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Late Effects of Exposure to Ionizing Radiation and Age on Human Thymus Morphology and Function

Reiko Ito^{a,1}, Laura P. Hale^{b,1}, Susan M. Geyer^c, Jie Li^d, Andrew Sornborger^{e,f}, Junko Kajimura^a, Yoichiro Kusunoki^a, Kengo Yoshida^a, Marcel R. M. van den Brink^g, Seishi Kyoizumi^g, Nancy R. Manley^d, Kei Nakachi^a, and Gregory D. Sempowski^{b,2}

^aDepartment of Molecular Biosciences, Radiation Effects Research Foundation, Hiroshima, Japan

^bDepartment of Pathology and the Duke Human Vaccine Institute, Duke University School of Medicine, Durham, North Carolina

^cDepartment of Pediatrics, Health Informatics Institute, University of South Florida, Tampa, Florida

^dDepartment of Genetics, Paul D. Coverdell Center, University of Georgia, Athens, Georgia

^eDepartment of Mathematics, University of California, Davis, California

^fDepartment of Mathematics and College of Engineering, University of Georgia, Athens, Georgia

^gDepartments of Medicine and Immunology, Memorial Sloan Kettering Cancer Center, New York, New York

Abstract

The thymus is essential for proper development and maintenance of a T-cell repertoire that can respond to newly encountered antigens, but its function can be adversely affected by internal factors such as pregnancy and normal aging or by external stimuli such as stress, infection, chemotherapy and ionizing radiation. We have utilized a unique archive of thymus tissues, obtained from 165 individuals, exposed to the 1945 atomic bomb blast in Hiroshima, to study the long-term effects of receiving up to ~3 Gy dose of ionizing radiation on human thymus function. A detailed morphometric analysis of thymus activity and architecture in these subjects at the time of their natural deaths was performed using bright-field immunohistochemistry and dual-color immunofluorescence and compared to a separate cohort of nonexposed control subjects. After adjusting for age-related effects, increased hallmarks of thymic involution were observed histologically in individuals exposed to either low (5–200 mGy) or moderate-to-high (>200 mGy) doses of ionizing radiation compared to unirradiated individuals (<5 mGy). Sex-related differences were seen when the analysis was restricted to individuals under 60 years of attained age at sample collection, but were not observed when comparing across the entire age range. This indicates that while females undergo slower involution than males, they ultimately attain similar phenotypes. These findings suggest that even low-dose-radiation exposure can accelerate thymic aging, with

²Address for correspondence: DUMC Box 103020, 909 S. La Salle St., Duke Human Vaccine Institute, Durham, NC 27710, greg.sempowski@duke.edu.

[†]These authors contributed equally to this work.

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decreased thymopoiesis relative to nonexposed controls evident years after exposure. These data were used to develop a model that can predict thymic function during normal aging or in individuals therapeutically or accidentally exposed to radiation.

INTRODUCTION

The thymus produces naïve, self-tolerant T cells from bone marrow-derived precursors for export to the periphery. However, the thymus is unusual compared with other organs because both its size and function peak in early life, and then it undergoes age-associated involution. The resulting decreases in cellularity, organization and function continue through old age, with corresponding decreases in export of naïve T cells and in thymic epithelial (TE) cell-derived mechanisms of selection that contribute to a reduction in T-cell antigen receptor diversity and maintenance of self-tolerance (1–3). Although the mechanisms that regulate thymus involution are still poorly understood, both TE-cell-specific mechanisms and noncell-autonomous crosstalk between TE cells, non-TE-cell stroma and developing T cells have been implicated (1). These mechanisms are primarily stroma-intrinsic and occur prior to age-related alterations in hematopoietic stem cell (HSC) function (4, 5). In addition to intrinsic age-related decline in thymus size and function, the thymus may also be affected by a variety of external stressors. These include insults such as severe infection (6), therapeutic radiation (7), chemotherapy (8, 9), pregnancy (10, 11) or emotional/physical stress that transiently increases glucocorticoid levels (12). However, it is less clear whether and how these external events affect the trajectory of aging-related involution.

Current evidence suggests that the thymus has a potential for “rebound” after acute damage that declines with age. However, most research to date has been performed in mice that are exposed to individual thymic insults under controlled conditions. The long-term impact of stress events that may occur during a human lifespan are rarely considered and have not been experimentally addressed. In particular, determining the late effects of ionizing radiation on human thymus function has not previously been possible, due in part to lack of an appropriate cohort for study.

The Radiation Effects Research Foundation (RERF) in Hiroshima, Japan houses an extensive archive of human tissue blocks collected from surgery and autopsy of individuals exposed to the 1945 Hiroshima atomic bomb blast, then followed until their natural deaths. These unique tissue samples are of great potential interest and scientific value to address questions relating to the effects of ionizing radiation on thymus morphology and function. We report results of a detailed morphometric analysis of thymus activity and architecture obtained from this autopsy archive through image analysis of tissue sections using bright-field immunohistochemistry (IHC) and dual-color immunofluorescence (IF). The aim of the study was to determine the long-term impact of up to ~3 Gy exposure to ionizing radiation on human thymus function.

METHODS

Human Subjects

Thymus tissues used for this study were derived from autopsied Life Span Study cohort subjects (13) previously exposed to A-bomb radiation in Hiroshima and were stored at RERF and Hiroshima University Hospital. Subjects were 20–91 years old at the time of sample collection (typically death). Estimates for radiation exposure to the bone marrow were used, where calculations were based on the revised DS02 A-bomb radiation dosimetry (13–15). An estimated dose of <5 mGy was defined as “nonexposure.” “Low-dose” exposure was defined as 5–200 mGy, while >200 mGy was defined as “moderate-to-high-dose” exposure. Autopsy reports were reviewed for all 296 cases that were potentially available for study. Forty-six subjects were excluded based on the potential influence of a subject’s history of selected diseases or treatments, including thymoma, solid cancer or lymphoma with infiltration into thymus, lymphoid leukemia, aplastic anemia, pregnancy, radiotherapy involving thymus or intensive chemotherapy or steroid therapy within the month before death. An additional 85 were found to have insufficient thymus tissue remaining in their thymus tissue blocks or the tissue obtained was not thymus. Thymus tissues from the remaining 165 subjects were examined in this study. All tissues were unlinkably anonymized prior to analysis and remained in Japan throughout the entire study.

For reference, 11 nonexposed pediatric and adult thymus tissues were selected from a Duke Human Vaccine Institute repository of anonymous discarded tissues for which consent was not required (Duke IRB exemption no. 474) to age-match with available RERF autopsy tissues from subjects that received a <5 mGy dose (i.e., unirradiated). To increase the sample size for unirradiated reference thymus, this set was further augmented by analysis of existing immunostained and anonymized slides from an additional 25 subjects (Duke IRB exemption no. 474). Demographic characteristics and radiation exposure for the RERF and Duke thymus tissues examined are shown in Table 1. Samples from RERF for IF/MiCASA analysis [see Supplementary Materials (Methods section); <http://dx.doi.org/10.1667/RR14554.1.S1>] were from individuals that received a <15 mGy dose due to limited availability of suitable age-matched blocks to the Duke tissue collection.

Study Approvals

The use of human thymus tissue specimens from RERF was approved by the Human Investigation Committee and the Ethics Committee for Genome Research at RERF. De-identified discarded human thymus tissues in the Duke Human Vaccine Institute repository were used under Duke IRB exemption no. 474.

Slide Preparation and Immunostaining

RERF thymus tissues were fixed in unbuffered formalin, processed into paraffin blocks, and stored at uncontrolled ambient temperatures (0–35°C) for 5–20 years, with only daytime temperature control for ~15 years, then with 24 h temperature control (20–25°C) for an additional ~15 years. Optimization of IHC and IF staining for these tissues is described in detail elsewhere (16).

For immunohistochemical studies, 4- μ m-thick sections of thymus were stained with hematoxylin and eosin (H&E) (17). Antihuman cytokeratin (CK) antibody cocktail (clones AE1/AE3, Dako; Glostrup, Denmark) or CD1a antibody (clone EP3622, Epitomics, Burlingame, CA) and immunoperoxidase reactions with 3,3'-diaminobenzidine (DAB) were, respectively, used to highlight thymic epithelium and immature thymocytes with a brown color for bright-field morphologic scoring. H&E and CK (AE1/AE3) and CD1a-immunostained slides were digitally scanned at 40 $\times$ magnification using a Leica SCN slide scanner (Leica Microsystems, Wetzlar, Germany), mounted on a server and examined by two independent pathologists (Duke and RERF) using virtual microscopy (Aperio eSlide Manager; Leica Biosystems, www.leicabiosystems.com). Independent scores (see below) and tissue comments were entered into a secure RedCap database hosted on an RERF server.

Bright-Field Morphometry and Histologic Scoring

Classification of a tissue as thymus required the presence of cytokeratin-positive epithelial cells in a configuration morphologically consistent with thymic epithelium (Fig. 1). The presence or absence of thymic capsule was noted and was used in some cases to aid in delineation of thymus tissue from surrounding mediastinal fat or other connective tissue. Histological scoring was performed independently by two pathologists (RI, LPH) using ImageScope software (www.leicabiosystems.com). This included morphological classification of the total area of thymus tissue on the slide ("total area") and areas containing lymphocytes ("lymphoid area", as defined by H&E stain, includes thymic epithelial area and perivascular space but not intrathymic adipose tissue), thymic epithelium ("TE area", as defined by CK stain), or immature thymocytes ("cortical area", as defined by CD1a stain) by outlining them using the "pen tool" provided by the ImageScope software (see Supplementary Materials Fig. S1; <http://dx.doi.org/10.1667/RR14554.1.S1>). Individual areas identified for each tissue and type of area (total, thymic epithelial or cortical) were imported into Microsoft Excel for summation. Areas were measured in μm^2 , which were used for all calculations and data analysis, then rounded to the nearest mm^2 for data presentation. To adjust for the differences in amounts of thymus tissue available for each subject, all lymphoid, TE and cortical areas were converted to percentage area by dividing by the total area of thymus tissue examined. The primary outcome measurement of thymopoiesis was defined by the percentage cortical area, i.e., the percentage of the tissue that contained immature thymocytes.

Hassall bodies were defined as swirls of cytokeratin-positive terminally differentiated thymic epithelial cells enclosing variable amounts of amorphous debris that may contain calcifications (see Supplementary Materials Fig. S2A and B; <http://dx.doi.org/10.1667/RR14554.1.S1>). Accordingly, calcifications that were present in adipose tissue adjacent to residual islands of thymic epithelium but were themselves not surrounded by epithelial cells (see Fig. S2C) were not counted as Hassall bodies. The number of Hassall bodies present on each section was categorized as: 0, 1–10, 11–50 or <50 for each tissue. The presence/absence of thymic epithelial pseudo-rosettes ("TE rosettes"), defined as a circular arrangement of thymic epithelial cells that excludes interaction with thymocytes (Fig. S2D) (18), was also recorded.

Initial nonconcordance was evaluated for all measurements for samples independently scored by both site pathologists (RERF and Duke). For continuous measurements of area, overall concordance correlation coefficients less than 0.99 for a given measurement were classified as insufficiently concordant (19). Given the categorical nature of the measurements, full concordance was expected in classification of Hassall body numbers or presence of TE rosettes. These scenarios of discordance prompted re-examination of the tissue by both pathologists. This re-examination and adjudication process resulted in 100% concordance for classification of Hassall body numbers and TE rosettes.

Analysis Approach and Statistical Methods

Overall concordance correlation coefficients, Bland-Altman plots and Kappa statistics were used to evaluate concordance between Duke and RERF scoring of each of the samples and corresponding measurements. After adjudication to achieve the concordance requirements, areas and area percentages were calculated as the mean of the measurements provided by the two pathologists for each of the continuous marker endpoints. Nonparametric tests were used to compare continuous measurements (Wilcoxon rank-sum test, Kruskal-Wallis test) and categorical measurements (Fisher's exact and chi-square tests) among groups of interest. Analyses were conducted within all nonexposed (<5 mGy) subjects as well as across all RERF subjects.

Generalized linear regression (continuous measurements) and logistic regression (dichotomous measurements) models were used to assess relationships of age, sex and dose and their interactions in relationship to thymic measurements of interest. Multivariable models were used and predictive accuracy was assessed using Monte Carlo cross validation techniques. Specifically, generalized linear and logistic regression models were used to evaluate the influence of factors such as age, dose and sex on the various thymic markers of interest. Dose was evaluated in these models as a categorical factor (no- vs. low- vs. intermediate-/high-dose exposure) and as a continuous measurement (including square-root transformed values). Age was also evaluated in these models using attained age at sampling for analyses of all of the subjects, and also subject's age at the time of the bombing for those with non-negligible exposure (defined here as ≥ 5 mGy). Patterns and relationships with the thymic markers held for these different included variables.

A simple prediction of whether the thymus is capable of making new T cells (i.e., has cortical area >0) may be important biologically, since the ability to generate new T cells that can fill "holes in the repertoire" is likely critical for optimal responses to newly encountered antigens. Logistic regression analysis was therefore used to identify which of the factors predicted whether or not a subject had cortical area >0 at sample collection. This analysis was focused to determine whether noninvasive general demographic factors such as age at sampling, dose exposure and sex could be effectively used to predict active cortical area.

All analyses were conducted using the R statistical programming software (R-3.1.2 for Windows). A value of $P < 0.05$ was required for statistical significance.

RESULTS

Impact of Attained Age on Thymic Morphology and Function in Nonexposed Subjects

Table 1 shows demographic features of subjects as well as the tissue area available for study. Since the larger pieces of thymus tissue (greater total thymus area) were available in the RERF cohort (Table 1), all comparisons between groups used percentage lymphoid, TE and cortical areas. The Duke cohort of nonexposed subjects was significantly younger at sample collection (Table 1) and the percentage lymphoid, TE and cortical areas measured in the Duke cohort were significantly greater than those from RERF in a univariate analysis based on collection site (Supplementary Table S1; <http://dx.doi.org/10.1667/RR14554.1.S1>). However, a multivariable analysis with covariates of attained age and site showed that the differences in percentage lymphoid, percentage TE and percentage cortical areas, presence of TE rosettes and number of Hassall bodies were all significantly correlated with attained age, independent of collection site (Supplementary Table S1). Thus, thymic architecture and activity were similar in nonexposed Duke and RERF subjects for all measurements (Supplementary Table S1) based on standard morphologic parameters, with no difference in the rate of age-related thymic involution based on race/country of residence (U.S. compared with Japan).

Significantly more Hassall bodies were observed in the Duke tissues compared with the RERF tissues in a univariate analysis, but these differences disappeared in a multivariable analysis that adjusted for differences in attained age between the cohorts (Supplementary Table S1; <http://dx.doi.org/10.1667/RR14554.1.S1>). Hassall body number was significantly negatively correlated with age across both cohorts, when considered as presence of any Hassall bodies (>0) or as presence of abundant Hassall bodies (>10) (Supplementary Table S1).

In areas of thymus with ongoing thymopoiesis, TE cells were relatively widely separated (particularly in the cortex) with many thin cellular processes that could potentially interact with developing thymocytes, creating a “light and lacy” pattern upon cytokeratin immunostaining (e.g., Fig. 1B). However, in thymus tissues that were devoid of thymopoiesis (percentage cortical area = 0), TE cells were typically arranged in a “condensed” pattern where TE cells appeared to be tightly adherent to neighboring TE cells and did not appear to interact with other cell types (e.g., Fig. 1E). The presence of structures that may represent an intermediate phase between the light and lacy and condensed stages of TE architecture, termed “thymic epithelial (TE) rosettes” (Supplementary Fig. S2D; <http://dx.doi.org/10.1667/RR14554.1.S1>), was significantly associated with increased attained age ($P < 0.0001$) and with decreased cortical area/thymopoietic activity ($P < 0.0001$; Supplementary Fig. S2E;).

Since TE-cell-thymocyte interactions are critical for thymopoiesis, we compared the thymic microarchitecture in males aged 25–49 years who were exposed to 0–15 mGy of radiation ($n = 4$ Duke and 7 RERF subjects) in more detail using the MiCASA technique, a highly sensitive assessment of cellular relationships (see Supplementary Methods and Figs. S3 and S4; <http://dx.doi.org/10.1667/RR14554.1.S1>). MiCASA showed largely similar red (CD1a) and green (CK14) spectra, with only minor differences at the <20 -micron scale (red/green =

CD1a/CK14 comparison) when comparing the Duke and RERF cohorts to each other (Supplementary Fig. S4). More importantly, the coherence plots for the Duke and RERF samples were overlapping at all scales. This similarity in coherence plots indicated that any minor differences in microarchitecture at distances similar to the size of a cell (i.e., $<20\ \mu\text{m}$) do not appear to affect spatial relationships between CD1a⁺ thymocytes and CK14⁺ TE cells. The observed similarities in thymic microarchitecture and thymocyte-TE cell spatial relationships between these cohorts of sex- and age-matched subjects with less than 15 mGy exposure are consistent with our finding of no differences in thymic activity between Duke and RERF nonexposed cohorts based on morphometric analysis of percentage cortical area.

Impact of Gender on Thymic Morphology and Function in Nonexposed Subjects

To assess whether age-related thymic atrophy in humans occurs more quickly in males than females, the morphometric data obtained for all nonexposed individuals in the combined Duke/RERF cohort ($n = 81$) was analyzed with respect to gender (Supplementary Table S1; <http://dx.doi.org/10.1667/RR14554.1.S1>). Univariate analyses showed no significant differences in percentage lymphoid, percentage TE and percentage cortical areas, presence of TE rosettes and numbers of Hassall bodies between males and females. Multivariable generalized linear regression (for percentage area measurements) or logistic regression (for dichotomized measurements) analyses that included both attained age and sex as covariates showed a significant difference between males and females only for the presence or absence of TE rosettes, which became nonsignificant when gender and its interaction with attained age were considered.

However, plots of the percentage lymphoid, percentage TE and percentage cortical areas as a function of attained age for the nonexposed subjects in the Duke and RERF cohorts (Fig. 2A, B, E, F, I, J) showed a marked transition in the rate of change around the age of 60. Therefore, we also determined whether the thymic areas differed between males and females during the age range where involution was occurring most rapidly (i.e., 60 years old). This analysis showed that, consistent with a previous report that included few older males (20), nonexposed males 60 years of age showed decreased percentage cortical area (increased thymic atrophy) compared to females (Supplementary Table S1; <http://dx.doi.org/10.1667/RR14554.1.S1>). Males in this age range were also significantly more likely to demonstrate TE rosettes, a marker of decreasing thymopoiesis (Supplementary Table S1).

Impact of Low- and Moderate-to-High-Dose Radiation on Thymic Involution

The effects of radiation on thymic involution were examined in RERF subjects exposed to low- or moderate-to-high-dose radiation compared to nonexposed Duke and RERF individuals (Fig. 2; Table 2). Univariate analysis showed significant differences between the groups only for TE rosettes, which were more common in subjects exposed to moderate-to-high doses of radiation (Table 2).

However, multivariable analyses that incorporated age (attained or ATB), radiation dose and their interaction revealed significant dose-based decreases in percentage lymphoid area, percentage TE area and percentage cortical area in radiation-exposed vs. nonexposed subjects. Age at the time of the bombing had the largest effect on these thymic

measurements. Coefficients were negative, indicating that the older a subject was at the time of exposure, the higher the likelihood of having reduced percentage lymphoid area, percentage TE area and percentage cortical area later in life (Supplementary Table S2, columns A–C; <http://dx.doi.org/10.1667/RR14554.1.S1>). Additional multivariable analyses that incorporated the interaction between age at the time of the bombing and the dose of radiation showed significant interactions between age at the time of the bombing and dose (Supplementary Table S2, columns D and E). The effect of having any (low or moderate to high dose) exposure was sufficient to decrease thymic measurements such that there was less incremental change with age compared with the nonexposed control group. The dose of radiation received thus had a higher impact on thymic function than the effect of age. These dose-based differences in thymic measurements also remained significant when the analysis tested for interactions between dose and attained age at sample collection rather than age at time of the bomb. Overall, thymopoiesis, as demonstrated by multiple measurements including the percentage lymphoid, TE and cortical areas, was significantly decreased in exposed individuals compared to the nonexposed cohort, when adjusted for age.

Focusing only on exposed subjects, there were no significant differences in thymic measurements when comparing low-dose vs. moderate-to-high dose exposed subjects, although there was a trend toward increased TE rosettes (indicating not yet fully involuted thymus) in individuals exposed to low-dose radiation (Supplementary Table S3; <http://dx.doi.org/10.1667/RR14554.1.S1>). Multivariable analyses that incorporated age at the time of the bombing and the interaction between age at time of the bombing and moderate-to-high radiation dose showed that any differences in thymic measurements between these groups with negligible exposure can be fully accounted for by differences in age at the time of exposure to the bomb. Model-derived coefficients for this impact of age were all negative, indicating that subjects who were older at exposure had decreased percentage lymphoid, TE and cortical areas and decreased probability of having Hassall bodies (indicative of recent thymopoiesis) observed in their thymus tissue (Supplementary Table S3). Taken together, these data indicate that exposure to even low-dose radiation is sufficient to result in decreased thymic function many years after exposure.

Prediction of Thymopoietic Activity as a Function of Age and Radiation-Dose Group

In the Japanese cohort, the median number of years from exposure to autopsy (sample collection) was 31 years (range: 9–41). Even with this delay from exposure to thymic assessment, the dose-exposure group still influenced prediction of the presence of active thymopoiesis. The influence of these factors and their interaction with attained age at sample collection are reflected through the prediction probabilities (multivariable model) for presence of cortical area shown in Fig. 3.

The overall cohort studied had a large number of older subjects, with 122 subjects >60 years old at time of the sampling. In exposed and nonexposed subjects, 60 years old, gender was a significant predictive factor in relationship to percentage cortical area in the multivariable model ($P = 0.011$). Specifically, when percentage cortical area was examined as a continuous measurement with age and exposure group, whether the subject was male conferred additional reduction on predicted percentage cortical area. When the model was

dichotomized to presence versus absence of cortical area, male sex was no longer a significant determinant of this more absolute thymic outcome. The same finding that male sex was associated with decreased predicted thymic areas was also seen in the multivariable models for percentage lymphoid area ($P = 0.034$) and percentage thymic epithelial area ($P = 0.032$), where attained age and exposure-dose group are included in the models.

DISCUSSION

These studies utilized a unique archive of thymus tissues obtained from individuals exposed to the atomic bomb blast in Hiroshima to study the long-term impact of radiation on human thymus function. A detailed histologic morphometric analysis using bright-field immunohistochemistry and dual-color immunofluorescence demonstrated decreased thymus activity (i.e., increased thymic involution) in individuals exposed to either low (5–200 mGy) or moderate-to-high (>200 mGy) doses of ionizing radiation compared to nonexposed individuals (<5 mGy). These data indicate that exposure to even low-dose radiation can accelerate thymic aging, with decreased thymopoiesis relative to nonirradiated controls still evident at a median of 30 years postirradiation. These results have implications for monitoring or management of people exposed to ionizing radiation from therapy or from other circumstances such as nuclear accidents or long-term space travel.

A number of methods have been previously used to assess thymus function in humans. These include radiographic assessment of thymus size, flow cytometric evaluation of thymocyte subsets and/or naïve peripheral blood T cells, molecular assessment of T cell diversity (e.g., V β spectratyping) or DNA double-strand breaks characteristic of ongoing T-cell receptor gene rearrangement (signal-joint T-cell receptor excision circles; sjTRECs) in lymphocytes derived from peripheral blood or other lymphoid organs and immunohistologic staining of thymus tissue for markers associated with ongoing generation of new T cells [reviewed in (21)]. Given that only archival FFPE tissue blocks were available, the studies reported here used immunohistologic staining for CD1a, a marker characteristic of immature thymocytes, to derive the percentage cortical area as the primary measurement of thymus function.

Hassall bodies are collections of terminally differentiated thymic epithelial cells that are typically present in normal thymus with ongoing thymopoiesis (21). The formation of Hassall bodies has been hypothesized to be triggered by interactions between the TE cells and developing thymocytes (22). If this hypothesis is correct, then numbers of Hassall bodies should be high in tissues with robust thymopoiesis and rare to absent in tissues that lack thymopoiesis. The data in this study support this hypothesis. Identification of a few to no Hassall bodies was an additional histologic correlate of decreased thymic function with age. As reported elsewhere (7, 22, 23), Hassall bodies that were present in thymus tissues with minimal to no thymopoiesis were often dilated (Fig. 2A) and/or calcified, and calcifications were sometimes observed in the intrathymic fat (Fig. 2C). We hypothesize that such changes may occur due to incomplete reabsorption/removal of older Hassall bodies and thus represent past but not current thymopoiesis. However, the mechanisms that govern Hassall body removal and the time this takes to occur remain unknown. This study also

confirmed that the presence of TE rosettes correlated with decreased thymopoiesis as previously hypothesized (22).

Exposure to radiation has long been known to result in the death of developing thymocytes and to at least transiently suppress thymic function [reviewed in (24)]. However, effects on the thymic architecture have been less studied, particularly in the context of a survival that is long enough to assess interactions induced by aging. Using a model where mice were exposed to low to moderate doses of ionizing radiation in early adulthood then followed to assess long-term effects with aging, Xiao *et al.*, found a life-long suppression in thymopoiesis when mice were exposed to a single whole-body dose of 500 mGy at 6 weeks of age (Xiao *et al.*, manuscript submitted for publication). These results mirror those obtained in this study of human thymus, in which thymic function was impaired in radiation-exposed subjects examined based on their age at natural death at a median of 30 years postirradiation (range: 9–41 years), thus integrating effects of radiation, thymus recovery and aging.

Several previous studies have also reported long-term effects of radiation on thymic function. Patients with Hodgkin disease who received mediastinal radiation therapy but not chemotherapy showed long-term decreases in peripheral blood naïve CD4⁺ and CD8⁺ T cells, although memory CD4⁺ and CD8⁺ T-cell numbers had returned to normal or slightly above normal, respectively, within 2.5 years (25). These decreases in naïve T cells (that would have been produced in the thymus) were independent of age at exposure or time since therapy, which was greater than 20 years in some cases (25). In a separate study, survivors of acute lymphoblastic leukemia (ALL) who were treated with both radiation and chemotherapy showed decreased numbers of naïve (CD45RA⁺) T cells, as well as decreased T-cell receptor signal joint excision circles (sjTRECs) compared to healthy siblings at a median of 8.7 years after chemotherapy, although their T-cell diversity did not decrease, as indicated by similar complexity of their V β spectratypes (26). Naïve T cells and sjTRECs continued to decline with time in these ALL survivors independent of the patient age, indicating compromised thymopoiesis that was not sustainable long term (26). The histologic evaluation performed in this study shows similar prolonged impairment of thymic function after exposure, without the confounding effects of malignancy or chemotherapy. Immunological studies performed at RERF have shown significant radiation effects on T-cell immunity, including significant radiation dose-dependent decreases in the relative number of naïve CD4 and CD8 T cells [reviewed in (27)].

The results of this study were limited by the numbers and distribution of A-bomb-exposed subjects in the various age at sample collection, sex and exposure groups. The unexposed, low-dose and moderate-to-high dose RERF cohorts were similar with respect to age at the time of the bombing, age at sampling and sex (Table 1), variables that were hypothesized to potentially affect thymic function. Male sex did not significantly affect the continuous outcome of percentage cortical area in multivariable models using data from all subjects, when age at sample collection and exposure group were also included in the model. However, when the model focused only on those subjects $\geq$ 60 years old at time of sample collection, gender remained a significant determinant of thymopoiesis in the multivariable model, with females undergoing slower involution than males. This result is consistent with

a previous study of human thymus that was smaller, had fewer males in the older age ranges and did not perform a multivariable analysis that could correct for the age when assessing potential sex effects (20) and with data from murine models (Xiao *et al.*, manuscript submitted for publication). Even in the younger 60 years old cohort of the current study, gender did not significantly affect the dichotomized outcome of presence of cortical area (e.g., at least some active thymopoiesis, >0) versus not, although few individuals had values of 0. Taken together, these results indicate that while males undergo involution more rapidly than females, both sexes attain the same thymic involution endpoints with age. It is also important to note that all tissues from RERF were obtained at autopsy after natural death. RERF subjects with a history of diseases or treatments that are known or hypothesized to affect thymic function were excluded from this study (see Methods section). However it was not possible to stratify subjects according to both dose and their specific cause of death (e.g., infection, accident, etc.) and retain adequate statistical power. That our morphologic studies of thymic function showed similar involution in the RERF tissues derived from autopsy and the Duke tissues that were derived from surgery makes it unlikely that the specific cause of death contributed specifically to our final conclusions.

Since many of the methods commonly used to evaluate thymic function require examination of matched tissue and blood specimens (21, 28), they may not be appropriate for use in individuals without specific clinical indications for study. We have shown that a multivariable model focused only on attained age at sample collection and exposure and their interaction can be used to noninvasively predict probability of functional cortical area (Fig. 3). After appropriate validation by an independent cohort (e.g., using analysis of peripheral-blood-lymphocyte phenotype and sjTRECs), this information may prove important for studies of normal aging as well as for individuals exposed to ionizing radiation, from therapies, nuclear accidents or long-term space flight.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG 1.

Representative histology of thymus tissues with mild vs. severe involution. Tissues varied widely in their percentage area containing lymphocytes (as defined by H&E stain; panels A and D), thymic epithelium (TE area, as defined by CK stain; panels B and E) and immature thymocytes (cortical area, as defined by CD1a stain; panels C and F). Mild involution is shown in thymus from a 28-year-old male who was 15 years old at time of exposure to <10 mGy (panels A–C). The thymus from a 76-year-old male who was not exposed to radiation shows severe involution with rare lymphocytes (panel D), thin strips of thymic epithelium (panel E) and complete lack of CD1a-positive immature thymocytes (panel F). Scale bar represents 0.2 mm. Cort = cortex; M = medulla; HB = Hassall body; P = perivascular space, a region outside of the thymic epithelial network that contains adipose tissue, vessels and peripheral lymphocytes rather than developing thymocytes.

FIG 2.

Changes in thymus morphology and architecture with aging and radiation exposure.

Changes in percentage lymphoid (panels A–D), percentage TE (panels E–H) and percentage cortical areas (panels I–L) are shown for the following cohorts of subjects: Panels A, E, I: Duke nonexposed; Panels B, F, J: RERF nonexposed; Panels C, G, K: RERF low dose; Panels D, H, L: RERF; moderate-to-high dose. In these scatterplots, each point represents a single subject and fitted lines are based on loess regression lines.

FIG 3.

Predicting thymopoiesis based on age and radiation exposure. Prediction probabilities for nonzero cortical area (percentage cortical area >0) based on attained age at sample collection and radiation dose group and the interaction between these covariates. Solid lines represent predicted values for the indicated dose groups; dotted lines represent the 95% confidence intervals. The form of this model was nonzero cortical area $\sim \beta_0 + \beta_1 \text{ age} + \beta_2 \text{ dose group} + \beta_3 \text{ age} \times \text{dose group}$ (interaction) and was analyzed using a multivariable logistic regression model.

TABLE 1**Demographics and Radiation Exposure for Thymus Tissues Examined**

	RERF subjects (n = 165)	Duke subjects (n = 36)	P value^a
Male	86 (52%)	13 (36%)	0.12
Female	79 (48%)	23 (64%)	
Median age at the time of the bombing, years	43 (1–71)	–	–
0–35 years, number (%)	53 (32%)		
36–45 years	40 (24%)		
46–59 years	64 (39%)		
60+ years	8 (5%)		
Radiation exposure, mGy			
Median (range)	36 (0–2,909)		–
Number of exposed subjects (males/females)			
<5 mGy	45 (27M/18F)	36 (13M/23F)	
5–200 mGy	80 (41M/39F)	–	
>200 mGy	40 (18M/22F)	–	
Years since exposure, median (range)	30 (9–41)	–	–
Median attained age at sampling, years	74 (11–100)	45 (0–71)	<0.0001
0–35 years, number (%)	15 (9%)	12 (33%)	
36–59 years	29 (18%)	19 (53%)	
60–75 years	44 (27%)	5 (14%)	
>75 years	77 (47%)		
Median area of available thymus, mm ² (range)	218 (3–629)	111 (6–534)	0.0004

^aP-values are based on the Fisher's exact test for comparisons of gender and the Wilcoxon rank-sum test for comparisons of age and total thymus area. The composition of the RERF subjects did not differ in gender, age at the time of the bombing, or age at sampling using the Kruskal-Wallis nonparametric test.

TABLE 2

Measurements of Thymic Activity for Nonexposed and Exposed RERF Subjects^a

	Nonexposed (<5 mGy)	Low dose (5–200 mGy)	Moderate-to-high dose (>200 mGy)
Dose, mGy			
Median	0	37	422
Range	0–4	5–184	201–2909
Total area, mm ²			
Median	204	222	227
Range	75–629	3–569	4–520
Lymphoid area, mm ²			
Median	4	3	2
Range	0.1–247	0–90	0.1–88
TE area, mm ²			
Median	2	1	1
Range	0.1–203	0.1–60	0–64
Cortical area, mm ²			
Median	0	0	0
Range	0–149	0–45	0–37
Percentage lymphoid area			
Median	2	2	1
Range	0.1–65	0–54	0–69
Percentage TE area			
Median	1	1	0.4
Range	0.1–54	0–45	0.01–50
Percentage cortical area			
Median	0	0	0
Range	0–40	0–32	0–29
T.E. rosettes?			
No	25 ^b	44 ^b	1 ^b
Yes	20	36	29
No. of Hassall bodies			
0	18	30	17
1–10	16	36	19
11–50	9	13	2
50+	2	1	2
0	18	30	17
1+	27	50	23
0–10	34	66	36
11+	11	14	4

^aThe reported values are based on morphometric analysis of immunostained tissue sections, as described in the Methods section and shown in Supplemental Fig. S1 (<http://dx.doi.org/10.1667/RR14554.1.S1>).

^b
 $p = 0.01$; Kruskal-Wallis nonparametric test. All other comparisons did not yield significant differences between groups in a univariate analysis.

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