survivors. We observed differences in baseline leukemia mortality rates between proximal and distal survivors, particularly in the first decade after the bombings. Adjustment for proximal or distal location ATB affected the estimates of radiation dose–leukemia mortality associations (Appendix Table A1). Differences in baseline mortality rates by location at time of bombing may also reflect selective survival among proximal survivors. Adjustment for baseline differences in mortality rates between proximal and distal survivors minimizes problems of confounding by proximal or distal location but does not address concerns that survivors of the atomic bombings may be a select group of people who are relatively less susceptibility to radiation-induced leukemia than the general population (*24*). While not easily evaluated empirically, theoretical work on the potential for bias on solid cancer risk estimates, considering a range of values for the magnitude of dose-related selective survival, suggested that the potential bias under the scenarios considered was modest (*25*).

In these analyses the magnitude of the radiation dose–leukemia mortality association was approximately threefold larger for Hiroshima survivors than for Nagasaki survivors. This could reflect differences between cities in co-exposures or other factors influencing susceptibility to radiation-induced leukemia, differences in exposure conditions, or differences by city in the accuracy of exposure or outcome classification. The shape of the dose–response association also appears to differ by city. The linear-quadratic shape of the dose response for leukemia in the LSS has been given substantial attention in the literature. Interpretations for a linear-quadratic dose–response function have been posited in the context of the theory of dual action of ionizing radiation and have played an important role in discussions of risk projections to low-dose (< 0.1 Gy) or low-dose-rate exposures. Evidence of heterogeneity by city in the shape of the radiation dose–leukemia mortality association suggests that caution is warranted when attributing a biological interpretation to the parametric form of this dose–response association.

Comparison to the National Academy of Science's Biological Effects of Ionizing Radiation (BEIR) VII Report

The findings of this mortality analysis complement the BEIR VII report, which examined leukemia mortality in the same cohort of LSS members followed over the same period (1950–2000) (*8*). The current report presents findings for specific types of leukemia. There are, in addition, a number of differences between the approach used in this report and that taken by the BEIR VII committee. While spanning the same period of follow-up, this analysis includes more deaths due to leukemia than the analysis in the BEIR VII report. The BEIR VII analysis of leukemia mortality in the LSS included some but not all deaths due to ATL; that analysis included only those deaths due to ATL that were coded to the 10th revision of the ICD, while we have included all deaths for which ATL was noted as the underlying cause of death (see Materials and Methods).

Unlike the recent BEIR VII report, our preferred model for the ERR of leukemia does not allow for sex differences in the radiation dose–mortality association (*8*). There was no evidence of such heterogeneity in our analyses, nor does such a product term appear to be necessary in the BEIR VII leukemia model. In the current analysis, control for potential confounders was achieved through background stratification, while the recent BEIR analyses employed a parametric model for baseline rates. In addition, we adjusted for confounding of the radiation dose–leukemia mortality association by proximal or distal location, a covariate not included in the BEIR VII analysis (see Appendix). Furthermore, we used a cubic spline function to model the modifying effect of time since exposure in analyses of leukemia of all types and acute myeloid leukemia. Our preferred models for mortality due to ALL and CML are simple time-constant ERR functions; the numbers of deaths due to these types of leukemia were quite small, and there was relatively little statistical support for fitting of more complex time-varying ERR models for those outcomes.

Although we have taken a somewhat different analytical approach from that taken in the BEIR VII report, we can compare predictions of the ERR/Gy for leukemia of all types to the values reported in the BEIR VII report. One complication of such a comparison is an apparently erroneous figure in the BEIR VII report. Figure 12-2 of the BEIR VII report was intended to illustrate the predicted ERR/Gy for leukemia of all types. However, the ERRs shown in that figure are roughly half the magnitude of the correct values obtained by direct calculation using the Committee's reported model coefficients. Appendix Fig. A4 presents a correct illustration of the ERR/Gy for leukemia calculated using the BEIR VII Committee's model coefficients; also shown are the values derived using the coefficient estimates for the preferred model in this paper.

Conclusion

Members of the LSS cohort have been followed for over half a century. For contemporary Japanese atomic bomb survivors, an important question is whether leukemia risk diminished entirely within the first decades after the bombings, or whether excess risk of leukemia continues to persist. These analyses suggest that the variation in ERR/Gy with time since exposure appears to diverge from the monotonic decay function posited in prior analyses of leukemia mortality and incidence (*8*). Under the model for leukemia that was fitted in the

current paper, in the most recent decade of observation (1991–2000), 34% of the deaths due to leukemia observed among members of the LSS with doses over 0.005 Gy were estimated to be radiation-associated excess deaths (Table 4). The majority of the predicted excess leukemia deaths in this period are due to AML (Table 4). While regression models that incorporate spline functions are empirical rather than biological models for analysis of epidemiological data, the temporal variation in the ERR of leukemia is interesting to consider in terms of biological models of radiation-related leukemia. Early onset of leukemia (or leukemia with short latency) may have occurred among those who are predisposed by the presence of spontaneously arising clonally expanded preleukemic cells (*26*). In contrast, late-onset leukemia, observed decades after irradiation, may be less related to clonal expansion of preleukemic cells and more related to induction of cancers by contributing to one step in the malignant transformation of cells (similar to the pattern observed for solid cancers).

The persistent evidence of excess leukemia among Japanese atomic bomb survivors underscores the importance of the LSS to understanding the long-term health effects of exposure to ionizing radiation. These observations should be of interest to contemporary A-bomb survivors and their caregivers and more generally to those interested in the human health effects of nuclear weapons and other sources of ionizing radiation exposures.

APPENDIX

There are several differences between the analysis of leukemia mortality in the current paper and the analysis of leukemia mortality reported in the National Academy of Science's BEIR VII report (*8*). The BEIR VII analysis of leukemia mortality in the LSS included only those deaths due to ATL that were coded to the 10th revision of the ICD while the current paper included all deaths for which ATL was noted as the underlying cause of death. In the current paper we adjusted for baseline differences in mortality by background stratification. We also allowed for mortality differences between proximal and distal atomic bomb survivors. In addition, in the current paper we employed a cubic spline model to describe temporal variation in the excess relative rate of leukemia. In the text below we discuss some of these differences.

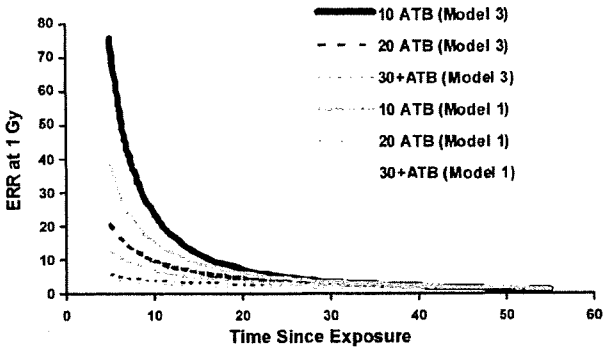

FIG. A1. Estimated sex-averaged excess relative rate of leukemia mortality as a function of bone marrow dose (in weighted Gy) among A-bomb survivors in the LSS exposed at ages 10 years, 20 years and 30+ years at time of bombing (ATB). Results shown for two models. Model 1 adjusts for baseline leukemia mortality rates using a loglinear subterm of the linear relative rate model that includes terms for city, sex and for each sex, a linear-quadratic spline function of log *age*, with a knot at age 70 years, and a linear-quadratic function of birth year. Model 3 employs background stratification on sex, city, attained age, age at exposure, and proximal or distal location.

Adjustment for Baseline Factors

The BEIR VII Committee's preferred ERR model for leukemia is of the form $ERR(d,s,e,t) = \beta_s(d + \theta d^2)\exp[\gamma e' + \delta \log(t/25) + \phi e' \log(t/25)]$, where d is the dose to the bone marrow (in weighted Gy), e is age at exposure (in years), e' is $(e - 30)/10$ for $e < 30$ and zero otherwise, and t is time since exposure (in years). The values for

TABLE A1
Estimated Model Coefficients for the Association between Bone Marrow Dose and Leukemia Mortality among A-Bomb Survivors in the LSS under an Excess Relative Rate Model of the Form
$$ERR(d, c, s, e, t) = \beta_s(d + \theta d^2)\exp[\gamma e' + \delta \log(t/25) + \phi e' \log(t/25)]$$

Model	Model 1[a]	Model 2[b]	Model 3[c]
Parameter	Estimate (95% CI)	Estimate (90% CI)	Estimate (90% CI)
β_M	1.1 (0.1, 2.6)	1.4 (0.3, 3.4)	1.5 (0.2, 3.9)
β_F	1.2 (0.1, 2.9)	1.6 (0.3, 3.8)	1.6 (0.3, 4.0)
γ	−0.40 (−0.78, 0.00)	−0.39 (−0.77, −0.00)	−0.32 (−0.77, 0.16)
δ	−0.48 (−1.10, 0.18)	−0.47 (−1.08, 0.17)	−0.47 (−1.29, 0.34)
ϕ	0.42 (−0.05, 0.96)	0.41 (−0.05, 0.94)	0.60 (−0.03, 1.38)
θ	0.88 (0.16, 15.27)	0.66 (0.10, 5.32)	0.75 (0.13, 6.72)
Residual deviation	2255.22	2250.10	1560.32
df	33200	33198	32133

Note. Results of fitting three models that employ different approaches to adjusting for baseline leukemia mortality rates.

[a] Model 1 adjusts for baseline leukemia mortality rates using a loglinear subterm of the linear relative rate model that includes terms for city, sex, and, for each sex, a linear-quadratic spline function of the natural log of attained age, with a knot at age 70 years, and a linear-quadratic function of birth year.

[b] Model 2 specifies the same parametric baseline model as Model 1 with the exception that the binary indicator variable for city was replaced by a four-level variable that indicated proximal compared to distal location ATB in each city.

[c] Model 3 employs background stratification on sex, city, attained age, age at exposure, and proximal or distal location.

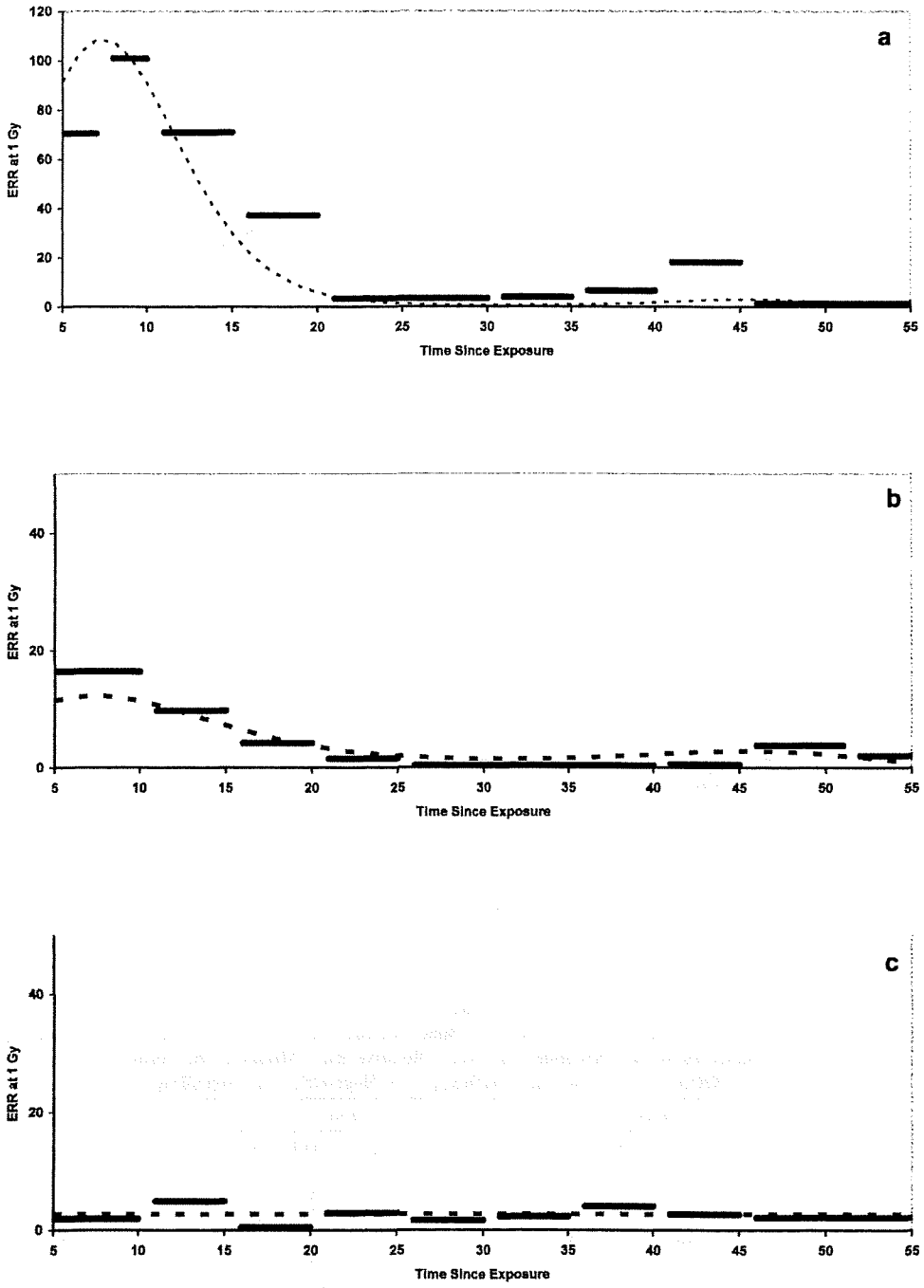

FIG. A2. Evaluation of modification of association between radiation and death due to leukemia of all types by age at exposure and time since exposure. Piecewise constant estimates of the excess relative risk at 1 Gy for people exposed at ages (panel a) <10, (panel b) 10-<30, and (panel c) 30+ years. Dashed lines indicate fitted cubic spline model.

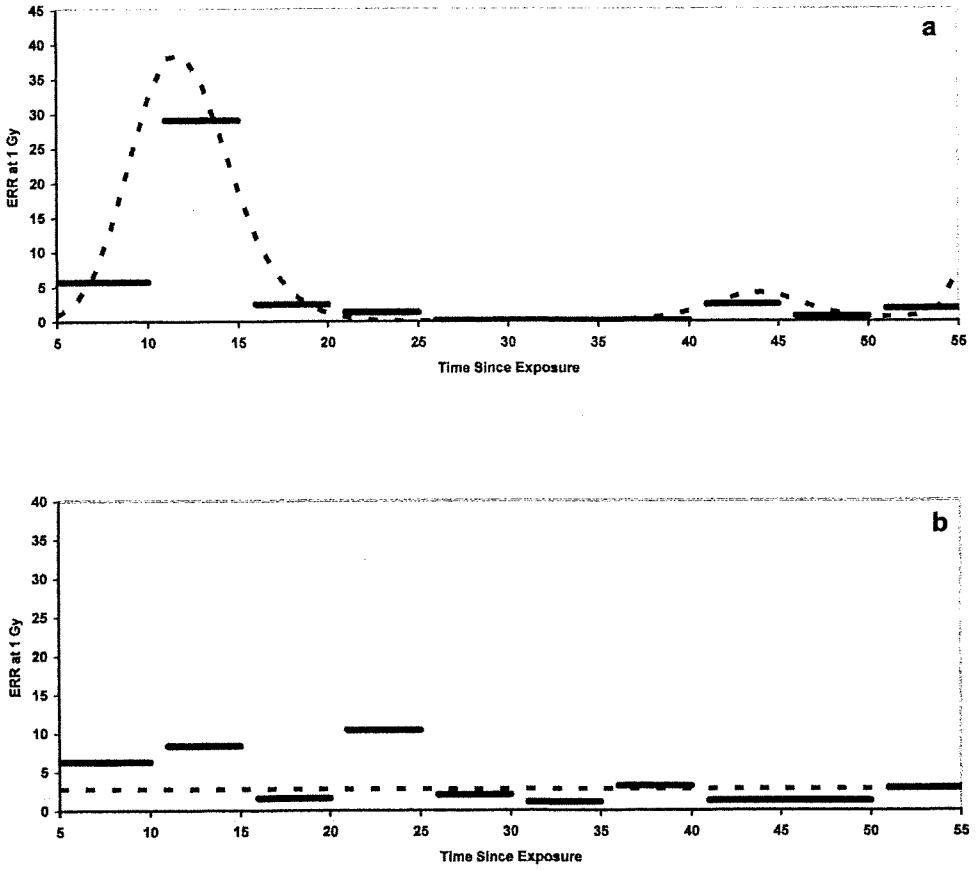

FIG. A3. Evaluation of modification of association between radiation and death due to AML by age at exposure and time since exposure. Piecewise constant estimates of the excess relative risk at 1 Gy for people exposed at ages (panel a) <30 and (panel b) 30+ years. Dashed lines indicate fitted cubic spline model.

these model parameters as provided in the BEIR VII report are as follows: $\beta_M = 1.1$ (95% CI: 0.1, 2.6), $\beta_F = 1.2$ (95% CI: 0.1, 2.9), $\gamma = -0.40$ (95% CI: −0.78, 0.0), $\delta = -0.48$ (95% CI: −1.1, 0.2), $\phi = 0.42$ (95% CI: 0.0, 0.96), $\theta = 0.87$ (95% CI: 0.16, 15).

Table A1 reports estimates of the parameters for the BEIR VII Committee's preferred ERR model for leukemia as obtained using three regression analyses; for comparability with the BEIR VII report, 95% confidence intervals are reported for these parameter estimates. To achieve comparability in the definition of the mortality outcome, the analysis reported in Table A1 is based upon the data set used by Preston *et al.* (*9*), which encompasses the same cohort of LSS members examined in the current paper with follow-up spanning the same period. However, the category of all leukemia deaths was defined by Preston *et al.* (*9*) to include only those deaths due to ATL that were coded to the 10th edition of the ICD. Model 1 specifies a parametric baseline model for the rate of leukemia that includes terms for city, sex and, for each sex, a linear-quadratic spline function of log of attained age, with a knot at age 70 years, and a linear-quadratic function of birth year. The parameter estimates obtained using Model 1 are equivalent to those reported in the BEIR VII report. Model 2 specifies the same parametric baseline model; however, the binary indicator variable for city was replaced by a four-level variable that indicated proximal or distal location ATB in each city. Evidence of confounding by proximal or distal location ATB is suggested by the change in the estimates of the parameters for the ERR model upon adjustment for this factor. Model 3 employs background stratification on sex (*s*), city (*c*), attained age (*a*), age at exposure (*e*), and proximal or distal location (*l*). Stratification allows the effect of proximal or distal location to vary over time, as a consequence of its cross-classification with attained age and age at exposure. Background stratification on *s*, *c*, *a*, *e* and *l* was employed in the current paper to obtain control for confounding by these covariates. Figure A1 illustrates the estimates of the age–time patterns of ERR at 1 Gy obtained with Model 1 and with Model 3.

Cubic Spline Models

Use of a cubic spline function to model effect modification by time since exposure is a departure from the approach employed in previous analyses of leukemia mortality among LSS survivors (*8, 19*). However, neither a model with a monotonic function of t nor $\log(t)$ fits these data as well (LRT = 9.3, 2.*df*, $P = 0.01$ and LRT = 6.9, 2 *df*, $P = 0.03$, respectively). Regression model development involves a balance between over-smoothing, thereby obscuring potentially important patterns in the data, and over-fitting, which may result in model parameters that are highly sensitive to minor perturbations in the data. Cubic spline functions are attractive because they accommodate a wide variety of functional forms; nonetheless, there are limitations to spline functions, such as a tendency for instability in the tails of the fitted function. Such concerns were addressed in part by assessing the fit of our model to the data by reference to model estimates of temporal variation in the

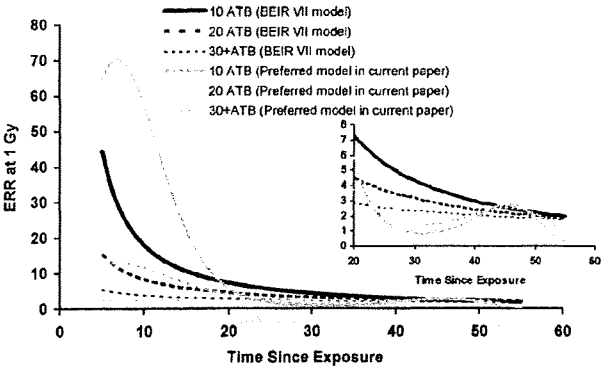

FIG. A4. Estimated excess relative rate of leukemia mortality as a function of bone marrow dose (in weighted Gy) among A-bomb survivors in the LSS exposed at ages 10 years, 20 years and 30+ years at time of bombing (ATB). Results shown for the BEIR VII model (sex-averaged) and the preferred model in the current paper (city-averaged). The BEIR VII model is the preferred excess relative rate model for leukemia reported in the National Academy of Science's BEIR VII report. The preferred model in the current paper is the model for leukemia: all types shown in Table 3.

ERR/Gy obtained with a piecewise constant model for time since exposure. Separate analyses were conducted for survivors exposed at ages 0–9 years, 10–29 years and 30+ years considering death due to leukemia of all types (Fig. A2) and death due to AML (Fig. A3). The horizontal line segments in each figure illustrate the estimates of the ERR at 1 Gy as a piecewise constant function of time since exposure; the dashed line in each figure illustrates the fitted cubic spline model, plotted at the mean age ATB of the decedents included in each analysis.

Figure A4 illustrates the ERR/Gy for leukemia derived using the coefficient estimates for the preferred model in this paper and the values calculated using the BEIR VII Committee's model coefficients.

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RESEARCH

Radiation exposure and circulatory disease risk: Hiroshima and Nagasaki atomic bomb survivor data, 1950-2003

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ABSTRACT

Objective To investigate the degree to which ionising radiation confers risk of mortality from heart disease and stroke.

Design Prospective cohort study with more than 50 years of follow-up.

Setting Atomic bomb survivors in Hiroshima and Nagasaki, Japan.

Participants 86 611 Life Span Study cohort members with individually estimated radiation doses from 0 to >3 Gy (86% received <0.2 Gy).

Main outcome measures Mortality from stroke or heart disease as the underlying cause of death and dose-response relations with atomic bomb radiation.

Results About 9600 participants died of stroke and 8400 died of heart disease between 1950 and 2003. For stroke, the estimated excess relative risk per gray was 9% (95% confidence interval 1% to 17%, P=0.02) on the basis of a linear dose-response model, but an indication of possible upward curvature suggested relatively little risk at low doses. For heart disease, the estimated excess relative risk per gray was 14% (6% to 23%, P<0.001); a linear model provided the best fit, suggesting excess risk even at lower doses. However, the dose-response effect over the restricted dose range of 0 to 0.5 Gy was not significant. Prospective data on smoking, alcohol intake, education, occupation, obesity, and diabetes had almost no impact on the radiation risk estimates for either stroke or heart disease, and misdiagnosis of cancers as circulatory diseases could not account for the associations seen.

Conclusion Doses above 0.5 Gy are associated with an elevated risk of both stroke and heart disease, but the degree of risk at lower doses is unclear. Stroke and heart disease together account for about one third as many radiation associated excess deaths as do cancers among atomic bomb survivors.

INTRODUCTION

The effects of radiation on incidence of or mortality from circulatory disease have large implications for public health, especially if effects occur at doses under 1 Gy. Given that the frequency of multiple computed tomography scans of the head or chest and of interventional radiographic procedures is increasing rapidly, information on whether these may confer risk for subsequent stroke or heart disease is essential.

Several studies, including randomised controlled trials, have found that high doses of radiation to the heart from radiotherapy for Hodgkin's disease or breast cancer cause an excess of deaths from heart disease in later years,[1-4] and other studies have suggested that radiotherapy for Hodgkin's disease, childhood leukaemia or brain tumours, and head and neck cancer increases the risk of stroke.[5-8] Several authors have suggested that lower doses from occupational, medical, and environmental exposures may be associated with excess mortality from circulatory disease,[9-14] although other studies have not found such low dose effects,[15-19] and information on doses and potential confounding lifestyle factors is limited in many of the studies of low doses. We examined the dose-response information on the risk of heart disease and stroke in the large Life Span Study cohort of atomic bomb survivors in Hiroshima and Nagasaki who have been followed up for 53 years, from 1950 to 2003.

METHODS

Study population

The Life Span Study cohort, defined on the basis of the Japanese national census in 1950 and special surveys between 1950 and 1953, consists of 86 611 atomic bomb survivors with estimated radiation doses. It includes a large proportion of the survivors who were within 2.5 km of the hypocentres at the time of the bombings and still resided in Hiroshima or Nagasaki in 1950, plus a random age and sex matched sample of people 2.5 to 10 km from the hypocentre who sustained small to negligible radiation doses.[20] This study population was of all ages and both sexes at the time of the bombings.

Individual doses have been carefully estimated using the recent improved DS02 dosimetry system,

primarily on the basis of people's location and shielding at the time of the atomic bomb.[21 22] We estimated risks by using weighted colon doses in gray (Gy) for all analyses. We used weighted doses, the sum of the γ dose plus 10 times the smaller neutron dose, to allow for the greater biological effectiveness of neutrons.

The follow-up of vital status took place from 1 October 1950 to the end of 2003 and was based on the nationwide obligatory family registration system (koseki) that documents mortality and is virtually 100% complete. Causes of death came from the official vital statistics death schedules based on the death certificates. Underlying and contributing causes of death were classified according to the ICD-7 (international classification of diseases, 7th revision) (for deaths in 1950-68), ICD-8 (in 1969-78), ICD-9 (in 1979-97), and ICD-10 (in 1998-2003). However, for the purposes of these analyses we converted them to ICD-9 codes 390-459 for all circulatory disease, 430-438 for stroke, and 393-429 (excluding 401, 403, and 405) for heart disease. We used only underlying causes of death in the primary analyses but examined underlying plus contributing causes in a subsidiary analysis.

Collection of covariate data and data from autopsy and tumour registry

A mail survey was sent to a defined sub-cohort of 51 965 Life Span Study cohort members in 1978. Information was obtained from 36 468 (response rate of 70%) on sociodemographic (education, type of occupation), lifestyle (smoking, alcohol intake), and health variables (obesity, diabetes mellitus), which enabled the evaluation of possible confounding by these variables. Between 1950 and 1985 autopsy data were also available on more than 1900 deaths that had an underlying cause of circulatory disease on the death certificate, which permitted evaluation of diagnostic accuracy. To identify pre-existing cases of cancer, we used the Hiroshima and Nagasaki tumour registries (available since 1958) and tissue registries (since 1974).

Statistical analysis

We based the analyses on a detailed summary table of the number of deaths and person years stratified by dose, city, sex, and five year intervals of age at exposure, attained age, and follow-up period. We divided participants into categories according to the weighted colon dose (in Gy=γ dose plus 10 times neutron dose): 0-, 0.005-, 0.02-, 0.04-, 0.06-, 0.08-, 0.1-, 0.125-, 0.15-, 0.175-, 0.2-, 0.25-, 0.3-, 0.5-, 0.75-, 1-, 1.25-, 1.5-, 1.75-, 2-, 2.5-, and ≥3. As described elsewhere, we truncated the colon doses to correspond to the 4 Gy shielded kerma level,[20] but this affected only 317 participants.

We used Poisson regression methods for grouped survival data to describe the dependence of risk on radiation dose and to evaluate the variation of the dose-response effects with respect to city, sex, age at exposure, time since exposure, and attained age,[23] essentially identical to the methods used previously to examine mortality from cancer in this cohort.[20] We used Epicure software for parameter estimation and tests,[24] and we based significance tests and 95% confidence intervals on likelihood profiles.

The primary models used here are excess relative risk (ERR) models of the form λ_0 (c,s,a,b) [1+ERR (d, e,s,a)], where λ_0 () is the baseline, or background death rate (that is, the rate for people with zero dose), which depended on city (c), sex (s), attained age (a), and birth year (b). The function ERR (d,e,s,a) describes the relative change in rates associated with dose (d), allowing for the effects of sex, age at exposure (e), and attained age. We examined effect modifiers by using models corresponding to those in Preston et al.[20 25] We examined both dose and dose squared terms to evaluate the degree of linearity or curvature in the dose-response forms. We also tested a linear threshold model. We used differences in maximum likelihood to compare nested models or the Akaike information criterion for non-nested models.[26] We evaluated a linear threshold model repeatedly for a wide range of possible values of a threshold dose (d_0), modelling the risk function ERR on doses d as $\beta(d-d_0)$ for $d>d_0$ or $d=0$ for $d\leq d_0$. We empirically determined the values yielding the maximum likelihood and 95% confidence bounds.

We examined the impact of the possible confounding factors of smoking (never, past, present <20/day, present ≥20/day), alcohol intake (regular, seldom/never), education (primary or less, secondary, college/university), occupation for household (professional/technical, clerical/sales, farmer/craftsman, transportation/service), obesity (body mass index <20, 20-24, ≥25), and diabetes (yes, no) on the estimates of radiation risk, including codes for missing information, for the Life Span Study participants included in the 1978 mail survey. We included Cox-type regression models fitted to the individual data, where radiation dose was modelled as a linear excess relative risk, and indicator variables for the potential confounders jointly in the models as conventional exponential relative risk terms by using the Peanuts program in Epicure.[24]

RESULTS

During follow-up, 19 054 deaths from circulatory disease occurred among the 86 611 Life Span Study members with DS02 dose available. Table 1 shows the numbers of participants and deaths from circulatory disease by age, sex, and radiation dose. The cohort covers a wide range of doses but is weighted towards low doses, which indicates that it has considerable capability to examine risks at low doses and to examine the shape of the dose-response curve. The deaths included 9622 from stroke, 8463 from heart disease, and 969 from other circulatory diseases. The excess relative risk per Gy for all circulatory disease based on the linear model over the full dose range was 11% (95% confidence interval 5% to 17%, P<0.001). This represents about 210 excess cases of death from circulatory disease associated with the exposure to radiation.

Table 1 | Number of participants and deaths from circulatory disease

Characteristics	No of people (n=86 611)	No of deaths: Circulatory disease (n=19 054)	Stroke (n=9 622)	Heart disease (n=8 463)	Other circulatory disease (n=969)
Sex:					
Male	35 687	7 607	3 958	3 261	388
Female	50 924	11 447	5 664	5 202	581
Age at atomic bomb exposure (years):					
0-9	17 833	428	176	238	14
10-19	17 563	951	404	508	39
20-29	10 891	1 551	652	831	68
30-49	25 774	9 712	4 735	4 575	402
≥50	14 550	6 412	3 655	2 311	446
Weighted colon radiation dose (mGy):					
<5	38 509	8 440	4 247	3 723	470
5-	23 427	5 089	2 637	2 205	247
50-	12 508	2 838	1 405	1 305	128
200-	6 356	1 485	735	680	70
500-	3 424	745	363	342	40
1000-	1 763	341	176	158	7
≥2000	624	116	59	50	7

Stroke

The excess relative risk per Gy for stroke based on the linear model over the full dose range was 9% (1% to 17%, P=0.02) (table 2). Figure 1 shows estimates of the shape of the dose-response curve for all stroke, including the fitted linear and linear-quadratic models. The test for non-linearity based on a comparison of linear and linear-quadratic dose-response models was not statistically significant (P=0.17), but the pure quadratic model, which suggests relatively little risk at lower doses, nominally provided a slightly better fit (difference in Akaike information criterion statistics of 1.87) than did the linear model. This was confirmed by analyses of lower dose ranges which showed excess relative risk per Gy of 3% (−10% to 16%) for 0-1 Gy and −7% (−28% to 16%) for 0-0.5 Gy. Figure 1 also shows no apparent risk for the lower part of the dose range; a non-negligible threshold may exist below which no excess occurs. The best estimate of a threshold dose was 0.5 Gy with an upper 95% confidence limit of about 2 Gy. However, the lower 95% confidence limit was not greater than 0, so no threshold dose may exist.

An analysis of effect modification of the risk of stroke by sex, attained age, and age at exposure showed a statistically significant difference for attained age (P=0.04): the radiation excess relative risk per Gy for stroke was higher before age 60 than after, especially among men (web table A). We also found a non-significant indication (P=0.23) that the risk of stroke associated with radiation may be highest after exposure at young ages: the excess relative risk per Gy were 36%, 9%, 15%, and 5% for ages <10, 10-19, 20-39, and ≥40. An evaluation of subtypes of stroke was not very meaningful because, before the 1990s, differential diagnosis was often not done, resulting in many cases being classified as stroke, not otherwise specified.

Heart disease

The excess relative risk per Gy for all heart disease based on the linear model in the full dose range was 14% (6% to 23%, p<0.001) (table 2). Figure 2 shows the results for the linear and linear-quadratic models. The test for non-linearity based on a comparison of linear and linear-quadratic dose-response models was not statistically significant (P>0.5). A pure linear model fitted the data nominally better than did a pure dose-squared model (difference in Akaike information criterion statistics of 2.47). The excess relative risks per Gy for heart disease over restricted dose ranges were similar to that for the full dose range. Specifically, the excess relative risk per Gy based on the linear model for the dose ranges under 2, 1, and 0.5 Gy were 14% (4% to 25%), 18% (3% to 33%), and 20% (−5% to 45%). In figure 2, the slope over the lower part of the dose range was almost identical to the one for the entire dose

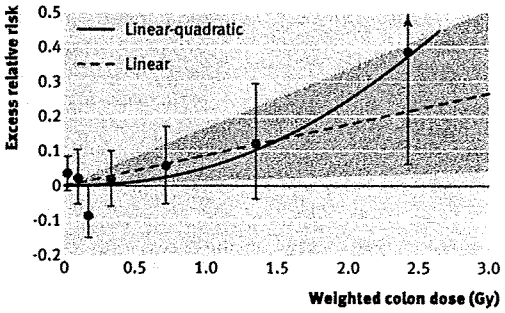

Fig 1 | Radiation dose-response relation (excess relative risk per Gy) for death from stroke, showing linear and linear-quadratic functions. Shaded area is 95% confidence region for fitted linear line. Vertical lines are 95% confidence intervals for specific dose category risks. Point estimates of risk for each dose category are indicated by circles

Table 2| Summary excess relative risks (ERR)* per Gy and excess additive risks per 10^4 person year Gy† (EAR/10^4 PY-Gy) for types of circulatory disease mortality

Circulatory disease	Indicated as underlying cause of death				Underlying or contributing cause of death	
	Deaths	P value	% ERR/Gy (95% CI)	EAR/10^4 PY-Gy (95% CI)†	Deaths	% ERR/Gy (95% CI)
Total	19 054	<0.001	11 (5 to 17)	5.5 (2.7 to 8.4)	25 113	15 (10 to 20)
Stroke	9 622	0.02	9 (1 to 17)	2.3 (0.4 to 4.4)	12 139	12 (5 to 19)
Heart disease	8 463	<0.001	14 (6 to 23)	3.2 (1.3 to 5.2)	14 018	18 (11 to 25)
Other	969	>0.5	2 (−18 to 29)	0.1 (−0.4 to 0.7)	5 846	58 (45 to 72)

*Estimates based on linear model, adjusted for city, sex, age at exposure, and attained age.
†Average EARs calculated directly from fitted ERR models.

range. The best estimate of a threshold dose was 0 Gy, with an upper 95% confidence limit of about 0.5 Gy. We found no significant modification of effect by sex, age at exposure, or age at risk (web table A).

Analyses of different subtypes of heart disease revealed some diversity in dose-response effects (web table B) but involve a variety of uncertainties, the articulation of which is beyond the scope of this report. The risk of ischaemic heart disease increased only in the higher dose categories, and the linear increase was not significant. We found stronger associations between radiation and other heart diseases, such as hypertensive heart disease and heart failure. However, unlike the relatively high accuracy in diagnosing the general category of heart disease, substantial misclassification of subtypes of heart disease occurs (see below), which limits the meaning that can be attached to the analyses of subtypes.

Confounding factors and misdiagnosis

We examined the impact of the possible confounding factors on the radiation risk estimates among the 51 965 Life Span Study participants included in the 1978 mail survey. Table 3 shows the excess relative risks unadjusted and adjusted for six potential confounding factors. Note that the excess relative risks in table 3 differ slightly from those in table 2, because the estimates in table 3 are based on only the subcohort of the Life Span Study, whereas those in table 2 are based on the full cohort (86 611 participants). Although smoking, alcohol intake, education, type of occupation, obesity (body mass index), and diabetes were risk factors for heart disease and stroke in their own right (for example, the relative risks for heart disease were 1.4 for smoking, 1.6 for diabetes, 1.1 for body mass index 25 or over, and 0.75 for university education), they showed virtually no confounding with dose of radiation. That is, adjustment for the six variables simultaneously produced inconsequential changes in the excess relative risk per Gy: only 0.1% for heart disease and −0.9% for stroke (table 3). Analyses limited to respondents similarly showed little impact of the confounder variables (data not shown). These results suggest that in the Life Span Study, the associations of dose of radiation with mortality from stroke and heart disease is unlikely to be an artefact of confounding by major lifestyle, sociodemographic, or disease risk factors.

We also examined the diagnostic accuracy of death certificates by comparing them with autopsy reports among the 1963 cases with death certificate designated circulatory disease for whom autopsies were available from our autopsy programme between 1950 and 1985.[27] For the broad categories of stroke and heart disease, the accuracy of the diagnoses on the death certificates was fairly good. For death certificates with stroke as the underlying cause of death, 86% of autopsies listed stroke as a cause; 92% of death certificates with heart disease as the underlying cause had heart disease listed as a cause on the autopsy report. Moreover, the accuracy of diagnoses on death certificates has probably improved since 1985. However, the corresponding accuracy was rather poor for the differential diagnosis of specific subcategories of stroke or heart disease (for example, 65% for cerebral infarction, 39% for cerebral haemorrhage, 69% for ischaemic heart disease, 22% for hypertensive heart disease, and 64% for rheumatic heart disease); web table B shows risk estimates for separate subcategories.

Deaths from circulatory disease based on death certificates may include misdiagnosed deaths from cancer or cases arising from cardiotoxicity due to chemotherapy or radiotherapy for cancer. To remove the effects of misdiagnosis of cases of cancer, we estimated risks after excluding people who had previous diagnoses of cancer, on the basis of our tumour registry data. These excess relative risks were reduced by about 30% compared with estimates based on the full cohort, but still

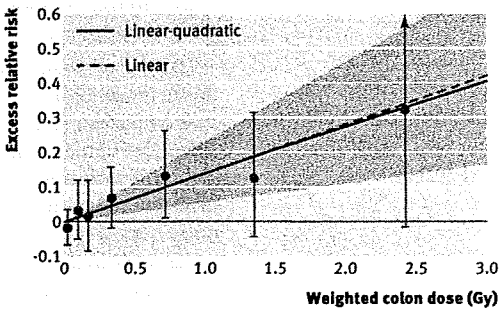

Fig 2| Radiation dose-response relation (excess relative risk) for death from heart disease, showing linear and linear-quadratic functions. Shaded area is 95% confidence region for fitted linear line. Vertical lines are 95% confidence intervals for specific dose category risks. Point estimates of risk for each dose category are indicated by circles

Table 3 | Effects of potential confounding factors on radiation risk estimates for types of circulatory disease mortality

Circulatory disease	No of deaths	% ERR/Gy unadjusted for confounders*	% ERR/Gy adjusted for all confounders*†
Total	7907	10.0	9.6
Stroke	3366	8.1	7.2
Heart disease	4204	12.2	12.3
Other	337	2.4	0.9

ERR=excess relative risks.
*All analyses adjusted for city, sex, age at exposure, and attained age.
†Additionally adjusted for smoking, alcohol intake, education, type of household occupation, obesity (body mass index), and diabetes mellitus (on basis of about 52 000 participants).

showed a tendency to significant dose-response effects. Excluding previous cases of cancer changed the excess relative risk per Gy from 10.8% to 7.3% (dose-response P=0.008) for all circulatory disease, from 8.8% to 6.2% (P=0.11) for stroke, and from 14.0% to 9.5% (P=0.03) for heart disease.

Although we used the designated underlying cause of death in the mortality analyses, selecting a single cause of death is difficult when several correlated diseases or conditions contributed to death. Therefore, we examined the risks on the basis of both underlying and contributing causes of death (table 2). The radiation dose-response effects were nominally higher than those based on underlying cause of death alone (12% *v* 9% excess relative risk per Gy for stroke, and 18% *v* 14% for heart disease), which lends additional support to the hypothesis of radiation risk.

DISCUSSION

This study found dose-response evidence for risk of heart disease and stroke among atomic bomb survivors over the radiation dose range 0-4 Gy (mostly 0-2 Gy) based on well characterised individual doses and essentially complete ascertainment of mortality over the period of five to 58 years after exposure to radiation. This report updates earlier brief reports of a dose related excess of circulatory disease among atomic bomb survivors.[20,28,29] These results, based on about 25% more deaths than the previous paper, are substantially stronger, and we now provide more elaboration of the associations.

As shown in table 1, at the youngest ages of exposure (more recent birth cohorts) the deaths from heart disease outnumber those from stroke, whereas the opposite is true of the earlier birth cohorts; this reflects the general secular trends in the Japanese population. The table also shows that the cohort covers a wide range of doses but is weighted toward low doses, indicating that it has substantial capability to examine risks at low doses and to examine the shape of the dose-response curve. Because several plausible disease mechanisms centre around systemic effects after whole body irradiation, we used colon doses as an approximation to whole body doses for all analyses, although analyses using brain dose for stroke and lung dose for heart disease produced very similar results (data not shown).

Summary of features and coherence of radiation risk data

Although the data were statistically consistent with linearity over the full dose range in this study, considerable uncertainty exists about the shape of the dose-response curve in the low dose range. The extent of curvature seemed to be larger for stroke than for heart disease; a pure dose-squared model fitted the stroke data slightly better than did a pure linear model, whereas the linear model provided a better fit for heart disease. However, the dose-response effect was not statistically significant for either end point when we limited the calculation to the dose range 0-0.5 Gy, implying that evidence is limited on the risk below about 0.5 Gy. For stroke, the estimated threshold dose was 0.5 Gy, with an upper 95% confidence limit of about 2 Gy. For heart disease, the estimated threshold dose was 0 Gy, with an upper 95% confidence limit of about 0.5 Gy.

Additional analyses supported the association of radiation with stroke and heart disease. Adjustment of the data for other potential risk factors for circulatory disease—obesity, diabetes, smoking, alcohol consumption, education, and occupation—had almost no impact on the associations with radiation, whereas an analysis for possible misdiagnosis of cancer as circulatory disease showed a small diminution of radiation risk. Because the underlying cause of death is often uncertain, we also did analyses of stroke and heart disease indicated as either an underlying or contributing cause; these showed nominally stronger associations with radiation than did the analyses of underlying cause alone.

The findings of the epidemiological study of circulatory disease among atomic bomb survivors are confirmed and extended by our Adult Health Study, which consists of biennial clinical and laboratory examinations since 1958 of about 15% of the Life Span Study cohort members. The Adult Health Study has found dose related increases in the incidence of stroke and myocardial infarction and in the incidence or prevalence of hypertension, elevated serum cholesterol concentrations, and aortic arch calcification.[30-35] Late radiation effects have also been found for potential biomarker precursors of circulatory disease, including biomarkers for inflammation,[36-38] deficient immunological responses,[39] and alterations in immune cell repertoire.[40,41] The findings present a reasonably coherent picture of preclinical and clinical risk of circulatory disease associated with exposure to radiation. However, this needs to be complemented by a risk assessment of low doses based on mechanistic and animal models.

Strengths and limitations of study

This study has several strengths, including a large population not pre-selected for existing disease or occupational fitness, a wide but relatively low dose range (0->3 Gy) and well characterised doses, a 53 year follow-up with virtually complete mortality ascertainment, and corroborative evidence from

more detailed clinical and biomarker studies of risk of circulatory disease on a random subsample of the cohort.[30-41] In addition, we believe medical surveillance bias to be minimal, as all of the cohort is eligible for free, special medical care, and many people have little idea of the doses they received, so that the level of radiation related medical concern is not highly correlated with the actual dose received. In addition, the analyses of radiation dose with stroke and heart disease mortality showed that the association is reasonably robust with respect to confounding by lifestyle, sociodemographic, or other health factors or misdiagnosis.

The study also has several limitations and uncertainties. Ascertainment of circulatory disease from death certificates is of limited diagnostic accuracy and represents only a fraction of cases of incident disease. Analyses for confounders, although very important, are incomplete, lacking information on, for example, blood lipids, physical activity, and nutrition. Some selection effects due to dose related early mortality from the bombs may have occurred, although the impact of these is likely to be small.[42] Other limitations include unclear dose-response effects below about 0.5 Gy, inadequate information about possible biological mechanisms, and uncertainty about the generalisability of these results to Western populations because of differences in genetic factors, dietary and lifestyle risk factors, and baseline levels of risk for stroke and heart disease.[43]

Comparison with other studies

Although epidemiological and experimental data are limited, several studies suggest the possibility of effects of radiation on circulatory disease. Among medically exposed cohorts, excess heart disease mortality has been shown among patients who received radiotherapy for Hodgkin's lymphoma or breast cancer.[1-4 44] At somewhat lower doses, an increase in coronary heart disease was seen among patients who received radiotherapy for peptic ulcer.[9] An association was also seen among patients with scoliosis who received multiple fluoroscopic examinations,[45] but not among patients with tuberculosis who received multiple fluoroscopic examinations of the chest,[16] nor among patients who received radiographic treatment for benign gynaecological disease.[46 47]

Studies of cohorts with occupational or environmental exposure to radiation have not provided clear evidence for or against a radiation associated increase in mortality from circulatory disease. In a long term follow-up of early US radiologists, circulatory disease mortality was higher than for a comparison group of other medical specialists,[48] but a similar increase was not seen among early UK radiologists.[17] Increased mortality from circulatory disease was found among US radiological technologists who worked before 1950, when radiation exposures tended to be higher, but individual people's doses were not documented.[10] Significant associations with radiation for both stroke and heart disease were reported for emergency workers at Chernobyl,[12] although the study may have limitations related to sample selection and to variations in circulatory disease risk factors and medical surveillance. Among workers for British Nuclear Fuels, a significant dose-response association was found for ischaemic heart disease but not cerebrovascular disease.[13] No association was found between radiation dose and circulatory diseases among German uranium miners.[19] Preliminary results for the Mayak nuclear workers show a statistically significant dose-response association for ischaemic heart disease and stroke.[49] A new update of the UK national registry for radiation workers shows a marginally positive association of dose of radiation with heart disease, but further analyses suggest that the finding might be due to variations in smoking habits.[14] An analysis of combined cohorts of 275 000 nuclear workers from 15 countries who were exposed to low, well documented doses of external radiation did not show a significant association between dose of radiation and either stroke or heart disease.[15]

With the exception of the study of US radiological technologists, the studies were not able to adjust for potential lifestyle factors or other confounding factors, and some of the studies had no or only crude estimates of individual doses. Most of the studies of low doses had limited statistical power and some potential for bias; consequently, the potential for both false positive and false negative results may be high. The United Nations Scientific Committee on the Effects of Atomic Bomb Radiation (UNSCEAR) concluded that little evidence, other than the atomic bomb studies, exists to support an association between circulatory disease and radiation in the dose range less than 1-2 Gy.[50] A recent review article reached a similar conclusion,[51] although additional suggestively positive data have appeared since these reviews were written.[13 14 49]

Mechanisms of circulatory disease

Knowledge of the mechanisms by which radiation doses of 2 Gy or less may cause circulatory disease is limited. Evidence suggests that pro-inflammatory responses to radiation, cellular loss or functional changes in the endothelium, or microvascular damage may be early events in the cascade of pathogenic changes that lead to radiation related heart disease.[52-55] These may augment other risk factors, such as hypertension, high serum cholesterol, smoking, diabetes, and infection, to promote heart disease.[56] Associations between dose of radiation and long term levels of inflammatory markers have been documented among atomic bomb survivors,[36-38] possibly because of damage to the immune system.[39] Radiation associated microvascular damage to the renal parenchyma and vascular endothelial cells may promote hypertension and ischaemia.[50 57]

Conclusions and implications

The effect of radiation on risk of circulatory disease is potentially a very important public health concern.

WHAT IS ALREADY KNOWN ON THIS TOPIC

High doses of radiation to the heart or head and neck from radiotherapy cause a subsequent excess of deaths from heart disease or stroke

Whether radiation exposures at dose levels under 1 Gy also increase the risk of heart disease or stroke is not known

WHAT THIS STUDY ADDS

Radiation may increase the rates of stroke and heart disease at moderate dose levels (mainly 0.5-2 Gy), although the results below 0.5 Gy were not statistically significant

The association was reasonably robust with respect to confounding by lifestyle, sociodemographic, or other health factors or misdiagnosis

Given the widespread use of multiple computed tomography scans,[58,59] and other relatively high dose diagnostic medical procedures, as well as radiotherapy that exposes the heart, the implications are substantial insofar as effects occur at doses under 1 Gy. The potential magnitude of the risk is shown by the fact that in the Life Span Study cohort, who received whole body irradiation, the radiation related excess of deaths from circulatory diseases (about 210) is about a third as large as the total excess number of deaths from cancer (about 625).

This study provides the strongest evidence available to date that radiation may increase the rates of stroke and heart disease at moderate dose levels (mainly 0.5-2 Gy), but robust confirmatory evidence from other studies is needed. Although our results below 0.5 Gy are not statistically significant, the additional cases occurring with further follow-up time should provide more precise estimates of the risk at low doses.

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Ethical approval: The research was conducted under the formal approval of RERF's Human Investigation Committee.

Data sharing: Detailed tabulation of data used for the analysis and the statistical code are available from the corresponding author.

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DNA Replication-Coupled PCNA Mono-Ubiquitination and Polymerase Switching in a Human *In Vitro* System

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Translesion DNA synthesis is a mechanism of DNA damage tolerance, and mono-ubiquitination of proliferating cell nuclear antigen (PCNA) is considered to play a key role in regulating the switch from replicative to translesion DNA polymerases (pols). In this study, we analyzed effects of a replicative pol δ on PCNA mono-ubiquitination with the ubiquitin-conjugating enzyme and ligase UBE2A/HHR6A/RAD6A–RAD18. The results revealed that PCNA interacting with pol δ is a better target for ubiquitination, and PCNA mono-ubiquitination could be coupled with DNA replication. Consequently, we could reconstitute replication-coupled switching between pol δ and a translesion pol, pol η, on an ultraviolet-light-irradiated template. With this system, we obtained direct evidence that polymerase switching reactions are stimulated by mono-ubiquitination of PCNA, depending on a function of the ubiquitin binding zinc finger domain of pol η. This study provides a framework for detailed analyses of molecular mechanisms of human pol switching and regulation of translesion DNA synthesis.

Keywords: DNA polymerase switching; DNA replication; PCNA mono-ubiquitination; translesion DNA synthesis

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Introduction

In living cells, DNA is exposed continuously to attack by endogenous reactive species, including oxygen radicals and metabolic intermediates, as well as environmental agents such as ionizing radiation, ultraviolet light (UV), and a variety of chemicals. Although resultant replication-blocking lesions are removed by nucleotide and base excision repair, significant numbers persist due to the balance between generation and excision. Therefore, cells need to be able to tolerate DNA damage during DNA replication.[1] The DNA damage tolerance pathway seems to act on single-stranded gaps[1,2] and therefore also is referred to as post-replication repair (PRR).[3] This process is separable into translesion DNA synthesis (TLS) and template switching (TS). In the TLS pathway, a number of nonessential DNA polymerases (pols) rescue stalled replication by extending the 3′-ends beyond the lesions. This process is essentially error-prone because of its utilization of a damaged template. With TS, the damage is bypassed by a copy choice mechanism using the newly synthesized sister chromatid as the template. For this reason, this process is deemed relatively error-free.[3]

In eukaryotes, a significant fraction of PRR is initiated by RAD6 (*Saccharomyces cerevisiae*)/UBE2A and UBE2B (in humans)- and RAD18-dependent ubiquitination at the lysine 164 residue of proliferating cell nuclear antigen (PCNA).[4–6] UBE2A and UBE2B, also known as HHR6A/RAD6A and HHR6B/RAD6B, respectively, are hereafter referred to as RAD6, applying the name of the yeast gene. RAD6, a ubiquitin-conjugating E2 enzyme, forms a tight complex with RAD18, a ubiquitin protein E3 ligase.[6–12] Mono-ubiquitination by the complex appears to be limited to PCNA already loaded onto DNA by replication factor C (RFC), demonstrated in *S. cerevisiae*[9,10] and humans.[13] It has been shown that Y-family translesion pols, which contain ubiquitin

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Abbreviations used: E1, ubiquitin-activating enzyme E1; PCNA, proliferating cell nuclear antigen; pol, DNA polymerase; PRR, post-replication repair; RFC, replication factor C; RF, replication factor; RPA, replication protein A; ssDNA, single-stranded DNA; TLS, translesion DNA synthesis; TS, template switching; UE, ubiquitin enzyme; UBZ, ubiquitin binding zinc finger; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid.

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binding motifs or ubiquitin binding zinc fingers (UBZs), are recruited by interaction with mono-ubiquitinated PCNA to allow TLS through damage sites.[14–18] Mono-ubiquitination can also be followed by poly-ubiquitination by the UBC13–MMS2 complex, an E2 ubiquitin-conjugating enzyme, and RAD5 (in *S. cerevisiae*)/SHPRH and HLTF (in humans) E3 ubiquitin ligases, leading to TS.[3,13,19–21]

Mono-ubiquitination of PCNA is considered to play a key role in regulation of PRR. In yeast, most TLS depends on PCNA mono-ubiquitination. However, in chicken DT40 cells, mono-ubiquitination of PCNA seems crucial, but not essential, for translesion synthesis.[22,23] The majority of the remaining TLS in the PCNA$^{(K164R)}$ background depends on REV1. The presence of distinct pathways in higher eukaryotes is very clear in PCNA$^{(K164R)}$ knock-in mice, in which only reduction of mutations at template A/T and a compensatory increase at G/C in immunoglobulin genes have been observed.[24] This phenotype is similar to that with pol η-deficient cells,[25–27] suggesting that PCNA mono-ubiquitination is closely linked to functions of pol η.

Mono-ubiquitinated PCNA accumulates on treatment of cells with DNA-damaging agents.[4,6,14,17,28,29] Recent reports have provided evidence that a significant proportion of PCNA mono-ubiquitination is constitutive, since accumulation is observed on inactivation of a de-ubiquitinating enzyme, USP1.[29–31] Importantly, in chicken DT40 cells, elevated levels of mono-ubiquitinated PCNA, which result from disruption of the *USP1* gene, do not increase mutagenesis at endogenously created DNA damage in the immunoglobulin locus.[31] This indicates that ubiquitination of PCNA is not sufficient for activating mutagenic translesion synthesis. In yeasts, it has been shown that PCNA is constitutively ubiquitinated during normal S phase.[28,32] For these reasons, the role of the RAD6–RAD18 complex in mono-ubiquitination of PCNA, its dependence on template damage, and its role in switching polymerases at stalled replication forks are not clear.

To address the molecular mechanisms underlying polymerase switching and its dependence on PCNA mono-ubiquitination by RAD6–RAD18 in mammals, we have established an *in vitro* reconstituted system. This system uses purified recombinant proteins, including pol δ, RFC, PCNA, replication protein A (RPA), ubiquitin-activating enzyme E1 (E1), the RAD6A–RAD18 complex, ubiquitin, and pol η. In this report, we described protein actions with this *in vitro* system.

Results

Reconstitution of PCNA mono-ubiquitination with recombinant human proteins *in vitro*

It has been suggested that mono-ubiquitinated PCNA mediates polymerase switching from replicative to translesion pols.[2] To address molecular mechanisms underlying mono-ubiquitination of PCNA and polymerase switching *in vitro*, we first established methods to obtain highly purified recombinant human replication factors (RFs, including RFC, PCNA, and RPA), ubiquitin enzymes (UEs, including E1, RAD6A–RAD18 complex, and ubiquitin), pol δ, and pol η (Fig. 1a)[33] from overproducing *Escherichia coli* cells by conventional column chromatography. The elution profile for each protein gave a sharp and symmetric peak from the gel-filtration column, demonstrating a quality sufficient for enzyme assays (data not shown).

Reactions for PCNA mono-ubiquitination *in vitro* have been established in *S. cerevisiae* and human systems.[9,10,13] Here, the reactions were reproduced using RPA-coated, singly primed mp18 single-stranded DNA (ssDNA) (Fig. 1b). The reaction condition was originally optimized for DNA replication,[33] and UEs were introduced to give saturated amounts with respect to mono-ubiquitination of PCNA. After

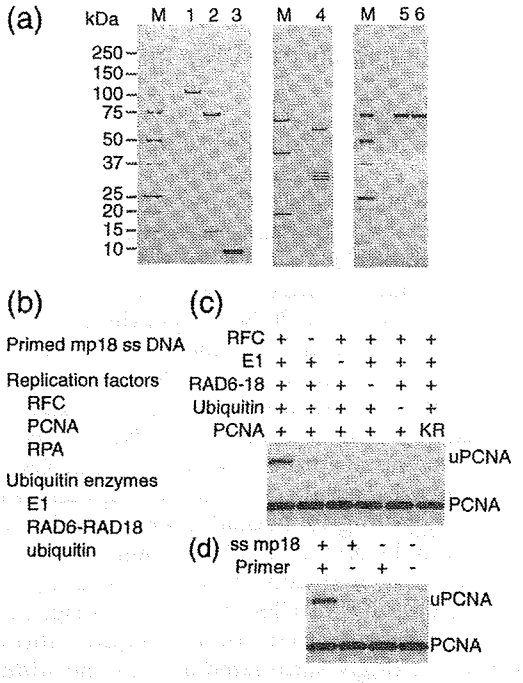

Fig. 1. Reconstitution of PCNA mono-ubiquitination. (a) Purified proteins in this study. Purified recombinant proteins, 500 ng (4.2 pmol) of E1 (lane 1), 650 ng (8.8 pmol) of RAD6A–RAD18 (lane 2), 500 ng (58 pmol) of ubiquitin (lane 3), 800 ng (3.6 pmol) of RFC$^{(p140N555)}$ (lane 4), 500 ng (6.4 pmol) of pol η (wild type) (lane 5), and 500 ng of pol η$^{(D652A)}$ (lane 6) were loaded on an SDS 5–20% gradient polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. (b) Summary of the assay system for PCNA mono-ubiquitination. (c) Requirement of protein components for PCNA mono-ubiquitination. KR represents a mutant of PCNA, PCNA$^{(K164R)}$, in place of the wild type. (d) Requirement of DNA components for PCNA mono-ubiquitination. The reactions were carried out for 30 min. The indicated components were omitted from the reactions, and reaction products were analyzed by Western blotting using anti-PCNA antibodies. uPCNA represents mono-ubiquitinated PCNA.

incubation for 30 min at 30 °C, PCNA molecules were visualized by Western blotting. This assay detected a slower migrating band, the size corresponding to the mono-ubiquitinated PCNA (Fig. 1c and d), which reacted with anti-ubiquitin antibody (data not shown), indicating it to be mono-ubiquitinated PCNA. The PCNA ubiquitination depended on the protein components, RFC and UEs (Fig. 1c), and singly primed mp18 ssDNA (Fig. 1d). Inefficient ubiquitination was detected without RFC (Fig. 1c) or the primer (Fig. 1d), probably due to nonspecific interactions of the proteins with mp18 DNA forming a secondary structure. When PCNA was replaced with a mutant, $PCNA^{(K164R)}$, no products were detected (Fig. 1c). Biochemical activity of the mutant was essentially identical with that of wild type with respect to DNA replication with pol δ (Supplementary Fig. S1), indicating that the mutation did not affect the integrity of the molecule. These results suggested ubiquitin to be specifically conjugated to the lysine 164 residue in these reactions, as detected earlier *in vivo*[4] and *in vitro*.[9,21]

Mono-ubiquitination of PCNA interacting with pol δ

Since pol δ forms a complex with PCNA at the 3′-end during elongation reaction, we asked the question of whether such PCNA could act as a target for ubiquitination. To address this question, we used poly(dA)–oligo(dT) as a DNA source. It is well established that poly(dA)–oligo(dT) is an excellent substrate for pol δ in PCNA-dependent and RFC-independent reactions,[34–42] since PCNA molecules spontaneously are loaded onto DNA from the ends without RFC[43] as illustrated in Fig. 2a. Indeed, we confirmed powerful stimulation of DNA synthesis of pol δ by only the addition of PCNA without RFC and RPA under our assay conditions (Supplementary Fig. S2), suggesting functional interactions between PCNA and pol δ on the poly(dA)–oligo(dT) without RFC. Then, we analyzed PCNA mono-ubiquitination in the presence of UEs. As shown in Fig. 2a, we surprisingly found that mono-ubiquitination of PCNA was strongly stimulated by only the addition of pol δ as well as RFC, but not pol β (Fig. 2b). Importantly, the required amount of pol δ for ubiquitination was stoichiometric, rather than catalytic, to that for PCNA (1 pmol as trimers was present in the reaction mixture) (Fig. 2b), suggesting the possibility that PCNA interacting with either RFC or pol δ is able to be a target for ubiquitination.

To obtain additional evidence, we used 5′-biotinylated oligo(dT) for the assay. First, loading of PCNA was monitored by stimulation of DNA synthesis (Fig. 2c). Neither the biotin moiety at the 5′-end nor streptavidin itself affects polymerase reactions (Fig. 2c, lanes 2 and 3, data not shown). When streptavidin was preincubated with template DNA, the stimulatory effect of PCNA was canceled (Fig. 2c, lanes 4 and 5), and it was partially restored by addition of RFC (Fig. 2c, lanes 5 and 6). Importantly, the results were identical with those when PCNA was preincubated with the template DNA before addition of streptavidin (Fig. 2c, lanes 7–12), indicating the amount of PCNA molecules on DNA without pol δ to be negligible. These results suggested that spontaneously loaded PCNA is basically unstable but can remain on the DNA by interaction with pol δ. Next, mono-ubiquitination of PCNA was analyzed by addition of UEs (Fig. 2d). The results showed that stimulation of PCNA mono-ubiquitination by pol δ was markedly reduced by addition of streptavidin (Fig. 2d, lanes 3 and 4) and was partially restored by addition of RFC (Fig. 2d, lanes 4 and 5). Identical results were obtained when PCNA was preincubated with the template DNA before addition of streptavidin (Fig. 2d, lanes 6–10). These results supported the possibility that PCNA molecules, interacting with pol δ, are able to be ubiquitinated.

To determine whether the interaction between pol δ and PCNA affected efficiency of mono-ubiquitination, we performed the following experiments with isolated encircled PCNA molecules on plasmid DNA as the substrate (Fig. 2e). After loading reactions, DNA–PCNA complexes were separated from DNA-free PCNA by gel filtration and then fractions containing DNA–PCNA complexes were subjected to ubiquitination assays using nicked circular plasmids, since they well support PCNA mono-ubiquitination[13] (data not shown). For PCNA loading, a mutant RFC, $RFC^{(p140N555)}$, which was formed with a truncated RFC1 subunit that lacked a nonspecific DNA binding domain,[44] was employed. The benefit of using $RFC^{(p140N555)}$ for loading reactions is prevention of contamination due to nonspecific interactions between the DNA binding domain of RFC and DNA on subsequent gel filtration.[45] When purified fractions were reacted with UEs, we detected a small amount of ubiquitinated PCNA (Fig. 2f, lanes 2 and 9), suggesting that encircled PCNA is capable of ubiquitination without pol δ or RFC. To see the effects of RFC or pol δ, we then introduced these proteins into the reactions. Here, we detected increased products depending on the amounts of RFC (Fig. 2f, lanes 2–7) and pol δ (Fig. 2f, lanes 9–14), suggesting that both have the ability to stimulate the reaction. In this experiment, it was critical to confirm that the PCNA–DNA complex is stable under our assay conditions, as an important control. If a significant fraction of PCNA dissociated spontaneously during incubation, the stimulation might be a consequence of stabilization of PCNA by interaction with pol δ. To assess the stability of encircled PCNA, we divided the purified complexes into three. One sample was immediately reacted with UEs for 30 min; others were preincubated for 30 min without RAD6A–RAD18 and E1 in the presence or absence of a restriction enzyme, HincII, for linearization of the plasmid, and then the reaction was carried out for a further 30 min by introduction of RAD6A–RAD18 and E1 (Supplementary Fig. S3a). The results of Western blotting showed that the capacities for ubiquitination were not changed before and after the incubation for 30 min but halted by linearization of DNA

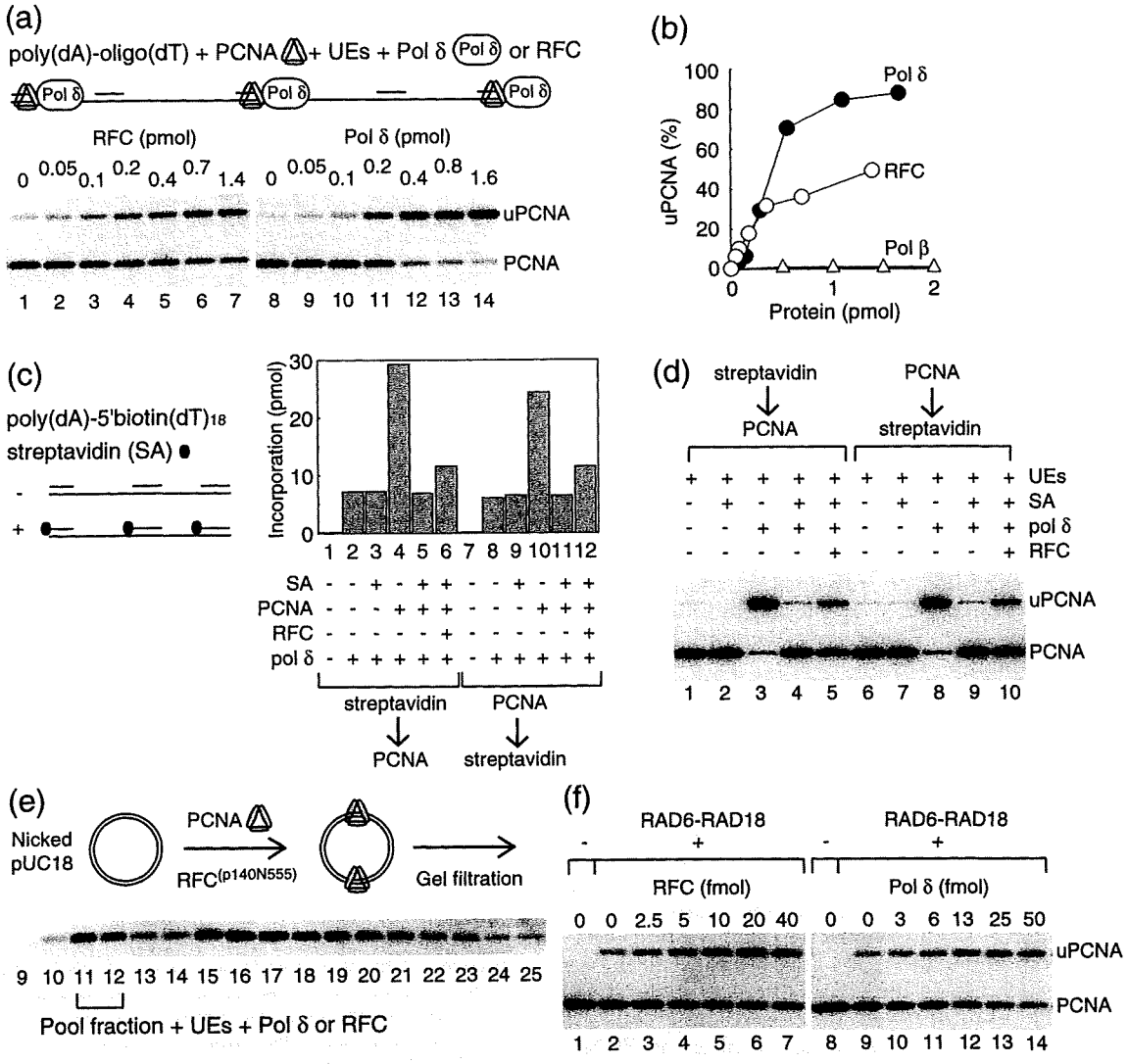

Fig. 2. Mono-ubiquitination of PCNA interacting with pol δ. (a) Titration of pol δ and RFC in the PCNA mono-ubiquitination reaction with poly(dA)–oligo(dT) as the DNA source. The experimental design is shown in the upper part. In this reaction, poly(dA)–oligo(dT) and PCNA were incubated with UEs. Amounts of oligo(dT) as primer termini were 0.9 pmol in the reaction. Indicated amounts of RFC or pol δ were introduced into the reaction mixtures followed by incubation for 30 min. Reaction products were analyzed by Western blotting using anti-PCNA antibodies. (b) Quantification of mono-ubiquitinated PCNA shown in (a), and with pol β. Titration of pol β was performed as for pol δ described in (a). The signal intensity detected by a CCD camera was quantified and plotted as the relative amount of the mono-ubiquitinated form. (c) DNA synthesis on poly(dA)–5′ biotinylated oligo(dT). The 5′ biotinylated oligo(dT), 18-mer, was annealed with poly(dA). In lanes 1 to 6, DNA was incubated with streptavidin (SA) on ice for 10 h and then mixed with PCNA. In lanes 7 to 12, DNA was incubated with PCNA on ice for 1 h and then incubated with streptavidin on ice for 10 h. Replication reactions were carried out for 10 min under standard reaction conditions with pol δ (220 fmol) and RFC (1.4 pmol) as indicated. (d) PCNA mono-ubiquitination assays with poly(dA)–5′ biotinylated oligo(dT). The substrate DNA was prepared as described in (c). Reactions were carried out for 10 min under standard reaction conditions with pol δ (840 fmol) and RFC (1.4 pmol) as indicated. Reaction products were analyzed by Western blotting using anti-PCNA antibodies. (e) Purification of PCNA assembled on nicked circular DNA by gel-filtration chromatography. A schematic representation of the experimental design is shown in the upper part. After loading PCNA on nicked circular DNA by RFC$^{(p140N555)}$, the products were passed through a gel-filtration column. Indicated fractions were analyzed by Western blotting. Encircled PCNA on DNA eluting in the void volume of the gel filtration in fractions 11 and 12 was subjected to ubiquitination reactions (f). (f) PCNA mono-ubiquitination assays with purified PCNA–DNA complexes. Encircled PCNA (e) was incubated with the indicated amounts of RFC or pol δ in the presence of UEs. Reaction products were analyzed by Western blotting using anti-PCNA antibodies. uPCNA represents mono-ubiquitinated PCNA.

(Supplementary Fig. S3b), indicating that encircled PCNA is the target for ubiquitination and spontaneous dissociation of PCNA was negligible under the reaction conditions. The result could also rule out the possibility of contamination of RFC$^{(p140N555)}$. Because RFC$^{(p140N555)}$ has the potential to unload encircled PCNA,[46] if it were present in the reactions, the result would be reduction of the capacity for

ubiquitination by preincubation for 30 min. Taking these results together, we suggest that PCNA interacting with either pol δ or RFC on DNA is a better target of RAD6A–RAD18, rather than PCNA just encircled on DNA.

PCNA mono-ubiquitination can be coupled to DNA replication

Next, we asked whether the PCNA molecules in the replication machinery consisting of pol δ and RFC during elongation reactions[33] are able to be a target. To address this question, we reconstituted the DNA replication reaction with pol δ in the presence of UEs under the ubiquitin assay conditions described in Fig. 1b. DNA synthesis was monitored in the additional presence of [α-^{32}P] dTTP (Fig. 3). The time course was analyzed by alkaline agarose gel electrophoresis of the products (Fig. 3a and b), and incorporation of radioactivity was determined (Fig. 3c). The results demonstrated that UEs exhibited no influence on the size of the product (Fig. 3a and b) and total amounts of DNA synthesis (Fig. 3c). This is consistent with findings in a yeast system.[9]

Then, mono-ubiquitination of PCNA was monitored by Western blotting under the same conditions as shown in Fig. 3b. The results demonstrated that PCNA molecules interacting functionally with pol δ during elongation were able to be ubiquitinated (Fig. 4a and b). In the absence of dCTP, further stimulation by addition of pol δ was not observed (Fig. 4a and b), suggesting the levels of stimulation by each of RFC and pol δ to be equivalent and not additive for the following reasons. In the reaction without pol δ, RFC forms complex with PCNA at apparently all the 3′-ends.[33] Such PCNA molecules interacting with RFC could be ubiquitinated efficiently (Fig. 2f). By addition of pol δ, the same number of PCNA molecules now could make complexes with pol δ at the 3′-ends. Those PCNA molecules interacting with pol δ could be again better targets for ubiquitination (Fig. 2f). In spite of addition of pol δ, the number of PCNA molecules interacting with either RFC or pol δ should be constant. Therefore, it was very surprising that the ubiquitination of PCNA was stimulated by DNA synthesis (Fig. 4a and b), since the number of PCNA molecules interacting with pol δ must be constant during elongation reactions in consideration of the fact that pol δ should interact with PCNA at only the 3′-ends.

To address why the elongation reaction stimulated the ubiquitination, we analyzed the status of PCNA molecules on the DNA by a previously established method to isolate PCNA–DNA complexes in reaction mixtures for DNA replication (Fig. 4c).[33] A primer containing an extended 5′ tail with one biotin molecule was annealed to mp18 ssDNA (Fig. 4c). The 5′ tail of the primer did not exert any influence on DNA synthesis (data not shown).[33] The primed mp18 ssDNA molecules were attached to magnetic beads, and then DNA replication reactions with RFs, UEs, and pol δ were carried out as

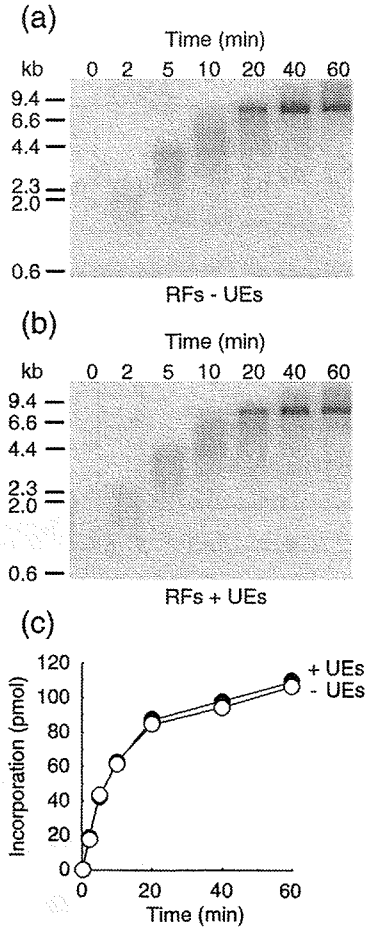

Fig. 3. Pol δ holoenzyme assays in the presence or absence of UEs. (a and b) Time courses of DNA synthesis in the presence (b) or absence (a) of UEs. Reactions were carried out for the indicated times under standard assay conditions with pol δ (380 fmol). Products were analyzed by 0.7% alkaline agarose gel electrophoresis. (c) Incorporation of dNMP was measured as described in Materials and Methods.

described in Fig. 4c. After reactions for 10 min, the beads were washed and bound PCNA was detected by Western blotting (Fig. 4d). Chemiluminescence signals detected with a CCD camera were quantified with reference to a standard curve for PCNA in the same blot (Fig. 4e). We have demonstrated previously that the assay detects PCNA molecules that are loaded on DNA in an RFC-dependent manner.[33]

First, general properties of loaded PCNA in DNA replication in the absence of RAD6A–RAD18 were analyzed (Fig. 4d and e, lanes 1–4).[33] The amount of PCNA detected in reactions with RFC alone was 54 (±7) fmol (Fig. 4d and e, lane 2). Since the background signal, which is the amount of PCNA detected after linearization of DNA with a restriction enzyme, HincII, was 29 (±4) fmol (Fig. 4d and e, lane 4), we estimated that the net amount of loaded PCNA could be about 25 fmol, equivalent to the amount of the primer template (33 fmol) (Fig.

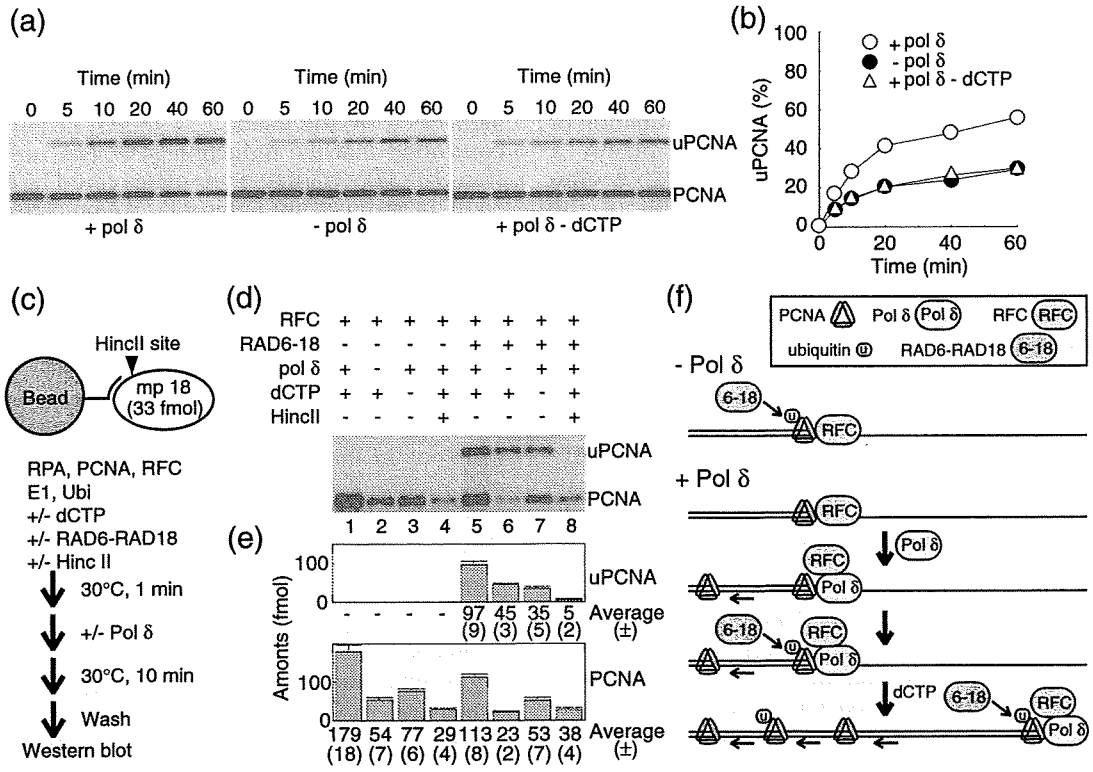

Fig. 4. PCNA mono-ubiquitination coupled with DNA replication. (a and b) Time courses of reactions for PCNA mono-ubiquitination in the presence or absence of pol δ (380 fmol) and/or dCTP. The reactions were carried out under the conditions described in Figs. 1b and 3b and in Materials and Methods. Reaction products were analyzed by Western blotting using anti-PCNA antibodies (a). The signal intensity detected by a CCD camera was quantified, and averages of three to five independent experiments were plotted as the relative amount of the mono-ubiquitinated form (b). Error bars are smaller than their symbols. (c) Outline of the assay to determine amounts of PCNA loaded on DNA. DNA was attached to magnetic beads via biotin–streptavidin linkage. The reactions were carried out for 10 min with the indicated factors in the presence or absence of pol δ (380 fmol). After termination of the reactions, the beads were washed and bound PCNA was analyzed by Western blotting (d and e). (d) A representative image of the Western analysis. uPCNA represents mono-ubiquitinated PCNA. (e) Chemiluminescence signals detected with a CCD camera were quantified with reference to a standard curve for PCNA in the same blot. Averages of four independent experiments with SD in parentheses were shown with graphs. (f) Conceivable protein actions based on the model proposed previously.[33] After loading by RFC, PCNA in the complex was ubiquitinated during 10 min incubation. Addition of pol δ induced dynamic actions so that additional PCNA molecules were loaded on DNA. Probably, the PCNA molecules interacting with pol δ were preferentially ubiquitinated as shown in Fig. 2f. Primer extension by addition of dCTP facilitated further accumulation of PCNA on DNA. PCNA molecules that were released behind the 3′-end before ubiquitination were not better substrates for ubiquitination, resulting in coexistence of ubiquitinated and ubiquitin-free PCNA molecules in a mosaic fashion.

4e, note the difference between lanes 2 and 4) with approximately one PCNA molecule loaded onto template DNA.[33,47,48] Addition of pol δ in the absence of dCTP raised the amount of PCNA slightly (Fig. 4d and e, lane 3). Consequently, approximately 48 fmol of PCNA was detected on the 90-mer primer (Fig. 4e, the difference between lanes 3 and 4) corresponding to one to two molecules on DNA, as illustrated in Fig. 4f.[33] Primer extension by addition of dCTP facilitated further accumulation of PCNA on DNA to about 150 fmol of the net amount of PCNA (Fig. 4e, lane 1, note the difference between lanes 1 and 4) corresponding to four to five molecules on DNA, as illustrated in Fig. 4f.[33,48]

Then, we analyzed ubiquitinated PCNA by introduction of RAD6A–RAD18 into the reaction mixture (Fig. 4d and e, lanes 5–8). In the absence of pol δ, almost all PCNA molecules were detected as ubiquitinated forms (Fig. 4d, lane 6). Introduction of pol δ in the absence of dCTP did not appreciably affect the amount of ubiquitinated PCNA (the slight reduction might be attributed to pull-down efficiencies of the experiment, which could be affected slightly by respective protein factors), even though the total amount was increased (Fig. 4d, lanes 3 and 7). Consequently, the significant fraction of PCNA on DNA persisted without ubiquitination, even in the presence of excess amounts of UEs. Primer extension with dCTP further increased the amount of ubiquitinated PCNA, but significant fractions of PCNA remained without ubiquitination (Fig. 4d, lane 5). These results are consistent with the observation in Fig.