Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population

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Summary

Background One of the most critical questions in immunosurveillance is whether differences between individuals with regards to natural immunological host defence can predict future development of cancer. Although this question has so far remained open, there are clear indications of significant roles of several naturally cytotoxic lymphocytes in preventing the development of cancer. We began a prospective cohort study among a Japanese general population in 1986, using various immunological and biochemical markers.

Methods Natural cytotoxic activity of peripheral-blood mononuclear cells was assessed by isotope-release assay in 3625 residents of a Japanese population mostly older than 40 years of age, between 1986 and 1990. Immunological and biochemical markers were also measured, and participants were given a questionnaire on lifestyle. We did an 11-year follow-up survey of the cohort members looking at cancer incidence and death from all causes, and analysed the association between cytotoxic activity of peripheral-blood lymphocytes assessed at baseline and cancer incidence found in the subsequent follow-up.

Findings 154 cancer cases were used in the analysis. When we categorised the cytotoxic activity of peripheral-blood lymphocytes by tertiles, age-adjusted relative risk of cancer incidence (all sites) was 0·72 (95% CI 0·45–1·16) for men with high cytotoxic activity, and 0·62 (0·38–1·03) for men with medium cytotoxic activity, taking the risk of those with low cytotoxic activity as reference. For women with high cytotoxic activity relative risk was 0·52 (0·28–0·95), and for those with medium cytotoxic activity 0·56 (0·31–1·01). For both sexes with high and medium cytotoxic activity risk was 0·63 (0·43–0·92) and 0·59 (0·40–0·87), respectively.

Interpretation Our results indicate that medium and high cytotoxic activity of peripheral-blood lymphocytes is associated with reduced cancer risk, whereas low activity is associated with increased cancer risk suggesting a role for natural immunological host defence mechanisms against cancer.

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Introduction

The concept of immunosurveillance against cancer was clarified in 1970 by Burnet,[1] who predicted that immunodeficient individuals or those being treated with immunosuppressive drugs would have an increased incidence of cancer, and that differences in immunological host defence among healthy individuals might influence the occurrence of cancer. However, clinical observation at first seemed to provide only marginal support for this concept. An enhanced incidence of cancer was reported among individuals with several types of general immunodeficiency[2,3] and among immunosuppressed patients with transplants,[4] but there is no clear evidence of any association between use of immunosuppressive drugs and increased risk of the more common cancers. Subsequently, however, various researchers have carried out in vitro and in vivo studies that have shown the significance of immunological host-defence mechanisms against cancer or its metastases.[5–7] Further longitudinal studies are needed to define immunological host defence against cancer in human beings, although intensive research has been done on the mechanisms of natural cytotoxicity.[8,9]

The initial mechanism in immunosurveillance for cancer is thought to be non-specific:[5,10] natural killer (NK) cells seem to play a key part at this stage, along with activated macrophages, K cells, and NKT cells, which have potent natural cytotoxic activity through recognition mechanisms of target cells that differ from those of NK cells.[11] Although the importance of natural cytotoxicity in immune surveillance against cancer has been indicated by a number of in-vivo studies,[6,7] few studies have investigated the levels of NK-cell activity among healthy individuals who have an increased risk of cancer. These studies found decreased NK-cell activity among relatives of patients with familial melanoma[12] and in individuals with high familial incidence of cancer,[13] compared with healthy controls, and an increased risk of hepatocellular carcinoma associated with low NK-cell activity in a prospective study of patients with liver cirrhosis.[14] The clinical significance of NK-cell activity has also been shown among patients with several cancers, specifically as an independent prognostic measurement.[15,16] These studies suggest that natural cytotoxicity does play a part in preventing the development of cancer, and recent studies have further implied that several lymphocytes other than NK cells also have natural cytotoxic activity. Whether differences in natural immunological defence between individuals without obvious defects in immune system can predict future development of common cancers remain unclear. Only an extended prospective cohort study in a general population can provide a reliable answer to this question, although the measurement of natural cytotoxic activity, which should be done in situ, may limit the number of participants in the study.

Since 1986, we have done such a study among general residents living in a Japanese town. At baseline, we measured various immunological and biochemical markers—including natural cytotoxic activity of

peripheral-blood lymphocytes against cultured cancer cells. In the subsequent 11-year follow-up we looked at cancer incidence and death from all causes in the cohort.

Methods

Study population

In 1986 we began a prospective cohort study among residents (mostly older than 40 years) in a town in Saitama Prefecture, Japan. The study is described in detail elsewhere.[17–20] An epidemiological survey was done with a self-administered questionnaire which covered 90 lifestyle factors. Of all individuals who completed the survey those who participated in this study gave peripheral-blood samples at health-screening checks between 1986 and 1990. All blood samples were collected between 1 pm and 3 pm after strict fasting of more than 12 h in July, August, or September and were used for immunological and biochemical assays. All assays were started within 5 h of the blood samples being taken.

Follow-up

We did a follow-up survey on cancer incidence and death from all causes among the people in the study. Cancer cases were identified primarily by death certificate from the local health centre and national health insurance receipts (incidence cases). With regard to national health insurance receipts, we screened all possible cancer cases upon initial diagnosis of cancer and every year thereafter, and registered only cases that were confirmed by primary site, histology, and date of diagnosis through inquiry at the hospitals.

Assay of cytotoxic activity

The cytotoxic activity of peripheral lymphocytes was determined by ^{51}Cr-release assay as described previously.[19] The effector cells were isolated from 5 mL of heparinised peripheral-blood samples. Target cells were K562, a human myeloid leukaemia cell line, which were labelled with ^{51}Cr. Effector cells were added to target cells with an effector-to-target (ET) ratio of 20, and incubated for 3 h 30 min. Percentage specific lysis was calculated according to the standard formula. Before the measurement in cohort members, we examined different ET ratios (5, 10, 20, and 40) in a pilot study with a small number of healthy volunteers including those with both high and low cytotoxic activity. We chose the ratio of 20, where differences between individuals in cytotoxic activity were most distinguishable: cytotoxic curves among individuals with low cytotoxic activity showed smaller differences between individuals at ET=5 or ET=10 than at ET=20; the curves among those with high cytotoxic activity reached plateau and showed smaller differences at ET=40 than at ET=20. In a parallel study we measured cytotoxic activity of peripheral-blood lymphocytes among women with two different target cells K562 and Molt 4 (human acute lymphoblastic leukaemia). Other immunological markers assessed were the subsets of T cell, with CD4 and CD8 antibodies, and the reactivity of peripheral-blood lymphocytes in the presence of phytohaemagglutinin.

Statistical analysis

We estimated relative risk of cancer incidence for three levels (low, medium, and high) of cytotoxic activity by the Cox proportional hazard model with software packages SPSS (version 8.0), adjusting for age and selected lifestyle factors and considering the interaction between the covariates. Cytotoxic activity among both men and women followed a normal distribution (a Lilliefors significance level for testing normality $p<0{\cdot}05$). Cytotoxic activity (in % units) was then categorised by tertiles: ⩽42%, 43–58%, and >58% for low, medium, and high respectively, among men; ⩽34%, 35–51%, and >51% for low, medium, and high respectively among women. Potent covariates used in this model for relative risk were chosen from all lifestyle factors in the questionnaire by multiple regression analysis, and five covariates, which showed close association with cytotoxic activity, were considered in the Cox proportional hazard model: age, relative body weight (% deviation from the standard weight), cigarette smoking (categorised as current, former, and never), alcohol consumption (categorised as current, occasional, former, and never), and frequency of green vegetables intake (categorised as ⩾5 times a week, 2 to 4 times a week, once a week, and less than twice a month). Of all lifestyle factors examined, only cigarette smoking was significantly associated with incidence of cancer in all sites along with age. Relative body weight was the ratio of observed individual weight to the standard calculated for individual height, sex, and age in the Japanese population. All individuals were used for estimation of age-adjusted relative risk; one cancer case and 47 healthy individuals, who did not have complete information for the covariates, were excluded from the analysis of lifestyle-adjusted relative risk.

Cytotoxic activity	Men	Women
Less than 20%	47 (3%)	177 (8%)
20–29%	139 (11%)	350 (16%)
30–39%	192 (15%)	441 (20%)
40–49%	260 (20%)	426 (19%)
50–59%	275 (21%)	440 (20%)
60–69%	244 (19%)	253 (12%)
70–79%	115 (9%)	79 (4%)
More than 80%	32 (2%)	30 (1%)

Table 1: **Distribution of cytotoxic activity at baseline**

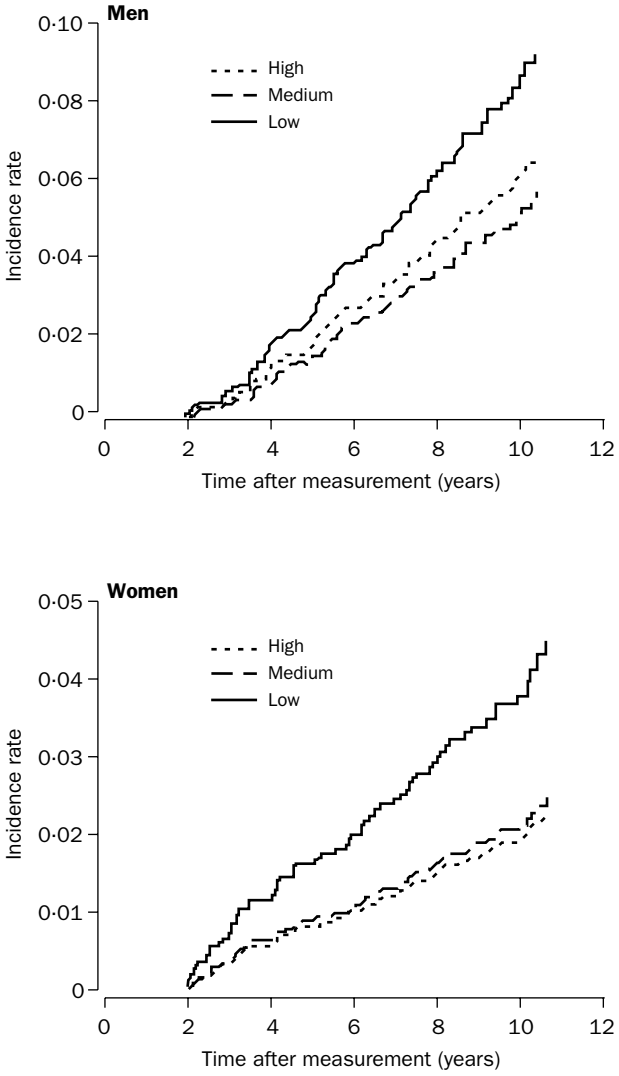
Results

8552 individuals (95% of all residents in Saitama older than 40 years) were sent the epidemiological survey, of which 3625 self-selected individuals (1371 men, 2254 women) agreed to give peripheral-blood samples.

	Cytotoxic activity							
	Men				Women			
	Low*	Medium	High	All categories	Low	Medium	High	All categories
Number of participants	n= 452	n=434	n=418	n=1304	n=746	n=750	n=700	n=2196
Mean (SE) age (years)	53·8 (0·6)†	54·3 (0·6)†	55·9 (0·6)†	54·7 (0·3)†	52·7 (0·4)†	53·6 (0·4)†	55·2 (0·4)†	53·7 (0·2)†
Number of cancer cases	39 (9%)	24 (6%)	29 (7%)	92 (7%)	29 (4%)	17 (2%)	16 (2%)	62 (3%)
Number of obese or lean participants‡	260 (58%)	252 (58%)	265 (63%)	777 (60%)	424 (57%)	441 (59%)	420 (60%)	1285 (59%)
Current smokers	306 (68%)	267 (62%)	246 (59%)	819 (63%)	70 (9%)	53 (7%)	43 (6%)	166 (8%)
Current drinkers	225 (50%)	231 (53%)	243 (58%)	699 (54%)	35 (5%)	42 (6%)	32 (5%)	109 (5%)
Daily intake of green vegetables§	133 (29%)	132 (30%)	124 (30%)	389 (30%)	221 (30%)	255 (34%)	263 (34%)	739 (34%)

*Categorised by tertiles; Low ⩽42%; medium 43–58%; high >58% for men; low ⩽34%; medium 35–51%; high >51% for women. †SE in parentheses. ‡Deviation from standard body weight > 10% or <–10%. §Intake of green vegetables ⩾5 times a week. All data are n (%) unless indicated.

Table 2: **The characteristics of cohort members**



Cumulative incidence rates of cancer by cytotoxic activity of peripheral-blood lymphocytes among men and women
Categorised by tertiles. Men—low: ≤42%; medium: 43–58%; high: >58%. Women—low: 34%; medium: 35–51%; high: >51%.

We identified 211 cancer cases (124 men, 87 women) in all sites among 3625 cohort members from September, 1986, to August, 1997 (488 cancer cases among 8552 cohort members surveyed); the 11-year follow-up study included 99·5% of cohort members (seven follow-up losses). In this analysis we first excluded 64 of the cohort members: 19 whose cytotoxic activity data were not available, 26 who were older than 80 years old (including two cancer cases), and 19 who had been diagnosed with cancer before the start of this survey. Additionally, 36 cancer cases that had been diagnosed within 2 years after the assay of cytotoxic activity were excluded, to avoid the possibility that cytotoxic activity measured at baseline might be affected by the preclinical stage of cancer. Among the cohort members, only participants who were still alive 2 years after the assay contributed to the person-years in this survival analysis. Accordingly, 25 cohort members who died from causes other than cancer within 2 years after the assay were also excluded. The final total was 154 cancer cases (92 men, 62 women) in all sites from 3500 cohort members, with the most frequent cancers being stomach (29), lung (17), and intestine (14) for men, and stomach (17), intestine (ten), and lung (eight) for women.

Cytotoxic activity of peripheral-blood lymphocytes showed wide variations between individuals in 1304 men and 2196 women. Cytotoxic activity in men and women can be seen in table 1. On the basis of this distribution, we divided the participants into groups of low, medium, and high cytotoxic activity levels by tertiles. The characteristics of each group at baseline are summarised in table 2. Cytotoxic activity was positively associated with age, keeping body weight closer to the standard (avoiding obesity and leanness), not smoking, alcohol consumption, and intake of green vegetables; a cross-sectional analysis of cytotoxic activity and lifestyles has been reported elsewhere.[19] The percentage of cancer cases among men and women with low cytotoxic activity was higher than in the groups with medium and high activity (table 2), although this was not statistically significant. The number of cancer cases (both sexes) diagnosed within 2 years from the date of assays and omitted from table 2 was eight, 19, or nine, for low, medium, or high cytotoxic activity, respectively.

We used one male and three female controls to examine variations in cytotoxic activity assay during months and/or years. Cytotoxic activity measured for the man was 53% and 55% on July 7, and July 21, in 1986, 54% and 51% on Aug 31 and Sept 11, in 1987, and 54% on Sept 4, 1988. The three women also showed stable cytotoxic activity measured at different times. Stable cytotoxic activity has also been reported in a study where individual cytotoxic activity was repeatedly measured during a 7 year period.[21]

We compared the observed cytotoxic activity of patients with cancer who were diagnosed with the disease on and after the third year from the date of assays, to that of participants who were free from cancer during the follow-up study (table 3). Since both cytotoxic activity and the incidence of cancer increase with age, the difference in activity in patients with cancer versus participants without cancer is partially masked unless the data are stratified by age in decades (<40 years, 40–49, 50–59, 60–69, and 70–79). The data in table 3 showing this effect as the differences in cytotoxic activity within decades are more substantial than the differences shown in "all ages". In age-ranks of 50–59 years, and 60–69 years, mean cytotoxic activity among participants with

	Age-groups (years)					
	<40	40–49	50–59	60–69	70–79	All ages
Men						
Without cancer	92; 46·7% (1·5)	342; 48·9% (0·9)	300; 48·1% (1·0)	358; 50·6% (0·9)	110; 51·9% (1·5)	1212; 49·3% (0·5)
With cancer	0	8; 47·9% (7·5)	24; 45·3% (3·8)	48; 48·7% (2·5)	12; 52·9% (4·0)	92; 48·3% (1·8)
Women						
Without cancer	146; 40·2% (1·4)	668; 40·8% (0·6)	605; 41·9% (0·7)	549; 45·7% (0·7)	166; 45·3% (1·2)	2134; 42·7% (0·4)
With cancer	0	14; 41·6% (4·1)	18; 39·6% (4·4)	21; 41·3% (4·3)	9; 45·6% (6·5)	62; 41·5% (2·3)

All data are n; mean cytotoxic activity (%); SE in parentheses.

Table 3: **Age distribution of cytotoxic activity by follow-up status**

	Cytotoxic activity (%)*		
	Low	Medium	High
Men			
Age-adjusted	1·0	0·62 (0·38–1·3)	0·72 (0·45–1·16)
Lifestyle-adjusted†	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			
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)‡

All data are relative risk (95% CI). *Categorised by tertiles; low ⩽42%; medium 43–58%; high >58% for men; and low ⩽34%; medium 35–51%; high >51% for women.
†Adjusted for age, relative body weight, cigarette smoking, alcohol consumption, and intake of green vegetables. ‡p<0·05. §p<0·01.

Table 4: **Relative risk of cancer incidence for cytotoxic activity levels**

cancer was lower than among participants without cancer, although this difference was not statistically significant.

Next we did a survival analysis on the basis of the follow-up study. Cumulative incidence rate and relative risk of cancer incidence for levels of cytotoxic activity were estimated by Cox proportional hazard model adjusting for age at entry. Cumulative cancer incidence rates are shown as functions of follow-up years (years after the assay of cytotoxic activity in the figure). Both men and women with low cytotoxic activity had a substantial increase in cancer incidence compared with those with medium or high cytotoxic activity. There were no distinguishable differences in cancer incidence between medium and high cytotoxic activity.

Relative risk of cancer incidence for levels of cytotoxic activity is estimated in table 4 adjusting for age only, and for both age and associated lifestyle factors (relative body weight, cigarette smoking, alcohol consumption, and intake of green vegetables). Women with high cytotoxic activity had approximately half the risk (0·52) of cancer compared with those with low cytotoxic activity ($p<0·05$). Men with high or medium cytotoxic activity also showed a reduction in risk, although it was not statistically significant. Combining the data for men and women, and adjusting for sex, both sexes with high cytotoxic activity had a significantly lower risk of cancer, a relative risk of 0·63 or 0·64 adjusted for age only or for age and lifestyle factors ($p<0·05$). Since there was no substantial difference in cancer risk between those with medium and high cytotoxic activity, our results indicate that individuals with low cytotoxic activity, who account for the lowest third in distribution of cytotoxic activity among a general population, have an enhanced risk of cancer development.

In a parallel study that assessed the cytotoxic activity of peripheral-blood lymphocytes among 52 women, using two different target cells, K562 and Molt 4 (human acute lymphoblastic leukaemia), cytotoxic activity showed a strong correlation (correlation coefficient 0·79 [95% CI 0·67–0·85], $p<0·001$, degrees of freedom=50), suggesting that relative cytotoxic activity among individuals may not be dependent on the type of target cell expressing low levels of major histocompatibility complex (MHC) class I antigens.

We compared the cytotoxicities of peripheral-blood lymphocytes among healthy individuals using K562 and Jurkat cells (a human acute T cell leukaemia cell line expressing MHC class I antigens as well as Fas) as target cells, along with measurement of cytotoxic activity, expression of killer-cell inhibitory receptor, and killer-cell activating receptor on NK cells, and production of cytokines. Cytotoxic activity against K562 showed a close correlation with that against Jurkat cells (correlation coefficient 0·56; $p<0·001$; degree of freedom=103), indicating that cytotoxic activity, assessed in our prospective cohort study, represents cytotoxicity against cancer cells, even highly expressing MHC class I antigens (there is a fuller paper in preparation). Moreover, we found that perforin and granzyme-dependent cytotoxicity, which was estimated using K562 or Jurkat cells with a neutralising antibody against Fas ligand, showed significant and positive correlation with the proportion of NK cells in peripheral-blood lymphocytes; the cytotoxicity per NK cell showed a significant and positive association with expression of NKRP1, an activation receptor, and a negative association with production of tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) in NK cells, implying that low cytotoxic activity in our prospective cohort study may be associated with decreased numbers of NK cells, decreased expression of activation receptors, and increased production of several cytokines.

Discussion

Our prospective cohort study showed that individuals with low cytotoxic activity are at a significantly higher risk of cancer, compared with those of medium or high activity. We show that there is an association between natural immunological defence and the incidence of cancer in a general population, but it has by no means answered all questions. In the future we plan to investigate organ-specific analysis between cytotoxic activity and cancer, which will require a longer follow-up of cohort members and a large number of cancer cases for analysis, along with a repeated survey to assess variations in cytotoxic activity and lifestyle since the baseline.

The large variations in cytotoxic activity among individuals, from 10% to 90% in this study, are thought to be due in part to selected lifestyle factors, with higher activity associated with keeping body weight closer to the standard, not smoking, increased intake of green vegetables, and moderate alcohol consumption. All factors, apart from alcohol consumption, are well known good health practices. As for the mechanisms underlying those good health practices,[19] cytotoxic activity of peripheral-blood lymphocytes may in part explain them. Although a small (at most 30%) portion of cytotoxic activity was attributable to lifestyle factors (data not shown), cytotoxic activity might be useful as a surrogate biomarker in lifestyle intervention studies for the purpose of cancer prevention. In fact, we have shown that cytotoxic activity can be increased by altering several lifestyle factors, in an intervention trial for women who had breast cancer more than 5 years before (unpublished data).

Given the potential implications of our epidemiological findings, factors apart from lifestyle that influence cytotoxic activity need to be investigated. Advances in immunology have provided more knowledge about the mechanisms of natural cytotoxicity.[8,9,22] The discoveries have included: two distinct mechanisms of cytotoxicity (perforin and granzyme-dependent and Fas ligand-dependent), receptors on NK cells, involvement of several cytokines in modulating cytotoxic activity, and the possibility that the cytotoxicity of NK cells may be restricted by MHC class I antigens on target cells.

Our findings show the association between natural immunological defence and the occurrence of common cancers, and argue for the need to do subsequent studies to clarify the mechanisms and also apply them for cancer prevention.

Contributors

K Imai and K Nakachi designed the study, collected the data, and write the paper. S Matsuyama, S Miyake, and K Suga collaborated on clinical follow-up and helped to write the paper.

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