chronic inflammation and hence to increased susceptibility to such noncancer diseases in heavily exposed survivors, resulting from unregulated and hence long-lasting inflammatory responses. In A-bomb survivors, however, no significant long-term effects of radiation on the antimicrobial functions of blood monocytes or granulocytes (e.g., phagocytosis, *in vitro* migration, etc.) have been noted (*32*). There is a growing interest in the role of pathogen recognition molecules, such as Toll-like receptors (TLRs), in the pathogenesis of chronic inflammatory diseases: TLRs play a key role in the host defense against exposure to microbial pathogens and also in the development and progression of atherosclerotic lesions (*33, 34*). It may be important and possible to conduct an integrated and systematic examination of the A-bomb survivors' innate immune systems in the future, including the analyses of TLR-mediated signaling as well as interaction of the signaling with T-cell immunity.

Conclusions and Perspectives

In summary, A-bomb radiation may have induced T-cell immunosenescence, resulting in attenuation of T-cell-mediated immunity. Such decrements in the T-cell system may cause chronic inflammation and in turn may be partly responsible for cardiovascular disease and other aging-associated diseases of importance. Although there is accumulating evidence of T-cell immunosenescence among A-bomb survivors, how ionizing radiation causes T-cell immunosenescence and how radiation-

induced T-cell immunosenescence interacts with ordinary aging remain to be explained. Mechanistic approaches using appropriate animal models will be necessary to depict a complete picture of radiobiological involvements in host immunological aging. Longitudinal analyses of the changes in the various immunological parameters may provide a suitable vehicle for a better understanding of the interaction between radiation-related and aging-associated immunological changes and for exploring causal relationships between these immunological changes and various noncancer diseases in A-bomb survivors.

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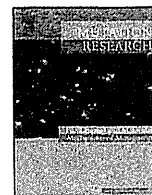

Increased DNA damage in hematopoietic cells of mice with graft-versus-host disease

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GVHD

ABSTRACT

Patients who received hematopoietic cell transplants have an increased risk for a new malignancy. In addition to genotoxic regimens such as radiotherapy and chemotherapy, graft-versus-host disease (GVHD) is a risk factor for development of new malignancies in long-term survivors. To understand mechanisms underlying this malignant transformation, we evaluated genomic damage in several murine models of GVHD by enumerating reticulocytes containing micronuclei (MN) in the blood after semi-allogeneic (parent-into-F1) hematopoietic cell transplantation. On day 40 after transplantation, MN frequencies were significantly increased in unirradiated (C57BL6 × DBA/2) F1 (BDF1) and (BALB/c × C57BL6) F1 (CBF1) mice that received cells from C57BL6 (B6) donors. MN frequencies were not significantly increased in F1 mice that received cells from DBA/2 or BALB/c donors. Serum levels of tumor necrosis factor-α (TNF-α) were higher after transplantation with B6 donors than with DBA/2 or BALB/c donors. The results indicate that GVHD, without irradiation, can induce genomic damage associated with inflammatory reactions manifested by increased TNF-α levels.

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1. Introduction

Second malignancies after hematopoietic cell transplantation (HCT) have been linked not only to DNA-damaging myeloablative treatments, such as ionizing irradiation, but also to graft-versus-host disease (GVHD) [1,2]. The immunological mechanisms for the association of GVHD with malignant transformation remain largely unknown. Current theory postulates that a gene mutation or a chromosomal aberration initiates the first step in a single cell towards malignant transformation and that this initiated cell, with time, clonally proliferates and accumulates further mutations to produce a fully malignant clone. Animal HCT models have suggested that one of the most important cytokines involved in the pathogenesis of GVHD is tumor necrosis factor-α (TNF-α) [3,4]. Tumor promotion activity of inflammatory cytokine TNF-α has also been indicated in several murine models [5,6]. TNF-α may also act as a cell surviving factor and may have endogenous tumor promoter effects by activating nuclear factor-κB (NF-κB) signaling pathways [7,8] that inhibit apoptosis of transformed cells [9–11] and by stimulating protein kinase Cα- and activator protein-1-dependent pathways that enhance clonal proliferation of transformed cells [12].

In the pathogenesis of a malignancy after HCT, exposure to DNA-damaging agents and chronic inflammation by GVHD likely serve as two important underlying risk factors, with the former causing tumor initiation and the latter causing tumor promotion [13]. On the other hand, inflammation by GVHD *per se* may induce genomic damage in cells that have not been genetically damaged by pretransplant conditioning regimens. Recent studies suggested that TNF-α not only induces anti-apoptotic signals through NF-κB activation, but also causes genomic instability through reactive oxygen species (ROS)-mediated DNA damage [14,15]. In the present study, we investigated whether or not GVHD causes genomic damage, particularly in relation to TNF-α production, using several parent-into-F1 transplantation models in which no DNA-damaging pretransplant conditioning was required to induce GVHD.

It has been suggested that radiation-induced inflammatory response is involved in the emersion of genomic instability in irradiated hosts [16,17]. We have previously analyzed the proportion of reticulocytes containing a micronucleus (MN) as a measure of genomic instability after non-lethal ionizing irradiation in mice. That measure of genomic instability can persist *in vivo* for prolonged periods after irradiation [18]. In this reticulocyte MN assay, the persistence of radiation effects (i.e., long-term genomic instability) can be distinguished from immediate effects on target cells, since reticulocytes have an *in vivo* lifespan as short as a few days [19]. Therefore, we used reticulocyte MN frequencies to evaluate

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genomic damage induced by GVHD in mice. Our present results indicate that GVHD after HCT even without ionizing irradiation can induce genomic damage, possibly through inflammatory effects mediated by TNF-α.

2. Materials and methods

2.1. Mice

A set of female C57BL/6 (B6), DBA/2, and (B6 × DBA/2) F1 (BDF1) mice eight weeks of age were purchased from Japan Clea Co. (Tokyo, Japan). Another set of female BALB/c, B6, and (BALB/c × B6) F1 (CBF1) mice eight weeks of age were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). All mice were housed in autoclaved microisolator cages and fed a sterile regular diet *ad libitum*. All animal handling procedures were approved by the Experimental Animal Care Committee of the Radiation Effects Research Foundation.

2.2. Induction of GVHD

Donor mice 9–16 weeks of age were used for transplantation. GVHD was induced by tail vein injection of either 50×10^6 splenocytes from B6 donors, or splenocytes (90×10^6) and lymph node cells (10×10^6) from DBA/2 donors, into BDF1 recipients 9 weeks of age. Similarly, CBF1 recipients 16 weeks of age were injected with splenocytes (100×10^6) and lymph node cells (12×10^6) from B6 donors, or with splenocytes (100×10^6) and lymph node cells (16×10^6) from BALB/c donors. Negative controls consisted of age- and sex-matched uninjected F1 and parental mice. Survival after HCT was monitored almost daily, and weight changes were assessed weekly. Blood cell chimerisms, reticulocyte MN frequencies, and serum cytokine levels were assessed 40 days after transplantation. Blood was obtained by orbital sinus puncture after ether-anesthesia, and the mice were euthanized by cervical dislocation.

2.3. Assessment of chimerism after transplantation

Heparinized blood was lysed with NH_4Cl buffer, and leukocytes were stained for two-color analysis with fluorescein (FL)-labeled CD3 antibody (BD Biosciences, Franklin Lakes, NJ, USA) and with biotinylated antibodies specific for H-2Kb (BD Biosciences) or H-2Kd (BD Biosciences). Binding of the biotinylated antibody was assessed by staining with streptavidin-conjugated phycoerythrin (PE) (BD Biosciences). The stained cells were subsequently analyzed by a FACScan flow cytometer (BD Biosciences). Bit maps for lymphoid cells and granulocytes were defined by forward- and side-scatter characteristics, and the percent of donor cells within each window was enumerated. Results were corrected for positive control samples and were rounded to the nearest integer. In the lymphoid and granulocyte gates, positive control samples showed 87.1–100% and 82.4–100% staining, respectively.

2.4. Reticulocyte MN assay

Blood reticulocyte MN frequencies were analyzed by flow cytometry with the use of the mouse MN analysis kit MicroFlow^{PLUS} Kit (Mouse) (Litron Laboratories, Rochester, NY, USA), as described previously [18]. Briefly, the blood was mixed with the anticoagulant solution supplied in the kit, and was fixed in absolute methanol at −80 °C and kept in an ultracold (−75 to −85 °C) freezer. After washing the fixed cells, aliquots of each cell pellet were added to tubes containing fluorescein-labeled anti-CD71 and phycoerythrin-labeled anti-CD61 antibodies and RNase A, and then resuspended in cold propidium iodide solution. More than 20,000 reticulocytes gated on $CD71^+/CD61^-$ populations were analyzed by a FACScan to determine MN frequencies. Data analysis was performed using Flowjo software (Tree Star, Ashland, OR, USA).

2.5. Cytokine assay

Serum samples were collected and preserved at −75 to −85 °C until testing. Cytokines, interleukin (IL)-6, IL-10, monocyte chemoattractant protein (MCP)-1, interferon-γ (IFN-γ), TNF-α, IL-12p70) were quantified using the Cytometric Bead Array Mouse Inflammation Kit (BD Biosciences) according to the manufacturer's instructions, with the use of a FACScan. The detection limits in these cytokine assays were 5 pg/ml (IL-6), 17.5 pg/ml (IL-10), 52.7 pg/ml (MCP-1), 2.5 pg/ml (IFN-γ), 7.3 pg/ml (TNF-α), and 10.7 pg/ml (IL-12p70). For data analysis, values under the detection limits were considered as 0 pg/ml. Because measured levels of IL-6, IL-10, and IL-12 were mostly under the detection limits, we did not use the results for these cytokines in this study.

2.6. Statistical analysis

Differences in the MN frequency and cytokine levels between experiment groups were examined with a Mann–Whitney's test, using SPSS (ver. 14.0) software (SPSS, Chicago, IL, USA), because the values were not normally distributed in each group. For all tests, a value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. GVHD and MN frequencies in unirradiated BDF1 recipients after HCT from parental B6 donors

In unirradiated BDF1 recipients with GVHD induced by parental B6 splenocytes, weight loss first appeared at two to four weeks after transplantation, and the weight of the transplanted mice was still lower than that of untreated controls 40 days after transplantation (Fig. 1A). On day 33, one of the 22 recipients died. On day 40, we assessed blood cell chimerisms, MN frequencies, and circulating cytokine levels in surviving mice, and compared these values with those of age-matched untreated BDF1 and B6 mice. Percentages of donor cells in blood granulocytes and T cells in the recipient mice were 0–80% (median: 25%) and 1–92% (median: 38%), respectively. Flow cytometry patterns of MN frequency assessment are exemplified in Fig. 2. As shown in Fig. 1B, MN frequencies of the transplanted mice were significantly higher than those of either untreated BDF1 or B6 mice ($P = 0.001$ or 0.005, respectively), indicating that this B6-into-BDF1 transplant model can induce a significant level of genetic damage in hematopoietic cells. In addition, serum TNF-α levels in recipients were significantly higher than in untreated BDF1 controls ($P = 0.001$) but did not significantly differ from those in parental B6 mice ($P = 0.14$, Fig. 1C). There was a positive correlation between MN frequencies and TNF-α levels in the BDF1 recipients, with correlation coefficient (r) of 0.48. On the other hand, we found no significant difference in either IFN-γ or MCP-1 levels among the three groups (data not shown). At 95 days after transplantation, we also found significant differences in MN frequencies and TNF-α levels between BDF1 recipients and untreated BDF1 mice (data not shown). Those results suggest that without irradiation, transplantation of B6 cells into BDF1 recipients can cause elevated MN frequencies in the circulating reticulocyte fraction as well as increased TNF-α levels in the serum.

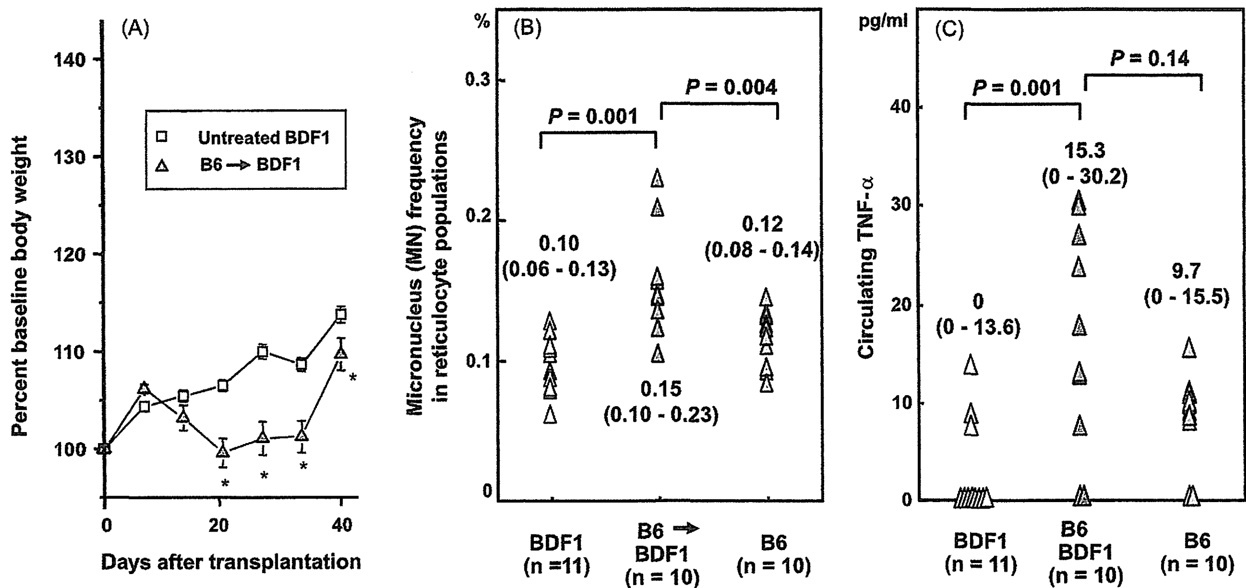

Fig. 1. Reticulocyte micronucleus (MN) frequencies and circulating tumor necrosis factor-α (TNF-α) levels in parental C57BL/6 (B6) mice, untreated (B6 × DBA/2) F1 (BDF1) mice, and BDF1 recipient after hematopoietic cell transplantation (HCT) from B6 donors. (A) Weight changes after transplantation (triangles) and in untreated (squares) BDF1 controls were monitored once weekly. Each symbol represents the mean in each group of mice. Error bars and asterisks denote standard errors (SE) and significant differences between the groups ($P < 0.05$, by t-test), respectively. (B) MN frequencies and (C) TNF-α levels were measured on day 40 after transplantation and plotted for each group. Numbers in the vicinity of the symbols denote the median and range (in parentheses).

3.2. GVHD and MN frequencies in unirradiated BDF1 recipients after HCT from parental DBA/2 donors

In unirradiated BDF1 recipients where GVHD was induced by injection of spleen and lymph node cells from parental DBA/2 donors, weight gain was retarded in comparison to untreated controls at least until day 40 (Fig. 3A). All the transplanted mice survived until 40 days after transplantation. On day 40, MN frequencies in blood samples from transplant recipients did not significantly differ from those of either untreated BDF1 or DBA/2 mice (Fig. 3B). Percentages of donor cells in blood granulocytes and T cells in the recipient mice were 1–36% (median: 9%) and 3–34% (median: 9%), respectively. Serum TNF-α levels were significantly higher in the transplant recipients in comparison to untreated BDF1 controls ($P = 0.033$) but not in comparison to untreated DBA/2 controls ($P = 0.085$) (Fig. 3C). In the DBF1 recipients, TNF-α levels showed no significant correlation with MN frequencies. We found no significant difference in either IFN-γ or MCP-1 levels among the three groups (data not shown). Therefore, unlike transplantation with B6 donors and BDF1 recipients, transplantation with DBA/2 donors and BDF1 recipients did not provide evidence causing genomic instability. This difference between these two transplantation models was associated with a difference in serum TNF-α levels, because TNF-α levels in DBA/2-into-BDF1 recipients were significantly lower than those in the B6-into-BDF1 recipients ($P = 0.039$).

3.3. GVHD and MN frequencies in unirradiated CBF1 recipients after HCT from parental B6 or BALB/c donors

In unirradiated CBF1 recipients with GVHD induced by injection of spleen and lymph node cells from parental B6, weight gain was retarded until at least day 34 (Fig. 4A). All recipients survived until 40 days after transplantation. On day 40, MN frequencies in CBF1 recipients transplanted with B6 cells were significantly higher than those in untreated CBF1 mice ($P = 0.015$). MN frequencies also showed a trend for higher values in CBF1 recipients when compared to age-matched parental B6 controls, but this trend was not statistically significant ($P = 0.11$) (Fig. 4B). Percentages of donor cells in

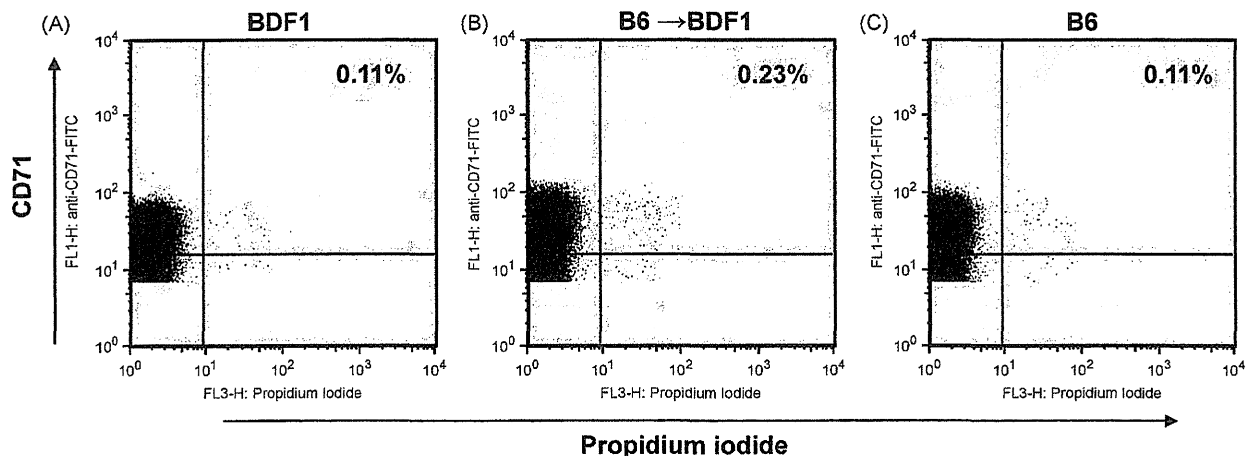

Fig. 2. Typical flow cytometry patterns of reticulocyte MN frequencies in (A) untreated BDF1, (B) BDF1 recipient after HCT from B6 donors, and (C) parental B6 mice. Number in the upper right corner of each cytogram denotes the frequency of reticulocyte MN.

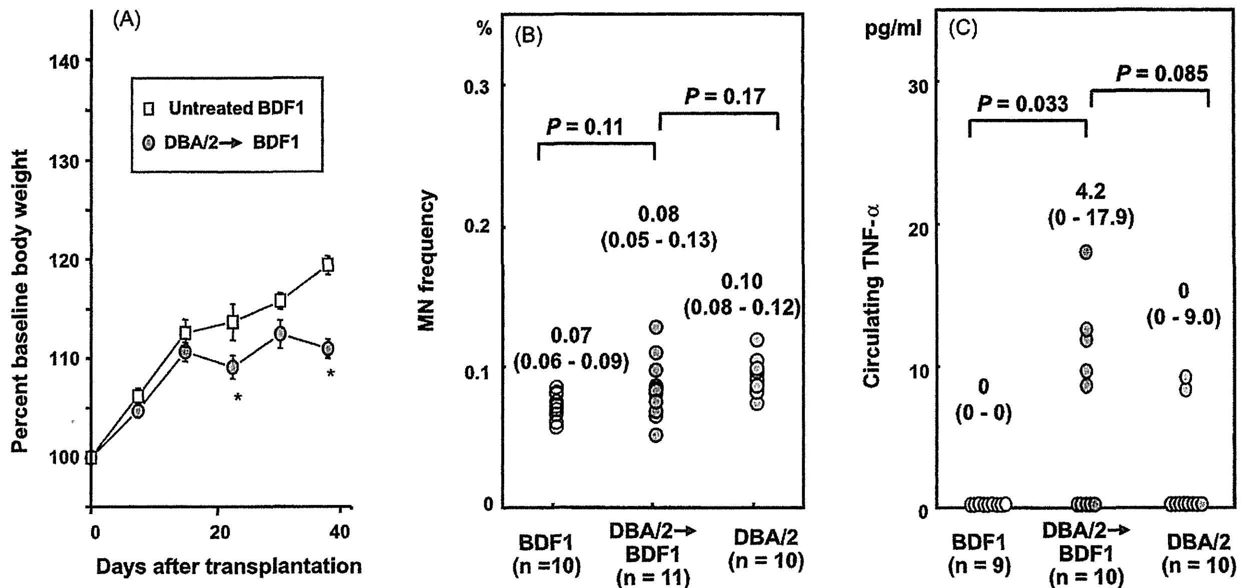

Fig. 3. MN frequencies and circulating TNF-α levels in parental DBA/2 mice, untreated BDF1 mice, and BDF1 recipients after HCT from DBA/2 donors. (A) Weight changes in after HCT (circles) and in untreated (squares) BDF1 controls were monitored once weekly. Each symbol represents the mean in each group of mice. Error bars and asterisks denote SE and significant differences between the groups, respectively. (B) MN frequencies and (C) TNF-α levels were measured on day 40 after transplantation and plotted for each group. Numbers in the vicinity of the symbols denote the median and range (in parentheses).

blood granulocytes and T cells in the recipient mice were 72–100% (median: 100%) and 68–94% (median: 91%), respectively. Circulating TNF-α levels in the B6-into-CBF1 transplant recipients were significantly higher than those in untreated CBF1 mice ($P=0.029$) (Fig. 4C), and positively correlated with MN frequencies ($r=0.67$). Transplantation of BALB/c cells into CBF1 recipients did not cause a significant increase in MN frequencies or serum cytokine levels when compared with untreated CBF1 mice. Percentages of donor cells in blood granulocytes and T cells in the recipient mice were 1–21% (median: 9%) and 11–29% (median: 26%), respectively. Therefore, like GVHD in BDF1 mice, transplants from B6 donors caused genomic instability in CBF1 recipients but transplants from BALB/c donors did not.

4. Discussion

We evaluated genomic instability in several parent-into-F1 transplantation models that could induce GVHD. Elevated MN frequency was observed in both BDF1 and CBF1 recipients with parental B6 donors but not in recipients with DBA/2 or BALB/c donors. Differences in types of GVHD may be at least partly involved in the different susceptibility to genomic dam-

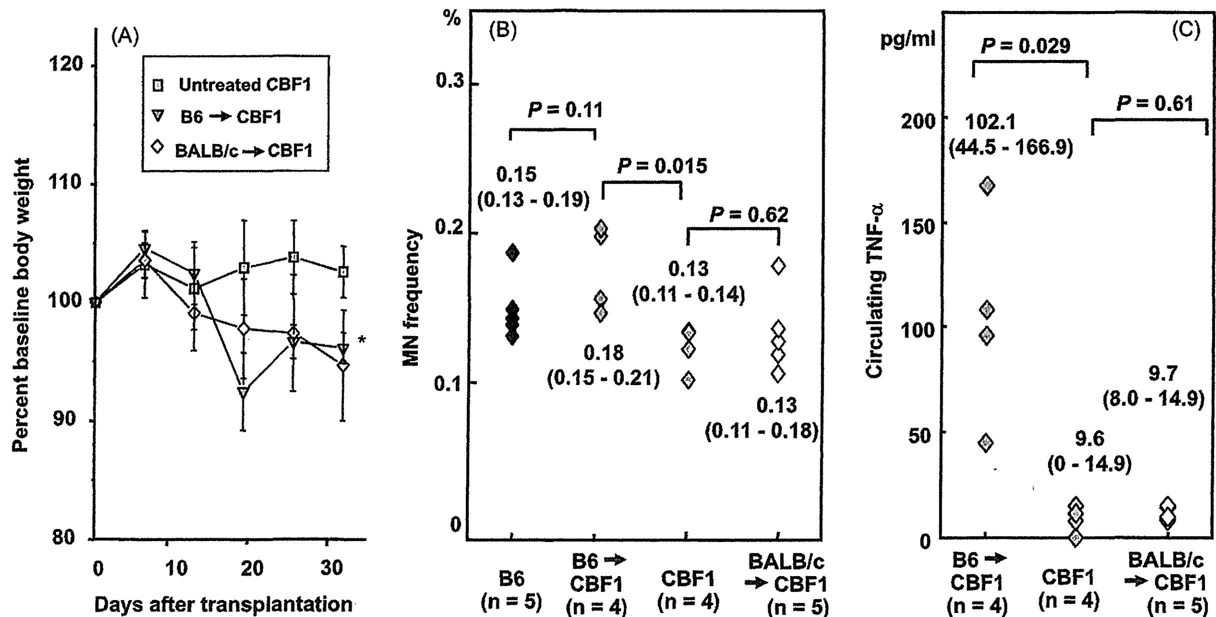

Fig. 4. MN frequencies and circulating TNF-α levels in parental B6 and BALB/c mice, untreated (BALB/c × B6) F1 (CBF1) mice, and CBF1 recipients after HCT from B6 or BALB/c donors. (A) Weight changes in CBF1 mice untreated (squares) and after HCT from B6 (triangles) or BALB/c (diamonds) donors were monitored once weekly. Each symbol represents the average in each group. Error bars denote SE, and the asterisk indicates a significant difference between CBF1 recipients on day 34 after HCT from B6 donors as compared to untreated CBF1 controls. (B) MN frequencies and (C) TNF-α levels were measured on day 40 after transplantation and plotted for each group. Numbers in the vicinity of the symbols denote the median and range (in parentheses).

age among the transplantation models. It is well known that B6-into-BDF1 transplantation induces acute GVHD associated with type-1 immune responses, whereas DBA/2-into-BDF1 transplantation induces chronic GVHD mediated by type-2 immune responses [20–23]. Similar differences in types of GVHD have been reported for B6-into-CBF1 and BALB/c-into-CBF1 models, respectively [24]. Such disease-type differences may also explain the reason why we observed elevated TNF-α levels in both BDF1 and CBF1 recipients with B6 donors but not in those with DBA/2 or BALB/c donors. The level of TNF-α production is genetically controlled, and H-2^b strains express higher levels of TNF-α than H-2^d strains [25]. Thus, TNF-α production appears to be higher when GVHD is caused by B6 donor cells as compared to DBA/2 or BALB/c H-2^d donor cells.

Type-1 immune responses involve inflammatory cells that are potent sources of reactive oxygen species causing DNA-damaging effects to bystander cells [26,27]. In many cell systems, TNF-α signaling is associated with an increase in oxidative stress. In the present study, levels of circulating TNF-α appeared to increase in GVHD mice with elevated MN frequencies, suggesting a possible role of TNF-α in causing MN induction. In support of this hypothesis, TNF-α has chromosomal damaging effects in some systems [14,15]. In addition, an increase in oxidative stress has been noted for hematopoietic cells, including blood erythrocytes and leukocytes, in the B6-into-CBF1 GVHD model [28]. Therefore, TNF-α production in the disease process may be a contributing cause of genetic damage at least in the hematopoietic system of mice with GVHD, although we base this suggestion on correlations and not on results of mechanistic experiments. In further support of that hypothesis, however, we recently found that MN frequencies were dose-dependently elevated in both BALB/c and B6 mice treated with potent TNF-α inducers, including Poly (I-C) and lipopolysaccharide (K.H. & Y.K., manuscript in preparation).

In B6-into-BDF1 model, granulocytes derived from the marrow of F1 recipients were not largely replaced by those derived from parental donors during the first 40 days after transplantation. In addition, the reticulocyte MN frequency was not associated with the level of granulocyte chimerism among the unirradiated F1 recipients (data not shown). We believe that the same holds true for erythroid cells. We found that expression of H-2 class I molecules on reticulocytes was too low to prove the point (data not shown), and we have concerns that assays based on polymorphic mRNA transcripts expressed in reticulocytes (e.g., α-globin) [29], might lack quantitative agreement with actual cell counts. Although the level of chimerism in reticulocytes remains to be determined, it is likely that inflammation by GVHD *per se* induced MN not only in donor cells, but also in recipient-derived cells. The appearance of GVHD-related genomic instability in donor hematopoietic cells might explain the well-documented, but infrequent development of leukemia in donor cells after allogeneic HCT [30–33].

The differences in MN frequencies between GVHD mice and controls appeared to be small as compared to those in MN frequencies between cells exposed and unexposed to genotoxic agents *in vitro*. This may be due largely to the host defense mechanisms that effectively eliminate genetically damaged cells. However, the differences (1.4–1.5-fold increase) that we observed in the present study are almost compatible to the level of genomic instability (1.3–1.6-fold increase) detected with same method in mice one year after whole-body irradiation with 2.5 Gy [18], and amply sufficient to evaluate *in vivo* genetic effects in terms of solid cancer risk. A recent meta-analysis showed a significant 1.45-fold increase of MN frequency in untreated cancer patients (95% confidence interval: 1.28–1.64), compared with a cancer-free population [34]. Evaluation of biomarkers indicating inflammation and genomic instability, and correlation with follow-up data may reveal important clinical associations. Recent advances in high-throughput and automated systems for detecting genetic damage could facilitate such studies [35].

Our study is limited by the reliance on a single method for assessment of genomic damage. The reticulocyte MN assay, however, has the advantages of high sensitivity and validated applicability for measuring genotoxicity *in vivo*. With this assay, acute effects of radiation doses as small as 0.1 Gy are detectable in mice [18]. Assessment of double strand breaks through phosphorylated histone H2AX (γH2AX) focus formation is highly sensitive as an indicator of double strand breaks induced *in vitro*, but the use of this assay for measurement of double strand breaks *in vivo* has not been validated [36]. The flow cytometric version of this assay and the comet assay are both less sensitive than MN frequency for measurement of double strand breaks *in vivo* (K.H. & Y.K., unpublished observation). Our study is also limited by the assessment of genomic damage only in hematopoietic cells. Although we have not shown that GVHD could also induce genomic damage in the recipient tissues affected by GVHD, including the skin and intestine, Faber et al. [37] reported increased microsatellite instability (MSI) in buccal and colon epithelial tissues after allogeneic HCT, but not after autologous HCT. The genetic damage reflected by MSI differs from the double strand breaks that induce MN, but these genomic instabilities are both related to oxidative stress and are thought to be involved in the development of malignancy [38].

In summary, we provide evidence of genomic instability as reflected by reticulocyte MN frequencies in certain strains of F1 mice with GVHD. Analysis of results with different parental donors suggests that type-I inflammatory responses manifested by increased TNF-α levels may be involved in the DNA-damaging process. More detailed understanding of the mechanisms underlying such genomic instability following allogeneic HCT might help to develop approaches for preventing second malignancies after HCT.

Conflict of interest statement

The authors declare no competing financial interests.

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Memory CD4 T-cell subsets discriminated by CD43 expression level in A-bomb survivors

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Abstract

Purpose: Our previous study showed that radiation exposure reduced the diversity of repertoires of memory thymus-derived cells (T cells) with cluster of differentiation (CD)- 4 among atomic-bomb (A-bomb) survivors. To evaluate the maintenance of T-cell memory within A-bomb survivors 60 years after radiation exposure, we examined functionally distinct memory CD4 T-cell subsets in the peripheral blood lymphocytes of the survivors.

Methods: Three functionally different subsets of memory CD4 T cells were identified by differential CD43 expression levels and measured using flow cytometry. These subsets consist of functionally mature memory cells, cells weakly responsive to antigenic stimulation, and those cells functionally anergic and prone to spontaneous apoptosis.

Results: The percentages of these subsets within the peripheral blood CD4 T-cell pool all significantly increased with age. Percentages of functionally weak and anergic subsets were also found to increase with radiation dose, fitting to a log linear model. Within the memory CD4 T-cell pool, however, there was an inverse association between radiation dose and the percentage of functionally mature memory cells.

Conclusion: These results suggest that the steady state of T cell memory, which is regulated by cell activation and/or cell survival processes in subsets, may have been perturbed by prior radiation exposure among A-bomb survivors.

Keywords: *A-bomb, CD4, immunological memory, CD43, flow cytometry, T cell*

Introduction

In humans, immunological memory resides in and is controlled by long-lived lymphocytes, with immunologic memory being maintained at an appropriate level by a constant proliferation of memory thymus-derived cells (T cells) (Dutton et al. 1998). Once subjected to antigenic stimulation, memory T cells tend to divide repeatedly, thus giving rise to greatly expanded clonal populations which may persist for very long periods of time (Maini et al. 1999). Clonally expanded T-cell populations are frequently observed not only in healthy aged persons (Posnett et al. 1994, Fitzgerald et al. 1995, Wack et al. 1998) but also in virally-infected individuals (Eiraku et al. 1998, Silins et al. 1998) and in patients with autoimmune diseases of various types (Fitzgerald et al. 1995, Musette et al. 1996, Waase et al. 1996).

In general, the peripheral blood pool of memory T cells with cluster of differentiation (CD)- 4 appear not to have been significantly affected by radiation exposure among atomic-bomb (A-bomb) survivors. However, there are significant dose-dependent deficits in the naïve T-cell pools (Kusunoki et al. 1998, 2002, Yamaoka et al. 2004). Further, clonal populations originating from peripheral T cells have been identified in blood samples from some of the A-bomb survivors primarily by tracking specific T-cell receptor (TCR) genes and/or chromosome aberrations in memory T-cell populations (Kusunoki et al. 1993, Nakano et al. 2004). In this regard, we have recently reported that the extent of deviation in the TCR repertoire of memory CD4 T cells significantly increased as the intensity of radiation exposure increased (Kusunoki et al. 2003). It seems reasonable, therefore, to assume that A-bomb radiation

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