Table III. Relationship between the methylation status and dietary factors in male patients

	CDX2 ($n = 58$)					*p16* ($n = 53$)					Any of *CDX2*, *p16* and *hMLH1* ($n = 53$)				
	Methylated ($n = 20$)	Unmethylated ($n = 38$)	Univariate[a] *P* value	Multivariate[b]		Methylated ($n = 8$)	Unmethylated ($n = 45$)	Univariate[a] *P* value	Multivariate[b]		Methylated ($n = 23$)	Unmethylated ($n = 30$)	Univariate[a] *P* value	Multivariate[b]	
				β^c	*P* value				β^c	*P* value				β^c	*P* value
Eating quantity															
moderate/light	10	25				4	28				11	21			
full/over	10	12	0.16	−0.37	0.02	4	17	0.39	0.06	0.73	12	9	0.09	−0.39	0.02
Green tea															
⩽6 cups/day	17	26				6	34				19	21			
⩾7 cups/day	2	12	0.08	0.31	0.02	2	10	0.60	−0.01	0.95	3	9	0.15	0.28	0.06
Cruciferous vegetables															
⩽twice/week	14	18				4	27				15	16			
⩾3 times/week	6	20	0.09	0.25	0.09	4	18	0.44	−0.20	0.23	8	14	0.28	0.15	0.34
Fruits															
⩽4 times/week	13	15				5	20				13	12			
⩾5 times/week	6	21	0.05	0.14	0.36	3	22	0.35	0.04	0.81	9	16	0.20	0.03	0.88
Beef															
⩽twice/month	13	16				7	21				15	13			
⩾once/week	6	22	0.06	0.01	0.93	1	23	0.04	0.29	0.10	7	17	0.07	0.02	0.93

[a]Used by Fisher's exact test.
[b]Adjusted for age, body mass index (BMI), histological classification, tumor size, smoking, alcohol drinking, eating quantity, and intake frequencies of green tea, cruciferous vegetables, fruits, and beef, using the categorical regression analyses.
[c]Regression coefficients in an optimal linear regression equation.

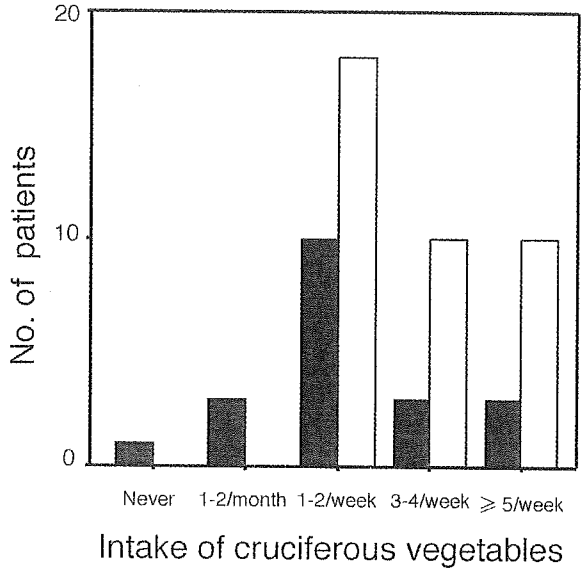

Fig. 4. Frequencies of the presence (closed bars) or absence (open bars) of *CDX2* methylation in gastric cancers stratified as to intake of cruciferous vegetables.

Table IV. *CDX2* methylation and combination of green tea and cruciferous vegetables

	CDX2		
	Methylated (n = 19)	Unmethylated (n = 38)	P value
Green tea ≤6 cups/day and cruciferous vegetables ≤twice/week	12 (52.2%)	11 (47.8%)	
Green tea ≥7 cups/day or cruciferous vegetables ≥3 times/week	7 (20.6%)	27 (79.4%)	0.01

with decreased frequency of *CDX2* methylation (P = 0.03). Distinct distribution of patients with the methylated and unmethylated *CDX2* is demonstrated for the intake of cruciferous vegetables (Figure 4).

Both green tea and cruciferous vegetables were inversely associated with *CDX2* methylation. Then we further analyzed the relationship between *CDX2* methylation and combination of these two factors. We found a stronger association in the combination (Table IV), i.e. the patients drinking more green tea or consuming cruciferous vegetables more often showed a lower methylation frequency than the patients drinking less green tea and consuming cruciferous vegetables less frequently.

Discussion

We reported here that cultured gastric cancer cells exhibiting no CDX2 expression showed methylation of the *CDX2* CpG island, while cultured gastric cancer cells exhibiting high CDX2 expression did not show methylation. Moreover, treatment of cultured cells with the demethylating agent 5-aza-2′-deoxycytidine activated *CDX2* expression. The data indicate that *CDX2* expression is silenced on methylation of the CpG island associated with the *CDX2* gene promoter region.

CDX2 gene methylation was found in 21 of the 73 (28.8%) primary gastric cancers. The methylation frequency in cancers with negative or partial CDX2 expression was significantly higher than that with positive expression, and five CDX2 expression-positive intestinal metaplastic tissues revealed no *CDX2* gene methylation as shown in Table I. Therefore, CDX2 expression may be silenced with gene methylation in primary gastric cancers, even though there may be also other unknown mechanisms. Because intestinal type gastric cancers are thought to develop through intestinal metaplasia (CDX2 expression-positive) (16,18–20), there may be more CDX2 expression-positive cancers in this type. On the contrary, many diffuse type gastric cancers may develop from the normal mucosa (CDX2 expression-negative) (16), and thus it is reasonable that there were more CDX2 expression-negative or -partial cancers in the diffuse type. The *CDX2* methylation frequencies were similar between the intestinal and diffuse type gastric cancers even in cancers with negative or partial expression. One reason might be that some gastric cancers develop as the intestinal type and then progress to the diffuse type with time (30).

The methylation frequency of *CDX2* did not show any significant relationship to pathologic characteristics, which may cause a bias in further analyses. We then analyzed the association between *CDX2* methylation and selected lifestyle factors known to be risky or preventive for gastric and colon cancers. The epidemiological analyses were carried out only on males, because *CDX2* methylation was found in only one female case. Univariate analysis revealed that none of the lifestyle variables was significantly associated with the methylation frequencies of *CDX2*, *p16*, *hMLH1* and any of the three genes except for beef intake and *p16*. However, multivariate analysis revealed significant differences between eating quantity and the methylation frequency of *CDX2* or any of the three genes, and also between the intake of green tea and *CDX2* methylation.

Full or overeating was associated with an increased frequency of methylation in *CDX2* and any of the three genes (P = 0.02 and P = 0.02, respectively). Although a few epidemiological studies have investigated eating quantity and gastric cancer, full eating is a consistent risk factor in the etiology of gastric cancer (31–33). One plausible interpretation may be that the gastric mucosa is physically damaged by repeated compulsory expansion of the gastric lining upon full eating, possibly resulting in an increased sensitivity of the cells to various exogenous compounds in food, which may include those promoting DNA methylation.

Among the 15 food groups and selected food items, an increased intake of green tea was independently and significantly associated with the *CDX2* methylation frequency (P = 0.02) and methylation in any of *CDX2*, *p16* and *hMLH1* (borderline significance, P = 0.06), after adjusting for confounding lifestyle and clinical variables. The effects of green tea seem to be dose-dependent: the *CDX2* methylation frequencies, 10/25 (40%), 7/18 (39%), 2/8 (25%), and 0/6 (0%) in three or less, four to six, seven to nine and ten cups or more a day, respectively.

Green tea contains several polyphenolic compounds, such as epigallocatechin gallate (EGCG). Significant inhibitory effects of EGCG or green tea extracts on carcinogenesis of rodents in various organs including the stomach have been demonstrated in many studies (34,35). Most epidemiological studies in Japan revealed cancer-preventive effects of drinking green tea

(36–39). Furthermore, it was reported recently that EGCG dose-dependently inhibited DNA methyltransferase activity in several cancer cells, resulting in the reactivation of methylation-silenced genes (*p16*, *retinoic acid receptor* β and *hMLH1*) (40). Taken together, our findings imply a novel mechanism of green tea in cancer prevention, i.e. inhibition of methylation of selected genes involved in gastric carcinogenesis.

Although we analyzed the association between dietary factors and DNA methylation using the categorical regression model, this analysis may overlook some factors due to a small number of study subjects. Therefore, we further reinvestigated the association by examining overall differences in the distribution of patients with methylated or unmethylated *CDX2* on the intake of food groups, using the non-parametric test, and found that the intake frequency of cruciferous vegetables in patients with unmethylated *CDX2* was distributed in higher categories than that in patients with methylated *CDX2* ($P = 0.03$, Figure 4). Cruciferous vegetables have been reported to be anticarcinogenic in a number of epidemiological and laboratory studies. Particularly, the active compounds in cruciferous vegetables, such as arylalkyl isothiocyanates (and their glucosinolate precursors) and indole-3-carbinol, have been extensively investigated, and the roles of isothiocyanates were presumed to suppress activating enzymes and induce detoxifying ones of carcinogens (41,42). There were several reports suggesting other cancer-preventive mechanisms of isothiocyanates, for example, dose-dependent inhibition of DNA methylation in nitrosomethylbenzylamine-induced esophageal tumorigenesis of rats (43), and inhibition of *Helicobacter pylori* and benzo[*a*]pyrene-induced stomach tumors in mice (44). Our findings provide evidence in humans supporting the cancer-preventive effects of cruciferous vegetables through the inhibition of DNA methylation.

We observed large differences in the methylation frequencies of genes examined, particularly *CDX2*, between male and female patients. Unexpectedly, gender comparison in dietary factors including eating quantity, and intake of green tea and cruciferous vegetables did not reveal any significant difference. However, male patients included more cases with advanced age at diagnosis (>60 years) than female ones ($P = 0.06$), and the intestinal type was more frequently found in male patients than in female ones ($P < 0.05$), implying a gender difference in the etiology of gastric cancer. These are in consistence with previous reports on gender difference in age at diagnosis and frequencies of gastric carcinoma and intestinal metaplasia (45,46). Thus, the gender difference in methylation frequency might be ascribed to gender-specific host factors, such as estrogens, not to lifestyle factors.

In this study, the methylation of *CDX2* and other genes involved in gastric carcinogenesis was investigated in relation to the clinicopathological and selected lifestyle factors of gastric cancer patients. We therein hypothesized that some of the lifestyle factors, particularly dietary ones, which have been reported to be risky or preventive for gastric cancer in epidemiological observation, may influence the development of gastric cancer through methylation of the selected genes. We for the first time found the inverse association of *CDX2* methylation with the intake of green tea and cruciferous vegetables, which have previously been suggested only by *in vitro* or animal studies, although a further study with an increased number of study patients should be required. Our findings may thus advance the chemoprevention of gastric cancer from a view of inhibiting gene methylation.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Drs Stephen B.Baylin, James G.Hermann and Satoshi Miyake for helpful discussion, and Yoshiko Ozawa for assistance in the preparation of this manuscript.

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Received July 22, 2004; revised September 5, 2004; accepted September 29, 2004

Identification of the *NKG2D* Haplotypes Associated with Natural Cytotoxic Activity of Peripheral Blood Lymphocytes and Cancer Immunosurveillance

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Abstract

We have previously shown that natural cytotoxic activity of peripheral blood lymphocytes was inversely related to cancer development based on a prospective cohort study. The genetic fraction of cytotoxic activity needs to be clarified, identifying individuals immunogenetically susceptible to cancer. A case-control study within the cohort members was designed: 102 cancer cases with peripheral lymphocyte DNA available and three control groups, each of which consisted of 204 subjects with each tertile level of cytotoxic activity. We first compared two control groups with high and low cytotoxic activity in terms of the single nucleotide polymorphisms in the natural killer complex gene region on chromosome 12p, identifying the haplotype alleles that were associated with the activity. Next, cancer risks were assessed for these haplotypes. We found two haplotype blocks, each of which generated two major haplotype alleles: low-activity-related *LNK1* (frequency 0.478 and 0.615 in groups with high and low activity, respectively; $P < 0.00008$) and high-activity-related *HNK1* (0.480 and 0.348; $P < 0.0001$), *LNK2* (0.711 and 0.821; $P < 0.0002$), and *HNK2* (0.272 and 0.174; $P < 0.0008$). These *NKG2D* haplotype alleles showed a significant difference between cases (0.632 for *LNK1* and 0.333 for *HNK1*) and controls (0.554 for *LNK1* and 0.406 for *HNK1*). The haplotype *HNK1/HNK1* revealed a decreased risk of cancer (odds ratio, 0.471; 95% confidence interval, 0.233-0.952) compared with *LNK1/LNK1*. Individuals who are genetically predisposed to have low or high natural cytotoxic activity can in part be determined by *NKG2D* haplotyping, which in turn reveals an increased or decreased risk of cancer development. (Cancer Res 2006; 66(1): 563-70)

Introduction

The initial mechanism of cancer immunosurveillance is thought to be a tumor-associated antigen nonspecific cytotoxicity that involves natural killer (NK) cells. In numerous past laboratory studies on cancer immunosurveillance, there were clear indications of significant roles played by the natural cytotoxicity of various lymphocytes in preventing the development of cancer (1–5) but it was a difficult task to extrapolate these results to yield an estimation of human cancer risk. One of the most critical questions in immunosurveillance against cancer has been whether interindividual differences of natural immunologic host defense could predict future development of common cancers, including those with no known viral etiology, among healthy individuals. To answer this question, we began a prospective cohort study among a Japanese general population in 1986 using various immunologic and biochemical markers measured at baseline. Using an 11-year follow-up of this cohort study—where the cytotoxic activity (measured at baseline by the isotope release method using K567 as target cells) was categorized into high, medium, and low levels by tertiles—we previously reported that individuals with high or medium levels of natural cytotoxic activity of peripheral blood lymphocytes had a decreased risk of cancer development compared with those with low cytotoxic activity (6). This was the first evidence of the vital role played by natural immunologic 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 immunoprevention and the usefulness of natural cytotoxic activity as a surrogate biomarker for this prevention (7).

It seems unlikely that the wide variations of natural cytotoxic activity among healthy individuals observed in this cohort study can be fully explained by environmental or lifestyle factors alone. A cross-sectional analysis of cohort members estimates the contribution of usual lifestyle to interindividual variations of natural cytotoxic activity to be ~30% and selected healthy lifestyle factors (e.g., not smoking, regular diet and sleep, proper body weight, moderate physical activity, and less mental stress) are in part associated with increased cytotoxic activity (8, 9). Given the important implications of our previous findings, we feel it is warranted to examine the genetic background underlying individual variations in natural cytotoxic activity, if such exists.

This study thus aims to identify the genetic factors associated with natural cytotoxic activity and then to assess the cancer risk of individuals who are predisposed to have low natural cytotoxic activity based on a phenotype-genotype association analysis and a case-control study within the cohort study. In this phenotype-genotype association analysis, we focused on a 270 kb region within an annotated region of ~2 Mb called the natural killer complex (NKC) gene region 12p13.2-p12.3 because this 270 kb region contains important NK receptor gene loci, such as *CD94* gene and killer cell lectin-like receptor family genes (10). Of these, we found that the *NKG2D* haplotypes revealed a significant association with the natural cytotoxic activity of individuals. The *NKG2D* gene encodes an activating homodimeric C-type lectin receptor, which is expressed on NK cells, $CD8^+\alpha\beta$ T cells, $\gamma\delta$ T cells, and activated macrophages, and is located at the NK complex gene locus (11, 12). The NKG2D triggers cell-mediated cytotoxicity in NK cells via the

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doi:10.1158/0008-5472.CAN-05-2776

DAP10-phosphoinositol 3-kinase signaling pathway, upon the recognition of their self-ligands, such as MICA, MICB, ULBP1, and ULBP2, which are distantly related to MHC class I (11–16). MICA and MICB are not usually expressed in normal cells but are found at low levels on intestinal epithelial cells; they are induced by cellular stress, typically in tumor or virus-infected cells (17, 18). Recently, NKG2D was reported to be a key factor in priming T-cell immunity as well as a primary cytotoxicity receptor (19).

Next, a case-control study was conducted within the cohort to assess the risk of cancer development on the basis of the *NKG2D* haplotypes: Results indicated that these haplotypes may be associated with immunogenetic susceptibility to cancer development. Along with these findings, our results also show an advantage of molecular epidemiology cohort studies (i.e., they make possible the measurement of phenotype biomarkers that would potentially be influenced by cancer and the subsequent genetic association analyses for both phenotype biomarkers and cancer risk).

Materials and Methods

Study population. We conducted a case-control study within the Saitama prospective cohort study, which began in 1986, with measurement of natural cytotoxic activity of peripheral blood lymphocytes and other immunologic markers among self-selected 3,625 individuals ages over 40 years living in a town in Saitama Prefecture, Japan, who participated in yearly health checks during 1986 to 1990 (accounting for ~40% of all residents of this age group). We did a follow-up survey on cancer incidence and death from all causes up to 2000: Cancer cases were identified primarily by death certificate and national health insurance receipts, followed by confirmation of primary site, histology, and date of diagnosis through inquiry at the hospitals. This study is described in detail elsewhere (6, 9, 20, 21). Briefly, the cytotoxic activity of peripheral lymphocytes was determined by ^{51}Cr-release assay with an effector-to-target ratio of 20 and incubation of effector and target cells for 3 hours 30 minutes, by using K562, a human myeloid leukemia cell line, as target cells. On the basis of a follow-up study from 1986 to 1997, we previously reported that individuals with high or medium cytotoxic activity revealed a decreased risk of cancer development, with a relative risk of 0.59 [95% confidence interval (CI), 0.40-0.87, estimated for both sexes] or 0.63 (95% CI, 0.43-0.92), respectively, when the cytotoxic activity (percent specific lysis) was categorized by tertiles: ≤42%, 43% to 58%, and >58% for low, medium, and high, respectively, among men; ≤34%, 35% to 51%, and >51% for low, medium, and high among women (corresponding tertiles for men and women were combined for the analysis of both sexes). Of 3,625 participants, a total of 2,063 individuals gave additional peripheral blood samples for DNA extraction.

In an extended follow-up study from 1986 to 2000, we identified 259 cancer incidence cases in all sites, 115 of whom have lymphocyte DNAs available. Of 115 cancer cases with their DNAs available, we further excluded 13 cancer cases who were ages over 75 years at the time of the assay of cytotoxic activity or who were diagnosed within 2 years after the assay of cytotoxic activity, as we had done in our previous analysis (6). The final total was 102 cancer cases (54 men and 48 women) in all sites, with the most frequent cancers being stomach (n = 19), lung (n = 8), and colorectum (n = 5) for men, and stomach (n = 10), colorectum (n = 6), and lung (n = 5) for women.

Assays for immunologic measurements and DNA extraction were done at the health screening checks. DNA was obtained from the participants at their second visit to the health screening checks during the baseline survey because all blood samples at the first visit had to be used for immunologic and biochemical assays. We compared cancer risk (based on tertile levels of the cytotoxic activity) and natural cytotoxic activity between the groups with and without DNA available. No significant differences were found between them (data not shown). Epidemiologic variables (smoking, alcohol consumption, physical activity, body mass index, etc.) in cancer cases and noncancer cohort members also showed no significant differences by the status of DNA extraction. Therefore, we think that a selection bias, even if it exists, did not significantly influence our results.

Two controls, who were individually matched to one case with respect to gender and age (±5 years), were randomly selected from each of the trisected groups with low, medium, and high cytotoxic activity. The final total was 612 controls comprising three groups (204 controls each) with low, medium, and high cytotoxic activity, who showed median 31% (range 5-42%), 51% (43-58%), and 68% (59-90%) among men; 26% (8-34%), 43% (35-51%), and 59% (52-85%) among women.

This case-control study has two purposes: (*a*) identification of genetic factors involved in individually differing cytotoxic activity and (*b*) estimation of cancer risk for these cytotoxic activity–related genetic factors. The former approach was undertaken by comparing the two control groups with low and high cytotoxic activity in terms of frequencies of single nucleotide polymorphisms (SNPs) in a 270 kb region within the NKC gene region on chromosome 12p, called the phenotype-genotype association analysis. The latter was undertaken by comparing cases and entire control groups (with low, medium, and high cytotoxic activity) in terms of odds ratios (OR). The baseline characteristics of cases and controls are shown in Table 1.

This study was approved by the Genome Ethical Committee at the Radiation Effects Research Foundation.

Identification and genotyping of SNPs. The Celera Genomic database (22, 23) was used to screen marker SNPs in the NKC gene region, along with

Table 1. Baseline characteristics of study subjects

Gender	Cases		Controls selected from cohort members with trisected natural cytotoxic activity					
			High		Medium		Low	
	Men (n = 54)	Women (n = 48)	Men (n = 108)	Women (n = 96)	Men (n = 108)	Women (n = 96)	Men (n = 108)	Women (n = 96)
Age at entry (y)								
40-49	3	9	6	18	6	18	6	18
50-59	15	20	32	40	34	40	32	40
60-69	31	17	63	34	60	34	62	34
70-74	5	2	7	4	8	4	8	4
Mean (SE)	61.6 (0.9)	57.1 (1.2)	60.6 (0.7)	57.3 (0.8)	60.7 (0.6)	57.3 (0.8)	60.8 (0.6)	57.4 (0.9)
Natural cytotoxic activity (percent specific lysis)								
Mean (SE)	48.6 (2.3)	41.4 (2.5)	68.5 (0.7)	60.1 (0.7)	51.2 (0.4)	42.5 (0.6)	29.6 (0.9)	23.8 (0.8)
Range	83-18	89-18	90-59	85-52	58-43	51-35	42-5	34-8
Smokers (%)	32 (60.4)	2 (4.2)	62 (57.4)	4 (4.2)	70 (64.8)	2 (2.1)	72 (67.3)	6 (6.3)

Table 2. Primers used for 20 SNPs

NKC	SNP ID (NCBI)	Forward primer	Variations	Reverse primer
1	rs3759272	TGGGCAAAACACAATGTTCAGAATT	T/G	GGGCGTCAACAAACGAATCTTG
2	rs2537752	TCTGGAGTCTATAAAATGTTTTTAAACAGTGTCA	A/T	TCTCAAATGTAGGTGAACGAATTTCATCA
3	rs1049174	CTGCCCATGAGGCAATTTCC	C/G	GGATCAGTGAAGGAAGAGAAGGC
4	rs2255336	CTGTAGCCATGGGAATCCGTTT	A/G	GCAATCTACTTCTCTGTTGTCACTTACA
5	rs2294148	AGAAACTAAACTAAACTACACAGAGGTTGC	A/G	GATGTGGAGTCAGACTTGAATTTTACTCA
6	rs2049796	AAGCATCTAAGAAACAATTAGAATTACCTTATAGTGTAAATAT	C/A	CAGGTGTGTGTATGTGTGTATGTGT
7	rs2617160	ATGACTAATGTAAAAGTAAAAAAGTCTGCAAACA	A/T	GCCTTGAGTTCATATAATTACAATACACCAGT
8	rs7972757	TGATTGCCATTAAACCTTCCATTTCCT	A/G	GTCGTTAAAGGCATCGTTCCATCTA
9	rs2246809	ACCCTTAAGAGAAAAGGCTTTCATGTAC	A/G	ACTGGTCATTCTGTATTGCCTGTTT
10	rs2617169	GGGATGCAAAATGATAATAAAATGTTTTGGG	A/T	GGAGAAAAGGACATGCCCTCATAT
11	rs2617170	TGACAATCATAATGTACCTTTCTGCATTCT	C/T	CACTTTAATTTTTCTAGGTATTGGAGTACTGGA
12	rs2617171	CCCAAGATAATATGCTGCTTCTGAAC	C/G	TCTCTTAAAACATGTCTTTGAGTCATGAAATCA
13	rs1971939	TCATTGCATATACCTAATGATACAAGTTCAACA	C/G	GGCTCACTGGCCTGTCTT
14	rs1915319	GTATTCTGTATTTGACATAATATTACTAGTGGGAACAAT	A/G	CTATTGGTGTTAAACATTTTTGAAGAATCTAACCTTA
15	rs4763525	AGACATGCCTTTCATGTAAGCATAAAGA	A/G	CCTGGGAGTGGGATTGCT
16	rs3003	TGTACTTTAGTAATTGTGTGCATCCTATTTCA	C/T	GCCCAGTGTGGATCTTCAATGATAT
17	rs1983526	GGCCCTCTGAGGCACTAAATAG	C/G	CAGAGTGGGATCTTTGGTTCATGAT
18	rs10772285	AGCCTCAGTAATGGCAGATGC	C/G	ACTGCCAGCAGAGCATTCT
19	rs1915325	TCACTGGTAAGTAAAGTGTAGTGTATCTGA	A/G	TGTTTATCATTTAGCCACACAAAAGAGC
20	rs2607893	CACCTTATCCCAAGTGCATCAACT	T/C	ACCAATGTAAAACCCATAGCACAGT

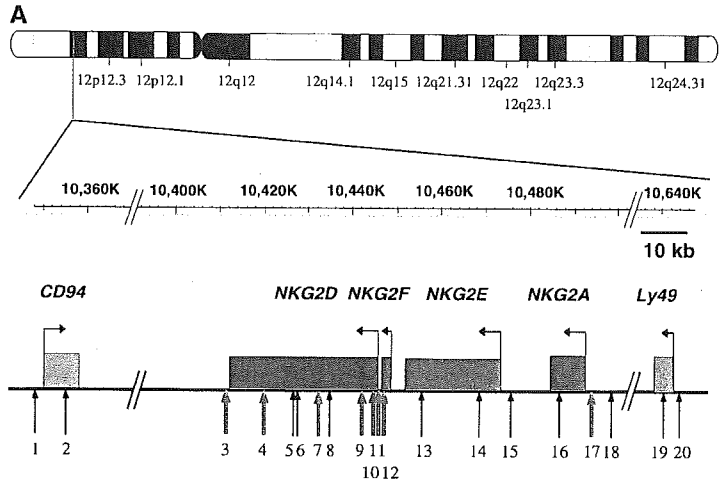
the detection of novel SNPs over the region using National Center for Biotechnology Information (NCBI) database: In this region, over 1,300 SNPs have been registered in the Celera Genomic database and NCBI database. We selected the 25 SNPs with allele frequency >10% among either Caucasian or Japanese. After examining allele frequency in the study population, we found that 20 of 25 SNPs actually showed a frequency >10%. We then selected these 20 SNP loci, named NKC-1 to NKC-20, which revealed variant allele frequencies >10% among our study population. The sequences of the primers used for 20 SNPs are listed in Table 2; the SNPs from NKC-1 to NKC-20 cover *CD94, NKG2D, NKG2F, NKG2E, NKG2A*, and *Ly49* genes, and the localization is shown in Fig. 1*A*. Primers and probes for these SNPs were designed using Primer Express software, version 2.1 (Applied Biosystems, Foster City, CA). The TaqMan-Allelic Discrimination method was used for the detection of SNPs. All of the assays were conducted in 384-well PCR plates. The principle of TaqMan Real-Time PCR assay system using fluorogenic probes and the 5′ nuclease is described by Livak (24). Amplification reactions (5 μL) were done in duplicate with 10 ng of template DNA, 1 × TaqMan Universal Master Mix buffer (Applied Biosystems), 300 nmol/L of each primer, and 200 nmol/L of each fluorogenic probe. Thermal cycling was initiated with a 2-minute incubation at 50°C, followed by a first denaturation step of 10 minutes at 95°C, and then by 40 cycles of 15 seconds at 95°C and of 1 minute at 60°C. After PCR was completed, plates were brought to room temperature, read in an ABI PRISM 7900 Sequence Detection System (Applied Biosystems), and results were analyzed using the Allelic Discrimination software.

Haplotype analysis and risk estimation. The linkage disequilibrium was estimated by relative linkage disequilibrium coefficients (D'), r^2 values, and the χ^2 values. Haplotype allele frequencies and haplotype distributions were estimated on the basis of multiple SNPs by the expectation-maximization algorism, using SNPAlyze (DYNACOM, Yokohama, Japan, http://www.dynacom.co.jp/). Statistical significance was examined by the χ^2 test. ORs were calculated along with 95% CI values using SPSS software program (version 11.1).

Results

Association between SNPs in the NKC region and natural cytotoxic activity. A genome approach was undertaken in the Saitama cohort study. Before case-control comparison, we did a phenotype-genotype association analysis done to identify the genetic factors involved in the natural cytotoxic activity of peripheral blood lymphocytes of individuals. Specifically, we examined the association between the 20 SNPs on the annotated 270 kb region within the NKC gene region and natural cytotoxic activity by comparing the allele frequency of the two control groups with high and low natural cytotoxic activity, together with ORs estimated for low natural cytotoxic activity versus high activity. Among these 20 SNPs, we found, in Table 3, that eight SNPs were closely associated with natural cytotoxic activity, having P values <0.001: NKC-3 (P = 0.00004), NKC-4 (0.0002), NKC-7 (0.00004), NKC-9 (0.0006), NKC-10 (0.0005), NKC-11 (0.00003), NKC-12 (0.00004), and NKC-17 (0.0002). It is notable that these natural cytotoxic activity–related SNPs are mostly located in the *NKG2D* gene region, except for NKC-17 that is located in the promoter region of the *NKG2A* gene (Fig. 1*A*).

Identification of haplotype blocks. We did linkage disequilibrium analysis on the basis of the 20 SNPs listed in Table 2. When looking at natural cytotoxic activity–related SNPs, many of these are closely linked to each other, with r^2 values >0.9, and this kind of close linkage is hardly ever found among other activity-nonrelated SNPs, except NKC-6 and NKC-8 (Fig. 1*B*). On the basis of the linkage disequilibrium analysis, Fig. 1*C* shows the relation between linkage disequilibrium (r^2) and the physical distance between the SNPs. All combinations of each pair of SNPs are plotted. An abrupt drop of r^2 values in the distance >80 kb in Fig. 1*C* implies that there are no haplotype blocks longer than 80 kb in this region. It is of much interest that most combinations of the natural cytotoxic activity–related SNPs revealed relatively strong linkage disequilibrium, whereas those of nonrelated SNPs showed weak or no linkage disequilibrium. When we divided the natural cytotoxic activity–related SNPs into two groups colored blue and orange, all combinations of blue-blue and orange-orange revealed a strong linkage disequilibrium, with r^2 values >0.9, whereas blue-orange combinations showed much weaker linkage disequilibrium, with r^2



B

Natural cytotoxic activity-related SNPs

NKC SNPs	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1																			
2	0.86																		
3	0.01	0.01																	
4	0.00	0.00	0.28																
5	0.01	0.02	0.29	0.05															
6	0.02	0.02	0.35	0.07	0.81														
7	0.01	0.01		0.36	0.28	0.33													
8	0.02	0.02	0.35	0.06	0.80		0.33												
9	0.00	0.00	0.30		0.06	0.08	0.38	0.07											
10	0.00	0.00	0.30		0.06	0.08	0.38	0.07											
11	0.00	0.01		0.36	0.27	0.32		0.32	0.38	0.38									
12	0.01	0.01		0.36	0.27	0.33		0.32	0.38	0.39									
13	0.02	0.02	0.28	0.05		0.79	0.26	0.78	0.06	0.06	0.27	0.27							
14	0.01	0.01	0.26	0.04	0.55	0.73	0.25	0.72	0.05	0.05	0.25	0.26	0.56						
15	0.00	0.00	0.46	0.22	0.08	0.11	0.50	0.10	0.24	0.24	0.51	0.51	0.08	0.15					
16	0.00	0.00	0.39	0.00	0.45	0.58	0.38	0.58	0.00	0.00	0.38	0.38	0.44	0.66	0.24				
17	0.01	0.01	0.86	0.34	0.26	0.31		0.30	0.36	0.37			0.25	0.24	0.55	0.36			
18	0.01	0.02	0.53	0.02	0.50	0.58	0.48	0.57	0.02	0.03	0.50	0.51	0.50	0.47	0.22	0.41	0.48		
19	0.01	0.00	0.02	0.02	0.01	0.00	0.03	0.00	0.02	0.02	0.03	0.03	0.01	0.01	0.08	0.00	0.04	0.01	
20	0.03	0.02	0.00	0.07	0.14	0.09	0.00	0.08	0.08	0.08	0.00	0.00	0.13	0.03	0.06	0.01	0.00	0.02	0.19

C

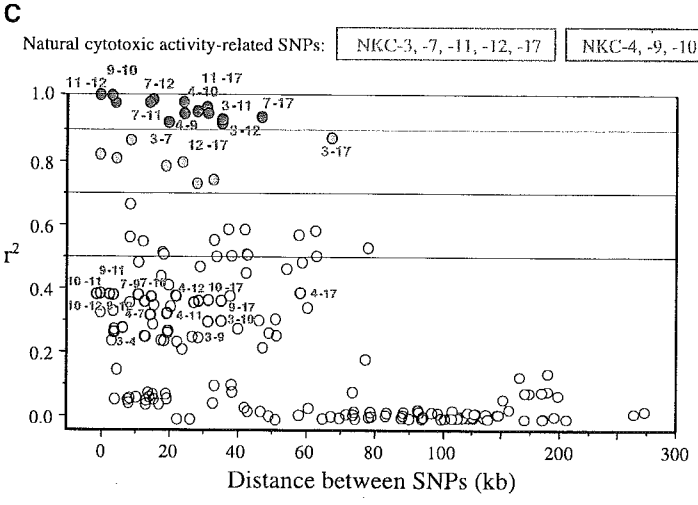


Figure 1. Identification of haplotype blocks. *A*, 20 SNPs examined in the 270 kb region within the NKC gene region (*arrows with numbers from 1 to 20*). *Red arrows*, eight SNPs closely associated with natural cytotoxic activity with $P < 0.001$. *B*, linkage disequilibrium analysis. Brown SNPs (*3, 4, 7, 9, 10, 11, 12*, and *17*) are natural cytotoxic activity-related SNPs with $P < 0.001$; *red elements in the lower triangle*, close linkage disequilibrium with $r^2 \geq 0.9$; *pink*, $0.9 > r^2 \geq 0.7$; *yellow*, $0.7 > r^2 \geq 0.5$. *C*, relation between linkage disequilibrium (r^2) and the physical distance between the SNPs. All combinations of every pair of SNPs among the 20 are plotted. *Redplot*, $r^2 \geq 0.9$; *pinkplot* $0.9 > r^2 \geq 0.7$; *yellow plot*, $0.7 > r^2 \geq 0.5$. *Numbers in plots*, the combined two NKC SNPs belonging to natural cytotoxic activity–related SNPs (*blue* or *orange*).

values <0.5 (Fig. 1*C*), indicating that five blue SNP sites belong to one haplotype block and three orange SNP sites to a different haplotype block. We finally identified the two haplotype blocks and named them NKG2D hb-1 and hb-2, each of which generated two major haplotype alleles related to low and high natural cytotoxic activity phenotypes (Fig. 2*A*).

Association between *NKG2D* haplotypes and natural cytotoxic activity. We estimated the haplotype allele frequencies in the groups with high and low natural cytotoxic activity and compared between groups (Fig. 2*B*). Respective low and high cytotoxic activity–related alleles *LNK1* and *HNK1* on NKG2D hb-1 revealed a close association with natural cytotoxic activity (P = 0.00008 and 0.0001, respectively) and this was also the case with *LNK2* and *HNK2* on NKG2D hb-2 (P = 0.0002 and 0.0008, respectively). To confirm the close association between natural cytotoxic activity and *NKG2D* haplotypes, we compared mean (±SE) natural cytotoxic activity of *LNK1/LNK1*, *LNK1/HNK1*, and *HNK1/HNK1* haplotypes among a total of 612 controls: The results were 42.1 ± 1.2 (n = 196), 47.8 ± 1.1 (260), and 50.1 ± 1.7 (109), respectively (P_{trend} < 0.001); 47 controls having heterozygous haplotypes other than *LNK1/HNK1* showed mean natural cytotoxic activity of 45.1 ± 2.7.

***NKG2D* haplotypes and cancer risk.** Finally, we estimated the risk of cancer development for the *NKG2D* haplotypes: *LNK1/LNK1*, *LNK1/HNK1*, and *HNK1/HNK1* from NKG2D hb-1 along with *LNK2/LNK2*, *LNK2/HNK2*, and *HNK2/HNK2* from NKG2D hb-2. A case-control study within the Saitama cohort study was done among those cohort members whose DNA of peripheral lymphocytes were available for this study. In Table 4, cases revealed increased and decreased frequencies (0.632 and 0.333, respectively) of *LNK1* and *HNK1* alleles, compared with those (0.554 and 0.406, respectively) in controls (Table 4). Individuals carrying *HNK1/HNK1* have a significantly reduced risk of cancer with an OR of 0.471 (crude, 95% CI, 0.233-0.952) or 0.482 (adjusted, 0.237-0.982), indicating that those with *LNK1/LNK1*, one third of the general population, have an enhanced risk of cancer development (Table 4). On the other hand, *LNK2* and *HNK2* alleles did not show any statistically significant differences between cases and controls because of the small number of subjects with *HNK2/HNK2*.

Discussion

Natural immunologic host defense plays the key role in occurrence of common cancers found in a general population, as we previously reported on the basis of an 11-year follow-up of the Saitama cohort study (6). This finding could lead us to a new field, cancer immunoprevention, which would aim to enhance the ability of the immune system to recognize and

Table 3. Eight SNPs closely associated with natural cytotoxic activity

NKC (reference SNP ID)	Genotype	No. subjects (%)		OR (95% CI)
		High activity	Low activity	
NKC-3 (rs1049174)	*C/C*	53 (26)	89 (44)	1.00
	C/G	102 (50)	88 (43)	0.514 (0.330-0.801)
	G/G	49 (24)	27 (13)	0.328 (0.184-0.568)
	Fr. of *C*-allele	0.510	0.652	P = 0.00004
NKC-4 (rs2255336)	*G/G*	107 (52)	139 (68)	1.00
	G/A	79 (39)	59 (29)	0.575 (0.377-0.876)
	A/A	18 (9)	6 (3)	0.257 (0.098-0.669)
	Fr. of *G*-allele	0.718	0.826	P = 0.0002
NKC-7 (rs2617160)	*T/T*	51 (25)	84 (41)	1.00
	T/A	101 (50)	93 (46)	0.559 (0.357-0.875)
	A/A	52 (25)	27 (13)	0.315 (0.176-0.563)
	Fr. of *T*-allele	0.498	0.640	P = 0.00004
NKC-9 (rs2246809)	*G/G*	107 (53)	137 (67)	1.00
	G/A	80 (39)	61 (30)	0.596 (0.392-0.905)
	A/A	17 (8)	6 (3)	0.276 (0.105-0.723)
	Fr. of *G*-allele	0.721	0.821	P = 0.0006
NKC-10 (rs2617169)	*T/T*	106 (52)	137 (67)	1.00
	T/A	81 (40)	61 (30)	0.583 (0.384-0.885)
	A/A	17 (8)	6 (3)	0.273 (0.104-0.717)
	Fr. of *T*-allele	0.718	0.821	P = 0.0005
NKC-11 (rs2617170)	*C/C*	49 (24)	83 (41)	1.00
	C/T	102 (50)	93 (45)	0.538 (0.343-0.845)
	T/T	53 (26)	28 (14)	0.312 (0.175-0.556)
	Fr. of *C*-allele	0.490	0.635	P = 0.00003
NKC-12 (rs2617171)	*C/C*	49 (24)	83 (41)	1.00
	C/G	103 (50)	93 (45)	0.533 (0.340-0.837)
	G/G	52 (26)	28 (14)	0.318 (0.178-0.567)
	Fr. of *C*-allele	0.493	0.635	P = 0.00004
NKC-17 (rs1983526)	*G/G*	47 (23)	78 (38)	1.00
	G/C	104 (51)	95 (47)	0.550 (0.349-0.869)
	C/C	53 (26)	31 (15)	0.352 (0.199-0.625)
	Fr. of *C*-allele	0.485	0.615	P = 0.0002

A

Alleles	Related activity	NKC-3	4	7	9	10	11	12	17
		Structure (htSNPs)							
NKG2D hb-1									
LNK1	Low	C		C			T	C	C
HNK1	High	G		T			A	G	G
NKG2D hb-2									
LNK2	Low		T		G	G			
HNK2	High		A		A	A			

B

	Allele frequency		
	High activity	Low activity	*P* value (χ^2)
LNK1	0.478	0.615	0.00008 (15.5)
HNK1	0.480	0.348	0.0001 (14.7)
LNK2	0.711	0.821	0.0002 (13.8)
HNK2	0.272	0.174	0.0008 (11.3)

Figure 2. Natural cytotoxic activity–related haplotype alleles. *A*, *LNK1* and *HNK1* are generated from haplotype block NKG2D hb-1, and *LNK2* and *HNK2* from NKG2D hb-2. *B*, allele frequencies are estimated for groups (n = 408 chromosomes for each group) with high and low natural cytotoxic activity.

eliminate nascent transformed cells in the body (7). The innate immune system, in its initial response to a pathogen, may also be involved in determining how long and how strongly inflammation will continue after pathogen infection, in some cases leading to a sequential process of infection to inflammation to cancer (25, 26).

The natural cytotoxic activity measured in the Saitama cohort study revealed wide variations among individuals, only a part of which can be explained by environmental factors. We thus investigated genetic determinants of this cytotoxic activity, where NK cells work as a major effector. Given that the varying cancer risk of individuals can be in part ascribed to natural cytotoxic activity, it is necessary to clearly assess the genetic/invariable fraction of the cytotoxic activity so that we can look at the variable fraction of the activity, which would be a surrogate marker for cancer immunoprevention. In this study, we succeeded in identifying haplotype alleles, which were constructed from five or three SNPs mostly located in the *NKG2D* gene region and closely associated with high and low natural cytotoxic activity of individuals. This was the first identification of

Table 4. Risk of cancer incidence for the *NKG2D* haplotypes

NKG2D hb-1

Haplotype	Cases *n* (%)	Controls *n* (%)	Crude OR (95% CI)	Adjusted OR* (95% CI)
LNK1/LNK1	42 (41)	196 (32)	1.00	1.00
LNK1/HNK1 †	42 (41)	260 (42)	0.754 (0.473-1.20)	0.694 (0.430-1.12)
HNK1/HNK1	11 (11)	109 (18)	0.471 (0.233-0.952)	0.482 (0.237-0.982)
	Allele frequency		*P*	
LNK1	0.632	0.554	0.036	
HNK1	0.333	0.406	0.049	

NKG2D hb-2

Haplotype	Cases *n* (%)	Controls *n* (%)	Crude OR (95% CI)	Adjusted OR (95% CI)
LNK2/LNK2	67 (65)	371 (61)	1.00	1.00
LNK2/HNK2 ‡	26 (25)	203 (33)	0.709 (0.437-1.15)	0.701 (0.425-1.16)
HNK2/HNK2	3 (3)	27 (4)	0.615 (0.181-2.09)	0.642 (0.188-2.19)
	Allele frequency		*P*	
LNK2	0.789	0.778	0.7	
HNK2	0.171	0.212	0.2	

*Adjusted for relative body weight, cigarette smoking, alcohol consumption, and intake of green vegetables.
†Seven cases and 47 controls with heterozygous haplotypes other than *LNK1/HNK1* were excluded.
‡Six cases and 11 controls with heterozygous haplotypes other than *LNK2/HNK2* were excluded.

individuals who are genetically predisposed to have low natural cytotoxic activity and consequent high risk of cancer development: It is they who will, therefore, be the logical targets for immunoprevention of cancer and virus-related diseases. Our preliminary analysis implied that the influence of lifestyle factors on the cytotoxic activity of individuals might depend on their haplotypes, e.g., cigarette smokers with *HNK1/HNK1* showed lower activity than nonsmokers with the same haplotype, although this decrease was not obvious in other haplotypes; increased intake of green vegetables was associated with increased cytotoxic activity among those with *LNK1/LNK1* but not *HNK1/HNK1* (data not shown). Although an intervention study is needed to confirm the influence of lifestyle factors, this preliminary finding suggests the possibility of individualized cancer prevention based on gene-environment interactions.

Because no strong linkage disequilibrium spanning over 80 kb was found in the 270 kb region, the five or three cytotoxic activity-related SNPs located on NKG2D hb-1 or hb-2, respectively, apparently include the SNP(s) carrying functional significance, although all these SNPs showed high significance levels of association. These five or three SNPs (Table 3) are located in the noncoding regions of the genes and it is likely that some of these SNPs may be involved in transcription regulation of the *NKG2D* or *NKG2A* gene; we excluded the possibility of as-yet-undiscovered SNPs in the coding region closely linked to the five or three SNPs by scanning the *NKG2D* gene region with denaturing high-performance liquid chromatography (data not shown). Further investigation is needed to identify which SNP(s) carries functional significance and to clarify the molecular mechanisms of individually differing cytotoxic activity.

Further investigation will also be needed of the genetic factors, other than the *NKG2D* haplotypes, involved in individual natural cytotoxic activity, specifically the genetic polymorphisms of killer immunoglobulin-like receptor (*KIR*) genes and human histocompatibility leukocyte antigen (*HLA*) class I genotypes (10, 27, 28). The involvement of HLA class I in NK cell repertoire selection leads to the hypothesis that HLA class I may play a role in determining individual NK cell activity, so we examined this hypothesis using the same cohort groups (with high and low natural cytotoxic activity) by comparing the frequency of *HLA class I* (*HLA-A, HLA-B,* and *HLA-C*) genotypes between the groups: Specific *HLA* genotypes of *B*1301, B*4403, B*5401, Cw*0401,* and *Cw*0702* showed significant association with cytotoxic activity (29). This implies that the polymorphisms of other immunorelated genes may also be associated with natural cytotoxic activity—immunogenetic susceptibility to cancer and other diseases. In the future, the combination of these genetic polymorphisms with the *NKG2D* haplotypes will provide more precisely defined, individually based descriptions of innate immune responses.

Our findings in this study show the advantage of molecular epidemiology cohort studies—a combination of phenotype and genotype markers. One possible combination would be to assess the cancer risk of genetic factors, which is modified by environment or other host factors described by phenotype markers, as was typically shown in the Shanghai prospective cohort study (30). This Saitama cohort study reveals another possibility: a phenotype-genotype association analysis combined with subsequent genome association analysis (risk estimation) done within the same cohort study. In a case-control study design within the cohort, we may be able to identify the genetic factors involved in a particular phenotype marker with a high degree of reliability by comparing the genome characteristics of two control groups who are matched to each other with major confounding factors (e.g., gender and age) and who show contrasting high and low values of this phenotype marker. We anticipate that this approach will provide useful information for future cancer prevention based on gene-environment interactions.

Acknowledgments

Received 8/9/2005; revised 10/11/2005; accepted 10/17/2005.

Grant support: Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan.

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Review Article

Perspectives on cancer immuno-epidemiology

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(Received August 19, 2004/Revised September 22, 2004/Accepted October 12, 2004)

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; NICI, 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.[5] 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 complexes 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-mediating 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

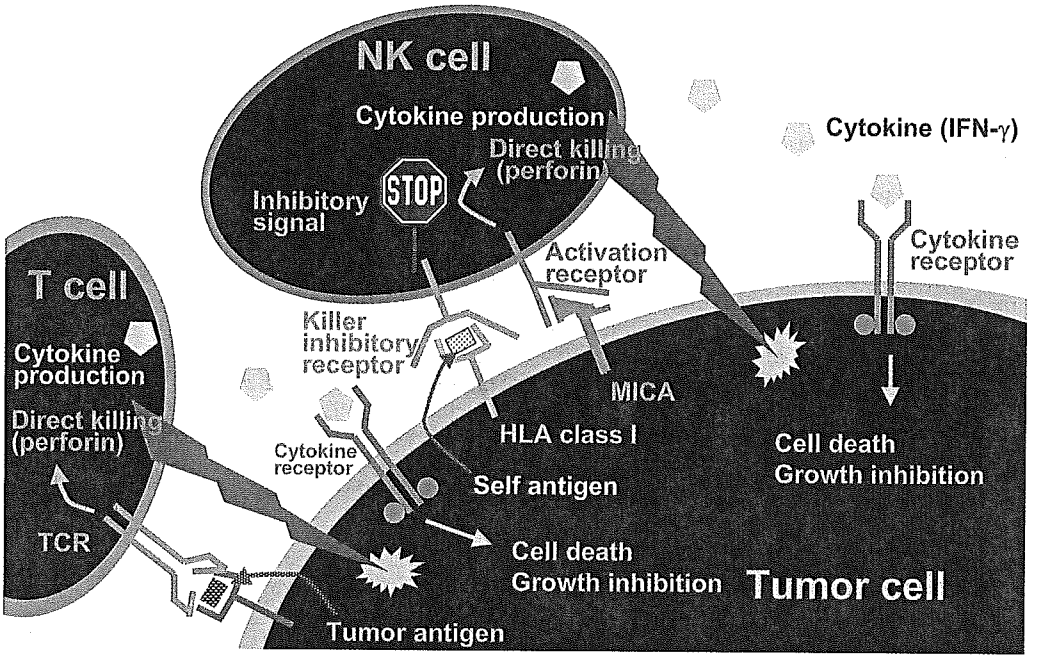

Fig. 1. Cells and molecules known to be involved in host immune responses to developing tumors. Tumor-specific cytotoxic T lymphocytes (CTL) recognize tumor antigens that are expressed in conjunction with HLA molecules and begin to directly kill tumor cells by secreting tumorcidal molecules (such as 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.

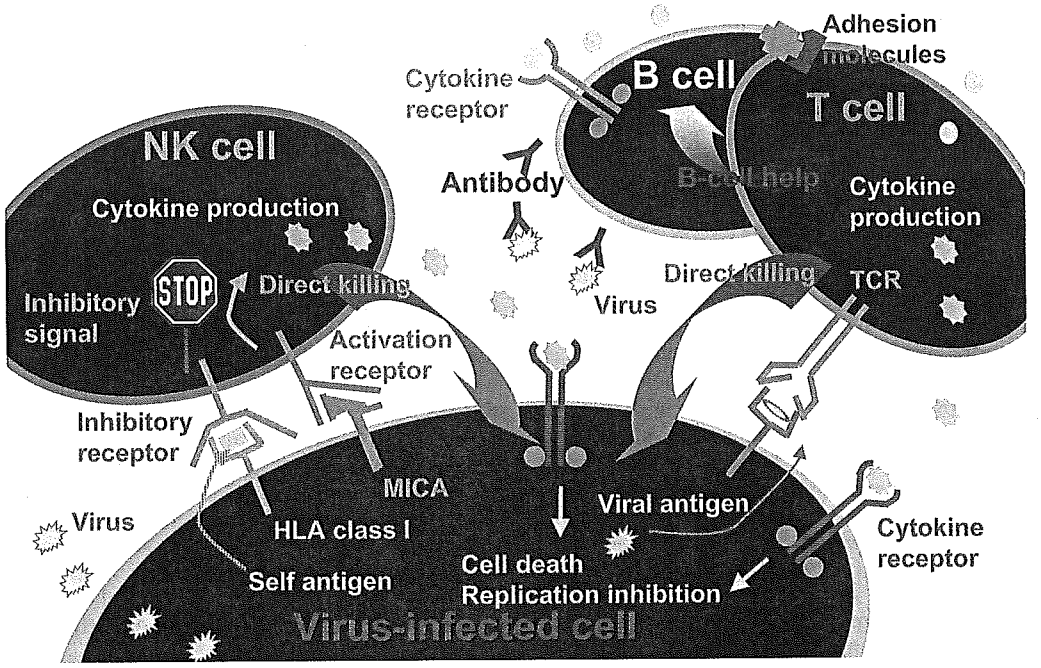

Fig. 2. In the case of immune responses to oncogenic virus infection, viral antigen presentation by HLA class I and II molecules to T cells initiates adaptive immune responses to the virus. Subsequent T-cell mediated cytotoxicity to virus-infected cells and T-cell cytokine production are key elements 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, γδT 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 commonly 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]

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.

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

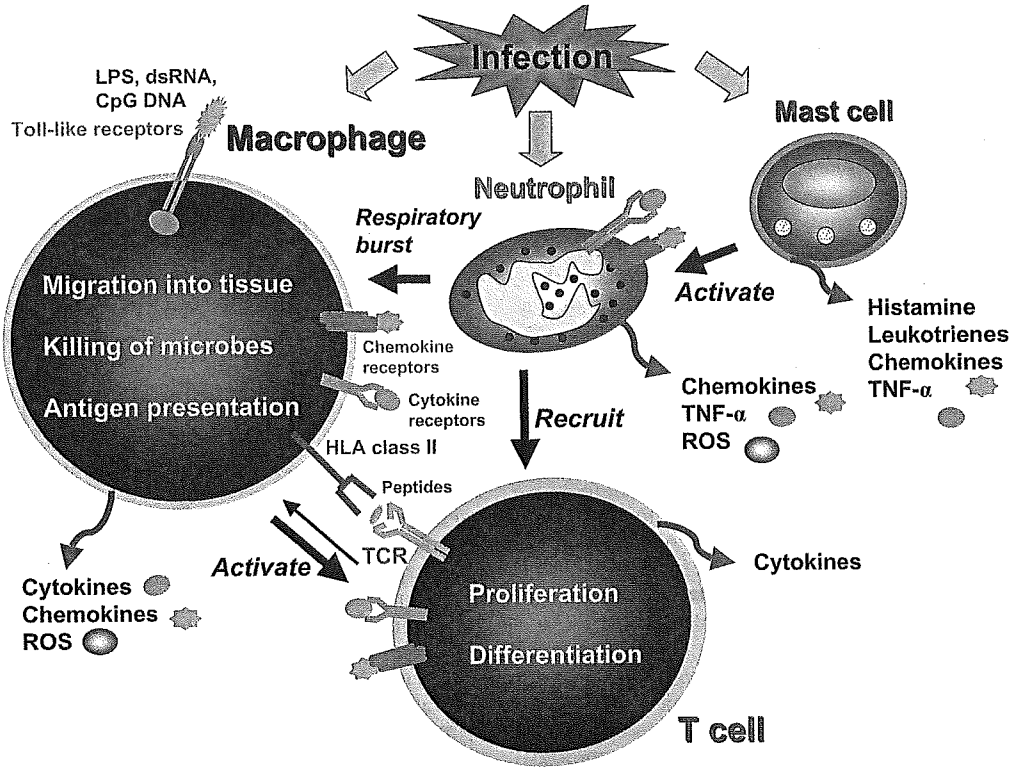

Fig. 3. Flow of inflammatory processes. 1) "Danger signals" from tissue trauma. The bioactive peptides released from neurons in response to pain activate mast cells, the intracellular proteins released from destroyed cells activate macrophages, and pathogen-associated patterns also activate macrophages through toll-like receptors. 2) Mast cells, the first responder, release histamine, luekotrienes, TNF-α, other cytokines/chemokines, and tryptases. 3) Neutrophils are activated by TNF-α and leukotrienes produced by mast cells; activation of matrix metalloprotenases; 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 cytolysis, 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.[33]

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-1β	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 immunoglobulins[1)]

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)	9 (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 knockout 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-1α and IL-1β 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 preclinical 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-1β* 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.

The work performed in our laboratory was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and for Cancer Research from the Ministry of Health, Labour and Welfare, Japan.

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