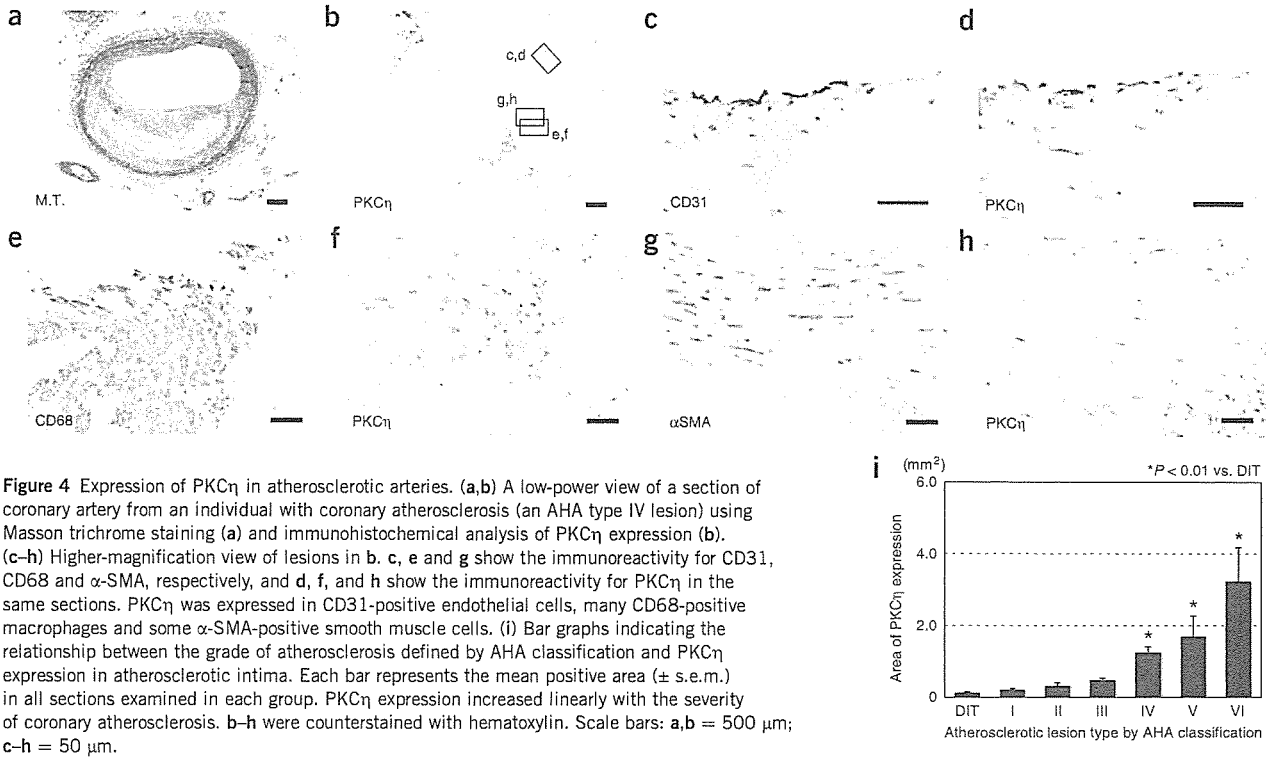

Figure 4 Expression of PKCη in atherosclerotic arteries. (**a**,**b**) A low-power view of a section of coronary artery from an individual with coronary atherosclerosis (an AHA type IV lesion) using Masson trichrome staining (**a**) and immunohistochemical analysis of PKCη expression (**b**). (**c**–**h**) Higher-magnification view of lesions in **b**. **c**, **e** and **g** show the immunoreactivity for CD31, CD68 and α-SMA, respectively, and **d**, **f**, and **h** show the immunoreactivity for PKCη in the same sections. PKCη was expressed in CD31-positive endothelial cells, many CD68-positive macrophages and some α-SMA-positive smooth muscle cells. (**i**) Bar graphs indicating the relationship between the grade of atherosclerosis defined by AHA classification and PKCη expression in atherosclerotic intima. Each bar represents the mean positive area (± s.e.m.) in all sections examined in each group. PKCη expression increased linearly with the severity of coronary atherosclerosis. **b**–**h** were counterstained with hematoxylin. Scale bars: **a**,**b** = 500 μm; **c**–**h** = 50 μm.

PKCη is known to be expressed predominantly in epithelial tissues in mouse[14], but its expression pattern in humans was unknown. Therefore, we carried out quantitative real-time PCR using mRNA obtained from various human tissues. PKCη was ubiquitously expressed and showed slightly higher expression in thymus and spleen, suggesting its physiological importance in white blood cells (**Supplementary Fig. 4** online). On the basis of this result and the association with atherosclerotic diseases, we subsequently investigated the expression of PKCη in human atherosclerotic lesions (**Fig. 4**). Immunohistochemical analysis of a coronary arterial specimen showed that PKCη was expressed in endothelial cells and some foamy macrophages in the intima and in a part of spindle-shaped smooth muscle cells in the intima and media (**Fig. 4b–h**). This immunohistochemical positivity was completely abolished by preabsorbing antibody with an excess of immunogenic peptide. To further investigate a role of PKCη in atherosclerosis, two independent pathologists carefully classified 60 coronary arterial specimens according to the type of atherosclerotic lesion[15]. The expression of PKCη increased in accordance with the progression of coronary atherosclerotic lesion type ($P < 0.0001$ for Spearman's rank correlation; **Fig. 4i**). These results suggest a role for PKCη in the development and progression of atherosclerosis in humans.

PKC is a serine-threonine kinase that regulates a wide variety of important cellular functions including proliferation, differentiation and apoptosis. PKCs are classified into three subfamilies based on their molecular structure and cofactor requirements. Classical PKC isoforms (α, βI, βII, and γ) are regulated by calcium, diacylglycerol and phospholipids. Novel PKC isoforms (δ, ε, η and θ) are regulated by diacylglycerol and phospholipids but are insensitive to calcium. Atypical PKC isoforms (ζ and λ or ι) are insensitive to either calcium or diacylglycerol[16]. Despite their high degree of sequence homology, different PKC isoforms mediate unique cellular functions and phosphorylate unique protein substrates[17]. Although PKCη was identified in 1990 (ref. 13), its specific substrates are not yet known. Therefore, it is unclear how PKCη is involved in the development and progression of atherosclerosis. Our pathological findings showed that PKCη was abundantly expressed in foamy macrophages that are essential in all phases of atherosclerosis. Macrophages contribute to the uptake of lipoproteins, release of reactive oxygen species and immune mediators that have important roles in the development of atherosclerosis[18]. Previous studies indicated that PKCη was involved in oxidative stress. Overexpression of PKCη in human monocytic cells resulted in the induction of inducible nitric oxide synthase and nitric oxide (NO) production after endotoxin exposure[19]. Antisense oligonucleotides for PKCη inhibited LPS-induced NO release in primary astrocytes[20]. The other possible downstream signal is the promotion of cell growth through suppression of cyclin E (ref. 21) and caspase-3 activity[22] as well as activation of the Akt pathway[23], which is involved in diverse cellular processes.

In conclusion, we have identified *PRKCH* as a new candidate gene for cerebral infarction using genome-wide SNP analysis and have replicated this association in an independent case-control sample. The nonsynonymous SNP (1425G/A) in *PRKCH* causes enhancement of PKC activity and is associated with a higher incidence of cerebral infarction in a Japanese population-based sample. Although the function and the signaling pathway of *PRKCH* are not fully elucidated, our findings may contribute new insights into the pathogenesis of atherosclerosis and suggest the possibility of new preventive measures for cerebral infarction.

METHODS

Study subjects. Details of the study subjects and the flow diagram of this study are shown in **Supplementary Figure 5** online. For the genome-wide case-control study, affected individuals with cerebral infarction were recruited from

seven affiliated hospitals of Kyushu University (Kyushu University Hospital, Hakujyuji Hospital, Fukuoka Red Cross Hospital, Kyushu Medical Center, Imazu Red Cross Hospital, Fukuoka Higashi Medical Center and Seiai Rehabilitation Hospital) in 2004. The diagnoses of cerebral infarction and its subtypes for all cases were made by stroke neurologists of the affiliated hospitals, referring to detailed clinical features and ancillary laboratory examinations: namely, cerebral angiography, brain imaging (including computed tomography and magnetic resonance imaging), echocardiography and carotid duplex imaging. Details of the diagnostic criteria for cerebral infarction and its subtypes have been described previously[24]. Briefly, subtypes of cerebral infarction were determined on the basis of the Classification of Cerebrovascular Disease III proposed by the National Institute of Neurological Disorders and Stroke[3]. Lacunar infarction was diagnosed as the presence, as observed by brain imaging, of a relevant brainstem or subcortical hemispheric lesion with a diameter of <1.5 cm and no evidence of cerebral cortical or cerebellar impairment. Atherothrombotic infarction was diagnosed when the subjects had significant stenosis or occlusion of a major cerebral artery with infarct size ≥ 1.5 cm on brain imaging. The diagnosis of cardioembolic infarction was made on the basis of primary and secondary clinical features suggestive of cardioembolism as reported by the Cerebral Embolism Task Force[25] with the TOAST classification of risk sources of cardioembolism[26]. The category of undetermined subtype included all cerebral infarction cases for which the subtype could not be determined because of insufficient clinical or morphological information.

Control subjects were enrolled from the participants in the Hisayama study, an ongoing prospective population-based epidemiological study of cardiovascular disease established in 1961. Details of this study have been described previously[1,2]. Between 2002 and 2003, a screening survey for the present study was performed. Briefly, a total of 3,328 individuals aged 40 years or older consented to participate in the screening survey and underwent a comprehensive assessment (participation rate of 78%). After excluding subjects with a history of stroke or coronary heart disease, we selected age-matched (within 5 years) and sex-matched control subjects from the participants by 1:1 matching using random numbers (**Supplementary Table 3** online).

The replication case-control samples were recruited from the BioBank Japan project. We selected lacunar infarction cases from subjects registered to have cerebral infarction in the BioBank. Cases with lacunar infarction were diagnosed on the basis of clinical findings, including neuroimaging results. Controls were randomly selected from the subjects registered in the BioBank Japan.

To investigate the impact of 1425G/A on the development of cerebral infarction, we used a cohort population of the Hisayama study established in 1988 (ref. 2). A total of 2,742 Hisayama residents aged 40 years or older participated in the health examination (participation rate of 81%) in 1988. After we excluded individuals with a history of stroke or coronary heart disease, we monitored 2,637 subjects for the occurrence of cardiovascular disease or death. Of these, 1,683 participated in the 2002–2003 examination.

We obtained written informed consent from all study subjects, and procedures were approved by the ethics committees of the Graduate School of Medical Sciences of Kyushu University and the Institute of Medical Science of the University of Tokyo.

SNP genotyping. We extracted genomic DNA from peripheral blood leukocytes using standard protocols. We genotyped SNPs using the multiplex PCR-based Invader assay (Third Wave Technologies) as previously described[27] or by direct sequencing of PCR products using ABI3700 capillary sequencers (Applied Biosystems) according to standard protocols.

Cell culture, transfection and immunoprecipitation. We maintained 293T cells in D-MEM supplemented with 10% (vol/vol) FBS and 1% (vol/vol) antibiotic/antimycotic solution (Sigma) at 37 °C under 5% CO_2. We constructed a plasmid designed to express full-length PKCη-374V by cloning human thymus cDNA into a p3xFLAG-CMV-14 expression vector (Sigma). A plasmid expressing full-length PKCη-374I was constructed from p3xFLAG-CMV-14-PKCη-374V vector using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's instructions. For transient transfection, we plated 293T cells in 15-cm dishes and transfected p3xFLAG-CMV-14-PKCη-374V or p3xFLAG-CMV-14-PKCη-374I using FuGENE6 (Roche) according to the manufacturer's instructions. After 48 h, we collected cells and lysed them at 4 °C in lysis buffer containing 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM phenylmethyl sulfonyl fluoride, 1 mM dithiothreitol and 0.1% protease inhibitor cocktail set III (Calbiochem). After a 30-min incubation on ice, the lysates were centrifuged at 20,000*g* for 15 min at 4 °C. The supernatant was incubated with anti-Flag M2 affinity gel (Sigma) for 3–4 h at 4 °C after preclearing with rec–Protein G–Sepharose 4B conjugate (Zymed) and mouse normal IgG for 30 min. After incubation, the gels were washed twice with lysis buffer and with 1× TBS buffer, and the Flag-tagged protein was eluted by addition of 15 μg of 3× Flag peptide (Sigma). We assessed the purity of immunoprecipitates by Coomassie brilliant blue staining. Equal amounts of immunoprecipitates were subjected to SDS-PAGE, and the amount of Flag-PKCη was assessed by protein blotting using antibody to PKCη (Santa Cruz) and the ECL protein blotting detection system (Amersham). The protein concentration was measured by the Bradford method.

PKC activity and autophosphorylation assay. PKC autophosphorylation activity and kinase activity were measured according to a previously described method[28]. For the PKC autophosphorylation assay, equal amounts of immunoprecipitates for mock (empty vector), PKCη-374V and PKCη-374I were incubated at 30 °C for the indicated times in a total volume of 50 μl of reaction mixture containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgSO_4$, 1 mM EGTA and 5 μCi [γ-^{32}P]ATP with 10 μM phosphatidylserine (Sigma) and 100 nM phorbol-12,13-dibutyrate (PDBu, Sigma) and were then subjected to SDS-PAGE and autoradiography. Protein kinase activity was measured by incorporation of ^{32}P from [γ-^{32}P]ATP into myelin basic protein (MBP) peptide (Sigma). The incubation mixture contained 20 mM Tris-HCl (pH 7.5), 5 mM $MgSO_4$, 1 mM EGTA, 100 μM ATP, 1 μCi [γ-^{32}P]ATP, 10 μg MBP peptide and PKCη immunoprecipitant in a total volume of 50 μl, with 10 μM phosphatidylserine and 100 nM PDBu. After incubation for 3 min at 30 °C, the reaction was terminated by direct application to P81 phosphocellulose squares (Upstate) followed by washing with 75 mM phosphoric acid and measurement of radioactivity. One unit of the activity was defined by incorporation of 1 nmol/min of radioactive phosphate from ATP into MBP.

Quantification of *PRKCH* expression using real-time PCR. We carried out real-time quantitative PCR using an ABI 7700 (Applied Biosystems) with SYBR Premix ExTaq (TaKaRa) in accordance with the manufacturer's instructions. We purchased total RNA from various human tissues (Clontech) and synthesized first-strand cDNA from 1 μg of total RNA using oligo $d(T)_{12-18}$ primers and Superscript III Reverse Transcriptase (Invitrogen). The relative expression of *PRKCH* mRNA was normalized to the amount of β-actin expression in the same cDNA using the standard curve method described by the manufacturer.

Immunohistochemistry and morphometric analysis of coronary arteries. Hearts were obtained at autopsy at the Department of Pathology of Kyushu University from 16 deceased Hisayama residents (eight men and eight women), ranging in age from 68–91 years old (81.1 ± 6.2), within 16 h of death. The coronary arteries were cannulated, washed with 0.1 mol/l PBS (pH 7.4) and perfused with 1 l of 4% (wt/vol) paraformaldehyde in 0.1 mol/l sodium phosphate (pH 7.4) at 100 mm Hg. Then, the heart was immersed in 4% paraformaldehyde for at least 24 h at 4 °C. The right coronary artery and left anterior descending coronary artery were dissected free from the surface of the heart, cut perpendicular to the long axis at 3-mm intervals and then embedded in paraffin. Sixty blocks were obtained and cut into 3-μm-thick serial sections at once. Serial sections from each block were separately subjected to hematoxylin and eosin staining, elastica–van Gieson staining and Masson trichrome staining, as well as immunohistochemistry. In accordance with the definitions proposed by the Committee on Vascular Lesions of the Council on Arteriosclerosis of the American Heart Association (AHA)[15], the atherosclerotic lesion type of each section was carefully classified by two independent pathologists (T.N. and K.S.).

Immunohistochemical examinations were performed as described previously[29]. In brief, deparaffinized sections were incubated with 3% nonfat milk; with primary antibodies against human PKCη (Santa Cruz), endothelial cells (anti-human CD31, Dako), monocytes/macrophages (anti-human CD68, Dako) and smooth muscle cells (anti-human α-SMA, Sigma); and then with peroxidase-labeled secondary antibody (Dako). The slides were incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and counterstained with

hematoxylin. As a negative control, we substituted nonimmune rabbit IgG or nonimmune mouse IgG of each isotype instead of primary antibody. As a second negative control, we used antibody preabsorbed with an excess amount (10:1 molar ratio) of immunogenic peptide. As PKCη is expressed in normal epithelium of human mammary glands[30], we used the tissue blocks retrieved from human mammary glands as positive controls. A single observer, blind to the atherosclerotic lesion types, quantified PKCη-positive lesions by determining the positive area in atherosclerotic intima. All images were captured and analyzed by US National Institutes of Health Image software.

Statistical analysis. Data are presented as mean ± s.d. unless otherwise stated. We assessed association and Hardy-Weinberg equilibrium by χ^2 test and Fisher's exact test. We calculated linkage disequilibrium index and Δ index and created **Figure 1d** as described previously[7]. For adjustment of multiple testing, we performed a random permutation test with 10,000 replications using the MULTTEST procedure in SAS9.12 software. In the 14-year follow-up cohort study, we estimated hazard ratios and 95% confidence intervals for the development of cerebral infarction among the candidate genotypes using Cox proportional hazards model after adjustment for age and sex. In adjustment for clinical risk factors, we used a conditional logistic regression model for the case-control study and Cox proportional hazards regression model for the prospective cohort study using SAS software. We compared the PKCη-positive area among the grade of coronary atherosclerosis using Spearman's rank correlation with Bonferroni correction. The difference in PKC activity between 374V and 374I was tested by Student's *t*-test.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

M.K., J.H., T.M. and K. Yamazaki performed SNP genotyping; T. Ninomiya and K. Yonemoto provided Hisayama samples and carried out statistical analyses; T. Nakano and K.S. performed immunohistochemical experiments; M.K., K. Yamazaki and K.M. performed protein blotting experiments; Y.O. and S.S. performed genotyping of genome-wide screening samples; T.K. and S.I. provided clinical information and samples of individuals with cerebral infarction; Y.N. provided BioBank Japan samples; M.K. performed all other experiments and wrote the manuscript with contributions from K.S., Y.N. and Y.K.; T. Ninomiya, K. Yonemoto, Y.K. helped with revisions and M.I., Y.N. and Y.K. jointly directed the project.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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3) 今後の研究計画

平成19年度には、大規模な断面調査を行い、40歳以上の同意が得られた住民を対象として新たなコホートを設定する予定である。久山町住民健診の、一般健診項目は以下の項目である：

既往歴、家族歴、生活歴、食事調査、身体活動度、身体計測（身長、体重、皮脂厚比、腹囲、腹囲/腰囲比）、体脂肪、血圧測定、眼底検査、血計、血液生化学、ヘモグロビンA1c、75g経口糖負荷試験、尿検査、心電図、胸写

過去に設定したコホート集団の追跡調査も継続して行う予定である。

また、1961年から現在までの間に、福岡県久山町の一般住民（40歳以上）を対象として行われた複数の断面調査とその後の追跡調査の成績より、今後も循環器疾患の実態および危険因子を検討していく予定である。

6. 放射線影響研究所成人健康調査コホート

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(1) 成人健康調査

放射線影響研究所(放影研)の成人健康調査は原爆被爆者とその対照からなるコホート調査集団について、疾病の発症や測定値等の情報を収集するため、2年毎の包括的な健康診断を1958年から現在まで継続して実施している。

統合コホートのベースラインデータとして1986年7月から2年間の健診データを提供した。2年毎の健診では1回の健診に付き最高12種類の診断が国際疾病分類（ICD）コードでコードされている。

(2) 2006年度の研究成果

肥満がインスリン抵抗性、高血圧、糖尿病、高脂血症を合併しやすいことは知られているが、欧米人に比べ肥満の程度が比較的軽度の日本人において肥満が糖尿病や心血管疾患の発症に対し、どの程度の危険度の上昇をもたらすかに関する報告は少ない。肥満の程度の指標は従来、Body mass index (BMI)が一般的に用いられてきたが、近年、内臓脂肪を反映するとされるウエスト周囲径やウエスト/ヒップ比が注目されてきた。

成人健康調査では1996-98年の健診サイクルで2999名(男性981名、女性2018名)の身長、体重、ウエスト周囲径、ヒップ周囲径を測定した。肥満指標測定時の年齢は50-95歳であった。2年毎の健診結果に基づき、糖尿病、心筋梗塞、脳卒中の新規発症を診断し、2006年4月までの追跡結果について解析した。平均追跡期間は6.8年で、その間に134名（男性50名、女性84名）の糖尿病、117名(男性36名、女性81名)の脳卒中、22名の（男性10名、女性12名）の心筋梗塞の新規発症を認めた。表1に1996-98年の対象者の特徴を示す。いずれの肥満指標も糖尿病のリスクの独立した危険因子であった(表2)。ウエスト周囲径75cm未満の群を1とした場合の階級別糖尿病発症の相対危険度を図1に示す。男性では対象者数が少なく信頼区間が広いが、男女ともウエスト周囲径の増加と共に糖尿病発症の危険度が増加した。脳卒中は肥満指標の内、体重、BMI、ウエスト周囲径、ヒップ径と負の関係を認めた(表3)。心筋梗塞は症例数が少なく、肥満指標と有意な関係は認められなかった。脂肪と筋肉の年齢に伴う変化のパターンは異なることから、肥満指標の健康リスクの予測における評価は年齢の影響を受けるであろう。また高齢者では健康状態の変化が肥満指標の変化に反映されている割合が高いと考えられる。これらの問題点について今後検討する予定である。

Table 1. Characteristics of Study Subjects

	Men	Women
Number of observation	981	2018
Age at baseline (years)	64.9 (10.0)	69.2 (9.6)
Height (cm)	163.0 (6.3)	149.5 (6.1)
Body weight (kg)	60.4 (9.6)	51.3 (8.8)
Body mass index (kg/m^2)	22.7 (3.1)	23.0 (3.6)
Waist circumference (cm)	83.4 (8.2)	82.5 (10.3)
Hip circumference (cm)	89.7 (5.8)	89.9 (6.9)
Current smoker (%)	43.8	8.3

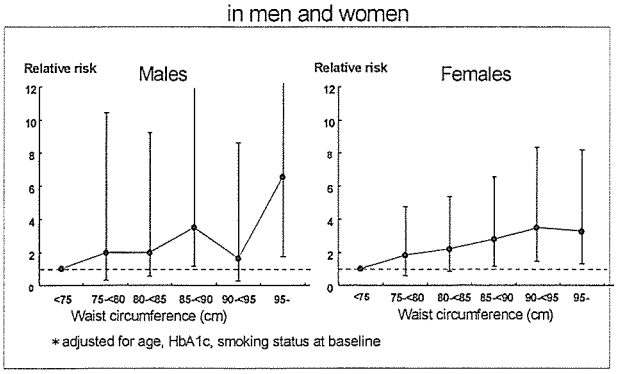
Values are expressed as mean (SD).

Table 2. Development of diabetes
(mean follow-up period 6.8 years)
134 cases (men 50 subjects, women 84 subjects)

Anthropometric measurements	relative risk of diabetes	95% confidence interval	*p*-value
body weight (per 5kg)	1.20	1.10, 1.31	<0.001
body mass index (per 2kg/m^2)	1.23	1.12, 1.36	<0.001
waist circumference (per 5cm)	1.27	1.16, 1.39	<0.001
hip circumference (per 3cm)	1.15	1.07, 1.25	<0.001
waist/hip ratio (per 2%)	1.16	1.10, 1.24	<0.001

* adjusted for age, sex, HbA1c, smoking status

Figure 1. Risk of diabetes by waist circumference in men and women



* adjusted for age, HbA1c, smoking status at baseline

Table 3. Development of stroke
(mean follow-up period 6.8 years)
117 cases (men 36 subjects, women 81 subjects)

Anthropometric measurements	relative risk of stroke	95% confidence interval	*p*-value
body weight (per 5kg)	0.80	0.71, 0.91	<0.001
body mass index (per 2kg/m^2)	0.82	0.72, 0.92	<0.001
waist circumference (per 5cm)	0.80	0.68, 0.93	0.016
hip circumference (per 3cm)	0.87	0.80, 0.96	0.004
waist/hip ratio (per 2%)	0.97	0.91, 1.04	0.381

* adjusted for age, sex, HDL-cholesterol, SBP, DM

（公表論文）

Levels of antibodies to microorganisms implicated in atherosclerosis and of C-reactive protein among atomic bomb survivors

大規模コホート共同研究に関連した研究成果

横断的研究デザインで動脈硬化への関連が示唆されている微生物感染指標として抗体レベルを、また炎症の指標としてC反応性蛋白質（CRP）を測定した。この研究は原爆被爆者とその対照者集団において放射線被曝の動脈硬化に対する影響を調べる研究の一環として実施されたが、抗体レベルならびにCRPに対する性、年齢、喫煙の影響についても検討した。2000年3月から2年の健診サイクルで4,068人のｸﾗﾐｼﾞｱ・ﾆｭｰﾓﾆｴに対するIgAとIgG抗体、ﾍﾘｺﾊﾞｸﾀｰ・ﾋﾟﾛﾘに対するIgG抗体、ｻｲﾄﾒｶﾞﾛｳｲﾙｽに対するIgG抗体、高感度CRP、身長、体重を測定し、問診により喫煙に関する情報を得た。対象者は年齢54歳以上、平均年齢は71歳であった。ｸﾗﾐｼﾞｱ・ﾆｭｰﾓﾆｴに対するIgAとIgG抗体価は共に年齢の増加ならびに喫煙本数の増加で高値を示し、女性では男性に比べ抗体価が低かった。ﾍﾘｺﾊﾞｸﾀｰ・ﾋﾟﾛﾘに対するIgG抗体価は年齢が増加すると減少し、男性ならびに喫煙者で高値であった。ｻｲﾄﾒｶﾞﾛｳｲﾙｽに対するIgG抗体高齢者、女性、喫煙者で高値であった。高感度CRPは高齢者、男性、喫煙者で高値であった。また、BMIと正の関係を認め、肥満に伴う炎症の増加が示唆された。

喫煙は微生物感染ならびに炎症を介して、また肥満は炎症を介して動脈硬化を進展させる可能性を示唆している。

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Levels of Antibodies to Microorganisms Implicated in Atherosclerosis and of C-Reactive Protein among Atomic Bomb Survivors

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Hakoda, M., Kasagi, F., Kusunoki, Y., Matsuura, S., Hayashi, T., Kyoizumi, S., Akahoshi, M., Suzuki, G., Kodama, K. and Fujiwara, S. Levels of Antibodies to Microorganisms Implicated in Atherosclerosis and of C-Reactive Protein among Atomic Bomb Survivors. *Radiat. Res.* 166, 360–366 (2006).

Although it has been suggested that cardiovascular disease incidence is increased among atomic bomb survivors, the existence of a causal relationship between radiation exposure and atherosclerosis is unclear. Microbial infections, including those caused by *Chlamydia pneumoniae*, *Helicobacter pylori* and cytomegalovirus, have recently been implicated in atherosclerosis. Since immune function is somewhat impaired among atomic bomb survivors, their immune defense against such infections might be diminished. To investigate this possibility, we measured antibody levels to the above microorganisms in the sera of survivors. We found that the levels of IgG and IgA antibodies to *Chlamydia pneumoniae* decreased significantly with radiation dose, whereas the levels of IgG antibodies to *Helicobacter pylori* or cytomegalovirus remained unchanged. The inflammation marker C-reactive protein was significantly and positively associated with level of antibodies to *Chlamydia pneumoniae* only in heavily exposed (≥1000 mGy) survivors. These results may suggest that among atomic bomb survivors, immune response to *Chlamydia pneumoniae* is diminished and chronic inflammatory reactions related to *Chlamydia pneumoniae* infection are present.

INTRODUCTION

Mortality and incidence studies have suggested that cardiovascular disease is increased with radiation dose among atomic bomb (A-bomb) survivors (*1–3*). Long-term investigation of patients treated with radiation therapy for Hodgkin's lymphoma confirmed earlier observations that the treatment may have induced coronary heart disease (*4, 5*). The total local dose received by such patients, however, was about 30 Gy, whereas for A-bomb survivors, the total dose typically did not exceed 3 Gy (*6*). The possibility exists, therefore, that the mechanism for development of cardiovascular disease in A-bomb survivors differs from that in radiation therapy patients.

Although various factors initiate the atherosclerotic process, of which coronary heart disease is a typical clinical expression, inflammation accompanying activation of macrophages and T lymphocytes plays a central role (*7, 8*). The causal mechanism for development of this inflammatory process is not fully understood, but infectious agents have been implicated repeatedly (*9–12*). Among several pathogens, *Chlamydia pneumoniae*, *Helicobacter pylori* and cytomegalovirus have been investigated extensively, with *Chlamydia pneumoniae* regarded as the most plausible cause for the pathogenesis of atherosclerosis (*9–12*). Since impairment of immune function, especially decreased T-lymphocyte function, is suggested for A-bomb survivors (*13–16*), infectious agents may provide a plausible explanation for the development of atherosclerotic disease among this population.

It has been reported that mortality due to pneumonia is increased with radiation dose (*1*) and that this may be a reflection of increased infections among A-bomb survivors. Another example of increased infections among the survivors is the increase in the prevalence of hepatitis B virus surface antigen with radiation dose (*17–19*). On the other hand, tuberculosis mortality is not increased among A-bomb survivors (*1*). To further investigate infectious agents implicated in atherosclerosis among A-bomb survivors, we measured serum levels of antibodies to *Chlamydia pneumoniae*, *Helicobacter pylori* and cytomegalovirus in this survivor population and evaluated the association of the antibodies with radiation dose.

MATERIALS AND METHODS

Study Subjects

The study population comprised Adult Health Study (AHS) participants who had undergone biennial clinical examinations since 1958 at

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the Radiation Effects Research Foundation (RERF) in Hiroshima and Nagasaki. The AHS was initiated to evaluate the long-term effects of ionizing radiation from the atomic bombs on human health (*20*). The original AHS cohort consisted of 19,961 individuals, approximately half of whom were exposed to the bombs proximally (<2,000 m from the hypocenter), with the other half exposed either distally (≥3000 m from the hypocenter) or not in the cities at the time of the bombings. The latter group of survivors was thus not exposed to substantial levels of A-bomb radiation. The design of the AHS has been described elsewhere (*20*). In 1977, 1,218 individuals whose estimated radiation dose was greater than 1 Gy, and the same number of controls distally exposed to the bomb, were newly added to the cohort. The 5,000 people who were not in the two cities at the time of the bombings were excluded from the AHS cohort in 1986. Thus there were 17,397 cohort members at that time. When this study began in March 2000, 10,030 cohort members had died and 1,058 had moved away from the two cities, so there were 6,309 AHS cohort members who were potentially able to visit the RERF clinic.

While almost 4,187 (66%) of the eligible cohort members agreed to visit RERF and undergo health examinations, 119 (3%) of these people did not consent to the antibody measurements for this study. Radiation dose estimates (DS86 whole-body kerma estimates) (*21*) were available for 3,476 (85%) of the 4,068 participants with antibody measurements. We obtained written informed consent from the subjects and approval from the institutional Human Investigation Committee. Body weight and height were measured during the participant's clinic visit. Body mass index (BMI) was calculated by dividing weight in kilograms by the square of height in meters. Smoking information (number of cigarettes smoked per day) was obtained through interview by nurses at the visit.

Laboratory Methods

Serum samples were obtained from each participant at the time of the clinic visit. Serum was stored at −80°C until use. Frozen serum samples obtained in Nagasaki were sent to Hiroshima for analysis. We used enzyme-linked immunosorbent assay (ELISA) kits to determine serum antibody levels. IgG and IgA antibodies to *Chlamydia pneumoniae* were measured using HITAZYME® *C. pneumoniae* Ab-IgG (Hitachi Chemical Co., Ltd., Tokyo, Japan) and HITAZYME® *C. pneumoniae* Ab-IgA (Hitachi Chemical Co., Ltd.), respectively. For IgG antibody to cytomegalovirus, we used CYTOMEGALO IgG (II)-EIA "SEIKEN" (DENKA SEIKEN Co., Ltd., Osaka, Japan), and for IgG antibody to *Helicobacter pylori*, we employed AUTOACE H. PYLORI G (AZWELL Inc., Osaka, Japan). Using these ELISA kits, we measured antibody levels in serum semi-quantitatively based on a comparison of optical density of the serum samples with that of the standard materials provided in the kits. The values obtained were expressed as units (U). In addition to sequential antibody levels, we used antibody status (positive or negative) for analysis. Following the manufacturers' instructions, we scored antibody level as positive if it measured ≥1.1 U for IgG or IgA antibody to *Chlamydia pneumoniae*, ≥16.5 U for IgG antibody to *Helicobacter pylori*, and ≥4 U for IgG antibody to cytomegalovirus.

We measured serum level of C-reactive protein (CRP), a sensitive marker for inflammation, using a validated, high-sensitivity assay kit (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) and an auto-analyzer (Clinical Analyzer 7170, Hitachi, Ltd., Tokyo, Japan), a robotic system used for the assay of many routine clinical biochemical parameters.

Statistical Analysis

The relationship between measurements of antibody levels (*response*) and radiation dose (*dose*) was analyzed by a multiple regression model, with adjustment for city, gender, age and smoking history, as follows:

$$\log(response) = \alpha + \beta_1 city + \beta_2 gender + \beta_3(age - 70) + \beta_4 smoking + \beta_5 dose,$$

where *city* = 0 for Hiroshima subjects, 1 for Nagasaki subjects; *gender* = 0 for males, 1 for females; *smoking* = number of cigarettes smoked per day; *dose* = exposed dose. Logarithmic transformation was applied to the dependent variable *response* to obtain a more normal distribution of antibody level. With this model, for any of the values of *city, gender, age* and *dose,* the increased rate of *response* per gray was calculated as e^{β_5} and its percentage change was $100(e^{\beta_5} - 1)$. The antibody levels for nonexposed nonsmoking males aged 70 years in Hiroshima were estimated by e^{α}.

For analysis of the relationship between antibody level and log-transformed CRP level, BMI centered at 23 kg/m² was included in the regression model as follows:

$$\log(CRP) = \alpha + \beta_1 city + \beta_2 gender + \beta_3(age - 70) + \beta_4 smoking + \beta_5(BMI - 23) + \beta_6 dose + \beta_7 response$$

The interaction between radiation dose and antibody level was tested by adding the term *dose* × *response* to the above model.

When the relationship was examined by dose category (0, 1–999 mSv, and ≥1000 mSv), the following model was applied:

$$\log(CRP) = \alpha + \beta_1 city + \beta_2 gender + \beta_3(age - 70) + \beta_4 smoking + \beta_5(BMI - 23) + \beta_6 response + \beta_7 d_1 \times response + \beta_8 d_2 \times response,$$

where $d_1 = 1$ for the 1–999-mGy group, = 0 otherwise; $d_2 = 1$ for the ≥1000-mGy group, = 0 otherwise. Accordingly, for any of the values of *city, gender, age* and *BMI,* the percentage change in *CRP* for 1 U of antibody level was estimated as $100(e^{\beta_6 \times unit} - 1)$ for 0 mGy, $100(e^{(\beta_6+\beta_7)\times unit} - 1)$ for 1–999 mGy, and $100(e^{(\beta_6+\beta_8)\times unit} - 1)$ for ≥1000 mGy. Heterogeneity among dose categories in the relationship between CRP and antibody level was tested by the null hypothesis, H_0: $d_1 = d_2 = 0$.

For the dichotomous summaries (positive or negative) of antibody measurements, binominal odds regression model was used to analyze relationship with radiation dose, and adjustment was made for age, gender and smoking history.

RESULTS

Table 1 shows the number of individuals in each radiation dose category and the mean and median serum levels of antibodies to *Chlamydia pneumoniae*, *Helicobacter pylori* and cytomegalovirus. The antibody level to cytomegalovirus was not measured for the subjects who came to RERF after about the halfway point of this study (14 months after the start of the study). Thus the antibody level to cytomegalovirus was measured for 2,049 subjects but was not for the rest of the subjects. This was because the proportion of subjects who had a positive response to cytomegalovirus was very high (more than 99%) among those whose antibody level had been measured before that time. A high prevalence of positive IgG antibody response to cytomegalovirus was also reported in a different region of Japan (*22*).

Table 2 shows the association of the level of IgG antibody to *Chlamydia pneumoniae* with radiation dose. Since antibody level was significantly associated with male gender, older age, and cigarette smoking, we adjusted for those factors in addition to city in the multivariate regression analysis. After the adjustments, the serum level of IgG an-

TABLE 1
Antibody Level and Positivity among Atomic Bomb Survivors

	Radiation dose (mGy)		
	0	1–999	≥1000
Number of individuals[a]	1,481	1,368	627
Mean age (years)	71.6	72.1	70.8
Mean dose (mGy)	0	315.8	1524.9
Male (%)	32.1	28.6	37.8
Mean BMI (SE)	23.0 (0.09)	23.1 (0.1)	22.4 (0.1)
Current smoker (%)	14.3	14.2	13.1
Antibody level[b]			
Chlamydia pneumoniae, IgG			
Median level (U)	2.31	2.19	2.00
Mean level (SE) (U)	2.69 (0.036)	2.59 (0.052)	2.49 (0.081)
Positivity (%)	77.7	75.4	71.1
Chlamydia pneumoniae, IgA			
Median level (U)	1.63	1.54	1.41
Mean level (SE) (U)	1.94 (0.036)	1.87 (0.038)	1.79 (0.055)
Positivity (%)	67.9	66.1	63.0
Helicobacter pylori, IgG			
Median level (U)	41.1	38.2	45.2
Mean level (SE) (U)	48.2 (1.1)	46.8 (1.0)	49.9 (1.7)
Positivity (%)	62.7	60.1	63.0
Cytomegalovirus, IgG			
Median level (U)	47.8	51.7	47.4
Mean level (SE) (U)	61.9 (1.8)	62.4 (1.8)	62.0 (2.6)
Positivity (%)	99.2	99.6	99.2
CRP level			
Median level (mg/dl)	0.062	0.066	0.062
Mean level (SE) (mg/dl)	0.11 (0.004)	0.12 (0.004)	0.12 (0.006)

[a] For the measurement of cytomegalovirus IgG, the numbers of subjects were: 0 Gy: 858, 1–999 mGy: 816, and ≥1000 mGy: 375.

[b] Antibody level is presented as median and mean values for quantitative measurements. Positivity is the proportion of individuals whose antibody level was defined as positive, as described in the Materials and Methods, for each dose category.

tibody to *Chlamydia pneumoniae* was significantly and negatively associated with radiation dose. The level of IgA antibody to *Chlamydia pneumoniae* was significantly and positively associated with male gender, older age and cigarette smoking and negatively associated with radiation dose (Table 2). This antibody level was significantly higher in subjects from Nagasaki than in those from Hiroshima (Table 2). The distribution of levels of IgG and IgA antibody to *Chlamydia pneumoniae* in relation to radiation dose is presented in Figs. 1 and 2, respectively. No significant

TABLE 2
Association of Levels of IgG and IgA Antibodies to *Chlamydia pneumoniae* with Radiation Dose (multiple regressions)[a]

	Chlamydia pneumoniae					
	IgG			IgA		
Factors (unit)	Change (%)	95% CI	*P*	Change (%)	95% CI	*P*
Dose (1,000 mGy)	−14.2	−19.7, −8.3	<0.001	−4.9	−8.9, −0.1	0.021
Age (10 years)	29.5	21.5, 38.0	<0.001	18.3	13.6, 23.3	<0.001
Gender (Male: 0, Female: 1)	−34.2	−41.7, −25.4	<0.001	−22.9	−28.6, −11.4	<0.001
Smoking (20 cigarettes/day)	20.1	1.9, 41.6	0.029	19.3	7.3, 32.7	0.001
City (Hiroshima: 0, Nagasaki: 1)	3.8	−6.8, 15.5	0.50	8.9	1.6, 16.7	0.016

[a] Association was examined by linear regression analysis, adjusting for each factor. Values are indicated as percentage change of the antibody level per unit (in parentheses) increase for each factor. Representative antibody level (intercept) (95% CI) for nonirradiated and nonsmoking males at 70 years of age in Hiroshima was calculated to be 2.06 (1.83, 2.32) U for IgG and 1.52 (1.41, 1.65) U for IgA. CI, confidence interval.

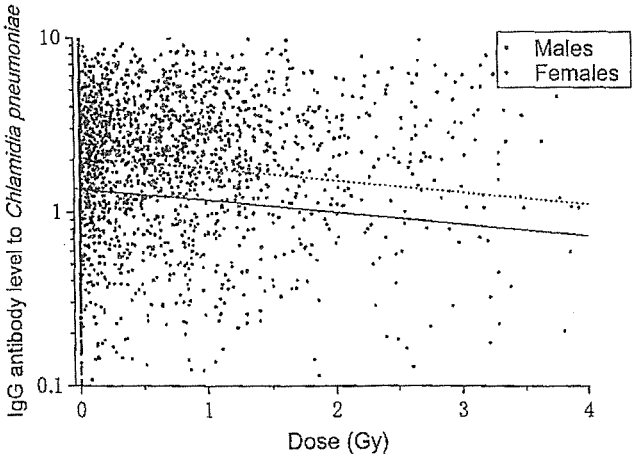

FIG. 1. Scatter diagram of IgG antibody level and radiation dose with regression lines fitted for nonsmoking male (dotted line) and female (solid line) at age 70 years in Hiroshima. Multiple R^2 value for the regression was 0.037.

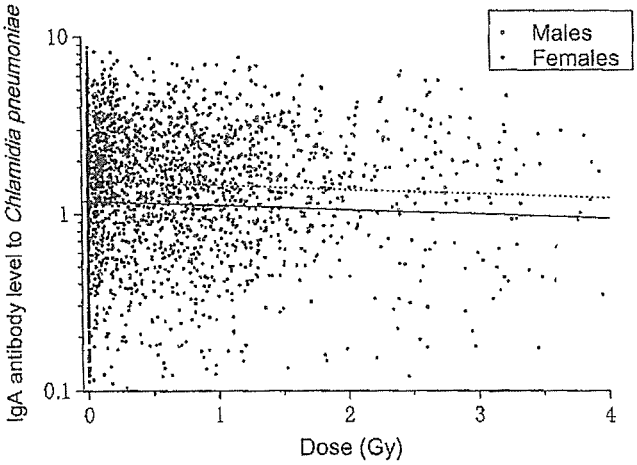

FIG. 2. Scatter diagram of IgA-antibody level and radiation dose with regression lines fitted for nonsmoking male (dotted line) and female (solid line) at age 70 years in Hiroshima. Multiple R^2 value for the regression was 0.035.

dose effect was observed for other antibody levels. The level of IgG antibody to *Helicobacter pylori* decreased with age and was not significantly associated with either cigarette smoking or radiation dose (Table 3). The level of IgG antibody to cytomegalovirus was significantly associated with female gender, older age and cigarette smoking but not with radiation dose (Table 3). In these analyses for association of antibody level with radiation dose, interactions between age, gender, smoking and radiation dose were examined, but no significant interactions were observed.

When antibody level was classified as either positive or negative, association with radiation dose was similar. Thus a negative association was observed between radiation dose and positive response to *Chlamydia pneumoniae* in both the IgG and IgA antibody classes (data not shown). Radiation dose was not significantly associated with positive IgG antibody response to *Helicobacter pylori* or cytomegalovirus (data not shown).

To investigate the biological significance of the decreased antibody level to *Chlamydia pneumoniae* in radiation-exposed survivors, the serum level of CRP, a sensitive marker of inflammation, was examined. In this analysis, samples with CRP levels greater than 1.0 mg/dl were excluded from analysis to avoid confounding by acute infectious disease and systemic inflammatory disease samples. Thus a total of 3,155 samples were analyzed. CRP level was positively associated with radiation dose, age, male gender, smoking and BMI (Table 4). CRP level also differed between Hiroshima and Nagasaki (Table 4). The distribution of the CRP level in relation to radiation dose is presented in Fig. 3.

After adjustment for the factors presented in Table 4, the CRP level was positively and significantly associated with the level of IgA antibody to *Chlamydia pneumoniae* (3.7% increase per 1 U increase in antibody level, $P = 0.006$) but only marginally with the level of IgG antibody to *Chlamydia pneumoniae* ($P = 0.057$) (Table 5). In these analyses, association of CRP level with radiation dose did not change substantially from the results presented in Table 4 [percentage increase in CRP level per 1,000 mGy was 7.0% ($P = 0.004$) and 7.1% ($P = 0.003$) when IgG and IgA anti-

TABLE 3
Association of Levels of IgG Antibody to *Helicobacter pylori* and Cytomegalovirus with Radiation Dose (multiple regressions)[a]

Factors (unit)	*Helicobacter pylori* IgG			Cytomegalovirus IgG		
	Change (%)	95% CI	*P*	Change (%)	95% CI	*P*
Dose (1,000 mGy)	8.9	−10.2, 32.0	0.39	1.9	−3.2, 7.1	0.46
Age (10 years)	−20.6	−34.7, −3.5	0.021	18.1	12.6, 23.8	<0.001
Gender (Male: 0, Female: 1)	−60.4	−72.9, −11.4	<0.001	37.9	26.0, 50.9	<0.001
Smoking (20 cigarettes/day)	8.8	−31.7, 73.2	>0.5	31.4	16.5, 48.2	<0.001
City (Hiroshima: 0, Nagasaki: 1)	19.1	−12.7, 62.4	0.271	2.1	−5.9, 10.8	>0.5

[a] Association was examined by linear regression analysis, adjusting for each factor. Values are percentage change of the antibody level per unit (in parentheses) increase of each factor. Representative antibody level (intercept) (95% CI) for nonirradiated and nonsmoking males at 70 years of age in Hiroshima was calculated to be 58.8 (42.1, 82.1) U for *Helicobacter pylori* IgG and 32.8 (30.0, 35.9) U for cytomegalovirus IgG. CI, confidence interval.

TABLE 4
Association of CRP Level with Radiation Dose (multiple regressions)[a]

Factors (unit)	Change (%)	95% CI	*P*
Dose (1,000 mGy)	6.9	2.1, 11.9	0.004
Age (10 years)	20.4	15.0, 26.1	<0.001
Gender (Male: 0, Female: 1)	−18.8	−25.1, −11.9	<0.001
Smoking (20 cigarettes/day)	27.5	14.1, 42.4	<0.001
BMI (1.0)	7.7	6.6, 8.8	<0.001
City (Hiroshima: 0, Nagasaki: 1)	−12.4	−18.5, −5.7	<0.001

[a] Association was examined by linear regression analysis, adjusting for each factor. Values represent the percentage change of CRP level per unit (in parentheses) increase of each factor. Representative CRP level (intercept) (95% CI) for nonirradiated and nonsmoking males at 70 years of age in Hiroshima was calculated to be 0.072 (0.67, 0.79) mg/dl. BMI, body mass index. CI, confidence interval.

body levels were included in the model, respectively]. In addition, when CRP was included in the adjustment factors for analysis of the relationship between antibody level and radiation dose, the results were similar to those presented in Table 2. Thus a 15.3% decrease in antibody level per gray was observed for IgG (when CRP was not included, a 14.2% decrease was observed, as indicated in Table 2) and a 5.1% decrease for IgA (when CRP was not included, a 4.9% decrease, as indicated in Table 2).

The response of CRP to the *Chlamydia pneumoniae* antibody level did not interact formally with radiation dose (significance level of the interaction between radiation dose and antibody level: $P = 0.36$ for IgG and $P \geq 0.5$ for IgA). To further investigate the decreased antibody levels in radiation-exposed survivors, however, the relationship between CRP level and antibody levels was examined in different dose categories. In this analysis, a significant association of CRP level with IgA antibody level was noted in the high-dose group (≥1000 mGy) but not in the 0-Gy or 1–999-mGy groups (Table 5). The percentage increase in CRP levels per unit of antibody in the high-dose group was approximately twice that in the nonirradiated and intermediate-dose groups (Table 5), although the difference between the groups was not statistically significant. The relationship between CRP level and the level of IgG antibody to *Chlamydia pneumoniae* was also significant in the high-dose group but not in the 0-Gy or 1–999-mGy groups (Table 5).

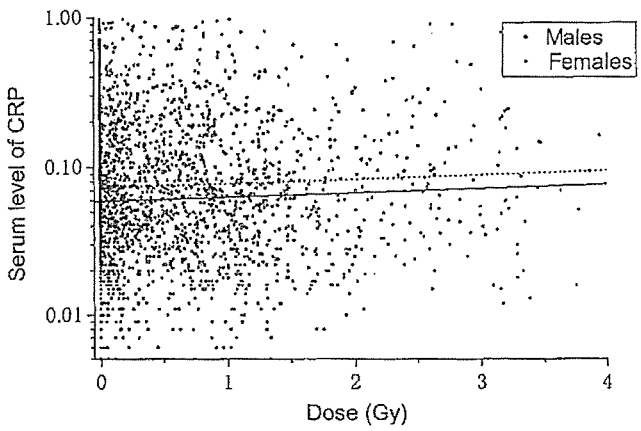

FIG. 3. Scatter diagram of CRP level and radiation dose with regression lines fitted for nonsmoking male (dotted line) and female (solid line) with BMI of 23.0 kg/m² at age 70 years in Hiroshima. Multiple R^2 value for the regression was 0.086.

DISCUSSION

In this cross-sectional study of A-bomb survivors, we observed a radiation dose-associated decrease in the level of antibody to *Chlamydia pneumoniae*. The effects of gender and smoking on level of antibodies to the three pathogens observed in the present study were similar to those reported previously (*23–25*). The limitations of our study may be as follows. Since more than half of the original cohort had died before the start of this study, the possibility cannot be excluded that persons with higher antibody levels might have been preferentially lost from the group of subjects with higher radiation doses. It is also possible that some persons could not participate in the study because of

TABLE 5
Association of CRP Level with Antibody Level to *Chlamydia pneumoniae* Stratified by Radiation Dose[a]

	CRP level			
	No. of subjects	Change (%)	95% CI	*P*
Chlamydia pneumoniae IgG				
Total samples	3,155	1.8	−0.01, 3.7	0.057
0 mGy	1,355	1.2	−1.0, 3.5	0.287
1–999 mGy	1,239	1.5	−0.8, 3.9	0.201
≥1,000 mGy	561	3.5	0.5, 6.6	0.024
Chlamydia pneumoniae IgA				
Total samples	3,155	3.7	1.0, 6.5	0.006
0 mGy	1,355	2.9	−1.3, 6.1	0.075
1–999 mGy	1,239	3.3	−0.1, 6.8	0.055
≥1,000 mGy	561	6.5	2.0, 11.2	0.005

[a] Association was examined by multiple regression analysis, adjusting for city, gender, age, body mass index, and smoking. Percentage change in CRP level is presented for one unit increase in antibody level. CI, confidence interval.

illness related to high or low antibody levels. Further, some other unknown factor(s) not adjusted for in the present analysis might have confounded the results.

Biologically, two possibilities exist for the lower antibody response to bacterial infection. One is a decrease in infectious agents; the other is a decrease in immune response to post-bombing bacterial infection. The former seems less likely in A-bomb survivors due to the exacerbation of hygiene conditions by the complete destruction of lifeline and medical systems after the bombings of the two cities. Diminished immune function has been suggested in radiation-exposed survivors. Thus lymphocyte proliferation induced by mitogens, alloantigens and bacterial superantigens (*13, 16*), the proportion of T lymphocytes, especially CD4-positive helper T lymphocytes (*14*), and the frequency of T lymphocytes producing interleukin 2 (*15*) are all diminished among radiation-exposed survivors. In addition to these nonspecific decreases in immune function, there may be some antigen-specific impairment among A-bomb survivors, as suggested by repertoire analysis examining the usage of divergent variable region genes of T-lymphocyte receptors in the blood (*26*). Since T lymphocytes are essential for antibody production of B cells, impairment in T lymphocytes in the survivors may affect antibody production.

Similarly, diminished immune response has been implicated in other infections in the survivors. It has repeatedly been shown, for example, that prevalence of hepatitis B surface antigen is increased with radiation dose in A-bomb survivors, whereas prevalence of its antibody is not (*17–19*). Although a smooth dose–response relationship was not observed between the prevalence of antibody to hepatitis C virus and radiation dose, prevalence of the antibody was lower in survivors with a positive dose estimate than in those with a dose estimate of 0 (*27*).

The mechanism behind the absence of a significant association between antibody levels to *Helicobacter pylori* and cytomegalovirus with radiation is not clear, but it may be related to the difference in the frequency of recurrent infection by these pathogens. The first infection among the three pathogens occurs early in childhood, and subsequent recurrent infection is common only for *Chlamydia pneumoniae* (*28*). If immune function at the time of *Chlamydia pneumoniae* infection is related to antibody level, diminished immune function due to A-bomb exposure at the time of the recurrent infection might cause decreased antibody response. Naturally, we cannot estimate either the timing of infection or the cause of the antibody level (i.e., past primary infection, re-infection or reactivation). Of interest is that the increased prevalence of hepatitis B surface antigen with radiation dose among A-bomb survivors is restricted to those who received blood transfusions after A-bomb radiation exposure (*19*). Since persistence of hepatitis B virus antigen is related to diminished immune response to the virus, the radiation dose-associated increase in the prevalence of hepatitis B antigen among the survivors who received blood transfusions suggests impaired immune function from exposure to A-bomb radiation.

Although interaction with radiation dose was not formally significant for the association of CRP level with antibody level, we found a tendency for CRP level to be associated with levels of both IgG and IgA antibodies to *Chlamydia pneumoniae* in the high-dose group. This finding may suggest the presence of infection-related inflammation in heavily exposed survivors. Since persistent infection in macrophages is typically found after the acute phase of *Chlamydia pneumoniae* infection ends, association of CRP level with antibody level may reflect an active state of chronic infection.

We also confirmed, with a larger number of subjects, our previous finding of a positive association of CRP level with radiation dose (*29*). This finding could prove to be important because many studies have demonstrated that a mildly elevated CRP level is a risk factor for cardiovascular disease (*8, 30*). Association of CRP level with male gender, BMI and smoking, which was found in the present study, was also reported previously (*31*). The reason for the city difference in CRP level found in the present study is unclear. Systemic technical error is not likely because all samples were measured in the Hiroshima laboratory. Since the prevalence of diabetes mellitus among A-bomb survivors is lower in Nagasaki than in Hiroshima (*32*), and since diabetes is associated with increased CRP levels (*33*), we adjusted for diabetes in our analysis, but the city difference remained significant (data not shown).

In conclusion, our study suggests that the immune response to *Chlamydia pneumoniae*, a microorganism implicated in cardiovascular pathogenesis, is diminished in radiation-exposed A-bomb survivors. However, inflammatory response to *Chlamydia pneumoniae* may be present, which would reflect an active state of infection in the survivors.

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（公表論文 2）

Prevalence of atherosclerosis in relation to atomic bomb radiation exposure: An RERF Adult Health Study

大規模コホート共同研究に関連した研究成果

大動脈弓の石灰化と頚動脈の内膜中膜肥厚（IMT: intima-media thickness）を指標として、関連するリスク要因を調べる横断的研究を実施した。成人健康調査の目的である放射線被曝の影響だけでなく、白血球数、ヘモグロビン A1c（HbA1c）、総ｺﾚｽﾃﾛｰﾙ、HDL ｺﾚｽﾃﾛｰﾙ、ｸﾚｱﾁﾆﾝ、収縮期血圧（SBP）、BMI、喫煙の影響についても検討した。2000 年 10 月から 2002 年 5 月に受診した男性 615 人、女性 1189 人に対し、胸部レントゲン検査、頚動脈超音波検査、末梢血検査、生化学検査、身長・体重測定と問診よる喫煙情報の聴取を行った。対象者の年齢は 54 歳以上、男性の平均年齢は 66 歳、女性の平均年齢は 71 歳であった。大動脈弓の石灰化は石灰化無し、軽度石灰化（点状石灰化または細い線状石灰化）、重度石灰化（1 つ以上の大きな石灰化）の 3 ｶﾃｺﾞﾘｰで評価した。IMT は 11MHz のﾌﾟﾛｰﾌﾞを用いて頚動脈分岐部の 15-20ｍｍ中枢側の総頚動脈を測定した。大動脈弓の軽度石灰化、重度石灰化共に女性の有病率が高く、リスク要因に関する解析は男女別に行った。男性の大動脈弓の石灰化には年齢、HbA1c、放射線線量が女性の大動脈弓の石灰化には年齢、喫煙、SBP、放射線線量が関係していた。IMT は高年齢、総ｺﾚｽﾃﾛｰﾙ高値、HDL ｺﾚｽﾃﾛｰﾙ低値、SBP 高値で高値を示した。大動脈弓の石灰化と IMT のリスク因子は異なっていた。この結果は個人の有するリスク因子の違いにより、動脈硬化がより進展した部位も異なるかもしれない事を示唆するが、縦断的研究により検討する必要がある。

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Prevalence of atherosclerosis in relation to atomic bomb radiation exposure: An RERF Adult Health Study

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Abstract

Purpose: To determine whether exposure to atomic bomb radiation altered the prevalence of asymptomatic atherosclerosis.
Material and methods: In a cross-sectional analysis, we examined aortic arch calcification by plain chest radiography and common carotid artery intima-media thickness (IMT) by ultrasonography among 1804 survivors of the atomic bombing in Hiroshima. We evaluated the association between atherosclerotic changes and radiation exposure, while adjusting for potentially confounding factors.
Results: Multivariate logistic regression analysis showed that aortic arch calcification was significantly associated with radiation exposure ($p < 0.05$). The odds ratio at 1 Gy was 1.30 (95% confidence interval [CI]: 1.05–1.53) for men and 1.31 (95% CI: 1.13–1.51) for women. Carotid artery IMT did not vary significantly with radiation dose ($p = 0.18$).
Conclusion: Radiation dose contributed to the prevalence of aortic atherosclerosis but not carotid artery atherosclerosis in atomic bomb survivors.

Keywords: *Atherosclerosis, atomic bomb radiation exposure, aortic arch calcification, carotid artery intima-media thickness, risk factors*

Introduction

The association between radiation and cardiovascular disease has been investigated in therapeutic and occupational settings (Trivedi & Hannan 2004). Therapeutic exposure of the neck, head, or thorax to a relatively high cumulative dose is a risk factor for cardiovascular disease (Levinson et al. 1973, Corn et al. 1990, Boivin et al. 1992, Rutqvist et al. 1992, Hancock et al. 1993, Franklyn et al. 1998) and subclinical findings such as accelerated carotid artery atherosclerosis are also observed (Chung et al. 1994, Dubec et al. 1998, King et al. 1999). A cardiovascular effect for low-dose radiation exposure, however, is not well established. Increased mortality from cardiovascular disease was reported among the Chernobyl emergency workers (Ivanov et al. 2001) and among US radiologic technologists with occupational radiation exposure before 1950, when radiation doses were likely to be high (Hauptmann et al. 2003), but no association between cardiovascular disease and radiation exposure was shown among British radiologists (Berrington et al. 2001). McGale and Darby (2005) reviewed the published literature and concluded that epidemiological data other than from the atomic bomb survivors do not provide clear evidence of a risk of circulatory system disease at doses of ionizing radiation in the range 0–4 Sv.

In atomic bomb survivors, statistically significant increases in mortality were seen for heart disease and stroke in the Life Span Study (LSS) by the Radiation Effects Research Foundation (RERF) (Shimizu et al. 1999, Preston et al. 2003). In the Adult Health Study (AHS), a subcohort of a high-exposure group and controls in the LSS, the incidence of myocardial infarction (MI) between 1968 and 1998 was increased among younger (less than 40 years old at the time of the bombings), heavily exposed subjects (Wong et al. 1993, Yamada et al. 2004).

Radiation effects on the conventional cardiovascular disease risk factors were reported in the AHS. Increased serum cholesterol levels were observed among exposed subjects for all birth cohorts

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(Wong et al. 1999), as was increased blood pressure among those born in or after 1930 (Sasaki et al. 2002).

Atherosclerosis of the aorta and carotid artery can be detected by non-invasive techniques. Aortic arch calcification has been estimated by chest X-rays taken during the RERF biennial health examinations since 1958 and intima-media thickness (IMT) in the common carotid artery has been measured by ultrasonography since 2000. Since atherosclerosis in the aorta or carotid arteries is a predictor of coronary heart disease (Nagai et al. 1998, O'Leary et al. 1999, Iribarren et al. 2000, Li et al. 2002, Symeonidis et al. 2002), we can detect the subclinical effects of atomic bomb radiation exposure on such diseases by measuring aortic arch calcification and carotid IMT.

Materials and methods

Subjects and procedure

The AHS cohort in Hiroshima and Nagasaki at its inception in 1958 consisted of 10,000 survivors who were within 2000 m from the hypocenter at the time of bombings, 5000 survivors who were beyond 3000 m, and 5000 controls who were not in the city at the time of the bombings. After 1976, follow-up of controls (not in the city group) was discontinued and about 3,500 survivors were added to the cohort. They have been followed by biennial clinical examinations. Details are available elsewhere (Wong et al. 1993). The clinical follow-ups include physical examinations, laboratory measurements, height and weight measurements, questionnaires regarding smoking habits, plain chest X-ray, and ultrasonography. The patients provided informed consent, and the study complied with institutional guidelines regarding research ethics and the welfare of human subjects.

The study subjects are the AHS subjects in Hiroshima. About 55% of the original AHS subjects had died and about 7% of them had moved away from the area by 2000. The participation rate for the AHS subjects living in Hiroshima was 74%. A total of 2267 Hiroshima subjects participated in the clinical examinations between October 2000 and May 2002. Excluded from the study were 101 subjects who were examined outside RERF and did not undergo chest radiography and ultrasonography, 186 subjects who refused chest radiography and/or ultrasonography, 162 subjects for whom dose estimates were lacking, and 14 subjects for whom height and weight measurements were missing. If a subject had two examinations during the study period, the first examination was selected for the analysis. The final sample consisted of 615 men and 1189 women.

The Dosimetry System 86 dose comprised the sum of gamma rays and included the use of neutrons. The dose component was truncated to 4 Gray (Gy), in consideration of the imprecision in dose assessment for high-dose subjects. The doses received were under 0.005 Gy for 269 men and 472 women (non-exposed), 0.005–0.499 Gy for 182 men and 408 women, 0.5–0.999 Gy for 66 men and 159 women, and $\geq$1.0 Gy for 98 men and 150 women. The mean dose for exposed subjects was 794 mGy for men and 701 mGy for women. There was no evidence that the participation rates of the clinical examinations, chest radiography, or ultrasonography differed in relation to radiation doses.

Posterior-anterior chest radiographs were obtained during deep inspiration in a standing position by Fuji Computed Radiography (Fuji Film Medical, Tokyo, Japan) with 17 × 17 in film. We reviewed the films for the purpose of this study without knowledge of the study participants. We graded aortic arch calcification into three categories: no calcification (no visible calcification), mild calcification (small spots or a single thin area of calcification), or severe calcification (one or more areas of thick calcification).

We performed carotid artery ultrasonography using GE Yokogawa LOGIQ500MDMR3 imaging units (GE Yokogawa Medical Systems, Hino, Japan) with an 11 MHz probe. We measured the IMT of the far wall of the common carotid artery at a 15–20 mm segment proximal to the bifurcation manually at three points using high-resolution images, and we calculated the mean value as the value representing individual carotid IMT.

Statistical analysis

We applied a polytomous logistic regression model with proportional odds assumption for evaluating the three category ordered outcome variables of aortic arch calcification. The multivariate models included covariates at the time of the evaluation of atherosclerotic changes for age, white blood cell count (WBC), hemoglobin A1c (HbA1c), total cholesterol, high-density lipoprotein (HDL) cholesterol, creatinine, systolic blood pressure (SBP), body mass index (BMI) calculated as weight in kilograms divided by height in meters squared, and number of cigarettes smoked per day. We treated all covariates as continuous values. Since subjects lacking dose or BMI were excluded from the study, there were no missing data. Independent risk factors were identified by sex, because previous reports showed sex differences in aortic arch calcification risk predictors (Iribarren et al. 2000, Li et al. 2002). Linear regression models were used to identify risk factors for carotid IMT. Sex was included as a covariate in the analysis of carotid IMT.

We used the LOGISTIC procedure for analysis of aortic arch calcification and the REG procedure for

analysis of carotid IMT in the SAS program (SAS version 6.11, SAS Institute Inc, Cary, NC, USA) for the estimation of the parameters and testing.

Results

Table I shows the characteristics of the subjects by sex and exposure category. The age range at the time of the evaluation of atheroscrelotic changes was 55–95 years.

Prevalence of mild aortic arch calcification was 26.1% for men and 31.9% for women and that of severe calcification was 8.8% for men and 19.1% for women. Prevalence of aortic arch calcification increased for older subjects ($p < 0.001$) and for women ($p < 0.05$). Proportion of aortic arch calcification severity by sex and dose category is shown in Table II.

Table III shows the results of multiple logistic regression analysis for aortic arch calcification in which age, dose, WBC, HbA1c, total cholesterol, HDL cholesterol, creatinine, smoking, SBP, and BMI were simultaneously entered as independent variables. The proportional odds ratio for 1 Gy increments of radiation dose was 1.30 for men ($p = 0.012$) and 1.31 for women ($p = 0.0003$). Older age was independently associated with aortic arch calcification in both sexes. Aortic arch calcification was related to higher HbA1c in men and smoking and higher SBP in women.

Quartile values of carotid IMT by sex and dose category are shown in Table II. The carotid IMT value was positively associated with age, total-cholesterol, and SBP, and negatively associated with HDL cholesterol (Table IV). Radiation dose was not associated with carotid IMT.

Table I. Characteristics of subjects by sex and radiation dose.

	Men		Women	
Dose (Gy)	<0.005	≥0.005	<0.005	≥0.005
Number	269	346	472	717
		Mean (SD)		
Age	65.9 (9.0)	67.4 (8.8)	71.0 (9.5)	71.7 (9.1)
WBC (coount/mm^3)	6070 (160)	6230 (190)	5420 (150)	5720 (160)
HbA1c (%)	5.5 (0.7)	5.7 (1.0)	5.6 (1.0)	5.5 (0.9)
Total-cholesterol (mg/dl)	200 (33)	201 (36)	216 (35)	217 (35)
HDL-cholesterol (mg/dl)	59 (17)	57 (15)	68 (18)	67 (17)
Creatinine (mg/dl)	0.9 (0.7)	0.8 (0.3)	0.6 (0.2)	0.7 (0.4)
BMI (Kg/m^2)	22.8 (3.1)	22.7 (3.0)	23.2 (3.5)	23.0 (3.9)
SBP (mmHg)	134 (18)	135 (19)	135 (21)	136 (20)
DBP (mmHg)	82 (10)	82 (10)	79 (10)	79 (10)
Current smoker (%)	44	38	6	8
Cigarettes/day	19	20	15	14

WBC, white blood cell count; HbA1c, HemoglobinA1c; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Table II. Aortic calcification and carotid intima media thickness by sex and dose category.

	Aortic calcification					
	Men			Women		
Dose (Gy)	no	mild	Severe	no	mild	severe
<0.005 Gy	68.8%	25.3%	5.9%	51.9%	30.3%	17.8%
0.005–0.499 Gy	68.1%	24.2%	7.7%	51.7%	30.4%	17.9%
0.5–0.999 Gy	53.0%	33.3%	13.6%	44.0%	33.3%	22.6%
≥1.0 Gy	58.2%	27.6%	14.3%	38.0%	39.3%	22.7%
	Intima Media Thickness (mm)					
	Men			Women		
Dose (Gy)	Lower quartile	Median	Higher quartile	Lower quartile	Median	Higher quartile
<0.005 Gy	0.60	0.70	0.77	0.60	0.70	0.83
0.005–0.499 Gy	0.60	0.70	0.83	0.63	0.70	0.83
0.5–0.999 Gy	0.60	0.70	0.80	0.60	0.70	0.83
≥1.0 Gy	0.63	0.70	0.83	0.63	0.70	0.87

Table III. Odds ratio and 95% confidence interval (CI) of aortic arch calcification.

Variable	Men		Women	
	Odds ratio	95% confidence interval	Odds ratio	95% confidence interval
Age (10 years)	2.54**	2.04, 3.20	3.00**	2.58, 3.49
Dose (1 Gy)	1.30*	1.05, 1.53	1.31**	1.13, 1.51
WBC (100 cells)	1.01	1.00, 1.02	1.00	0.99, 1.01
HbA1c (1%)	1.29**	1.06, 1.56	1.08	0.94, 1.22
Total cholesterol (10 mg/dl)	0.99	0.94, 1.05	0.99	0.96, 1.03
HDL cholesterol (10 mg/dl)	1.01	0.90, 1.14	1.02	0.95, 1.10
Creatinine (1 mg/dl)	1.35	0.94, 2.00	0.95	0.69, 1.27
Smoking (10 cigarettes)	0.96	0.81, 1.13	1.64**	1.24, 2.19
SBP (10 mmHg)	1.06	0.96, 1.16	1.14**	1.07, 1.21
BMI (Kg/m^2)	1.01	0.95, 1.07	1.00	0.96, 1.03

The abbreviations are the same as Table I. $^{**}p < 0.01$ $^{*}p < 0.05$.

Table IV. Linear regression analysis of carotid intima media thickness.

Variable	regression coefficient	p value
Age (years)	0.00705	<0.0001
Sex (women/men)	−0.01732	0.08
Dose (Gy)	0.00719	0.18
WBC (100 cells)	−0.00012	0.66
HbA1c (%)	0.00101	0.82
Total cholesterol (mg/dl)	0.00029	0.02
HDL cholesterol (mg/dl)	−0.00085	0.001
Creatinine (mg/dl)	0.01222	0.24
Smoking (1 cigarette)	0.00056	0.30
SBP (mmHg)	0.00103	<0.0001
BMI (Kg/m^2)	0.00056	0.65
R^2	0.158	

The abbreviations are the same as Table I.

Discussion

Although mortality from circulatory system diseases increased with radiation dose among atomic bomb survivors, other epidemiological data investigating the association between low doses of ionizing radiation and circulatory diseases have not provided clear evidence of such a relationship (McGale & Darby 2005). Few subclinical markers of atherosclerosis for low-dose radiation exposure have been examined; meanwhile, the development of carotid atherosclerosis after radiation therapy with high-dose exposure has been reported (Chung et al. 1994, Dubec et al. 1998, King et al. 1999). In one study (Kawamura et al. 1992), severe aortic arch calcification significantly increased with atomic bomb radiation dose, but observer bias for detection of aortic arch calcification was large and the detection rate was low (less than half the prevalence of severe calcification in the present study), probably because the X-rays used were taken to detect lung lesions. In the present study, we reviewed films taken for the specific purpose of detecting aortic arch calcification, but it was still sometimes difficult to determine the level of calcification. To justify the results of analysis based on dose as a continuous value, we also estimated odds ratios for categories of dose. The relatively lower dose category (0.005–0.499 Gy) did not show any significant effects on aortic arch calcification based on comparison with the controls (<0.005 Gy). Odds ratio for the relatively high dose category (≥0.5 Gy) were 1.63 for men ($p = 0.02$) and 1.48 for women ($p = 0.007$), based on an age adjusted model. Estimated odds ratios were similar after adjusting for all categorical confounders. Our finding shows a stronger association between radiation dose and aortic arch calcification in both sexes.

Oxidation (Sakurai & Sawamura 2003) and inflammation (Ross 1993, Hallahan et al. 1996) are implicated in atherosclerosis. Since significant association between inflammation in atomic bomb survivors and radiation dose was observed (Neriishi et al. 2001) and increased expression of cell adhesion molecules such as E-selectin (1 Gy) (Hallahan et al. 1996) and intercellular adhesion molecule-1 (2–5 Gy) (Hong et al. 1995) was induced by irradiation, aortic arch atherosclerosis may develop through these mechanisms.

In this study, radiation dose was not related to the carotid IMT. Some reports show regional differences in the development of atherosclerosis (Tanaka et al. 1988), with the aorta showing the most rapid disease progression. Atherosclerotic changes in the aorta are frequently observed in the arch, where changes of shear stress and turbulence occur (Friedman & Ehrlich 1975). The hemodynamic differences between the aortic arch and the common carotid artery might be a reason for the apparent difference in the effect of atomic bomb radiation exposure. Capillaries and small arteries are the most radiosensitive vessels (Hopewell et al. 1993, Fajardo 1999). Microscopic

observation of the superficial minute vessels of atomic bomb survivors in the 1950s showed increased capillary abnormalities among subjects exposed proximally to the hypocenter (Tsuya et al. 1969). Atherosclerosis in the aorta might be caused by injury to the vasa vasorum.

Conventional risk factors for cardiovascular disease, such as high blood pressure, high serum cholesterol level, and smoking, are associated with carotid artery atherosclerosis (Heiss et al. 1991, Crouse et al. 2002) and aortic arch calcification (Iribarren et al. 2000, Li et al. 2002). Although few studies have investigated risk factors for different arterial regions simultaneously, risk factors for carotid artery and aortic arch calcification differ by study population and study design. Although no other study has investigated risk factors for aortic arch calcification among Japanese, the results of this study, showing that risk factors for aortic arch calcification were age, HbA1c, smoking, and SBP, were consistent with the result that risk factors for coronary artery calcification assessed by cinefluoroscopy were age, hypertension, and diabetes mellitus but not total cholesterol, HDL cholesterol, or obesity in Japanese clinical data (Yamanaka et al. 2002). In middle-aged working Japanese men, age, blood pressure, total cholesterol, and HDL cholesterol were associated with carotid IMT (Fujii et al. 2003), as indicated by this study. This study did not include certain other conventional risk factors, such as alcohol consumption, menopausal status, and family history, because the information was not available for some of the participants.

The present study has several limitations. First, it was cross-sectional study, thus introducing the possibility of a sample bias such as a lower participation rate among subjects with severe atherosclerosis. A longitudinal analysis using long-term AHS data is needed to avoid this kind of participation bias and to elucidate causal relationships with risk factors. Second, grading of aortic arch calcification was subjective. Third, some important risk factors were not included in the analyses.

In this study, we investigated the relationship between atomic bomb radiation exposure and subclinical markers of atherosclerosis. After we adjusted for cardiovascular risk factors, atomic bomb radiation exposure remained a significant risk factor for aortic, but not carotid artery, atherosclerosis. This may suggest an association between atherosclerotic changes and low-dose radiation exposure.

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