

BRIEF OBSERVATION

Long-term effects of radiation dose on inflammatory markers in atomic bomb survivors

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Atomic bomb survivors have a persistently increased risk of cancer, hepatitis, and cardiovascular and autoimmune diseases.¹⁻⁴ There is no clear explanation for these late effects of radiation exposure. One hypothesis is that radiation causes chronic low-grade inflammation, with elevated circulating levels of cytokines. Proinflammatory cytokines, such as interleukin 6, tumor necrosis factor (TNF) α , and interferon γ , and anti-inflammatory cytokines, such as interleukin 10, are synthesized predominantly by macrophages and lymphocytes, and regulate the inflammatory response.^{5,6} Interleukin 6 in turn induces the synthesis of acute-phase plasma proteins, such as C-reactive protein.⁷ Increased levels of inflammatory cytokines, even within the normal range, have been associated with an increased risk of cardiovascular disease.⁸ Chronic low-grade inflammation may also influence the production of immunoglobulins by B cells.⁹

We therefore analyzed the effects of presumed radiation dose on inflammatory parameters in atomic bomb survivors.

Methods

Subjects

We studied subjects from Hiroshima who had participated in an epidemiological follow-up study of atomic bomb survivors, which collected health information from 2436 survivors during biennial medical examinations.¹⁰ Peripheral blood samples were collected between March 1995 and April 1997. We obtained institutional approval from the human investigation committee and informed consent from participants. We excluded subjects with a history of cancer or diseases that have been associated with inflammation (e.g., current upper respiratory tract infection, chronic bronchitis, collagen disease, arthritis, or myocardial infarction). We classified the other participants into four radiation dose groups: nonexposed, low dose (0.005 to 0.7 Gy), medium dose (0.7 to 1.5 Gy), and high dose (>1.5 Gy). Estimated bone marrow doses were based on the 1986 Dosimetry System.¹¹ Doses were for whole-body exposure, mainly from gamma rays but with a small neutron component. We selected 180 subjects from the nonexposed group and 90 from each of the other

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Table 1 Characteristics of the study subjects*

Characteristic	Radiation Exposure (Gy)			
	Nonexposed (n = 179)	0.005–0.7 (n = 87)	0.7–1.5 (n = 88)	>1.5 (n = 88)
	Number (%) or mean \pm SD			
Radiation dose (Gy)	0	0.3 \pm 0.2	1.1 \pm 0.2	2.1 \pm 0.5
Age (years)	68 \pm 11	69 \pm 11	67 \pm 10	68 \pm 10
Female sex	96 (54)	50 (58)	52 (59)	47 (53)
Body mass index (kg/m ²)	23 \pm 3	23 \pm 3	22 \pm 4	23 \pm 4
Current smokers	44 (25)	17 (20)	23 (26)	21 (24)

*Among atomic bomb survivors from Hiroshima, Japan.

groups, such that the age and sex distributions were similar in the four groups. Data were missing for 8 subjects; these subjects were excluded from all analyses.

Measurements

We measured plasma TNF- α , interferon γ , and interleukin 10 levels in duplicate using a highly sensitive enzyme-linked immunosorbent assay kit (Quantikine HS; R&D Systems, Minneapolis, Minnesota). Mean values of duplicate measurements were reported for all assays. We quantitated immunoglobulin levels using standard kits (Bethyl Lab. Inc., Montgomery, Texas). The interassay and intra-assay coefficients of variations of these enzyme-linked immunosorbent assay kits were lower than 10%. The erythrocyte sedimentation rate was measured using standard methods.

Statistical analysis

We estimated the effects of changes in several predictor variables (linear radiation dose, age, and sex), adjusted for current smoking and body mass index (in kg/m²) using a multivariate linear regression model based on the log of the outcome variables (biological markers). We present results as percentage changes in the outcome variables with 95% confidence intervals. All analyses were performed using SAS software (Cary, North Carolina).

Results

There were no significant differences in age, sex, body mass index, or current smoking among the four groups (Table 1). Interferon γ levels and the erythrocyte sedimentation rate increased significantly with radiation dose (Figure). Tumor necrosis factor α and interleukin 10

levels also increased slightly but not significantly with radiation dose. The levels of immunoglobulin (Ig) A and IgM increased significantly with radiation dose, but those of IgG and IgE did not.

In multivariate models, the levels of TNF- α , interferon γ , and interleukin 10, and the erythrocyte sedimentation rate, increased significantly with radiation dose, as did IgA, IgM, and total immunoglobulin levels (Table 2). The levels of TNF- α , interleukin 10, IgG, IgA, and total immunoglobulins, and the erythrocyte sedimentation rate, increased significantly with age.

Discussion

Tumor necrosis factor α , interleukin 6, interferon γ , and interleukin 10 coordinate the inflammatory response. In the present study, plasma levels of inflammatory cytokines and biomarkers (TNF- α and the erythrocyte sedimentation rate) increased with radiation dose and with age. Plasma levels of other cytokines (interferon γ and interleukin 10) and immunoglobulins (IgA and IgM) increased with radiation dose. Combined with previous results on other inflammatory signs, such as increased white blood cell counts, and sialic acid and C-reactive protein levels,^{12,13} our results provide evidence of persistent inflammatory responses in atomic bomb survivors more than 50 years after radiation exposure.

In light of these studies, we hypothesized that radiation exposure accelerated aging. To test the hypothesis, we calculated radiation exposure as a function of age using inflammatory status as an index. We estimated that exposure to 1 Gy was equivalent to an increase in age of about 9.0 years. Noting that the mean exposure among atomic bomb survivors was about 0.2 Gy, we inferred that mean accelerated aging among atomic bomb survivors was about 2 years (range, 1 to 2.5 years). Others have reported that the decrease of CD4-expressing T cells was about 4% per 10 years and 2% per Gy, implying that the decrease per Gy is equivalent to about 5 years of aging.¹⁴ Furthermore, interleukin 6 levels correlate

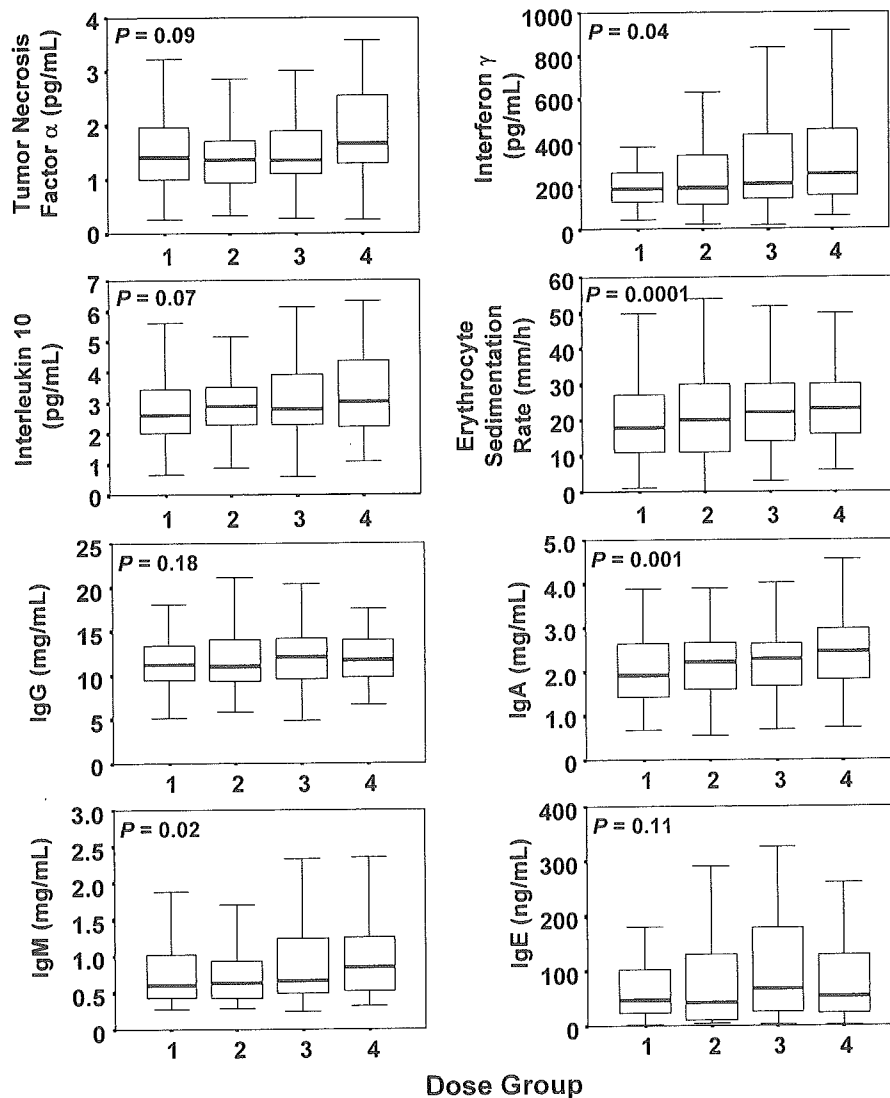


Figure 1 Box plot of inflammatory biomarker levels and erythrocyte sedimentation rate among atomic bomb survivors. The horizontal line inside the box represents the median. Lower and upper boundaries represent the 25th and 75th percentiles. Whiskers represent the smallest and largest values that are less than 1.5 box-length from the 25th and 75th percentiles. 1 indicates nonexposed; 2 indicates radiation exposure of 0.005 to 0.7 Gy; 3 indicates exposure of 0.7 to 1.5 Gy; and 4 indicates exposure of >1.5 Gy. Ig = immunoglobulin.

negatively with the percentage of CD4 T cells.¹³ Thus, acceleration of immunological aging may also be involved in radiation effects on the inflammatory status in humans.

Increased mortality and morbidity from cardiovascular disease has been observed in atomic bomb survivors,¹⁰ and elevated plasma levels of inflammatory markers, including interleukin 6, have been associated with an increased risk of cardiovascular disease.⁸ Indeed, C-reactive protein and complement are mediators of ischemic myocardial injury.¹⁵ Further, the percentage of CD4 T cells in the blood is markedly lower among atomic bomb survivors who have a history of myocardial infarction.¹⁶ We hypothesize that modification of cytokine production may be involved in the onset or progression of some of

the conditions, such as hepatitis and cardiovascular disease, which are more common in atomic bomb survivors.

Several studies have shown that radiation causes short-term inflammatory effects, such as increased plasma levels of proinflammatory cytokines, among patients who received radiation therapy.¹⁷⁻¹⁹ In addition, radiation for cancer or Hodgkin's disease leads to long-term depletion of naïve CD4 T cells,^{20,21} and pathologic cardiac changes.²² Our results suggest that radiation exposure may also produce long-term adverse effects by generating a persistent inflammatory status, manifested by cytokines and other inflammatory markers along with long-lasting impairment of CD4 T cells. Given the potential implication of our findings, follow-up of radiotherapy-treated patients is warranted to assess the asso-

Table 2 Multivariate models of the effects of age, sex, and radiation dose on inflammatory biomarkers and immunoglobulins

Variable	Tumor Necrosis Factor α		Interleukin 10		Erythrocyte Sedimentation Rate		Total Ig	IgG	IgA	IgM	IgE
	Percentage	Increment (95% Confidence Interval)	Percentage	Increment (95% Confidence Interval)	Percentage	Increment (95% Confidence Interval)					
Age per 10 years	15 (9 to 20)	4 (-4 to 12)	8 (4 to 13)	15 (9 to 20)	3 (1 to 6)	3 (1 to 6)	5 (2 to 9)	-6 (-11 to 14)	2 (-11 to 14)		
Female sex*	15 (2 to 30)	-8 (-23 to 10)	6 (0 to 12)	17 (9 to 24)	7 (1 to 13)	5 (0 to 10)	-9 (-17 to -1)	14 (1 to 28)	-51 (-63 to -34)		
Radiation dose per Gy	7 (1 to 15)	12 (2 to 23)	6 (0 to 12)	17 (9 to 24)	2 (-1 to 5)	3 (1 to 6)	8 (3 to 13)	9 (2 to 15)	14 (-3 to 32)		

Ig = immunoglobulin.

*Compared with men.

ciation between inflammatory status and the occurrence of inflammation-associated diseases.

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Perspectives on cancer immuno-epidemiology

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Estimating human cancer risk based on host-environment interaction is one task of epidemiology, and it has provided indispensable knowledge for prevention of cancer. The recent development of gene-engineered mice has also provided solid evidence about the relationship between cancer development and immunity. The aim of this review is to discuss the possible contribution of epidemiology to understanding the role of immunity in host defense against cancer, and also to assess the involvement of inflammation in the occurrence of selected cancers. Here we look at the concepts of cancer immunosurveillance and infection-inflammation-cancer, and include a brief introduction to recent studies in humans and experimental animal models. It has been postulated for many years that the immune system has the ability to recognize and eliminate nascent transformed cells in the body (so-called cancer immunosurveillance hypothesis), and this idea has recently obtained strong support from animal experiments. In humans, follow-up studies among immunosuppressed transplant recipients revealed a remarkably increased risk of not only selected malignancies, but also cancers with no known viral etiology. On the other hand, a prospective cohort study among the general population revealed that individuals with low natural cytotoxic activity of peripheral blood lymphocytes had an increased risk of cancer development. More studies are warranted to allow the construction of a model for the interaction between host immunity, aging, and the environment. The host immune system is also involved in inflammatory responses to pathogen infection: insufficient immune function of the host, or repeated infection, may result in persistent inflammation, where growth/survival factors continuously act on initiated cells. The combined use of biomarkers will be necessary to define low-grade persistent inflammation in future cohort studies; and, in addition to these phenotype marker-based cohort studies, one plausible future direction will be a genomic approach that can be undertaken within cohort studies, looking at the genetic background underlying individual variations in phenotype markers. (Cancer Sci 2004; 95: 921-929)

Epidemiological studies investigate the association between cancer development and various environmental or/and host factors in human populations, providing models to estimate cancer risk as a quantitative function of these factors (e.g., exposure levels, physiological status) among individuals. We anticipate that epidemiological studies will work well under the following conditions: 1) the intensity of factors varies among individuals (being expected to produce substantial differences in cancer risk); 2) adequate measurements are available to evaluate the intensity or grade of factors (in the case of biomarkers); 3) a relevant basic biological concept or laboratory evidence-supported working hypothesis describing the relation between cancer and these factors is available; and 4) the association between cancer and these factors, if it exists, will contribute to cancer prevention. In this review, we discuss whether

“cancer development and immunity” is a proper object of epidemiology from the above viewpoint.

The concept of multi-stage carcinogenesis implies that cancer prevention with different strategies at each stage is feasible. Recently, emphasis has been placed on defense mechanisms existing in different stages of carcinogenesis, such as detoxification of reactive metabolites derived from environmental carcinogens, trapping or decomposition of reactive oxygen species, DNA repair enzymes, and natural inhibitors of proliferating initiated cells.¹⁾ The immune system may be the body's last line of defense against cancer development, and the concept of cancer immunosurveillance-routinely eliminating nascent transformed cells in the body-was first proposed by Burnet and Thomas.^{2,3)} However, despite accumulating evidence from *in vivo* studies that the immune system dominates the development of spontaneous tumors, observations in human populations have been limited, providing only marginal support for this concept. Since cancer immunosurveillance targets preclinically existing, nascent transformed cells, it is difficult to directly evaluate the immunological effects on cancer or pre-cancerous cells just emerging in the human body. Thus, epidemiological approaches such as long-term follow-up studies of human populations may be the most suitable way to assess the relation between host immunological status and future development of cancer in humans. Efficient epidemiological evaluation of host tumor immunity is thus different from efficient cancer immunotherapy, which targets clinically recognized cancer cells that are evading natural immunosurveillance and thereby acquiring a survival advantage.⁴⁾

Another area where the host immune system is involved in cancer development may be in the sequential processes of infection-inflammation-cancer. Immunological features in the initial response to a pathogen in the host may in part determine how long and how strongly inflammation will continue after pathogen infection. The host will face chronic infection leading to persistent inflammation in the case of incomplete elimination of the corresponding pathogen, but, on the other hand, may retain homeostasis after successful eradication of the pathogen. Numerous observations of virus-related cancers have provided

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Abbreviations: STAT, signal transducer and activator of transcription; RAG, recombination activating gene; IFN, interferon; TNF, tumor necrosis factor; MHC, major histocompatibility complex; NK, natural killer; CTL, cytotoxic T lymphocyte; IL, interleukin; APC, antigen presenting cell; HLA, human leukocyte antigen; SNP, single nucleotide polymorphism; PGE, prostaglandin E. In Tables 1 and 2: WBC, white blood cell; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse-transcriptase polymerase chain reaction; RANTES, regulation on activation, normal T cells expressed and secreted; MIG, monokine induced by interferon- γ ; IP-10, interferon- γ -inducible protein-10; MCP-1, macrophage chemoattractant protein-1; CRP, C-reactive protein; ROS, reactive oxygen species; ESR, erythrocyte sedimentation rate; 8-OH-dG, 8-hydroxydeoxyguanosine; HPLC, high-performance liquid chromatography; ECD, electrochemical detector; MS, mass spectrometry; GC, gas chromatography; NICl, negative ion chemical ionization; HMdU, 5-hydroxymethyl-2'-deoxyuridine; Ig, immunoglobulin.

evidence that persistent inflammation involving repeated viral infection is a key step in carcinogenesis, although the immunological mechanisms underlying this process largely remain to be established.⁹ Specifically, the environment-caused modification of host immune responses needs to be investigated in relation to cancer as well as other inflammation-related diseases: this might provide new and important insights into cancer prevention.

Cancer immunosurveillance

Cancer immunosurveillance may involve adaptive immune responses specific for antigens on malignant cells, as well as innate immune responses to non-self status or stress-induced ligands of transformed or malignant cells. Molecular changes that consistently occur in carcinogenesis of the cells may be recognized by the immune system as "flags" on target cells, and these aberrant molecules (neoantigens) may include: 1) products of oncogenes or tumor suppressor genes that are often mutated or products of other genes mutated due to genetic instability (e.g., Ras, Bcr/abl, p53),⁶ 2) normal cellular proteins that are overexpressed or aberrantly expressed (e.g., MAGE, tyrosinase, gp100),⁷ 3) oncogenic virus products (e.g., papillomavirus E6 and E7, EBNA-1, SV40 T antigen),⁸ and 4) overexpression of stress-inducible proteins (e.g., NKG2D ligands: MICA, MICB, ULBPs).⁹ Several mechanisms in which numerous other normal cellular molecules are involved can work to recognize, suppress, and/or eliminate tumor cells (Fig. 1). One of the key mechanisms in adaptive immunity for cases 1) to 3) involves the recognition of MHC/peptide com-

plexes by T cells: tumor cells expressing mutated oncogene products can be eliminated *in vivo* by tumor-specific T cells that recognize MHC/peptide complexes in which the peptide components are encoded by mutant DNA sequences. However, some tumor cells can escape detection and survive when the mutated gene products in question are not presented as MHC/peptide complexes.¹⁰

On the other hand, innate immune responses for case 4) target a great variety of abnormal cells showing cellular transformation, infection, and distress, specifically in cases where the expression of MHC class I molecules is lost or downregulated ("missing-self"): NK cells can recognize and kill cells which overexpress the ligands of NKG2D, an activating NK receptor.¹⁰ Here, NK cell effector functions are regulated by a balance between inhibitory receptors specific for MHC class I and activating receptors, although this NKG2D-mediated activation may be able to overcome the MHC class I-mediated inhibitory signaling in responding NK cells.¹¹ Clearly these two immunological mechanisms are complementary and work at different stages of the tumor-host interaction, providing as they do *in vivo* protection against the persistence of different types of tumor cells.

Granzymes, perforin, FasL and cytokines (such as IFN- γ) act as effector molecules for both T and NK cells to eliminate tumor cells; chemokines and their receptors are responsible for infiltration of lymphocytes into tumor tissue. In cases of infection by oncogenic viruses, such as hepatitis virus and HTLV-1, viral antigen presentation by HLA class I and II molecules to T cells, and subsequent T-cell mediated cytotoxicity and cytokine

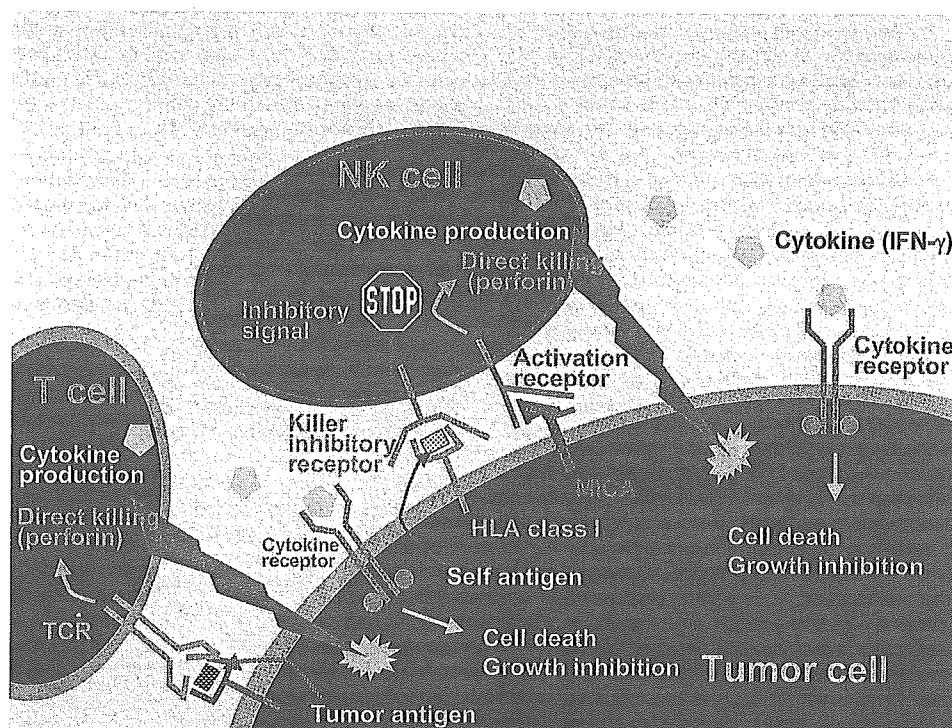


Fig. 1. Cells and molecules known to be involved in host immune responses to developing tumors. Tumor-specific cytotoxic T lymphocytes (CTL) recognize tumor antigens that are expressed in conjunction with HLA molecules and begin to directly kill tumor cells by secreting tumoricidal molecules (such as perforin) or to produce cytokines (such as IFN- γ) that suppress the growth of tumor cells. NK cell recognition is mediated by the opposing effects of two sets of NK receptors, activation and inhibitory receptors. Activation receptors recognize ligands (such as MICA) expressed on the target cell and transmit intracellular signals that initiate cytotoxicity; inhibitory receptors recognize cell-surface HLA class I molecules and generate counter-activating signals that block the induction of cytotoxicity. NK cell effector functions that kill or suppress tumor cells are almost identical to those of CTL. In the course of tumor progression, tumor cells tend to lose expression of HLA molecules and escape T cell recognition. Loss of HLA-class I expression (missing-self) on tumor cells engages NK cells to kill these cells.

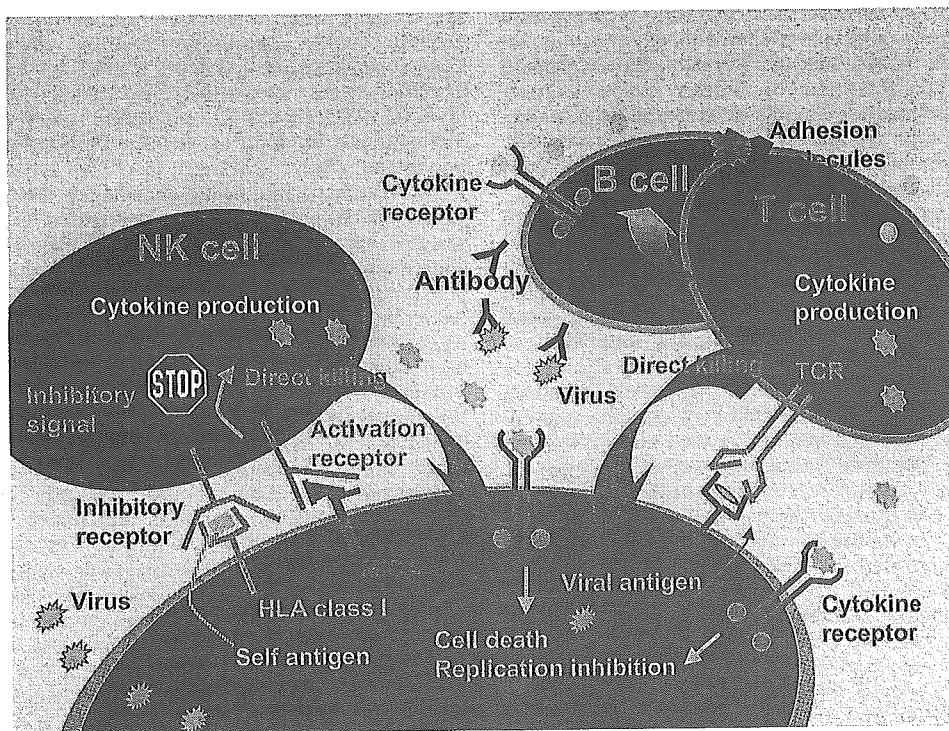


Fig. 2. In the case of immune responses to oncogenic virus infection, viral antigen presentation by HLA class I and II molecules to T cells initiates adaptive immune responses to the virus. Subsequent T-cell mediated cytotoxicity to virus-infected cells and T-cell cytokine production are key elements in control of the infection. NK cells can recognize virus-induced cellular antigens (such as MICA) and engage in eradication and suppression of virus-infected cells. T cells help B-cell production of virus-specific antibodies that can block viral replication by releasing cytokines and through direct cell-to-cell interaction.

production, are also key elements in the control of infection (Fig. 2). NK cells are also known to play important roles in eradication and suppression of virally infected cells.¹⁰ Virus-specific antibodies that are produced by B cells with T-cell help may block infection of adjacent cells and thereby suppress viral replication. T and B cell interaction is mainly mediated by cytokines and cell adhesion molecules; and minor T-cell subsets, $\gamma\delta$ T and NKT cells, are also known to act as effector cells in the cancer elimination phase. Although the role of these immune effector mechanisms in tumor protection is not well defined, epidemiological approaches to investigating the association between cancer development and individual variations in the ability to mount these immune defense mechanisms are essential to establish the concept of cancer immunosurveillance and to develop a new basis for cancer prevention.

Observations in humans. One logical approach to examining the immunosurveillance hypothesis in humans is to determine whether patients with immunodeficiency, or immunosuppressed transplant recipients, show a greater incidence of cancer. A consistent finding in various follow-up studies of transplant recipients is a remarkably increased risk ratio (observed/expected ratio) of selected malignancies, many of which are associated with viruses such as Epstein-Barr virus (Hodgkin's disease), human papilloma virus (cervix cancer, anogenital cancer, and some skin cancers), human herpes virus 8 (Kaposi's sarcoma), and hepatitis B and C viruses (hepatocellular cancer).^{12, 13} These observations have demonstrated that one relevant function of immunosurveillance is eradication of viruses, some of which may cause cancers, although it is still not clear whether the immune system can eliminate cancer cells with no known viral etiology. Recent studies have shown that transplant recipients have an increased risk of developing various cancers com-

monly observed in general populations, including those of the respiratory organs, digestive organs, and endocrine glands, which clearly demonstrates the role of immunological defense mechanisms in preventing the development of cancer.^{12, 13} Of malignancies that develop in transplant recipients, the portion transmitted from donors is estimated to be less than 1%. In patients with various immunodeficiencies—such as Chediak-Higashi syndrome, X-linked lymphoproliferative syndrome, ataxia-telangiectasia, and the Wiskott-Aldrich syndrome—an increased incidence of selected cancers, such as non-Hodgkin's lymphoma, was observed; patients with adaptive immunodeficiency syndrome also showed 100-fold increase of Kaposi's sarcoma and non-Hodgkin's lymphoma.

However, these studies of immunodeficient populations have several limitations: 1) study subjects were relatively young and had therefore not reached the age when solid cancers are frequently seen (e.g., the mean age at transplantation was 43 years, and the mean age for diagnosis of malignancies was 48 years in the Cincinnati Transplant Tumor Registry) and the follow-up periods were short (in part, due to the patients' shortened lifespans and medical complications)¹³; 2) since immunodeficient people seem to carry widespread dysfunctions of the immune system, including both innate and adaptive immunity, it is difficult to assess the involvement of a specific immune function in cancer immunosurveillance, which also causes difficulty in extrapolating results obtained with immunodeficient people to the general population, who do not have obvious defects in the immune system and who have reached the "cancer-prone age." Since aging is the most important factor in the development of cancer, it is important to know how inter-individual differences in a particular immune function are associated with future development of common cancers among the

general population. In addition, the existence of pre-clinical cancer in the body may influence the immune function, so case-control studies seem to be inadequate for assessing the relation between cancer and immunological defense. Therefore, prospective cohort studies, using specific immunological biomarkers that are measurable with peripheral lymphocytes and stable during long periods of follow-up, are needed. Unfortunately, very few such studies are available.

In one prospective cohort study of the Japanese general population (the Saitama cohort study), an 11-year follow-up study recently revealed that individuals with medium and high natural cytotoxic activity of peripheral-blood lymphocytes—measured by the isotope-release method using K562 as target cells—had a reduced risk of developing cancer in all sites, whereas those with low cytotoxic activity had an increased cancer risk (Table 1).¹⁴⁾ This is the first evidence of the vital role played by natural immunological defense in the occurrence of common cancers among the general population who do not have obvious defects in their immune systems, indicating the possible feasibility of cancer immuno-prevention. Since natural cytotoxic activity is in part associated with selected lifestyle factors as well as mental stress, this cytotoxic activity will be a useful surrogate marker for future cancer prevention studies.^{15, 16)} The findings also imply that individual variations in innate immune responses seen in the general population may generate large differences in cancer incidence with advancing years, specifically when people reach cancer-prone age. To date, though, no clear results have been obtained from studies using biomarkers of adaptive immunity. However, in one promising on-going cohort study, a subcohort of atomic-bomb survivors (the RERF immunological cohort study) has revealed a significant dose-dependent association between past experience of radiation exposure and attenuated immunity measured in terms of T-cell repertoire and functions, and cytokine levels, all of which are also associated with aging.^{17–20)} It is anticipated that a baseline measurement of various immunological markers of adaptive immunity in this unique cohort will answer some questions on cancer immunosurveillance and will provide a model for the interactions among host immunity, aging, and environment.

In addition, a genomic approach was recently undertaken in the Saitama and RERF cohort studies. To find genetic factors involved in individual variations of natural cytotoxic activity,

age- and sex-matched Saitama cohort groups with high and low natural cytotoxic activity were compared in terms of *HLA class I* genotype frequencies: *B*1301*, *B*4403*, *B*5401*, *Cw*0401*, and *Cw*0702* were significantly associated with the activity ($P=0.02$, 0.02 , 0.04 , 0.03 , and 0.004 , respectively).²¹⁾ Specifically, *Cw*0702* is relatively frequent (11%) among the Japanese population. This phenotype-genotype association analysis within cohorts is now being extended to the genetic polymorphisms of NK cell receptors, a new genomic approach unique to cohort studies. In the RERF immunological cohort study, radiation effects on risk of type II diabetes were studied in terms of *HLA class II* haplotyping, indicating that individuals with a particular *HLA* haplotype, either *DQA1*03-DRB1*09* or *DQA1*0401-DRB1*08*, revealed an increased risk of type II diabetes dependently on their atomic-bomb radiation dose (trend $P=0.0003$).²²⁾

Experimental animal studies. The cancer immunosurveillance hypothesis has been tested using numerous immunocompromised animals in which spontaneous and/or carcinogen-induced tumor development was assessed. Several lines of experiments using athymic nude mice found no significant increase in tumor formation as compared with euthymic immunocompetent mice,²³⁾ and these negative results initially gave some tumor immunologists an unfavorable view of this hypothesis. However, as modern immunology has begun to explain abnormalities in the immune system in terms of deficiencies of particular genes, various gene-knockout mice have become available for testing the immunosurveillance hypothesis. Mice deficient in one of several key molecules (IFN- γ , IFNGRI, and STAT-1) involved in the IFN- γ system more frequently developed spontaneous and/or carcinogen-induced tumors than did wild-type mice.^{24, 25)} *Rag2* gene ablation, which results in lack of lymphocytes mediating adaptive immunity, also appeared to increase susceptibility to spontaneous and/or carcinogen-induced cancers.²⁴⁾ Interestingly, mice deficient in both *RAG2* and *STAT-1* did not differ in overall incidence of tumors from those deficient in only one, suggesting that the IFN- γ system may be a major effector mechanism for tumor suppression through adaptive immunity.²⁴⁾

Another key effector molecule for immunological tumor control has been identified from studies with perforin-knockout mice, which also show increased susceptibility to tumor development^{25, 26)}: perforin is a component of cytolytic granules of CTL and NK cells, and mice deficient in both perforin and IFN- γ showed a small increase in tumor induction compared with those lacking only one of the two immune mediators, suggesting the existence of cross-talk between innate and adaptive immunity for resisting tumor formation.²⁵⁾ IL-12 is a potent inducer of Th1, which produces IFN- γ and exerts anti-tumor immunity by activating both CTL and NK cells, and mice defective in one of the IL-12 subunits are also known to be more susceptible to chemical carcinogenesis.²⁷⁾ This anti-tumor cytokine is produced by macrophages and dendritic cells and plays a key role in the transition from innate to adaptive immunity, again suggesting cross-talk between these immune systems in cancer immunosurveillance.

Another important step in experimental animal studies on cancer immunosurveillance was demonstrating the possible involvement of NK-mediated effector mechanisms in the suppression of tumor formation. Previous observations with nude mice,²³⁾ which challenged the cancer immunosurveillance hypothesis, ignored the fact that nude mice have a potential innate immune system including NK cell function. NK-deficient beige mice, which have a defect in cytolytic granule formation that also affects CTL and macrophages, have an increased incidence of spontaneous and induced primary oncogenesis.²⁸⁾ In addition, antibody depletion studies using anti-NK1.1 or anti-asialo-GM1 antibody, which can deplete NK cells as well as NKT cells or

Table 1. Relative risk of cancer incidence for cytotoxic activity levels

	NK cell activity ¹⁾ (%)		
	Low	Medium	High
Men			
Age-adjusted	1.0	0.62 (0.38–1.03) ²⁾	0.72 (0.45–1.16)
Lifestyle-adjusted ³⁾	1.0	0.61 (0.37–1.02)	0.71 (0.44–1.16)
Women			
Age-adjusted	1.0	0.56 (0.31–1.01)	0.52 (0.28–0.95)
Lifestyle-adjusted	1.0	0.56 (0.31–1.04)	0.52 (0.29–0.98)
Both sexes			
Age-adjusted	1.0	0.59 (0.40–0.87)	0.63 (0.43–0.92)
Lifestyle-adjusted	1.0	0.60 (0.41–0.87)	0.64 (0.44–0.94)

1) Categorized by tertiles. Low: less than 42%, medium: 42–58%, high: more than 58% for men; low: less than 34%, medium: 34–51%, high: more than 51% for women.

2) 95% confidence interval.

3) Adjusted for age, relative body weight, cigarette smoking, alcohol consumption, and intake of green vegetables.

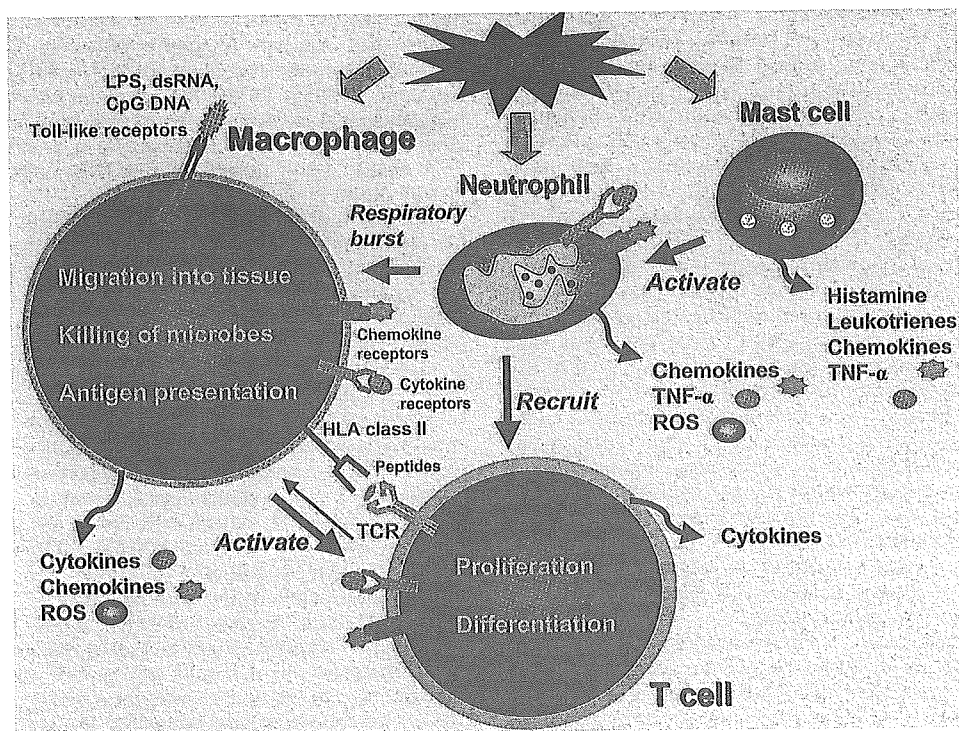


Fig. 3. Flow of inflammatory processes. 1) "Danger signals" from tissue trauma. The bioactive peptides released from neurons in response to pain activate mast cells, the intracellular proteins released from destroyed cells activate macrophages, and pathogen-associated patterns also activate macrophages through toll-like receptors. 2) Mast cells, the first responder, release histamine, leukotrienes, TNF- α , other cytokines/chemokines, and tryptases. 3) Neutrophils are activated by TNF- α and leukotrienes produced by mast cells; activation of matrix metalloproteases; tissue breakdown. 4) Macrophages are activated by pathogen-associated patterns; macrophage-derived TNF- α and chemokines activate more neutrophils and recruit lymphocytes, in conjunction with PGE₂, from mast cells and defensins from neutrophils. 5) Inflammatory responses (activation of mast cells and neutrophils) evolve into immune responses, i.e., activation of macrophages, dendritic cells (DCs), T cells and B cells.

activated macrophages, revealed that NK cells are important in preventing tumor induction by a chemical carcinogen.²⁷⁾ Although determining the specific roles of NK cells in cancer immunosurveillance is hampered by the lack of mouse models completely defective in NK cells but normal in T and B cells, it is likely that NK cells participate in various stages of tumor immunity, including cancer immunosurveillance, as has been indicated by a follow-up study in humans. NK cell recognition is mediated by the opposing effects of two different types of NK receptors, activation and inhibitory receptors: activation receptors recognize stress-induced ligands that are expressed on the target cell, and then transmit intracellular signals that initiate cytotoxicity; inhibitory receptors recognize cell-surface MHC class I molecules and generate counter-activating signals that block the induction of cytotoxicity. Besides being a vital player in innate immunity, NK cells influence subsequent adaptive immune responses by releasing cytokines and chemokines that induce growth and differentiation of various immune cells.

Infection, inflammation, and cancer

Cell proliferation does not by itself cause cancer: growth/survival factors enriched at sites of inflammation specifically promote the proliferation of initiated cells. Once tissue trauma has healed, the inflammation associated with cell proliferation (required for tissue-regeneration) ends. However, when inflammation is sustained and becomes chronic, continuous growth stimuli work on initiated cells and reactive oxygen species cause genotoxic damage, generating dysplastic changes (atypical cells). Therefore, for cancer cell development at sites of inflammation, it is critical whether the inflammation becomes persistent or not.

Inflammation is a sequential process of responding to the trauma often caused by microbial infection; in the process, various soluble factors and infiltrating or recruited cells (such as lymphocytes and leukocytes) become involved while interacting with each other in several steps: 1) recognition of tissue penetration by pathogens or tissue injury; 2) beckoning, instruction, and dispatch of cells (infiltration of lymphocytes); 3) eradication of pathogens and killing of infected cells; 4) liquefaction of surrounding tissue to prevent microbial metastasis; 5) healing of damaged tissue. Throughout, several checkpoint signals determine the advance or standstill of inflammatory response; if this sequential process is hindered at any step, or if repeated infections occur within the host, the inflammatory process may become stalled, resulting in persistent inflammation.²⁹⁾

Innate immune responses are induced by pathogen-associated patterns (e.g., lipopolysaccharide (LPS), double-stranded (ds) RNA, CpG DNA), which are recognized by toll-like receptors on macrophages,³⁰⁾ and/or by the NKG2D and other NKp receptor ligands expressed on infected cells.³¹⁾ The cytokine cascade plays an important role in augmentation and suppression of immune response to pathogens: cytokines released from APC or T cells, such as IL-6, TNF- α , and IFN- γ act as effector molecules in inflammation induced by microbial infection; chemokines and their receptors are then involved in migration of immune cells into inflammation sites (Fig. 3).^{5,29)} Hence, insufficient immune functions of the host may result in persistent inflammation because of failure to completely eradicate pathogens or infected cells, resulting in repeated destruction and regeneration of tissue. When initiated cells at sites of persistent inflammation continue to proliferate—interacting with inflam-

matory cells and growth factors that specifically act on the initiated cells (e.g., TNF- α)—the inflammatory process becomes a crucial step in carcinogenesis. In fact, many cancers are thought to be associated with inflammation caused by immunologically uncontrolled infections: colon carcinogenesis arising in individuals with inflammatory bowel diseases, chronic ulcerative colitis and Crohn's disease, esophageal carcinoma with reflux esophagitis, gastric cancer with atrophic gastritis, liver cancer with hepatitis, lung cancer with interstitial pneumonia, etc.

Observations in humans. It is said that 18% of cancer cases worldwide can be ascribed to infections with various pathogens, which include *Helicobacter pylori* (gastric cancer, 490,000 cases a year), human papillomavirus (cancer of the cervix and other sites, 550,000), hepatitis B and C viruses (hepatocellular carcinoma, 390,000), Epstein-Barr virus (lymphomas and nasopharyngeal cancer, 99,000), human herpes virus 8 (Kaposi's sarcoma, 54,000), *Schistosoma haematobium* (bladder cancer, 9000), human T-cell lymphotropic virus (adult T-cell leukemia, 2700), and *Opisthorchis viverrini* (cholangiocarcinoma, 800).³² For some of these cancers, the immunogenetic status of the host has been investigated in terms of HLA typing and SNPs in cytokine genes.³³ Specifically, the identification of HLA class II types that are sensitive or resistant to human T-cell lymphotropic virus has demonstrated a role of host immunity in virus-associated carcinogenesis.

The oncogenic processes in virus-related cancer are greatly influenced by a series of immune effector mechanisms: virus-infected cells that have encountered the immune system eventually go through processes involving escape from immunological recognition and cytotoxicity, and the cell transformation that accompanies rapid proliferation causes frequent gene mutations. How an individual's defense system undertakes these processes is thought to depend on individual ability to mount immunity in response to infection with a particular virus. Decreased immunity to infection with such a virus when complete elimination of the extrinsic antigen has failed may be closely related to carcinogenesis that results from continuous inflammation, and repeated destruction and regeneration of tissue, causing mutations. Among many cancers in which inflammation is considered to be involved, some may also be associated with production of carcinogenic proteins by infected microbes, e.g., oncoprotein CagA by *H. pylori* in gastric cancer, oncoproteins X by hepatitis B virus (HBV) and core protein by hepatitis C virus (HCV) in hepatocellular carcinoma, oncoprotein E6/7 by human papilloma virus (HPV) in cervical cancer, and oncoprotein Tax by human T-cell lymphoma virus (HTLV-I) in adult T-cell leukemia. HLA molecules play an important role in the recognition of antigens derived from carcinogenic proteins that have the potential to transform cells infected with these microbes, possibly ensuring surveillance of transformed cells.³⁴ In some cases, a particular HLA class II molecule may lack the capacity for binding to the peptide anchor motif needed to recognize an oncoprotein, and thereby fail to induce CTL responses to transforming cells and allow generation of a specific type of cancer. In support of this notion, there are numerous reports suggesting an association between susceptibility to cancer and HLA class II genotype.³⁵

Apart from infection-related cancers, many cancers have been associated with persistent inflammation: lung cancer associated with asbestosis or silicosis; colon cancer with inflammatory bowel disease, Crohn's disease, and chronic ulcerative colitis; pancreas cancer with chronic pancreatitis; esophageal cancer with reflux esophagitis or Barrett's esophagus; MALT lymphoma with Sjögren syndrome; melanoma with UV-caused skin inflammation; bladder cancer with chronic cystitis or bladder inflammation; oral squamous cell carcinoma with gingivitis.⁵ These findings may imply that persistent inflammation itself has carcinogenic activity, due to production of reactive

oxygen species, tumor promotion activity of inflammatory cytokines, and induction of genetic instability. One recent cohort study revealed that plasma levels of C-reactive protein were an excellent predictor of the risk of colon cancer, demonstrating that subclinical persistent inflammation may underlie colon carcinogenesis in general.³⁵ Interestingly, C-reactive protein levels were unchanged by administration of nonsteroidal anti-inflammatory drugs.

One problem in designing epidemiological studies to examine the relation between low-grade inflammation and cancer seems to be adequate selection of biomarkers that can define low-grade, persistent inflammation. It may be desirable to use a combination of inflammation-related markers such as plasma levels of IL-6, IL-10, TNF- α , and IFN- γ , along with C-reactive protein (CRP), together with erythrocyte sedimentation rate (ESR), whose validity has been demonstrated in the cohort of atomic-bomb survivors.²⁰ Since all these biomarkers are closely related to aging, the effect of aging and environmental factors on inflammatory status can be investigated in relation to occurrence of aging-related diseases, such as cancer. Candidate biomarkers which have been or could be used in studies of immuno-epidemiology are listed in Table 2. Environmental factors or events that potentially influence the immunological/inflammatory status of the host should be identified, and their relationship to the incidence of cancer should be intensively investigated. In addition to various pathogens, exposure to chemical carcinogens and radiation may induce impairments in the immune system on some occasions, resulting in low-grade chronic inflammation and eventually leading to enhanced risk of cancer development.

One long-term prospective cohort study has examined the effects of radiation on the health of atomic-bomb survivors. To our surprise, even now, more than 50 years after the bombings, impairments in T-cell immunity are radiation-dose-dependently observed among a sub-cohort of atomic-bomb survivors, along with increased levels of plasma inflammatory cytokines and other inflammation markers.^{17, 18, 20} In fact, atomic-bomb survivors even today continue to suffer from increased risk of cancer, cardiovascular disease, and hepatitis. These late effects pose serious, as yet unanswered, questions about the mechanisms involved. We hypothesize that T-cell impairments caused by radiation may generate age-associated chronic low-grade inflammation, which may in part be responsible for increased risk of diseases among atomic-bomb survivors. Decreased CD4 helper T-cell counts of the survivors appeared to be significantly associated with increased levels of IL-6 and CRP.³⁶ We found that both radiation exposure and increased age were associated with increases in selected plasma inflammatory biomarkers (Table 3), indicating that the effect of radiation could be further estimated in terms of acceleration of aging.^{20, 36} Among the inflammatory biomarkers we examined for the effects of increased age and radiation dose, the increased levels of TNF- α , IL-10, IL-6, ESR, CRP, and IgA per Gy corresponded, on average, to an increase in age of 10 years (range, 5 to 15); atomic-bomb survivors' average radiation dose was 0.2 Gy, corresponding to about 2 years (range, 1 to 3) of aging. This may provide a hint as to why the incidence of cancer and some inflammation-associated diseases among atomic-bomb survivors remains high even when so much time has elapsed, as well as a model for understanding the effects of various environmental factors on aging-related diseases in general. This cohort study clearly shows the significance of repeated clinical examination, measurement of various immunological markers (some of which are listed in Table 2), and preservation of biological materials, through more than 50 years of follow-up.

Experimental animal studies. Although numerous factors and cells are involved in the complicated process of inflammation, cytokines are assumed to play a key role in the crossover of in-

Table 2. Candidate biomarkers for immuno-epidemiology

Phenotype	Marker(s)	Function(s)	Method	Reference(s)
Cell numbers	WBC	Inflammation	Cell counting	46
	Neutrophils	Innate immunity/inflammation	Cell counting	46
	CD4	Cellular immunity/helper T cell	Flow cytometry	47
	CD8	Cellular immunity/cytotoxic T cell	Flow cytometry	47
	CD19	Humoral immunity/B cell	Flow cytometry	47
	CD16/CD56	Innate immunity/NK cell	Flow cytometry	47
	CD45RA/CD45RO	Naïve/memory T cell	Flow cytometry	47
	Th1	Cellular immunity/helper T cell	Intracellular staining of IFN- γ	48
	Th2	Cellular immunity/helper T cell	Intracellular staining of IL-4	48
Cell activities	NK activity	Innate immunity	Isotope release	14
	T cell proliferation	Blast formation of T cell by mitogen	[3 H]thymidine incorporation	49
Cytokines	IL-6	Pro-inflammation	ELISA, Real time RT-PCR	20
	IL-8	Pro-inflammation	ELISA, Real time RT-PCR	50
	TNF- α	Pro-inflammation	ELISA, Real time RT-PCR	20, 50
	IL-1 β	Pro-inflammation	ELISA, Real time RT-PCR	50
	IL-10	Anti-inflammation	ELISA, Real time RT-PCR	20, 50
	IFN- γ	Pro-inflammation	ELISA, Real time RT-PCR	20, 50
Chemokines	RANTES	Inflammation/recruitment of lymphocytes	ELISA, Real time RT-PCR	51
	MIG	Inflammation/recruitment of lymphocytes	ELISA, Real time RT-PCR	52
	IP-10	Inflammation/recruitment of lymphocytes	ELISA, Real time RT-PCR	52
	MCP-1	Inflammation/recruitment of lymphocytes	ELISA, Real time RT-PCR	53
Plasma/serum inflammatory markers	CRP	Inflammation	ELISA	35
	Metabolites of ROS	Inflammation/ROS production	Total ROS assay system	54
	ESR	Inflammation	Wintrobe method	46
	Sialic acid	Inflammation	Enzyme assay	46
	Haptoglobin	Inflammation	Nephrometry	46
	HMdU	Inflammation/DNA damage	ELISA	55
Tissue/cell inflammatory marker	8-OH-dG	Inflammation/DNA damage	HPLC/ECD	56
	Etheno DNA adduct	Inflammation/DNA damage	HPLC/MS, GC/MS, GC/NICI/MS	57

Table 3. Multivariate model of the effects of age, sex, and radiation dose on inflammatory biomarkers and immunoglobulins¹⁾

Variable	Percent increments (95% confidence intervals)										
	TNF- α	IFN- γ	IL-10	IL-6	CRP	ESR	Total Igs	IgG	IgA	IgM	IgE
Age per 10 years	15 (9, 20)	4 (-4, 12)	8 (4, 13)	24 (19, 30)	25 (13, 38)	15 (9, 20)	3 (1, 6)	3 (1, 6)	5 (2, 9)	-6 (-11, 14)	2 (-11, 14)
Female sex ²⁾	15 (2, 30)	-8 (-23, 10)	6 (0, 12)	8 (-41, 18)	0 (-25, 33)	17 (9, 24)	5 (0, 10)	7 (1, 13)	-9 (-17, -1)	14 (1, 28)	-51 (-63, -34)
Radiation dose per Gy	7 (1, 15)	12 (2, 23)	6 (0, 12)	13 (6, 20)	39 (20, 62)	17 (9, 24)	3 (1, 6)	2 (-1, 5)	8 (3, 13)	8 (2, 15)	14 (-3, 32)
Estimated aging by radiation (years per Gy) ³⁾	5 (0, 10)	29 (-29, 88)	6 (-1, 14)	5 (2, 8)	14 (4, 24)	11 (5, 17)	12 (-1, 26)	6 (-4, 17)	15 (1, 29)	-14 (-29, 2)	90 (-682, 861)

1) Subjects were a total of 442 atomic-bomb survivors who did not have a history of cancer or inflammatory-associated diseases (e.g., current cold, chronic bronchitis, collagen disease, arthritis, myocardial infarction).

2) Percentage change, female versus male.

3) Estimated by the δ -method.

flammation and cancer. Development of cytokine-gene knock-out mice has demonstrated the vital role of pro-inflammatory cytokines in carcinogenesis: TNF- α -deficient mice developed a significantly smaller number of tumors than did wild-type mice in two-stage skin carcinogenesis experiments, demonstrating that TNF- α is the key cytokine by which inflammation acts as a tumor promoter.^{37, 38)} The IL-1 knockout mouse model implies

that host-derived IL-1 α and IL-1 β are required for control of tumor angiogenesis and invasiveness in a melanoma model.³⁹⁾ In a urethane carcinogenesis experiment, TNF- α and IL-10 deficiencies showed contrasting effects on lung tumor susceptibility,⁴⁰⁾ and the pro-inflammatory cytokines, TNF- α , IL-1, and IL-6, seem to play different roles in tumor promotion and cell transformation.⁴¹⁾ In addition to these cytokines, macrophage

migration inhibitory factor (MIF) has been reported to amplify carcinogenic DNA damage by suppressing the transcriptional activity of p53 and by-passing p53 regulatory functions.⁴²⁾ We thus anticipate that a network of inflammatory signals, with discrete roles of cytokines/chemokines and their interactions, will be intensively studied in relation to cancer development.

Macrophages sense a variety of microbes through toll-like receptors that recognize pathogen-associated patterns, while NK cells recognize host proteins expressed after infection through NK-activating receptors, such as NKG2D. Recently, the interaction between innate/adaptive immunity and inflammatory response has been delineated: murine macrophages, which are activated with LPS through toll-like receptor, express ligands (RAE-1) that are recognized by NKG2D receptor on NK cells, thus implying a mechanism by which NK cells and infected macrophages directly interact during an innate immune response to infection.⁴³⁾ With HBV transgenic mice, CTL-mediated destruction of infected hepatocytes reportedly induces long-lasting hepatocellular regeneration, oxidative DNA damage, and clonal expansion, eventually resulting in hepatocellular carcinoma.⁴⁴⁾ This study leads to the quintessential question: are pathogen-specific functions essentially required for cancer development, in addition to persistent inflammation itself (including induction of inflammatory cytokines)?

Conclusions and perspectives

It is anticipated that cancer epidemiology will eventually clarify the roles of immunity in protecting the host from nascent transformed cells and in regulating inflammatory responses to pathogens. Although the recent development of gene-engineered mice has provided solid evidence for cancer immunosurveillance and for the inflammation-cancer sequence, reliable estimation of cancer risk for individually differing immunological competence can be performed only in epidemiology, which could also identify high-risk individuals and aim at cancer prevention based on immunological up-regulation. One advantage of immuno-epidemiology may be the array of biomarkers listed in Table 2, which demonstrates that peripheral blood can reflect the systemic status of host immunity. On the other hand, the fact that the immune system is easily influenced by the existence of cancer in the body, even when it is in a pre-clinical stage, narrows down the study methods to prospective cohort studies. Although only a few such cohort studies are

available at present (e.g., the Saitama cohort study and the RERF immunological cohort study), these studies should be expanded and extended in the future to answer the numerous questions concerning the roles of immune cells in cancer surveillance and inflammation, the characteristics of inflammation associated with cancer development, the effects of environment/lifestyle factors on the immune system, and the interaction between aging and immunity in the occurrence of cancer and other diseases.

Another important issue to be considered is the genetic background underlying individual variations in immune and inflammatory responses. *HLA* haplotyping has been intensively studied in relation to cancer among different races, and genetic polymorphisms of various cytokines and their receptors have also been investigated, mostly in case-control study design. One representative study is a large-scale case-control study which revealed that genetic polymorphisms of inflammatory cytokines including *IL-1 β* influenced the risk of gastric cancer by modulating the pH of gastric juice and the growth environment of *Helicobacter pylori*.⁴⁵⁾ A possible advantage of this genomic approach is that the involvement of immune-related genes can be readily examined in case-control studies, although any mechanistic interpretation (or conclusion on the functional significance of particular genetic polymorphisms considered in studies) must be made separately. However, risk estimation in these studies is made for particular polymorphisms of genes, not for the function or role of the genes.

On the other hand, cohort studies seem to have an advantage over case-control studies for the genomic approach: genomic analysis comparing cohort members with high and low values of particular phenotype markers can readily be performed, along with follow-up studies that reveal the association between these markers and cancer development. This phenotype-genotype association analysis within cohort studies may clarify the genetic background of those phenotype markers that are directly related to cancer risk, and possibly lead to their use as surrogate biomarkers for cancer prevention.

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Radiation-induced apoptosis of stem/progenitor cells in human umbilical cord blood is associated with alterations in reactive oxygen and intracellular pH

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Abstract

To investigate the sensitivity of human hematopoietic stem cell populations to radiation and its relevance to intracellular events, specifically alteration in cellular energy production systems, we examined the frequency of apoptotic cells, generation of superoxide anions ($O_2^{\bullet-}$), and changes in cytosol pH in umbilical cord blood (UCB) CD34⁺/CD38⁻, CD34⁺/CD38⁺ and CD34⁻/CD38⁺ cells before and after 5 Gy of X-irradiation. Human UCB mononucleated cells were used in this study. After X-irradiation and staining subgroups of the cells with fluorescence (FITC, PE, or CY)-labeled anti-CD34 and anti-CD38 antibodies, analyses were performed by FACScan using as stains 7-amino-actinomycin D (7-AAD) for the detection of apoptosis, and hydroethidine (HE) for the measurement of $O_2^{\bullet-}$ generation in the cells. For intracellular pH, image analysis was conducted using confocal laser microscopy after irradiation and staining with carboxy-SNARF-1. The frequency of apoptotic cells, as determined by cell staining with 7-AAD, was highest in the irradiated CD34⁺/CD38⁻ cell population, where the level of $O_2^{\bullet-}$ detected by the oxidation of HE was also most highly elevated. Intracellular pH measured with carboxy-SNARF-1-AM by image cytometer appeared to be lowest in the same irradiated CD34⁺/CD38⁻ cell population, and this intracellular pH decreased as early as 4 h post-irradiation, virtually simultaneous with the significant elevation of $O_2^{\bullet-}$ generation. These results suggest that the CD34⁺/CD38⁻ stem cell population is sensitive to radiation-induced apoptosis as well as production of intracellular $O_2^{\bullet-}$, compare to more differentiated CD34⁺/CD38⁺ and CD34⁻/CD38⁺ cells and that its intracellular pH declines at an early phase in the apoptosis process.

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1. Introduction

Apoptosis is an important mechanism in the selective elimination of mammalian cells: It is distinct from the process of the cell death by necrosis [1], and is basically characterized by cellular shrinkage, marked condensation and margination of chromatin, and nuclear and cellular fragmentation with well preserved cell organelles. Radiation-induced cell death has been studied extensively in a wide variety of cell types and cell lines. Ionizing radiation causes breaks in chromosomal DNA resulting in upregulation of stress-activated proteins including p53, p21, Gadd45, and manganese superoxide dismutase [2–6]. Apoptosis and classical necrosis are two genetically, biochemically and morphologically different types of cell death, and the differences have been recognized as depending not only on the cell type but also on the radiation dose. In apoptosis by radiation, following localization of pro-apoptotic proteins, such as Bax, to the mitochondria, there is a decrease in mitochondrial membrane permeability and release of cytochrome-C into the cytoplasm, followed by induction of typical apoptotic features including chromatin condensation and nuclear fragmentation accompanied with caspase-3 activation and poly-ADP-ribosyl polymerase cleavage [7–9]. The mechanism by which these subcellular events occur has remained unclear.

The reactive oxygen species (ROS), especially superoxide anion ($O_2^{\cdot-}$), play an important role in signaling mechanisms and are also in chromosomal aberrations, cell proliferation and cell death [10–13]. Radiation-induced $O_2^{\cdot-}$ generation among different cell types may be of interest for understanding sensitivity to radiation-induced chromosomal aberrations in the survived cells. Hydroethidine (HE), a non-fluorescent lipophilic marker, is oxidized by superoxide anion to the fluorescent hydrophilic product ethidium (Eth), and indicates the accumulation of ROS in the cells [14,15]. In several models of apoptosis, increased formation of ROS has been noted as an early event in apoptosis and as a main cause of chromosomal aberrations in survived cells [15,16]. A decline in the pH of the cell interior (pHi) has also been reported as a concomitant of apoptosis in HL-60 cells and CTL cells [17–20], and is thought to be an early feature of apoptosis in neutrophils [21]. However, whether ROS or pHi is the primary trigger/inducer of the radiation-induced apoptotic pathway has not been determined.

The hemopoietic stem cell seems to be one of the most radiosensitive cells in the body [22]. However, the death-signaling mechanism and susceptibility of hemopoietic stem cells to ionizing radiation have not been well understood. The stem cell marker CD34 is expressed on the surface of stem and early progenitor cells, and hemopoietic progenitor cells in bone marrow or umbilical cord blood (UCB) have proved divisible into various subpopulations in terms of CD34 and CD38 antigen expressions [23]. Thus, for example, $CD34^+CD38^-$ cells were found to be multilineage stem cells, whereas $CD34^+CD38^+$ cells and $CD34^-CD38^+$ cells were differentiated cells, respectively [24]. In this study, we examined the sensitivity of human stem/progenitor/differentiated cells to radiation-induced apoptosis and determined the relationship between $O_2^{\cdot-}$ generation and intracellular pH in the apoptotic process.

2. Materials and methods

2.1. Human UCB cells

Human UCB mononucleated cells were obtained from BioWhittaker Inc. (Walkersville, MD). After thawing cryopreserved cells by stepwise dilution in Earle's Balanced Salt Solution (EBSS) containing 2.5% fetal calf serum (FCS), the cells were washed with EBSS containing 2.5% FCS and cultured with 10% heat-inactivated FCS, and 90% RPMI 1640 (Nikken Biomedical Lab., Kyoto, Japan) until used for flow cytometric analysis or subset purification.

2.2. Irradiation

UCB cells or sorted cells were X-irradiated using a Shin-ai II unit (Shimadzu Corp., Tokyo, Japan) at 200 kVp, 20 mA, with 0.5 mm Al and 0.5 mm Cu filters. Dose rate was 0.61 Gy/min. Immediately after irradiation, cells were suspended in RPMI 1640-10% FCS and cultured at 37 °C humidified air containing 5% CO_2 .

2.3. Cytofluorometric analyses of $O_2^{\cdot-}$ generation and apoptosis (cell viability)

Many different methods have been described previously for the quantification of programmed cell death

at an individual cell level by flow cytometry [25,26]. Most of these techniques, however, do not permit the simultaneous evaluation of FITC and phycoerythrin (PE) cell-surface staining due to either a fixation or a sample preparation method that could not preserve cell surface staining and/or to the use of DNA dyes with fluorescence emission spectra that overlap extensively with PE. One technique for measuring apoptosis has been developed with 7-amino-actinomycin D (7-AAD), whose fluorescence emission can be clearly discriminated from PE fluorescence emission [27]. We therefore decided to use a combination of 7-AAD, FITC-CD34 and PE-CD38 to evaluate the apoptotic cell proportion in each hemopoietic cell subset.

For staining of CD34 and CD38 antigens in human UCB cells, 20 μ l each of FITC-conjugated antibody against CD34 (FITC-CD34) and CY or PE-conjugated antibody against CD38 (CY-CD38 or PE-CD38, respectively, PharMingen, San Diego, CA) in 100 μ l of phosphate buffered saline (PBS without Ca^{2+} and Mg^{2+} , Nikken Biomedical Lab., Kyoto, Japan) containing 1% BSA were added to 1×10^7 UCB cells followed by incubation for 30 min on ice. In the case of double staining, either combination of HE and CY-CD38 or PE-CD38 and 7-AAD were selected. After one wash with 1 ml of PBS, the supernatant was removed, and the cell pellet was resuspended in 1 ml of PBS. To measure $\text{O}_2^{\cdot-}$ generation, the cell suspension was stained for 15 min at 37 °C with 2.5 μ l of a 63.5 mM HE (Polysciences, Inc., Warrington, PA) solution in N,N-dimethylformamide (DMF), washed once with PBS containing 1% BSA and 0.01% sodium azide (PBS-BSA/AZ), and resuspended in 1 ml of PBS-BSA/AZ. To measure cell viability, the cell suspension was stained with 20 μ g/ml 7-AAD (Wako pure chemicals, Tokyo, Japan) in PBS-BSA/AZ for 15 min on ice protected from light; the cells were then analyzed with a FACScan flow cytometer (Beckton Dickinson Immunocytometry Systems, BDIS, San Jose, CA) in their staining solution.

2.4. Subset purification for pH_i analysis

CY- or PE-stained cells could not be used for pH_i analysis due to the interference of the fluorescent dye. To overcome this technical difficulty, we used the high-gradient magnetic cell sorting (MACS) system (Miltenyi Biotech GmbH, Bergisch Gladbach, Ger-

many) [28] and fluorescence-activating cell sorting (FACS) system for the collection of each cell subset before the analysis of pH_i. Briefly, human UCB cells were fractionated into CD34⁺ and CD34⁻ cell populations by positive and negative selection using MACS system. The purified CD34⁺ cells and CD34⁻ cells were stained with FITC-CD38 for 30 min on ice in RPMI 1640 containing 1% FCS. After incubation, the cells were washed twice, resuspended in RPMI 1640 containing 1% FCS, and sorted into CD34⁺/CD38⁻, CD34⁺/CD38⁺ and CD34⁻/CD38⁺ cell populations using a FACStar (BDIS).

2.5. Image cytometric analysis of intracellular pH

Cells were cultured for the indicated times in serum-free RPMI 1640 medium, then loaded with 10 μ M Carboxy-SNARF-1-AM (Molecular Probes Inc., Eugene, OR) for 30 min in HBSS, centrifuged, and resuspended in HBSS containing 20 mM Hepes (pH 7.4). Image analysis was performed on an Ultima Interactive Laser Cytometer (Meridian Instruments, Inc., Okemos, MI) with excitation at 488 nm and emission ratio analysis at 575 and 620 nm. Ten to twenty cells were analyzed, and emission ratios were converted to pH values by comparison to ratios observed in cells treated with nigericin in high potassium buffer at a defined pH [29].

3. Results

3.1. CD34⁺ cells from human UCB are sensitive to radiation-induced apoptosis

Separated human UCB cells were irradiated in vitro, harvested after 16 h, and subsequently flow-cytometrically analyzed with 7-AAD after staining with FITC-CD34 and PE-CD38. The cells investigated were gated on 2-color flow cytometry as described in Fig. 1, with the exclusion of monocytes, platelets and cell debris in a light-scattering profile. FITC-CD34/PE-CD38 stained cells are shown for a 16 h culture, demonstrating the plots obtained for unirradiated and 5-Gy irradiated cell samples, respectively.

Most unirradiated cells were negative for the 7-AAD staining, whereas about 60% of irradiated CD34⁺/CD38⁻ cells and 40–50% of irradiated CD34⁺/CD38⁺ cells were stained strongly with 7-

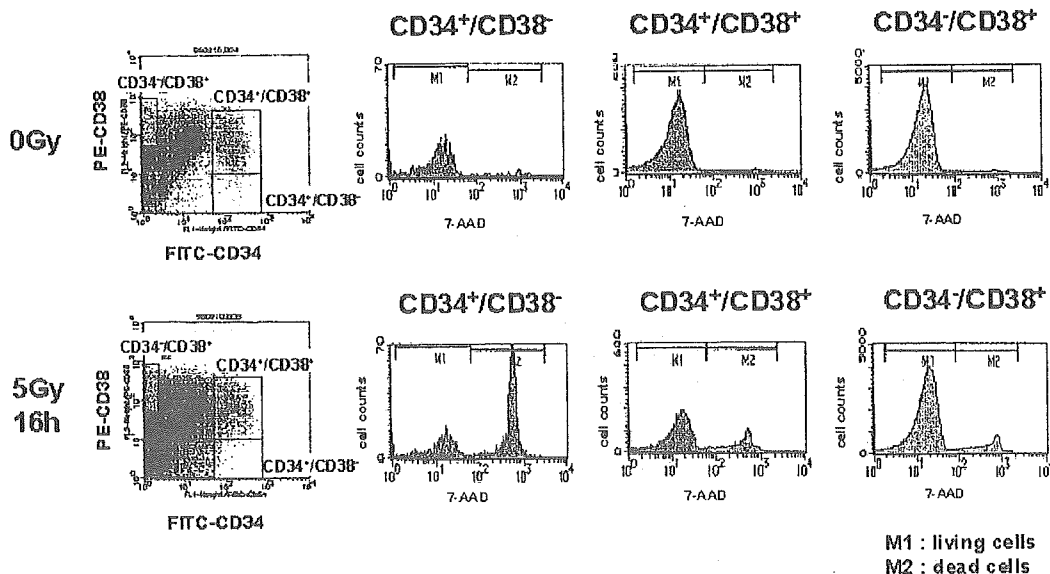


Fig. 1. Triple staining of cells for the simultaneous determination of apoptotic cells among different cell populations. Human UCB cells were stained with FITC-CD34, PE-CD38, and 7-AAD, and subjected to cytometric analyses. Cells were analyzed at 16 h post-irradiation with 5 Gy. Flow cytometer histograms show 7-AAD stained cells. M1: living cells; M2: apoptotic cells.

On the other hand, only a small fraction of irradiated CD34⁻/CD38⁺ cells were stained. Fig. 2 shows the percentages of living cells of each cell population at 4 and 16 h post-irradiation with 5 Gy: It is obvious that CD34⁺/CD38⁻ stem cells were more sensitive to radiation than more differentiated CD34⁺/CD38⁺ cells. Also of note is that few cells appeared to reach the stage of apoptosis at 4 h after 5-Gy irradiation.

3.2. A higher level of O₂⁻ generation in irradiated CD34⁺/CD38⁻ cells

O₂⁻ generation in irradiated cells (4 or 16 h post-irradiation with 5 Gy) was analyzed by flow cytometry with FITC-CD34, CY-CD38, and HE. The levels of O₂⁻ generation in unirradiated cells depended on the cell type, i.e., CD34⁺/CD38⁺ > CD34⁺/CD38⁻

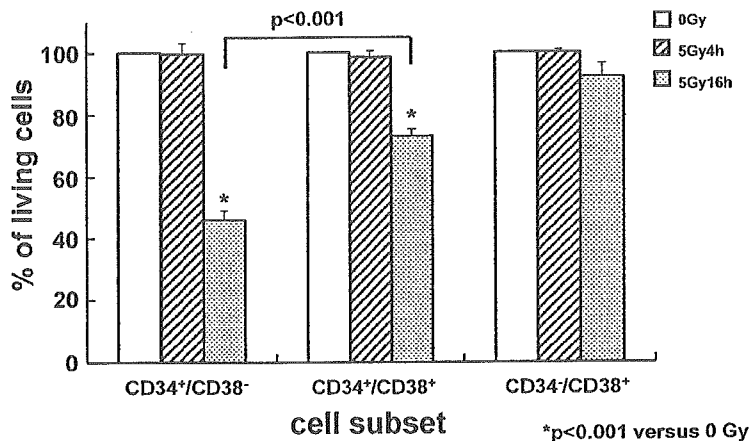


Fig. 2. The percentage of living cells in irradiated human UCB cell subsets. Human UCB cells were stained with FITC-CD34, PE-CD38, and 7-AAD, and subjected to cytometric analyses. Cells were analyzed at 4 and 16 h post-irradiation with 5 Gy. The percentage of living cells was calculated from the 7-AAD no-stained fraction in each UCB subset cells. The values represent mean and S.D. of three experiments.

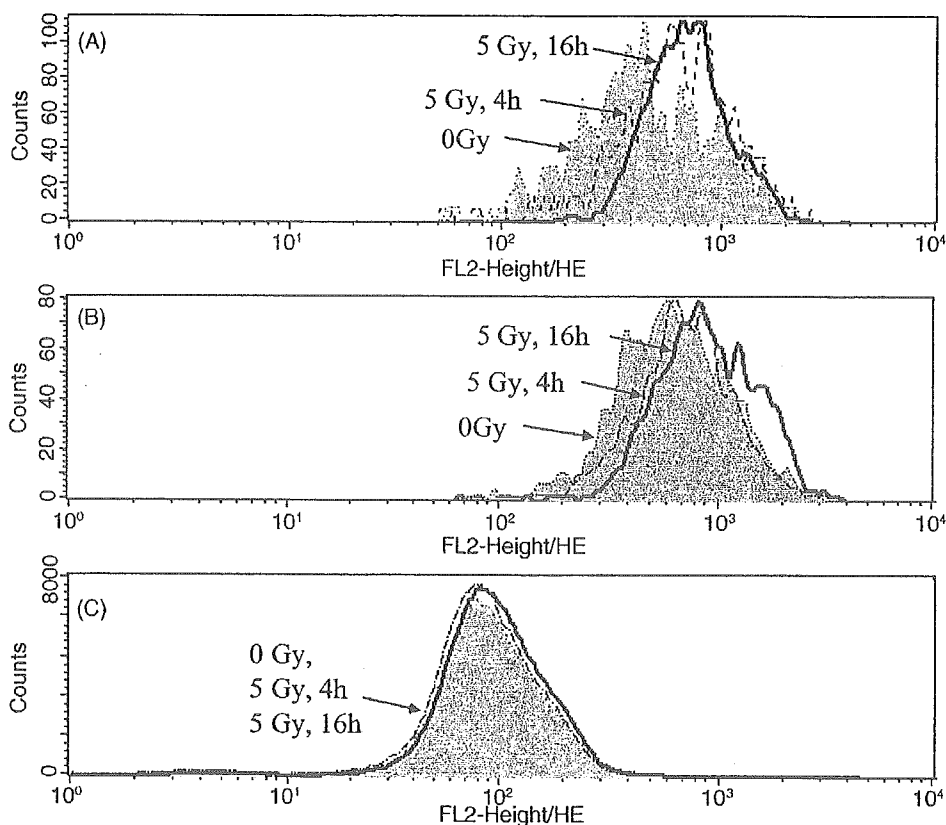


Fig. 3. Changes of $O_2^{\bullet-}$ generation in irradiated human UCB cell subsets. Irradiated human UCB cells were stained with FITC-CD34, CY-CD38, and HE, and subjected to cytometric analyses. (A) $CD34^+/CD38^-$ cells, (B) $CD34^+/CD38^+$ cells, (C) $CD34^-/CD38^+$ cells.

$> CD34^-/CD38^+$ (Figs. 3 and 4). $O_2^{\bullet-}$ generation levels in the $CD34^+/CD38^-$ and $CD34^+/CD38^+$ cell populations appeared to rise as early as 4 h after irradiation and had significantly increased 16 h after irradiation. In contrast, the levels of $O_2^{\bullet-}$ generation in the $CD34^-/CD38^+$ cell populations had not increased at even 16 h after irradiation. Overall changes and fold-induction in $O_2^{\bullet-}$ generation levels 16 h after irradiation were largest for the $CD34^+/CD38^-$ among these three cell populations. These results on cell-type-dependent $O_2^{\bullet-}$ generation after irradiation appear to be consistent with observations of radiation-induced apoptosis in the same cell types.

3.3. Intracellular pH of the $CD34^+/CD38^-$ cell population decreases in an early stage post-irradiation

We took note of intracellular pH as an indicator of the events taking place in live cells. Each of the

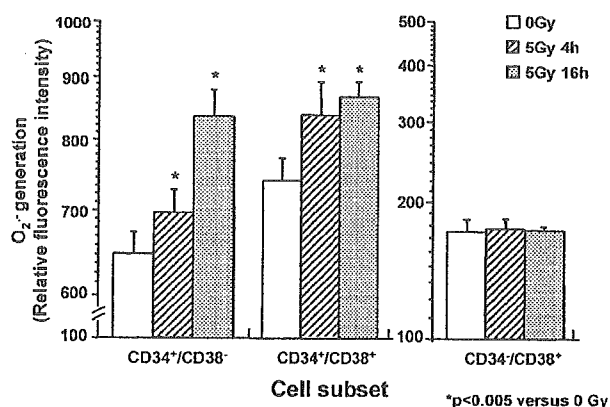


Fig. 4. $O_2^{\bullet-}$ generation in irradiated human UCB cell subsets. Irradiated human UCB cells were stained with FITC-CD34, CY-CD38, and HE, and subjected to cytometric analyses. The level of $O_2^{\bullet-}$ generation was monitored by the geometric mean fluorescence intensity of ethidium derived from HE. Cells were analyzed at 4 and 16 h post-irradiation with 5 Gy. The values represent mean and S.D. of three experiments.

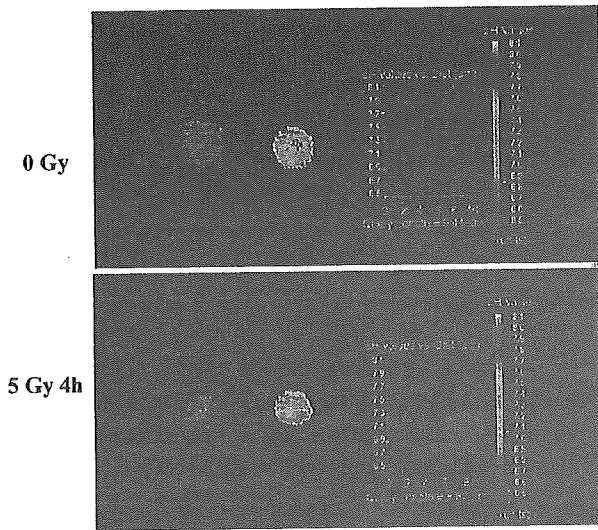


Fig. 5. Visualized imaging analysis of intracellular pH of CD34⁺/CD38⁻ cells using Carboxy-SNARF-1-AM. Purification of CD34⁺ and CD34⁻ cells was performed by positive selection using MACS system. Purified CD34⁺ cells and CD34⁻ cells were stained with FITC-CD38 and separated into CD38⁺ and CD38⁻ cells by a FACStar. Emission ratios were converted to pH values by comparison with ratios observed in cells treated with nigericin in high potassium buffer at a defined pH [29].

CD34⁺/CD38⁻, CD34⁺/CD38⁺, and CD34⁻/CD38⁺ cell fractions was sorted using magnet beads and FACStar, and intracellular pH in each cell population was evaluated using image analysis before, and 4 h after, 5-Gy irradiation (Fig. 5). The pH in irradiated cells was much lower than that in non-irradiated cells, and this radiation effect was more obvious in the CD34⁺/CD38⁻ cell population than in the CD34⁺/CD38⁺ cell population. As for the CD34⁻/CD38⁺ cell population, there was no significant radiation effect on the intracellular pH. These results indicate that a remarkable decline of intracellular pH in the CD34⁺/CD38⁻ cell population occurs along with the O₂⁻ generation that appears in the same cell population (Fig. 6).

4. Discussion

In the present study, we found that the CD34⁺/CD38⁻ stem cell population was more sensitive to radiation-induced apoptosis than were more differentiated cell populations, and that a decline in intracellular pH and an accumulation of intracellular

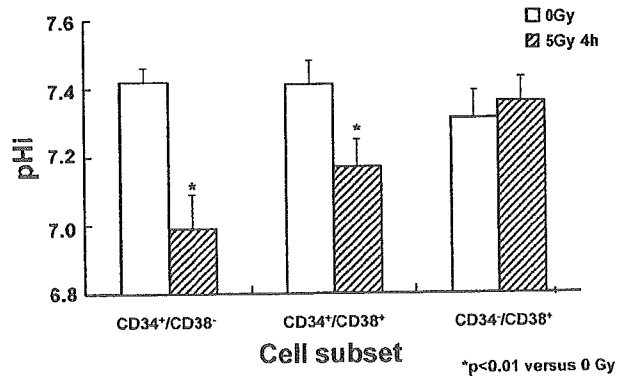


Fig. 6. Intracellular pH of irradiated UCB cell subsets. Ten to twenty cells were analyzed as depicted in Fig. 5. Emission ratios were converted to pH values by comparison with ratios observed in cells treated with nigericin. The values represent mean and S.D. of three experiments.

ROS in the stem cell population were early features prior to apoptosis.

Susceptibility to cell-death signal in early hemopoietic stem-progenitor cells has been partially clarified: It has been reported that hemopoietic stem-progenitor cells express membrane-bound FasL (mFasL) as well as Fas, which suggests autocrine and/or paracrine regulation of the Fas/FasL system in hemopoiesis [30–32]. It has also been reported that growth factor deprivation induces apoptosis of hemopoietic stem-progenitor cells through the Fas/FasL system, and that radiation-induced apoptosis can be prevented by the addition of a combination of KIT ligand, FLT-3 ligand, thrombopoietin, and interleukin-3 [33,34]. The radiation-induced apoptotic pathway, however, is different from the Fas-mediated apoptosis pathway: the apoptotic stimuli resulting from ionizing radiation induce a rapid decline of cytosol pH, followed by cytochrome c (cytC) release, caspase activation and mitochondrial swelling and depolarization, events that are not involved in the mitochondria-independent apoptosis found in Fas-mediated apoptosis [35]. Although there is general agreement that active mitochondria are needed for the variety of energy-required processes occurring during apoptosis, it seems apparent that energy charge is a key control point of cell death by either apoptosis or necrosis, and that cells with impaired mitochondrial energy metabolism reach a bioenergetic threshold that triggers apoptosis [36–41]. The background level of ROS in CD34⁺/CD38⁺ cells appeared to be higher than that

in CD34⁺/CD38⁻ cells. This may be due to that ROS generation during the continuous energy production for cell proliferation is pronounced in the former type of cells than in the latter. Such a difference in the background ROS generation may not be involved in different radiosensitivity between these cell populations. Our observation that declining intracellular pH accompanied ROS accumulation in irradiated CD34⁺/CD38⁻ cells may account for the significance of cellular energy charge that is decisive of radiation-induced apoptosis.

The accumulation of ROS is known to be associated with reduced levels of the cellular antioxidants GSH and NADPH, and the O₂^{•-} generated by the xanthine/xanthin oxidase system in mitochondria, where generation of a large amount of O₂^{•-} causes rapid cytC release to the cytosol. Madesh and Hajnóczky [42] examined the molecular machinery used by ROS to trigger apoptosis in the mitochondria phase. They found that cytC release induced by O₂^{•-} may have widespread significance in apoptosis, since in a number of pathological conditions – such as ischemia/reperfusion injury, drug insults, and inflammatory responses – large amounts of O₂^{•-} are produced by the xanthine oxidase-mediated catabolism of purine nucleotides, by increased electron transport chain activity or by activation of NADPH oxidase, particularly in neutrophils [43]. On the other hand, it has been reported that the efficiency of caspase activation by cytC is dependent on intracellular pH, with a pH optimum of 6.3–6.8 in vitro [35]. Furthermore, when changes in cytosolic pH induced by Bax were prevented by a use of F₀F₁-ATPase inhibitors or protonophores, caspase activation was impaired and fewer cells underwent apoptosis. Thus, mitochondria-induced cytosol acidification may promote cytC-mediated activation of caspases, a notion which our present results support.

When exploring the reasons for differences in cellular radiosensitivity, factors such as bcl-2 and p53 will need to be taken into account. In this study, decreased intracellular pH was closely associated with increased levels of O₂^{•-} generation as well as increased radiosensitivity among different types of blood cell populations. Thus, the ability of cells to control cellular energy charge may partly determine their susceptibility to radiation-induced apoptosis, and the molecules involved in this cellular control mechanism may play important roles in cell death. In addition, the significant elevation of intracellular ROS observed in the

CD34⁺/CD38⁻ stem cells, in response to radiation, suggested that the survived stem cells may proliferate bearing increased chromosomal aberrations. Further investigations of the molecular mechanisms by which DNA-damaged cells control their energy charges are therefore warranted.

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