Green PCR Core Reagent kit (Applied Biosystems, Foster City, CA). Real-time detection of the emission intensity of SYBR Green bound to double-stranded DNAs was by the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). *POT1* cDNA and internal control cDNA (β-actin gene, *ACTB*) were PCR amplified separately. Relative gene expression was determined by the threshold cycles for the *POT1* gene and *ACTB* gene. Reference samples (GC cell line, HSC-39) were included on each assay plate to verify plate-to-plate consistency. Plates were normalized to each other by these reference samples. PCR amplification was performed according to the manufacturer's instructions in 96-well optical trays with caps with a 25-μl final reaction mixture. Quantitative RT-PCRs were performed in triplicate for each sample primer set, and the mean of the three experiments was used as the relative quantification value. *POT1* primer sequences were 5′-TCAGTCTGTTAAACTTCATTGCCC-3′ and 5′-TGCACCATCCTGAAAAATTATATCC-3′. *ACTB* primer sequences were 5′-TCACCGAGCGCGGCT-3′ and 5′-TAATGTCACGCACGATTTCCC-3′.

Telomere Restriction Fragment Length Analysis. High molecular weight genomic DNA was extracted with a DNA Extraction kit (Stratagene Cloning System, La Jolla, CA). Tissue DNA was digested with *Hin*fI, electrophoresed on 0.6% agarose gels, and blotted onto nitrocellulose filters. The filters were hybridized with a telomeric DNA probe and then autoradiographed. We estimated the telomere length as the peak signal using Kodak Digital Science 1D software (Eastman Kodak Company, New Haven, CT).

3′ Telomeric Overhang Assay (In-Gel Hybridization). In-gel hybridization to measure 3′ telomeric overhangs was carried out as described in Wellinger *et al.* (25). DNA samples were digested with *Hin*fI and electrophoresed on 0.6% agarose gels. The gels were dried with a gel dryer for 24–28 min at room temperature. The very thin gels were then hybridized for 16 h at 37°C to end-labeled oligonucleotides in hybridization buffer. $[TTAGGG]_4$ and $[CCCTAA]_4$ oligonucleotide probes were end-labeled with γ-^{32}P-ATP and T4 polynucleotide kinase. After removal of excess hybridization buffer, gels were washed twice with 0.25× SSC for 1.5 h at room temperature, followed by 2-h washes at 30°C. After sequential native gel hybridization, dried gels were alkali-denatured in 0.15 M NaCl, 0.5 M NaOH for 25 min, neutralized in 0.15 M NaCl, 0.5 M Tris-HCl (pH 8.0) for 20 min, and reprobed. Image analysis and quantitation were performed with a Fuji Film BAS 2000 Bio-Imaging Analysis System and NIH Image.

Telomeric Repeat Amplification Protocol Assay. Telomeric repeat amplification protocol assay was done with a TRAPEZE Telomerase Detection kit (Intergen Company, Oxford, United Kingdom). Intensity of the telomeric repeat amplification protocol product bands and of the internal control bands was determined with the use of NIH Image.

Telomere Shortening Assay. We harvested MKN-28 and MKN-74 cells maintained at 37°C under 5% CO_2 in RPMI 1640 and 10% fetal bovine serum. Cells were pretreated for 5 and 10 days with medium exchange and addition of AZT (100 μM) each day before being used for experiments.

***POT1* Antisense Oligonucleotides.** The 24-mer phosphorothioate oligonucleotide antisense sequence of the first 24 nucleotides of *POT1* was synthesized and purified by reverse-phase high-performance liquid chromatography (Espec Oligo Service, Tsukuba, Japan). The sequence was 5′-TTGTTGCTGGAACCAAAGACATTG-3′, and for control, complementary (sense) oligonucleotides were synthesized as 5′-CAATGTCTTTGGTTCCAGCAACAA-3′. We harvested MKN-28 cells maintained at 37°C under 5% CO_2 in RPMI 1640 and 10% fetal bovine serum. Cells were pretreated with 2.5 μM antisense or sense oligonucleotides in Lipofectamine (Invitrogen-Life Technologies, Inc., Carlsbad, CA) for 4 and 8 days by medium exchange and addition of antisense or sense oligonucleotides every 2 days before being used for experiments.

Anaphase Bridges. H&E-stained cultures or tissue sections were examined for anaphase bridges under a light microscope at ×100 magnification. The anaphase bridge index (ABI) was determined by dividing the number of anaphases with bridges by the total number of anaphases. Anaphase bridging was defined as reported previously (26). Two investigators independently scored a minimum of 10 anaphases/sample.

Statistical Analysis. Statistical significance was assessed by Fisher's exact test, Mann-Whitney *U* test, Spearman's rank correlation test, or unpaired *t* test. Statview 5.0 Macintosh software was used. All tests were two-sided. A P of <0.05 was regarded as statistically significant.

RESULTS

***POT1* Expression Levels Increase with Tumor Stage.** Expression levels of *POT1* were measured by quantitative RT-PCR in the 51 cases of GC. We calculated the ratio of *POT1* mRNA expression levels in GC tissues relative to levels in nonneoplastic mucosae (T/N ratio). The T/N ratios were significantly higher in stage III/IV cancers than in stage I/II cancers ($P = 0.005$, Mann-Whitney *U* test; Fig. 1*A*). We considered a T/N $>$ 2.0 to represent up-regulation and a T/N $<$ 0.5 to represent down-regulation. Up-regulation of *POT1* was found in 12 (23.5%) of the 51 cases, and down-regulation was found in 18 (35.3%) of the 51 cases. Down-regulation of *POT1* was more frequent in stage I/II tumors (52.4%, 11 of 21) than in stage III/IV tumors (23.3%, 7 of 30; $P = 0.033$, Fisher's exact test; Table 1),

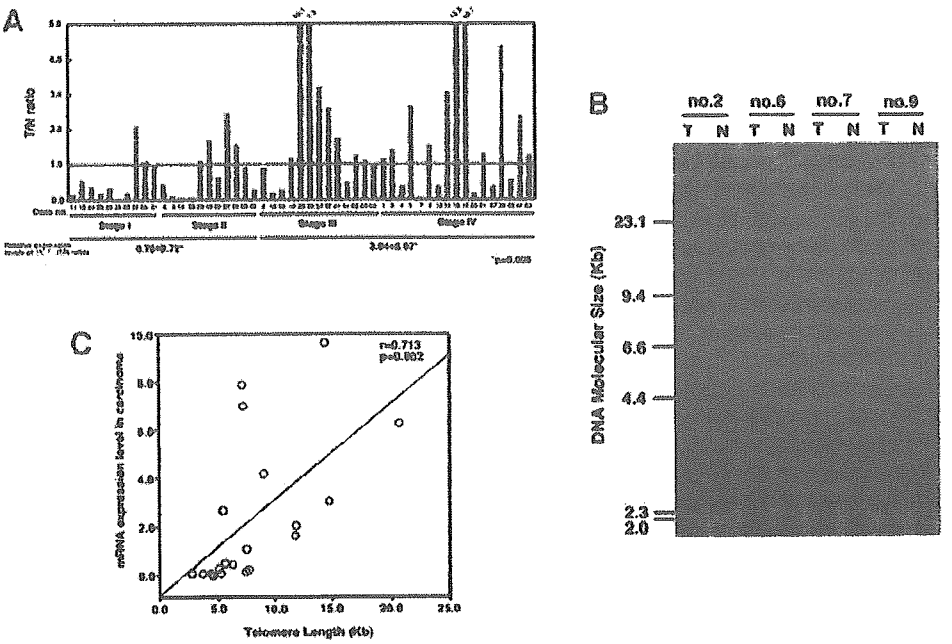

Fig. 1. *POT1* expression levels in gastric cancer (GC) and association with telomere length. *A*, distribution of *POT1* expression in the 51 cases of GC. T/N ratio stage I/II: 0.75 ± 0.72; T/N ratio stage III/IV: 3.84 ± 8.02, $P = 0.005$ by Mann-Whitney *U* test. *B*, representative telomere restriction fragment length analysis. Telomere lengths were determined in tumor tissues (T) and corresponding nonneoplastic mucosae (N). Horizontal *white bars* show the average length. *C*, *POT1* expression levels in tumor tissues correlate positively with telomere length ($r = 0.713$, $P = 0.002$ by Spearman's rank correlation test). T/N ratio = *POT1* mRNA expression levels in GC tissue relative to levels in corresponding nonneoplastic mucosa.

Table 1 *Clinicopathological features of gastric cancers (n = 51) in relation to POT1 expression levels*[a]

	Down-regulation (T/N <0.5)	No change (T/N = 0.5–2.0)	Up-regulation (T/N >2.0)	P
Histology[b]				
Intestinal	9 (36.0%)	9 (36.0%)	7 (28.0%)	
Diffuse	9 (34.6%)	12 (46.2%)	5 (19.2%)	n.s.[c]
T-grade[d]				
$T_{1,2}$	12 (50.0%)	9 (37.5%)	3 (12.5%)	
$T_{3,4}$	6 (22.2%)	12 (44.5%)	9 (33.3%)	0.038[e]
N-grade[d]				
N_0	6 (46.2%)	5 (38.4%)	2 (15.4%)	
$N_{1,2,3}$	12 (31.6%)	16 (42.1%)	10 (26.3%)	n.s.
Stage[d]				
I, II	11 (52.4%)	8 (38.1%)	2 (9.50%)	
III, IV	7 (23.3%)	13 (43.4%)	10 (33.3%)	0.033[e] 0.048[f]

[a] T/N ratio = *POT1* mRNA expression levels in GC tissue relative to levels in corresponding nonneoplastic mucosa.
[b] According to the Lauren criteria (23).
[c] n.s., not significant.
[d] According to the criteria of the tumor-node-metastasis stage classification system (24).
[e] By Fisher's exact test, for down-regulation *versus* no change and up-regulation.
[f] By Fisher's exact test, for down-regulation and no change *versus* up-regulation.

whereas up-regulation of *POT1* was more frequent in stage III/IV tumors (33.3%, 10 of 30) than in stage I/II tumors (9.5%, 2 of 21; $P = 0.048$, Fisher's exact test; Table 1). In addition, down-regulation of *POT1* was observed preferentially in low T grade (depth of invasion) cancers ($P = 0.038$, Fisher's exact test; Table 1). No association was found between *POT1* expression level and N grade (degree of lymph node metastasis) or histological type (Table 1).

Positive Correlation between *POT1* Expression Levels and Telomere Length. For association between *POT1* expression levels and telomere length in GC tissues, telomere length was examined by Southern blotting analysis in 20 of the 51 cases (Fig. 1*B*). *POT1* expression levels in GC tissue decreased in accordance with telomere shortening ($r = 0.713$, $P = 0.002$, Spearman's rank correlation test; Fig. 1*C*).

Positive Correlation between *POT1* Expression Levels and 3′ Telomeric Overhang Signals. 3′ overhang signals were examined by in-gel hybridization analysis in the same 20 GC tissues in which telomere lengths were measured (Fig. 2*A*). 3′ overhang signals reduced in accordance with reduced *POT1* expression levels ($r = 0.696$, $P = 0.002$, Spearman's rank correlation test; Fig. 2*B*) and telomere shortening ($r = 0.570$, $P = 0.013$, Spearman's rank correlation test; Fig. 2*C*).

AZT Inhibition of *POT1* Expression in GC Cells. To confirm the positive correlation between *POT1* expression levels and telomere length, we measured *POT1* expression levels in GC cells in which telomeres were shortened by AZT. AZT inhibited telomerase activity and shortened telomeres (Fig. 3, *A* and *B*). Telomerase activity was inhibited with AZT by 68% after 5 days and 82% after 10 days in MKN-28 cells and by 23% after 5 days and 94% after 10 days in MKN-74 cells. AZT reduced telomere length from 3.9 to 3.7 Kb after 5 days and to 3.4 Kb after 10 days in MKN-28 cells and from 3.6 to 3.1 Kb after 5 days and to 2.7 Kb after 10 days in MKN-74 cells. Quantitative RT-PCR analysis showed *POT1* expression to be down-regulated in AZT-treated cells (Fig. 3*C*). Expression of *POT1* in AZT-treated MKN-28 cells was 29% of that in nontreated cells after

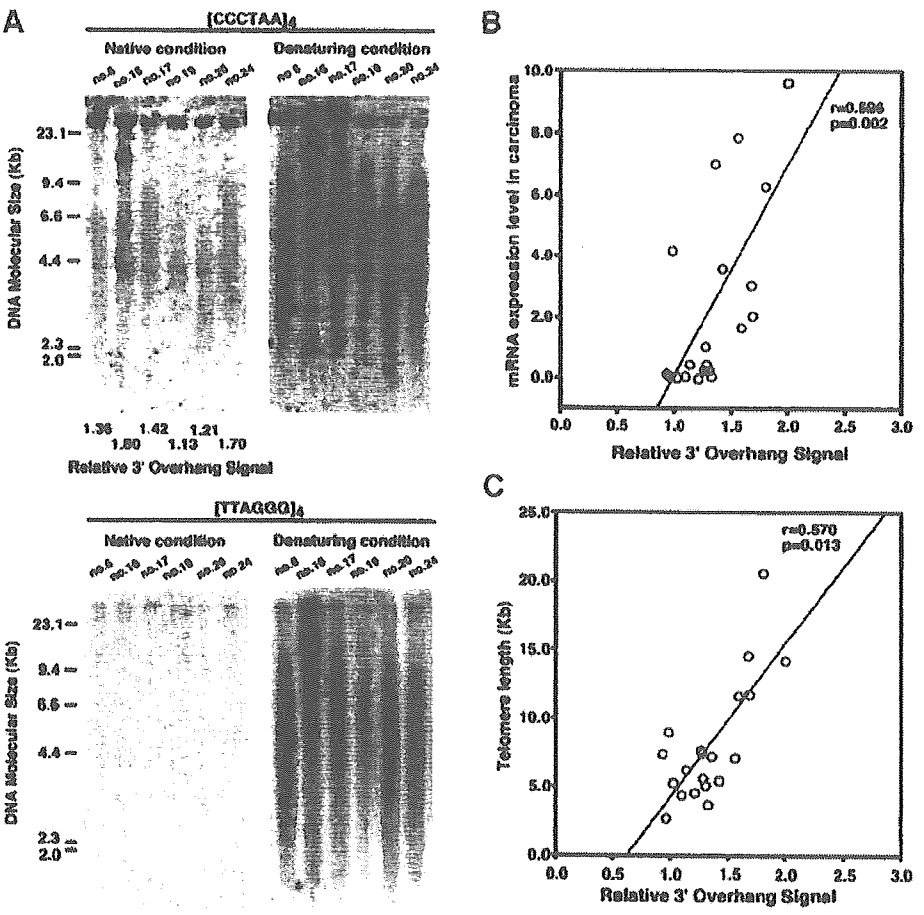

Fig. 2. 3′ Telomeric overhang signals were associated with *POT1* expression levels and telomere lengths. *A*, representative in-gel hybridization assay. The *top left panel* shows native gel probed with $[CCCTAA]_4$, and the *top right panel* shows denatured gel probed with $[CCCTAA]_4$. The *bottom left panel* shows native gel probed with $[TTAGGG]_4$, and the *bottom right panel* shows denatured gel probed with $[TTAGGG]_4$. Signals were expressed relative to the signal in MKN-74 gastric cancer cells. *B*, *POT1* expression levels in tumor tissues correlate positively with 3′ telomeric overhang signals in gastric cancer tissues ($r = 0.696$, $P = 0.002$ by Spearman's rank correlation test). *C*, telomere lengths correlate positively with 3′ telomeric overhang signals in gastric cancer tissues ($r = 0.570$, $P = 0.013$ by Spearman's rank correlation test).

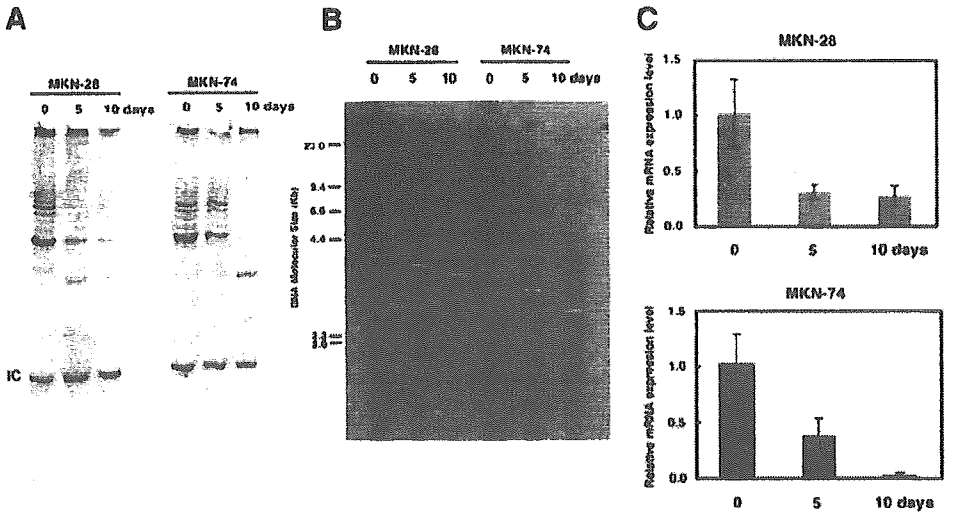

Fig. 3. *POT1* expression levels in relation to telomere shortening. *A*, reduced telomerase activity in MKN-28 and MKN-74 cells is observed after 5 and 10 days' treatment with azidothymidine (AZT). IC = internal control (36 bp). *B*, Telomere shortening is observed in MKN-28 and MKN-74 cells after 5 and 10 days' treatment with AZT. *C*, quantitative reverse-transcription-PCR analysis showed reduced *POT1* expression in MKN-28 and MKN-74 cells treated with AZT. *POT1* expression levels are the mean ± SD and relative to levels in nontreated cells.

5 days and 26% after 10 days. And expression of *POT1* in AZT-treated MKN-74 cells was 36% of that in nontreated cells after 5 days and 1% after 10 days.

Inhibition of *POT1* Induces Telomere Shortening. For additional analysis, we examined MKN-28 cells treated with *POT1* antisense oligonucleotides. Decreased *POT1* expression was confirmed by quantitative RT-PCR in MKN-28 cells after treatment with antisense oligonucleotides (Fig. 4*A*). Expression of *POT1* in antisense oligonucleotide-treated MKN-28 cells was 40% of nontreated cells after 4 days and 34% after 8 days. Treatment with *POT1* antisense oligonucleotides resulted in telomerase inhibition (by 58% after 4 days and 83% after 8 days; Fig. 4*B*) and telomere shortening (from 3.8 to 3.3 Kb after 4 days and to 3.0 Kb after 8 days; Fig. 4*C*). Treatment with sense oligonucleotides did not produce any alterations in *POT1* expression levels, telomerase activity, or telomere length.

Inhibition of *POT1* Reduces 3′ Telomeric Overhang Signals. To test whether *POT1* expression levels are associated with 3′ overhang signals, 3′ telomeric overhang signals were examined by in-gel hybridization in MKN-28 cells treated with *POT1* antisense oligonucleotides. A reduction in 3′ overhang signals was found in antisense

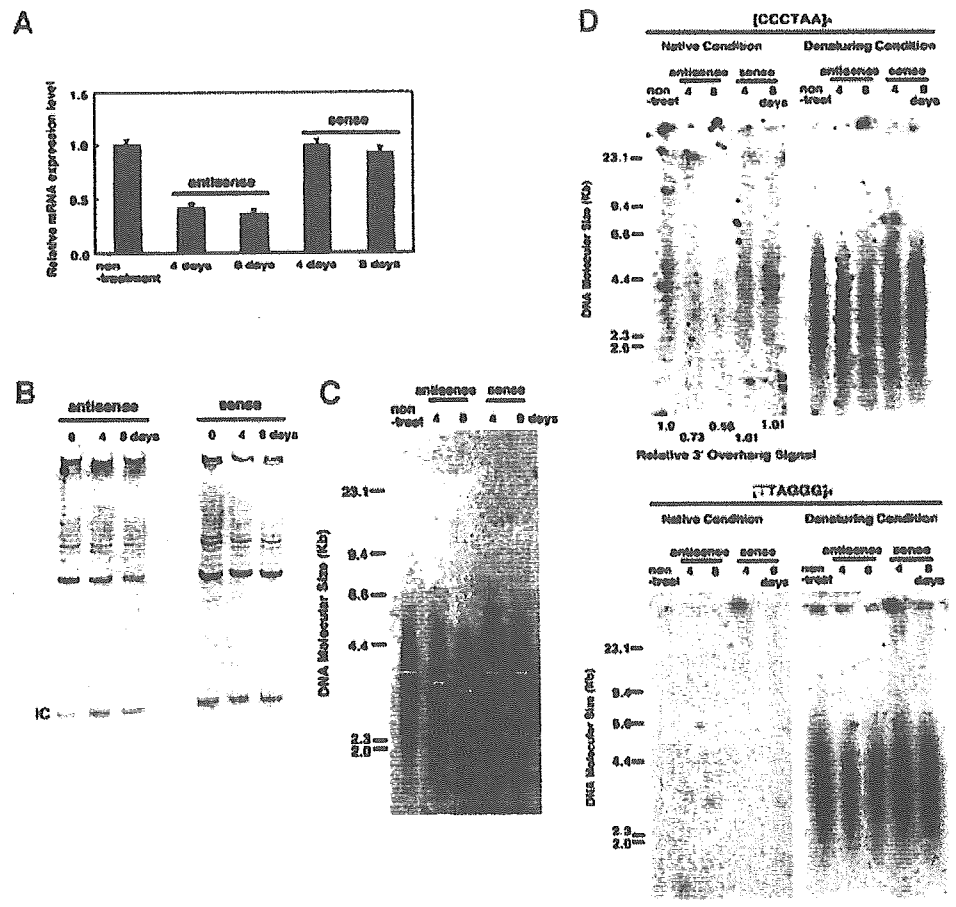

Fig. 4. Telomere shortening, telomerse inhibition and the reduction of 3′ telomeric overhang signals in association with reduced expression of *POT1* in antisense oligonucleotides-treated MKN-28 cells. *A*, reduction of *POT1* expression was confirmed in MKN-28 cells after antisense oligonucleotide treatment for 4 and 8 days. *B*, reduced telomerase activity was detected in antisense oligonucleotides-treated MKN-28 cells, whereas telomerase activity was not changed in sense oligonucleotides-treated MKN-28 cells. *C*, telomere shortening was detected in MKN-28 cells treated with antisense oligonucleotides. *D*, inhibition of Pot1 reduced 3′ telomeric overhang signals. The *top left panel* shows native gel probed with $[CCCTAA]_4$, and the *top right panel* shows denatured gel probed with $[CCCTAA]_4$. The *bottom left panel* shows native gel probed with $[TTAGGG]_4$, and the *bottom right panel* shows denatured gel probed with $[TTAGGG]_4$. Signals were expressed relative to the signal in the nontreated MKN-28 gastric cancer cells and normalized to the amount of DNA (10 μg) loaded based on denatured gels.

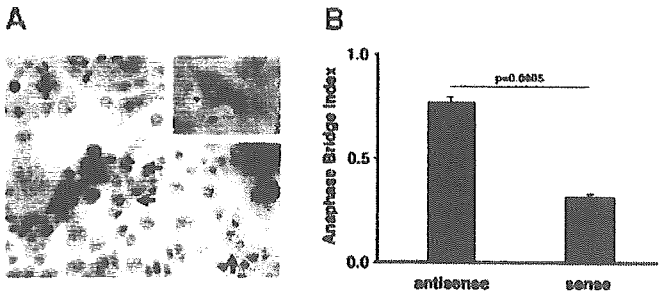

Fig. 5. Inhibition of Pot1 increases the incident of anaphase bridge. *A*, photomicrograph of typical anaphase bridge (*arrows* and *inset*) in MKN-28 treated with *POT1* antisense oligonucleotides H&E-stained section. (magnification, ×400; inset magnification, ×1000). *B*, inhibition of Pot1 by antisense oligonucleotides increases the number of anaphase bridge in MKN-28 gastric cancer cells.

oligonucleotide-treated cells (to 73% after 4 days and to 56% after 8 days; Fig. 4*D*). Signals in sense oligonucleotide-treated cells were almost the same as signals in nontreated cells (101% after both 4 and 8 days).

Inhibition of *POT1* Expression Increases the Frequency of Anaphase Bridges. To examine association between *POT1* expression levels and telomere dysfunction, we determined the ABI in the 51 GC tissues. No significant association was found between *POT1* expression levels and the ABI. Moreover, the ABI did not correlate with telomere length or 3′ telomeric overhang signals (data not shown). We then treated MKN-28 cells with *POT1* antisense and sense oligonucleotides for 8 days and determined the ABI. Treatment with *POT1* antisense increased the ABI in MKN-28 cells to about twice that of sense-treated cells (0.76 for antisense treatment and 0.31 for sense treatment; $P = 0.0005$, unpaired t test; Fig. 5, *A* and *B*).

DISCUSSION

Pot1 is thought to have two functions. One is mediating recruitment of telomerase, and the other is protecting the 3′ telomeric overhang from degradation and DNA repair activities (13, 16–18). We studied *POT1* mRNA expression in GC tissues with respect to both functions. We showed expression levels of *POT1* in the GC tissues relative to levels in nonneoplastic mucosae to be significantly associated with tumor stage and that up-regulation of *POT1* occurs preferentially in late-stage GC. Pot1 is thought to protect telomeres (13). In fact, inhibition of *POT1* by antisense oligonucleotides led to an increase in the frequency of anaphase bridge in MKN-28 cells. Thus, inhibition of Pot1 is associated with telomere dysfunction. Previous studies in a mouse model revealed that severe telomere dysfunction impaired tumor progression (26–31). Severe telomere dysfunction is shown to be reduced in advanced tumors that survive after crisis (26, 32, 33). Thus, up-regulated *POT1* may participate in protection of the telomere ends in late-stage GC. In addition, we found *POT1* mRNA expression levels to be associated with telomere length, as well as 3′ telomeric overhang signals in GC tissues. However, we did not find significant association between telomere length or 3′ telomeric overhang signals and tumor stage (data not shown). High-level *POT1* expression, as well as telomerase activity, may be required for maintenance of telomere function.

Telomere dysfunction appears to occur in cancer precursor lesions and increase the frequency of genetically initiated neoplasms (26, 30, 31, 34–39); therefore, in early-stage GC after neoplasm initiation, down-regulation of *POT1* may occur preferentially. We showed that down-regulation of *POT1* was observed preferentially in low T grade cancers, which might also reflect telomere dysfunction in early-stage GC. Approximately half of our GC patients (21 of 51) showed no changes in the expression level of *POT1*, perhaps because telomere dysfunction is circumvented by chromosomal rearrangement such as in decentric and ring chromosomes in these patients.

The ABI did not correlate with *POT1* expression levels, telomere length, or 3′ telomeric overhang signals. Anaphase bridges are chromatin bridges that are not resolved after anaphase, and they result in breakage-fusion-bridge cycles that produce rapid and widespread changes in the gene (40–46). Anaphase bridges are a hallmark of telomere dysfunction (26) but form not only as a result of defects in telomere structure or length (6, 34) but also as a result of defects in DNA replication (47), recombinations (48), or translocations that introduce a second centromere into the chromosome (46, 49). Therefore, we could not show significant association between ABI and *POT1* expression levels, telomere length, or 3′ telomeric overhang signals.

It was reported recently that the 3′ telomeric overhang is shortened at senescence and that progressive overhang loss occurred in cells that avoided senescence through inactivation of p53 and Rb (50). Evidence indicates that 3′ telomeric overhang shortening is the result of continuous cell division and that it is associated with telomere shortening. We showed telomere length to be associated with 3′ telomeric overhang signals, consistent with previously reported findings (50).

To confirm the association between *POT1* expression levels and telomere length, we measured telomere length in AZT-treated GC cells and showed reduced *POT1* expression as well as telomere shortening. Loayza *et al.* (51) also reported the amount of Pot1 to be correlated with telomere length. Our data support their findings. However, we cannot fully rule out the possibility that the down-regulation of *POT1* may have been because of a direct effect of AZT or other factors. We examined association between *POT1* expression levels and telomere length, 3′ telomeric overhangs, and the frequency of anaphase bridges using GC cells treated with *POT1* antisense oligonucleotides. The inhibition of *POT1* by antisense oligonucleotides was found to shorten the telomere, reduce the 3′ overhang signals, and increase the frequency of anaphase bridges. Colgin *et al.* (52) reported that overexpression of *POT1* led to telomere elongation. In yeast, Pot1-like protein Cdc13 recruits telomerase to the 3′ telomeric overhang (14); thus, inhibition of *POT1* may lead to telomere shortening through inhibition of recruitment of telomerase to the 3′ telomeric overhang. Furthermore, our study showed that inhibition of telomerase activity occurred via *POT1* antisense oligonucleotides. We are unable to explain this phenomenon at the present. However, inhibition of telomerase activity by *POT1* antisense oligonucleotides may participate partly in telomere shortening. We examined the viability of antisense-treated MKN-28 cells (4- and 8-day treatments) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and observed no change in the viability of treated cells compared with nontreated cells (data not shown). Thus, it seems there is no association between telomerase inhibition and decreased viability of cells. We found inhibition of *POT1* by antisense oligonucleotides to reduce 3′ overhang signals. Therefore, it is possible that *POT1* expression levels may generally depend on the size of the 3′ telomeric overhang. However, it remains a possibility that the reduction of 3′ overhang signals may be because of end-to-end fusion. Indeed, inhibition of *POT1* led to an increase in the frequency of anaphase bridges. In this study, no signal was present at the position of the larger terminal fragments representing the fused telomeres (53). Therefore, at least some of the loss of 3′ overhangs in GC cells must have taken place on unfused chromosome ends, and end-to-end fusion may have little effect on 3′ overhang signals.

Loayza *et al.* (51) observed telomere elongation using Pot1 mutant lacking the oligosaccharide/oligonucleotide-binding (OB) fold required for DNA binding. We are unable to fully explain the discrep-

ancy between the Loayza *et al.* (51) findings and those of Colgin *et al.* (52) along with ours. There are several possible explanations: we and Colgin *et al.* (52) altered whole expression levels of wild-type *POT1* with antisense oligonucleotides and expression vectors (52); thus, the discrepancy may be because of methodological differences. We did not perform any mutation analysis in the oligosaccharide/oligonucleotide-binding (OB) fold of Pot1 in the present study. This should be done in further study. Although telomere length may be regulated by the interaction between Pot1 and TRF1 complex, we did not examine TRF1 expression status in the present study. In yeast, the interaction between Cdc13 and telomerase has been previously described (14), but in human cells, the interaction between Pot1 and telomerase has not been examined. We presume that such investigation is most essential to understanding the function of Pot1.

Adequate telomere length, telomerase activity, and T-loop formation are required for maintenance of telomere function, and when only one mechanistic factor is compromised such as in a lack of functional telomerase or telomere shortening, the other components of the capping system can compensate (16). The association we observed between telomere length and telomerase activity indicates that Pot1 may play an important role in the maintenance of telomere function. Telomere dysfunction leads to genetic instability at the chromosome ends, and such instability is associated with the initiation of carcinogenesis (26, 30, 31, 34–39). We consider reduced levels of *POT1* expression to reflect telomere dysfunction and that they may serve as a useful screening tool for identifying individuals at greatest risk of carcinogenesis. Additional studies are needed to establish Pot1 as a clinical indicator of cancer risk.

ACKNOWLEDGMENTS

We thank Masayoshi Takatani and Mutsumi Ueda for their excellent technical assistance and advice, and Dr. Keiko Hiyama, Department of Translational Cancer Research, Research Institute for Radiation Biology and Medicine, Hiroshima University, for helpful discussions.

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Vegetable and Fruit Intake and Stroke Mortality in the Hiroshima/Nagasaki Life Span Study

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Background and Purpose—Fruits and vegetables are known for their beneficial effects on chronic diseases. The purpose of the present study was to investigate the protective effect of a diet rich in fruits and vegetables on total stroke mortality and its 2 main subtypes in men and women separately.

Methods—A prospective cohort study of 40 349 Japanese men and women was initiated in 1980–1981 and followed until 1998. Fruit and vegetable intake was assessed at baseline on the basis of the response to a food frequency questionnaire. During the 18-year follow-up period, deaths from stroke were registered.

Results—A total of 1926 stroke deaths were identified during the follow-up period. An increasing frequency of intake of green-yellow vegetables and fruit was associated with a reduced risk of death from intracerebral hemorrhage and cerebral infarction. Daily intake of green-yellow vegetables was associated with a significant 26% reduction in the risk of death from total stroke in men and women compared with an intake of once or less per week. The protective effect associated with daily fruit and vegetable intake was observed for both cerebral infarction and intracerebral hemorrhage mortality but was slightly stronger and clearer for infarction than for hemorrhage, with a 32% reduction in men and a 30% reduction in women. Daily fruit intake was associated with a significant 35% reduction in risk of total stroke in men and a 25% reduction in women and was equally strong for both intracerebral hemorrhage and cerebral infarction.

Conclusions—Daily consumption of green-yellow vegetables and fruits is associated with a lower risk of total stroke, intracerebral hemorrhage, and cerebral infarction mortality. The protective effects are similar in both men and women. **(*Stroke*. 2003;34:2355-2360.)**

Key Words: cerebrovascular disorders ■ fruit ■ Japan ■ longitudinal studies ■ mortality ■ vegetables

Stroke incidence and mortality rates have declined dramatically in the past 3 decades in Japan. The age-standardized mortality rate in Japan is now comparable to the rates observed in Western countries (Japan, 56.7/100 000 per year; United Kingdom, 51.8/100 000 per year; United States, 34.8/100 000 per year).[1] Stroke is a heterogeneous disease[2]; the 2 most common subtypes are intracerebral hemorrhage and cerebral infarction, and with the exception of increasing age and high blood pressure, they do not share the same risk factors. Risk factors for cerebral infarction have been well established and include diabetes mellitus, atrial fibrillation, elevated serum cholesterol level, and hypertension, leading to cerebral atherosclerosis.[3] In contrast, little is known about the etiology of intracerebral hemorrhage, although a low serum cholesterol level and a low intake of animal protein and fat are hypothesized to be risk factors.[4] Unlike in Western countries, tobacco smoking has not been identified as a strong determinant of total stroke mortality in Japanese populations.[5,6] The trend toward a more Western-style dietary pattern[7] is consistent with the 20% reduction in total stroke mortality rates observed over the past 20 years, largely due to a dramatic decline in hemorrhagic stroke, associated with a concomitant, although slow, increase in the rates of cerebral infarction.[8] Indeed, as in Western countries, cerebral infarction is now the most common stroke subtype diagnosed in Japan.

The beneficial effects of a high intake of vegetables and fruits on chronic diseases, especially on the prevention of cancer, have been studied extensively.[9] Recently, some prospective studies have reported a possible protective effect of vegetable and fruit consumption on stroke.[10–14] However, the study populations tended to be limited to either 1 sex,[10,11] total stroke,[12,13] or 1 stroke subtype.[14] One study in Japan suggested that both vegetables and fruits might be protective against the incidence of cerebral infarction as well as hemorrhagic stroke.[15]

The aim of the present study was to investigate the effect of a diet rich in fruits and vegetables on the risk of stroke mortality and its main subtypes, in men and women separately, in a large cohort in Japan.

Subjects and Methods

Study Population

The Life Span Study is an ongoing longitudinal cohort study of 120 321 persons exposed and not exposed to radiation from Hiro-

Received February 17, 2003; final revision received May 13, 2003; accepted June 10, 2003.

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Stroke is available at http://www.strokeaha.org

DOI: 10.1161/01.STR.0000089293.29739.97

TABLE 1. Distribution of Cerebrovascular Deaths in Men and Women

Type of Cerebrovascular Disease	Assigned ICD Codes		Men		Women		Total	
	9th Revision	10th Revision	No.	%	No.	%	No.	%
Subarachnoid hemorrhage	430	I60, I69.0	31	4.5	121	9.8	152	7.9
Intracerebral hemorrhage	431, 432	I61, I62, I69.1, I69.2	166	24.0	287	23.3	453	23.5
Cerebral infarction	433, 434	I63, I69.3	348	50.3	572	46.4	920	47.8
Other cerebrovascular diseases	435 to 438	I64 to I68, I69.4, I69.8	147	21.2	254	20.6	401	20.8
Total			692	35.9	1234	64.1	1926	100.0

shima and Nagasaki atomic bombings, which began in 1950, from June to November 1979. A mail survey was conducted among the 55 650 Life Span Study participants who were alive on September 1, 1978,[16] of whom 40 349 persons returned a completed questionnaire (response rate, 72.5%). The nonrespondents did not differ from the respondents in terms of sex (38% men in both groups), age (mean age: nonrespondents, 53 years; respondents, 54 years), city of radiation exposure (nonrespondents, 74% in Hiroshima; respondents, 73% in Hiroshima), and stroke death rate during the follow-up (5% in both groups).

After exclusion of self-reported history of stroke, the study population consisted of 39 337 participants (14 966 men and 23 471 women). The mean age at baseline was 54 years (range, 34 to 97 years) for men and 58 years (range, 35 to 103 years) for women.

Dietary Assessment

The mail questionnaire included items regarding sociodemographic information, lifestyle factors, past medical history, and a 22-item food frequency questionnaire.[17] Food items referred to beef and pork, chicken, pork products, dairy products, milk, eggs, fish (except broiled and dry), broiled fish, dry fish, green-yellow vegetables, fruit, seaweed, tofu, miso soup, salted foods, confectionery, rice, bread, black tea, coffee, fizzy drinks, and green tea. The validity of the food frequency questionnaire has been reported previously, and vegetable and fruit items showed a moderate correlation with a 24-hour diary.[18] On the questionnaire, vegetable intake was limited to green-yellow vegetables, and fruit intake represented total fruit consumption. Each food had a 4-level precoded answer: "once or less per week," "2 to 4 times a week," "almost daily," and "do not eat." Since those who responded "do not eat" were few for both green-yellow vegetables (n=449) and fruit (n=772), this category was merged with the "once or less per week" category. Participants with missing data were also included in this category (n=3533 for vegetables; n=2286 for fruit) because the food frequency questionnaire validation study found the mean intake of those with missing data, as estimated from a 24-hour diary, to be between the values for people of "do not eat" and of "once or less per week."[18]

Follow-up and Ascertainment of Stroke Death Cases

Stroke death cases were ascertained by linkage with the nationwide family registration system in Japan,[19] since the mortality ascertainment was complete for the Life Span Study participants residing in Japan. Copies of death certificates are regularly obtained by the Radiation Effects Research Foundation for all deceased Life Span Study participants, and trained coders enter the appropriate codes into the database. Causes of death were coded according to the *International Classification of Diseases, Ninth Revision*, and *International Statistical Classification of Diseases, 10th Revision*.[20] The follow-up was started on January 1, 1980, for men and February 1, 1981, for women and continued until the date of death or March 31, 1998, whichever came first.

Statistical Analysis

Characteristics at baseline were compared according to green-yellow vegetable and fruit intake with the χ^2 test (for categorical variables) and ANOVA (for continuous variables).

Associations between dietary factors and stroke mortality were examined with a Cox proportional hazards model with age as the primary time scale and stratified by sex and birth cohort. In a previous study we examined the nondietary risk factors of total stroke mortality and its 2 main subtypes among the present population.[21] Significantly increased risks of stroke death were observed among current smokers, past alcohol drinkers, and those with a self-reported history of hypertension and diabetes. Body mass index presented an L-shaped relationship with stroke mortality. According to these previous results, relative risks were first adjusted for smoking habits (never, current, past), then for additional potential confounders: city (Hiroshima, Nagasaki), radiation dose (continuous), alcohol habits (never, current, past), education level (junior high school or less, high school, junior college or more), self-reported body mass index (continuous), history of myocardial infarction (yes, no), history of diabetes (yes, no), and history of hypertension (yes, no). Additional adjustments were also made for dietary factors found to be independent risk factors for intracerebral hemorrhage mortality, such as animal products, eggs, dairy products, and fish, the results of which are presented elsewhere.[21] A test for trend was used to assess statistical significance across exposure categories by including ordinal terms for each of the 3 categories and entering the variable as a continuous term in the model. Relative hazards of stroke death compared with staying alive were calculated with the SAS PHREG procedure.[22]

Results

During the median follow-up period of 16 years, there were 1926 stroke deaths, comprising 920 deaths from cerebral infarction (48%), 453 deaths from intracerebral hemorrhage (24%), 152 deaths from subarachnoid hemorrhage (8%), and 401 deaths from other cerebrovascular diseases (21%) (Table 1).

Baseline characteristics by fruit and vegetable consumption are shown in Table 2. In general, women consumed green-yellow vegetables and fruits more frequently than men. Green-yellow vegetable and fruit intake was less frequent among current smokers and drinkers and among those with a low or moderate education level. Radiation dose was not associated with fruit and vegetable consumption. No appreciable difference was observed for body mass index across the consumption levels of green-yellow vegetables and fruits.

The association between green-yellow vegetable and fruit intake and risk of stroke mortality in men and women is shown in Table 3. Overall, an increasing frequency of both green-yellow vegetables and fruits was associated with a reduced age-stratified risk of mortality from total stroke, as well as intracerebral hemorrhage and cerebral infarction. The dose-response relationships were significant for all these associations, except for vegetable intake and hemorrhagic stroke mortality. Compared with consumption of vegetables and fruits once or less per week, a daily consumption of

TABLE 2. Baseline Distribution of Stroke Risk Factors According to Category of Green-Yellow Vegetable and Fruit Intake

	Green-Yellow Vegetables				Fruit			
	Serving Frequency				Serving Frequency			
	0–1/week	2–4/week	Daily	*P* Value*	0–1/week	2–4/week	Daily	*P* Value*
No. of Subjects	12 633	17 083	9621		9396	12 325	17 616	
Men, %	45.5	36.6	30.9	0.001	52.7	45.5	25.1	0.001
Hiroshima City, %	72.8	73.2	74.7	0.004	73.7	71.4	74.7	0.001
Radiation dose, mSv (mean)	119.2	115.5	110.0	0.078	118.5	117.7	112.0	0.136
Age, y (mean)	57.2	54.9	56.9	0.001	57.4	54.7	56.5	0.001
BMI, kg/m^2 (mean)	22.1	22.2	22.0	0.001	22.1	22.1	22.2	0.033
Current smokers, %	38.4	30.1	23.2	0.001	44.3	37.0	19.9	0.001
Current drinkers, %	48.0	44.9	39.5	0.001	52.1	50.0	36.7	0.001
Low and moderate education level, %	91.8	89.4	86.0	0.001	91.6	89.1	88.3	0.001
Hypertension, %	27.4	25.5	24.6	0.001	28.4	25.6	24.6	0.001
Diabetes, %	11.0	8.8	8.4	0.001	12.0	9.1	8.2	0.001

*Chi-square (sex, smoking, drinking, education, hypertension, and diabetes) and ANOVA.

vegetables and fruits was associated with a risk reduction of between 20% and 40%; this was equally strong for both men and women. Adjustment for smoking status did not alter the associations appreciably. Additionally, these protective associations remained after we controlled for other potential confounders such as radiation dose, city, body mass index, education level, alcohol drinking, and past history of hypertension, diabetes, and myocardial infarction. Additional adjustment for intake of animal products slightly attenuated the association between vegetables consumption and intracerebral hemorrhage, but the associations between fruit consumption and the risk of death from stroke remained unchanged. Furthermore, mutual adjustment for intake of fruits and green-yellow vegetables did not alter the relationships (data not shown).

Exclusion of individuals with missing information on green-yellow vegetable and fruit intake did not alter the associations materially. Fruits and vegetables remained protective factors against stroke mortality and its 2 main subtypes in men and women, although the association between vegetable intake and total stroke and 2 main subtypes was not statistically significant among men (Table I, available online at http://stroke.ahajournals.org).

Discussion

This cohort study in Japan, with a median follow-up time of 16 years and a total of 1926 deaths from stroke, showed that daily intake of fruits was significantly associated with a lower risk of mortality from total stroke as well as from cerebral infarction and intracerebral hemorrhage. Daily intake of green-yellow vegetables was significantly related to a decrease risk of total stroke and infarction. However, the relation between vegetable intake and intracerebral hemorrhage was less clear, especially in men. The protective effects of green-yellow vegetable and fruit consumption appeared to be similar for both men and women.

Compared with Western populations, Japanese people have different exposures to potential dietary and lifestyle risk factors and have different stroke subtypes and etiopathology. However, longitudinal studies in both Japanese and American populations have consistently shown fruit and vegetable consumption to be inversely associated with stroke incidence and mortality,[10,11,13,14,23] although these studies have not considered potential differences between men and women or between stroke subtypes. The present study adds to evidence that vegetable and fruit consumption is protective against intracerebral hemorrhage and cerebral infarction in both men and women.

The potential protective effects of fruits and vegetables are thought to be mediated through their effects on lowering blood pressure and/or their antioxidant effects.[24] Fruits and vegetables are rich sources of vitamins and minerals such as vitamin C, beta carotene, potassium, magnesium, and calcium, in addition to other nutrients such as folate and fiber.[24] Potassium has been shown to increase natriuresis and vasomotricity, thereby lowering blood pressure.[25–27] High serum vitamin C levels have also been associated with a reduced risk of stroke among hypertensive men,[28] and fiber may be involved in lowering blood pressure.[29,30] Magnesium, calcium, and other minerals may also play a role in preventing hypertension,[31] and randomized controlled trials have shown fruit and vegetable consumption to significantly lower systolic and diastolic blood pressure.[32–34] Consequently, vitamins and phytochemicals from fruits and vegetables may help to prevent and control high blood pressure, thus preventing both hemorrhagic and infarction strokes.

Although other mechanistic effects of vegetable and fruit consumption on the prevention of intracerebral hemorrhage are unclear, there is additional evidence on their preventive association with cerebral infarction, in particular through their ability to reduce atherosclerosis. For example, potassium has been shown to inhibit platelet aggregation and arterial thrombosis.[25–27] High folate levels may lower serum homocysteine levels, a risk factor for arterial endothelial dysfunction,[35–37] and fiber may have effects that act to lower cholesterol.[29,30] Antioxidants such as vitamin C and beta

TABLE 3. Relative Hazards and 95% CI According to the Level of Consumption of Green-Yellow Vegetables and Fruits by Sex and Stroke Subtype

	Men							Women						
		Serving Frequency							Serving Frequency					
		0–1/Week	2–4/Week		Daily				0–1/Week	2–4/Week		Daily		
	Total Cases, n	RH*	RH*	95% CI†	RH*	95% CI†	*P* Value for Trend	Total Cases, n	RH*	RH*	95% CI†	RH*	95% CI†	*P* Value for Trend
Green-yellow vegetables														
Total stroke														
Cases	692	310		242		140		1234	421		511		302	
Age-stratified risk		1.00	0.78	(0.65–0.92)	0.74	(0.60–0.91)	0.0015		1.00	0.88	(0.77–1.01)	0.73	(0.62–0.85)	0.0001
Age-stratified and smoking-adjusted risk		1.00	0.78	(0.66–0.93)	0.76	(0.62–0.94)	0.0034		1.00	0.90	(0.79–1.03)	0.74	(0.64–0.87)	0.0002
Age-stratified and multivariate-adjusted risk 1‡		1.00	0.82	(0.69–0.98)	0.77	(0.62–0.94)	0.0068		1.00	0.92	(0.80–1.05)	0.78	(0.66–0.91)	0.0016
Age-stratified and multivariate-adjusted risk 2§		1.00	0.83	(0.69–0.99)	0.77	(0.62–0.95)	0.0113		1.00	0.96	(0.83–1.10)	0.81	(0.68–0.96)	0.0125
Cerebral hemorrhage														
Cases	166	75		53		38		287	94		120		73	
Age-stratified risk		1.00	0.65	(0.45–0.95)	0.74	(0.49–1.11)	0.0785		1.00	0.87	(0.66–1.15)	0.74	(0.54–1.02)	0.0634
Age-stratified and smoking-adjusted risk		1.00	0.67	(0.46–0.97)	0.86	(0.57–1.30)	0.2685		1.00	0.89	(0.67–1.17)	0.78	(0.56–1.05)	0.0983
Age-stratified and multivariate-adjusted risk 1‡		1.00	0.68	(0.47–0.99)	0.84	(0.55–1.29)	0.2596		1.00	0.88	(0.67–1.17)	0.79	(0.57–1.09)	0.1469
Age-stratified and multivariate-adjusted risk 2§		1.00	0.72	(0.49–1.06)	0.90	(0.58–1.40)	0.4665		1.00	0.94	(0.70–1.26)	0.85	(0.60–1.19)	0.3367
Cerebral infarction														
Cases	348	156		131		61		572	210		225		137	
Age-stratified risk		1.00	0.87	(0.69–1.11)	0.71	(0.53–0.96)	0.0256		1.00	0.79	(0.65–0.96)	0.64	(0.52–0.80)	0.0001
Age-stratified and smoking-adjusted risk		1.00	0.90	(0.71–1.15)	0.73	(0.54–0.99)	0.0458		1.00	0.80	(0.66–0.97)	0.66	(0.52–0.82)	0.0002
Age-stratified and multivariate-adjusted risk 1‡		1.00	0.90	(0.70–1.15)	0.70	(0.51–0.95)	0.0265		1.00	0.83	(0.68–1.01)	0.69	(0.54–0.86)	0.0013
Age-stratified and multivariate-adjusted risk 2§		1.00	0.88	(0.68–1.14)	0.68	(0.50–0.94)	0.0223		1.00	0.85	(0.69–1.05)	0.70	(0.55–0.90)	0.0054
Fruits														
Total stroke														
Cases	692	271		222		199		1234	345		348		541	
Age-stratified risk		1.00	0.81	(0.67–0.97)	0.62	(0.51–0.75)	0.0001		1.00	0.92	(0.79–1.07)	0.69	(0.60–0.79)	0.0001
Age-stratified and smoking-adjusted risk		1.00	0.79	(0.66–0.95)	0.64	(0.53–0.78)	0.0001		1.00	0.92	(0.79–1.08)	0.71	(0.62–0.82)	0.0001
Age-stratified and multivariate-adjusted risk 1‡		1.00	0.81	(0.67–0.98)	0.66	(0.54–0.80)	0.0001		1.00	0.96	(0.82–1.12)	0.73	(0.64–0.85)	0.0001
Age-stratified and multivariate-adjusted risk 2§		1.00	0.81	(0.67–0.99)	0.65	(0.53–0.80)	0.0001		1.00	0.97	(0.83–1.15)	0.75	(0.64–0.88)	0.0001
Cerebral hemorrhage														
Cases	166	65		60		41		287	74		93		120	
Age-stratified risk		1.00	0.78	(0.54–1.12)	0.54	(0.36–0.81)	0.0025		1.00	1.01	(0.74–1.38)	0.61	(0.45–0.82)	0.0002
Age-stratified and smoking-adjusted risk		1.00	0.79	(0.54–1.15)	0.61	(0.41–0.91)	0.0160		1.00	1.04	(0.76–1.42)	0.64	(0.47–0.86)	0.0007
Age-stratified and multivariate-adjusted risk 1‡		1.00	0.83	(0.57–1.22)	0.61	(0.40–0.93)	0.0202		1.00	1.09	(0.79–1.49)	0.65	(0.48–0.88)	0.0012
Age-stratified and multivariate-adjusted risk 2§		1.00	0.90	(0.61–1.34)	0.63	(0.41–0.97)	0.0381		1.00	1.12	(0.81–1.56)	0.68	(0.49–0.94)	0.0046
Cerebral infarction														
Cases	348	131		111		106		572	163		152		257	
Age-stratified risk		1.00	0.90	(0.70–1.17)	0.63	(0.48–0.82)	0.0006		1.00	0.84	(0.67–1.06)	0.68	(0.56–0.83)	0.0001
Age-stratified and smoking-adjusted risk		1.00	0.90	(0.69–1.17)	0.66	(0.50–0.86)	0.0020		1.00	0.84	(0.67–1.06)	0.70	(0.57–0.86)	0.0005
Age-stratified and multivariate-adjusted risk 1‡		1.00	0.88	(0.68–1.15)	0.64	(0.49–0.85)	0.0016		1.00	0.89	(0.71–1.13)	0.74	(0.60–0.92)	0.0040
Age-stratified and multivariate-adjusted risk 2§		1.00	0.86	(0.65–1.13)	0.63	(0.47–0.83)	0.0012		1.00	0.92	(0.72–1.17)	0.77	(0.61–0.96)	0.0175

*Relative hazards (RH).

†95% confidence intervals.

‡Relative hazards 1: age-stratified, and adjusted for radiation dose, city, BMI, smoking status, alcohol habits, education level, and medical history of hypertension, myocardial infarction, and diabetes.

§Relative hazards 2: age-stratified, and adjusted for radiation dose, city, BMI, smoking status, alcohol habits, education level, medical history of hypertension, myocardial infarction, diabetes, and consumption of animal products (egg, dairy, fish).

Missing data on vegetable and fruit consumption were considered as zero consumption.

carotene may reduce lipid oxidation of LDL cholesterol, preventing atherogenesis and its progression.[31,38] However, a study among the elderly showed that the plasma level of antioxidant vitamins was not clearly associated with extracranial carotid atherosclerosis status.[39] Moreover, intervention studies of intake of vitamins A, C, and E, carotenoids, and beta carotene have failed to show any beneficial effect on total stroke incidence or mortality.[40,41] This may be because

the mixture of phytochemicals contained in fruits and vegetables has a greater effect than a single antioxidant. Indeed, 1 study has demonstrated that nearly all of the antioxidant activity of fresh apples on cell proliferation was derived from a mixture of phytochemicals rather than vitamin C alone.[42]

It is well established that a diet rich in fruits and vegetables is associated with lower rates of smoking, higher levels of physical exercise, better health management, and a relatively low intake of cholesterol, saturated fat, and dietary sodium.[14,43] However, adjustment for potential confounders of stroke such as body mass index, smoking habits, drinking habits, education level, animal products, and history of high blood pressure did not alter the protective association of vegetable and fruit consumption, suggesting that vegetable and fruit intake may have independent effects on stroke prevention.

Limitations of the study relate to the baseline dietary assessment. Although the questionnaire used had some validity,[18] its reproducibility has not been examined, and it assessed only 1 point in time. The absence of information on portion size and the limited number of foods included in the questionnaire meant that total energy intake could not be calculated or adjusted for in the analysis and may have therefore caused an underestimation of the present results. On the other hand, during the present study period, the 1980s and 1990s, the use of CT scanning for diagnosis of stroke was widespread in Japan, and therefore the death certificate information is likely to be reliable not only for total stroke but also for stroke subtypes.[44]

Although the study participants are unique in the fact that they were exposed to the atomic bombings of Hiroshima and Nagasaki in 1945, no association was observed between stroke mortality and radiation exposure in the Life Span Study cohort.[19] Additionally, dietary patterns were not related to radiation dose.[17] Consequently, the present results are likely to be generalizable to the whole Japanese population.

In conclusion, the results suggest that daily consumption of fruits and vegetables has a protective effect against both cerebral hemorrhage and infarction and that these effects are seen in both men and women. Future research needs to clarify the mechanisms through which a high intake of vegetables and fruits may protect against stroke.

Acknowledgments

This publication is based on research performed at the Radiation Effects Research Foundation (RERF), Hiroshima and Nagasaki, Japan. RERF is a private nonprofit foundation funded equally by the Japanese Ministry of Health, Labor, and Welfare and the US Department of Energy through the National Academy of Sciences. We are indebted to the persons in charge of the Life Span Study. We are also grateful to the staff of the Master File Sections in Hiroshima and Nagasaki for data collection and mortality coding and to Mikiko Hayashi for data set preparation.

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P02/1051: Cancer and non-cancer mortality risks in atomic bomb survivors, 1950-1997: lSS report 13

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The present report continues the series of general reports on mortality in the Life Span Study (LSS) cohort followed by the Radiation Effects Research Foundation (RERF). The present report deals with solid cancer and non-cancer disease mortality during the period from 1950 through 1997, updating with seven additional years of follow-up LSS Report 12. The LSS cohort includes 86,572 people with individual dose estimates. There have been 9,335 deaths from solid cancer and 31,881 deaths from non-cancer disease during the 47 years follow-up. 19% of the solid cancer and 15% of the non-cancer disease deaths occurred during the newly added follow-up period.

While excess rates for radiation-associated solid cancers are seen to increase throughout the study period regardless of age at exposure, relative risks are highest for those exposed as children but appear to decline with increasing age. For those exposed at age 30 the solid cancer risk is elevated by 47% at age 70. There are interesting variations by site of age and age at exposure pattern, though the patterns of the risk do not differ statistically from those for solid cancer as a group. However, the interpretation of age at exposure effects on the ERR or the EAR is complicated by changes in background rates with birth cohort or time trend.

The evidence for radiation effects on non-cancer mortality remains strong with risks increased by about 14% per Sv during the last thirty years of follow-up. Statistically significant increases are seen for heart disease, stroke, digestive diseases, and respiratory diseases. Although the non-cancer data are consistent with some non-linearity in the dose response, the evidence against linearity is weaker than past. However, there is no direct statistical evidence of radiation effects for doses less than 0.5 Sv.

Elevated Interleukin-9 Receptor Expression and Response to Interleukins-9 and -7 in Thymocytes During Radiation-Induced T-Cell Lymphomagenesis in B6C3F1 Mice

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Dysregulation of cytokine receptor expression and responsiveness to cytokines is hypothesized to play an important role in the development and expansion of preneoplastic cells or progression of neoplastic cells during the early and late stages of leukemogenesis. To determine the crucial changes in initiated cells that confer significant growth during the early stage of radiation-induced lymphomagenesis, we examined both the expression of receptors for thymus-derived cytokines and thymocyte response to cytokines before the onset of T cell lymphomas in B6C3F1 mice after split-dose irradiation. After irradiation, thymic T cell subsets underwent delayed regeneration consisting of two phases as determined by receptor expression. The first phase occurred within 1 week post-irradiation and was accompanied by transient expansion of T cell subsets strongly expressing receptor genes for *IL-1*, *IL-2*, *IL-6*, *IL-7*, *IL-15*, and *TNFα*. The second phase occurred 12 weeks after irradiation and was characterized by increased expression of *IL-9Rα*. Thymocytes from non-irradiated control mice were unresponsive to IL-9. However, IL-9 acted synergistically with IL-7 and PHA to stimulate the proliferation of irradiated cells during the second post-irradiation phase. Moreover, these cells showed hyperresponsiveness to IL-7 or PHA alone compared to age-matched non-irradiated control thymocytes. These results suggest that the unusual expression of *IL-9* receptors and/or increased responsiveness of thymocytes to cytokines are key processes in the development of radiation-induced T cell lymphomas. J. Cell. Physiol. 198: 82–90, 2004. © 2003 Wiley-Liss, Inc.

T-lineage lymphoblastic leukemia can be defined as the deregulated proliferation and expansion of hematopoietic cells that have undergone malignant transformation at distinct stages of proliferation (Reinherz et al., 1980; Rabbitts, 1991, 1994). Since the responsiveness of normal progenitor cells to cytokine-mediated proliferation signals depends on their lineage commitment and differentiation state, the characterization of cytokine responsiveness in leukemic cells may provide valuable information about malignant cell development and its dependence on tissue microenvironments. Earlier studies have demonstrated that, as with normal immature T progenitor cells, the growth of both human T acute lymphoblastic leukemia (T-ALL) and mouse thymic lymphomas are stimulated by several cytokines that are secreted from thymic microenvironments in which the malignant T cells develop. The abnormal production of cytokines and expression of their receptors accompanies the onset of certain lymphomas and leukemias, and these cytokines include IL-2, IL-4, IL-5, IL-7, IL-9, and SCF (Touw et al., 1990; Digel et al., 1991; Masuda et al., 1991; Gruss et al., 1992; Vink et al., 1993;

Abbreviations: IL, interleukin; SCF, stem-cell factor; TNF, tumor necrosis factor; PHA, phytohemagglutinin; T-ALL, T acute lymphoblastic leukemia; DEX, dexamethasone.

Contract grant sponsor: National Institute of Radiological Sciences; Contract grant sponsor: The Ministry of Education, Science, and Culture, Japan; Contract grant number: 12670337.

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Received 12 March 2003; Accepted 4 June 2003

DOI: 10.1002/jcp.10390

Tomeczkowski et al., 1998). IL-7, but not IL-2 or IL-4, functions as a potent inhibitor of spontaneous apoptosis in T-ALL blasts, and IL-7 receptor α (IL-7Rα) expression correlates well with the extent of inhibition (Karawajew et al., 2000). IL-7/IL-7R, a non-redundant cytokine loop for T cell development, appears to be the most important loop for the growth and survival of human T-ALL. Therefore, we hypothesize that an imbalance in cytokine production and cognate receptor expression (e.g., the IL-7/IL-7R loop) plays an important role in the development and expansion of leukemic T cells.

The conversion of a normal cell into a preleukemic and ultimately into a leukemic cell is a multistep process. New information on the molecular processes that occur during early stages of leukemogenesis will provide a better understanding of the origin and accumulation of initial lesions. Although the molecular and cellular changes of transformed leukemic T cells have been extensively studied (Rabbitts, 1994), the events that occur in the early stages of leukemia development are not fully understood. During the past decade, the factors that promote or inhibit the growth and survival of intrathymic T progenitor cells in a differentiation-stage-dependent manner have been thoroughly elucidated (Rodewald and Fehling, 1998). Therefore, we are now in a position to broach the question as to which thymus-derived cytokines are involved in the early stages of T cell leukemogenesis.

We examined time-dependent changes of thymocytes with regard to the expression of cytokine receptors and the responsiveness to thymus-derived cytokines before the onset of overt lymphomas using a mouse radiogenic T cell lymphoma model. This model is useful in that the time-dependent appearance of preleukemic cells and lymphoma after irradiation have been well characterized (Sado et al., 1991; Humblet et al., 1997). Extensive analysis of the molecular and cellular changes of lymphoma development suggests that regenerating radioresistant thymic cells may undergo neoplastic transformation via dysregulated growth control and differentiation arrest (Sado et al., 1991). Consistent with this proposal, our present work shows that *IL-9Rα* expression in thymocytes increases abruptly prior to the onset of T cell lymphoma. However, IL-9 alone neither stimulates cell growth nor prevents apoptosis. Rather, IL-9 along with either IL-7 or PHA act synergistically to promote cell proliferation in irradiated mice, and this synergism is not observed in cells from non-irradiated control mice. These results indicate that T cells at this stage acquire the ability to respond to co-stimulation involving IL-9 and that this responsiveness may be due to increased *IL-9Rα* expression. Interestingly, induction of cell proliferation and prevention of apoptosis are also apparent in cells treated with either IL-7 or PHA alone. We propose that selective expansion of T cell subsets that are highly responsive to thymus-derived cytokine(s) represents a key element in the early development of T cell lymphoma.

MATERIALS AND METHODS

Animals

Female B6C3F1 mice were obtained from Charles River, Inc. (Yokohama, Japan) and housed in a temperature- and light-controlled facility with food and water available ad libitum. The B6C3F1 mouse is a cross between T cell lymphoma (TL)-susceptible C57BL/6 and TL-resistant C3H mice (Kamisaku et al., 1997). Animal care and experimental schedules were approved by the National Institute for Radiological Sciences of Japan and were in strict accordance with the guidelines of the Institute.

Irradiation

Four to five-week old female mice were exposed to whole-body irradiation (1.61 Gy per exposure at 0.6 Gy/min) once a week for 4 consecutive weeks in a perforated Plexiglas apparatus using an X-ray-generator (Pantak Ltd., East Heaven, CT; 200 kVp, 20 mA with filters of 0.5 mm Cu and 0.5 mm Al). Age-matched control animals were sham irradiated.

Preparation of thymocytes

Mice were sacrificed under ether anesthesia on day 2, or at 1, 4, 8, and 12 weeks after the last irradiation. The thymus was surgically removed and squeezed between slide glasses in order to disperse thymocytes. Thymocytes were suspended in ice-cold phosphate-buffered saline, filtered twice through nylon mesh, and prepared for cell surface marker and molecular analyses as described (Shimada et al., 2001).

FACS analysis

A monoclonal antibody to TL-2 was purchased from Meiji Nyugyo, Ltd. (Tokyo, Japan). PE-conjugated antibodies to CD3, CD4, IL-2Rα (CD125), and FITC-conjugated anti-CD8, anti-c-kit were from Pharmingen (San Diego, CA). After dispersal of thymus tissue to single cells, 10^6 cells were resuspended in ~50 μl phosphate-buffered saline containing FITC- and/or PE-conjugated antibodies at an optimal dilution for 20 min on ice. For the TL-2 antibody, cells were stained sequentially with the secondary antibody (FITC-conjugated anti-mouse Ig). Appropriate control fluorescence was determined by staining with isotype Igs or with second step reagent alone. The relative fluorescence of 10,000 cells was measured by FACScan using Lysis software (Becton Dickinson, Mountain View, CA).

RT-PCR

Given that the amount of mRNA from each irradiated thymus was limited, a semi-quantitative RT-PCR assay was used to quantitate transcripts instead of Northern analysis. Total RNA was prepared using a single step guanidine thiocyanate/phenol method. Reverse transcription of 1 μg total RNA proceeded at 42°C for 30 min in a 20 μl volume of 10 mM Tris-HCl pH 8.3, 5 mM $MgCl_2$, 1 U RNase inhibitor, 2.5 U MMLV-RT, and 2.5 μM random hexamers.

Amplification of RT cDNA was performed in a final volume of 25 μl of 10 mM Tris-HCl pH 8.3, 2 mM $MgCl_2$, 200 μM each dNTP, 1 U AmpliTaq DNA polymerase (Roche Applied Sciences, Indianapolis, IN). Each tube contained 100 nM of both sense and antisense primers for the gene of interest. The sequences of the primers and annealing temperature used for the analysis of *G3PDH*, *IL-1RI*, *IL-2Rα*, *IL-2Rβ*, *IL-2Rγ* (common gamma), *IL-4Rα*, *IL-6Rα*, *IL-7Rα*, *IL-9Rα*, *IL-15Rα*, *c-KIT*, *IGF-IR*, *TNFαR1*, *TNFαR2*, and *FLT*-3 are listed in

Table 1. Aliquots (2 μl) were removed from the PCR mixture after various cycle numbers and electrophoresed through a 3% NuSieve agarose gel. The RT-PCR reaction was optimized for each set of primers so that the assumption could be made that, within the linear portion of the curve, the amplification kinetics were proportional to the amount of cDNA. RT-PCR kinetics curves were different for individual genes and the linear portion comprised between 25 and 35 cycles for the majority of genes. For each gene, a cycle number (within the linear portion of the curve) was chosen such that the amount of PCR product reflected the amount of mRNA in the respective thymocytes. The luminescence of gel bands was measured with the BioImage laser scanner (Genomic Solutions™, Ann Arbor, MI), and the OD recorded for each gene was scaled to that of the concurrently amplified internal control (*G3PDH* fragment) to give an OD ratio. Three thymuses were examined for each time point and an overall relative ratio was calculated by dividing the experimental OD ratio by that of control thymocytes.

Thymocyte proliferation assay

Samples of 1×10^5 thymocytes were cultured with or without murine IL-7 (10 ng/ml) (Biosource Int., Camarillo, CA), IL-9 (100 ng/ml) (Genzyme, Cambridge, MA), or PHA (20 μg/ml) (Sigma, St. Louis, MO) in 200 μl of RPMI medium (Gibco BRL, Rockville, MD) containing 10% FCS in a round bottom 96-well culture plate for 3 days. The concentrations of IL-7 and IL-9 were at least 10-fold higher than the ED_{50} doses for the factors in their respective bioassays described in the attached analysis sheet. Cultures were then pulsed overnight with 0.5 μCi [^{3}H]-thymidine (Amersham Biosciences Corp., Piscataway, NJ). Cells were then harvested and radioactivity incorporation was measured by a scintillation counter. Mean thymidine incorporation (cpm) of triplicate cultures was determined.

Viability assay

Cell viability was determined by propidium iodide incorporation as described (Renauld et al., 1995). Briefly, thymocytes from control or irradiated mice were cultured for 24 h with or without a combination of dexamethasone (DEX) (0.25 μM) and cytokines (IL-7 and IL-9). The cells were then incubated for 30 min with propidium iodide (25 μg/ml) at room temperature before fluorescence-activated cell sorting (FACS) analysis. Under these conditions, dead cells are brightly stained while live cells are not. A minimum of 10,000 cells was counted for each sample.

Statistical analysis

Wilcoxon test was used to evaluate the significance of the difference between control and cytokine or PHA treated cultures. Mann–Whitney *U* test was utilized to analyze the difference in thymocyte response to cytokines or PHA between non-irradiated control and irradiated mice. Statistical analysis of data on cell survival after exposure to DEX was performed by the Student's *t*-test. The chosen level of significance was $P < 0.05$.

RESULTS

Changes in thymus size and thymocyte subsets after irradiation

Thymus weight decreased markedly 2 days after completion of the irradiation regimen, and thymuses

TABLE 1. Sequence of PCR primers and amplification condition

	Sense	Antisense	PCR cycle	Number of cycle
G3PDH	5′ TGA AGG TCG GTG TGA ACG GAT TTG GC 3′	5′ CAT GTA GGC CAT GAG GTC CAC CAC 3′	94°C (45 sec)–60°C (45 sec)–72°C (90 sec)	24
IL-1RI	5′ AAA TAA TGA GTT ACC CGA GGT CCA GTG G 3′	5′ AGG CAT CGT ATG TCT TTC CAT CTG AAG C 3′	94°C (45 sec)–60°C (45 sec)–72°C (90 sec)	33
IL-2Rα	5′ ATG GAG CCA CGC TTG CTG ATG TTG 3′	5′ CCA TTG TGA GCA CAA ATG TCT CCG 3′	94°C (45 sec)–60°C (45 sec)–72°C (90 sec)	30
IL-2Rβ	5′ CAA TGT CTC TTG CAT GTG GAG CCA T 3′	5′ AAG ACG TCT ACG GGC CTC AAA TTC CAA 3′	94°C (45 sec)–60°C (45 sec)–72°C (90 sec)	33
IL-2Rγ	5′ CCA GAG GTT CAG TGC TTT GTG T 3′	5′ ATC CAC ACT AGG CAG GGA GAA T 3′	94°C (45 sec)–60°C (45 sec)–72°C (90 sec)	33
IL-4R	5′ CTA CTA TAC GGC GCG TGT GA 3′	5′ GGC ACC TGT GCA TCC TGA AT 3′	94°C (30 sec)–58°C (30 sec)–72°C (60 sec)	35
IL-6R	5′ AAT GCG TCA TCC ATG ATG CCT TGC GAG G 3′	5′ GTG GTT TAC GGT ATT GTC AGA CCC AGA GC 3′	94°C (45 sec)–60°C (45 sec)–72°C (90 sec)	30
IL-7R	5′ CAA AGT CCG ATC CAT TCC CCA TAA C 3′	5′ GTT TTC TTA TGA TCG GGG AGA CTA GG 3′	94°C (45 sec)–60°C (45 sec)–72°C (90 sec)	30
IL-9R	5′ CAC AAA TGC ACC TTC TGG GAC A 3′	5′ TCA CTC CAA CGA TAC GGT CCT T 3′	94°C (30 sec)–58°C (30 sec)–72°C (30 sec)	35
IL-15R	5′ CTA CTG TTG CTC CGC TGA G 3′	5′ TGT CTC TGT GGT CAT TGC GGT AT 3′	94°C (45 sec)–60°C (45 sec)–72°C (90 sec)	35
TNFαRI	5′ GCC CGA AGT CTA CTC CAT CAT TTG TAG GG 3′	5′ CAT CCA CCA CAG CAT ACA GAA TCG CAA GG 3′	94°C (45 sec)–60°C (45 sec)–72°C (90 sec)	32
TNFαRII	5′ ATA CTA TGA CAG GAA GGC TCA GAT GTG C 3′	5′ CCC TTG GTA CTT TGT TCA ATA ATG GGG G 3′	94°C (45 sec)–60°C (45 sec)–72°C (90 sec)	30
IGF1R	5′ GAC ATC CGC AAC GAC TAT CAG 3′	5′ GAT GTT ATT GGA CAC CGC ATC C 3′	94°C (45 sec)–60°C (45 sec)–72°C (90 sec)	29
c-KIT	5′ GGG CAA GAG TTC CGC CTT CTT 3′	5′ GCT GCG ACC ACA AAG CC 3′	94°C (30 sec)–58°C (60 sec)–72°C (120 sec)	33
FLT-3	5′ ACC ATG GAT TCG GGC TCA CCT 3′	5′ CTG GGC GTA TCA TTT TCT GC 3′	94°C (45 sec)–60°C (45 sec)–72°C (90 sec)	33

from 8-week-old non-irradiated mice weighed ~60 mg while those of irradiated mice decreased to ~20 mg (Fig. 1A). Histologically, irradiated thymuses displayed a very thin cortex (data not shown). The thymocyte population from irradiated thymuses showed a striking decrease in $CD4^+8^+$ cells and a relative increase in $CD4^+CD8^-$ cells (Fig. 1B(a,b)). An elevated level of highly TCRαβ-positive cells was also observed after 2 days, and these cells were also coincident with mature $CD4^+CD8^-$ cells (Fig. 1B(b)). By the end of post-irradiation week one, thymus weight was slightly restored; and thymocytes became repopulated and the normal T cell subsets (Fig. 1B(c)) and cortico-medullary architecture (data not shown) were reconstituted. A slight decrease in thymus weight occurred again after 4 weeks. During post-irradiation weeks 8–12, the thymus weight of irradiated mice began to increase to nearly normal levels. This recovery, as observed in 9 out of 14 of thymuses, was due to an enlargement of the unilateral lobe of the thymus in agreement with a previous report involving the B6 mouse (Siegler et al., 1966). We previously reported that the first death due to thymic lymphoma in B6C3F1 mice occurred at ~14 weeks post-irradiation, and the mean life expectancy for all mice was 26 ± 4 weeks (Shimada et al., 2001). In the present study, we found that the thymus from one mouse at 12 weeks post-irradiation weighed 207 mg, or ~fourfold that from age-matched control mice (Fig. 1A). Therefore, we considered the 12th post-irradiation week as the period just prior to the onset of overt lymphoma cell expansion. When examined by fluorocytometry at 12 weeks post-irradiation, thymocytes from four of seven thymuses were indistinct from control cells (Fig. 1B(d)). However, the expression of T cell differentiation antigens in the other three thymuses was distorted suggesting phenotypic alterations in thymocyte subpopulations during the early phase of leukemia (Fig. 1B). The cells from one thymus were strongly CD8-positive (85%) and weakly IL-2R-positive (18%) (Fig. 1B(e)). The cells from the other two thymuses showed a single peak of TCRαβ, one of which was also weakly c-kit-positive (22%) (Fig. 1B(f)).

Time-dependent changes in cytokine receptor expression after irradiation

A variety of cytokines expressed in intrathymic microenvironments regulate development of T cells (Zlotnik and Moore, 1995). Elucidating the cytokine/receptor networks that regulate thymocyte proliferation and survival constitutes an important conceptual advance towards understanding the mechanism of thymus regeneration as well as lymphoma development. Thus, we examined the kinetics of cytokine receptor gene expression in thymocytes within the irradiated thymus. Using semi-quantitative RT-PCR, we initially analyzed 14 cytokine receptor genes that regulate T cell growth and differentiation (Fig. 2A,B). Time-dependent changes in expression were classified into four distinct patterns, the first of which included *IL-1RI*, *IL-2Rα*, *IL-2Rβ*, *IL-6R*, *IL-7R*, *IL-15R*, and *TNFαRII*. The expression of these receptors was maximal on post-irradiation day 2 and returned to basal or sub-basal level thereafter. The second pattern included *IL-2Rγ* (common-gamma), *IL-4R*, *TNFαRI*, c-*KIT*, and *FLT*-3 whose expression

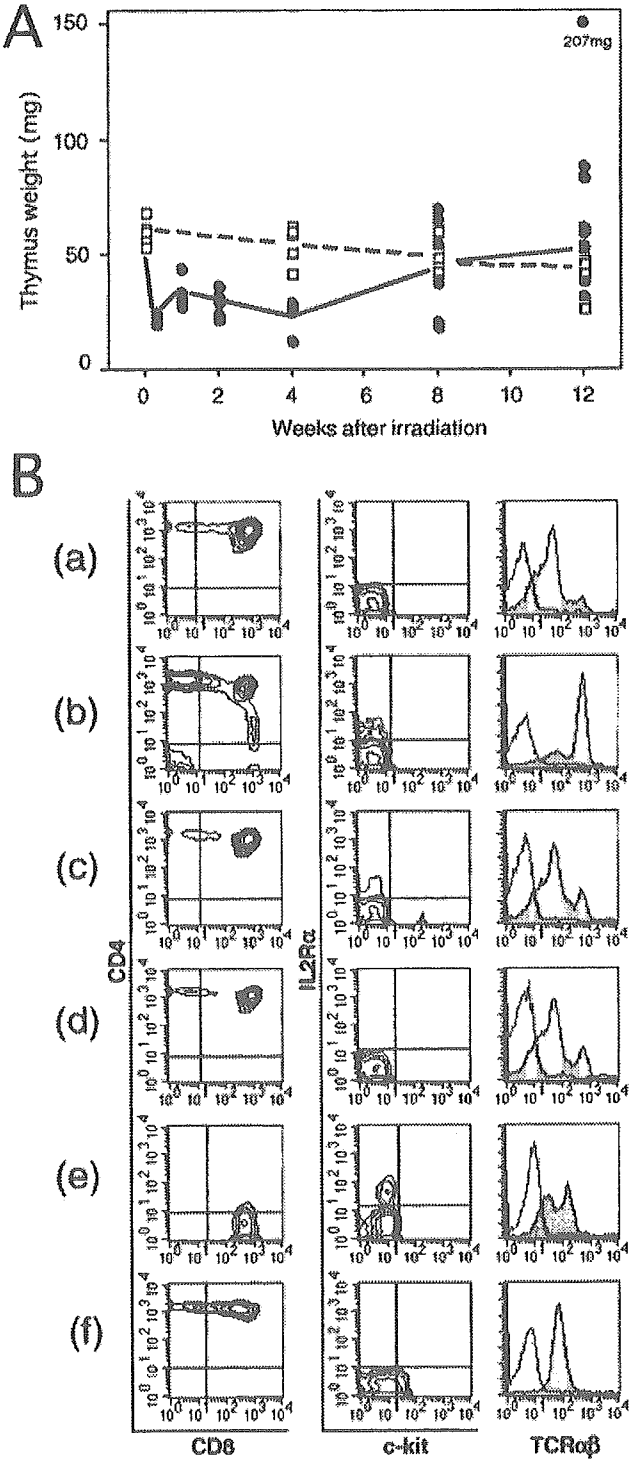

Fig. 1. Changes in thymocyte subpopulations after split-dose irradiation. **A**: Thymus weight following split-dose irradiation (●) or sham irradiation (□). **B**: Representative flow cytometry profiles of the cell-surface expression of CD4, CD8, TCRαβ, IL-2Rα, and c-kit in control thymocytes (a) and thymocytes on day 2 (b) and weeks 1 (c) and 12 (d–f) following irradiation. Shaded profiles were compared to unshaded profiles staining with isotype-matched control antibodies.

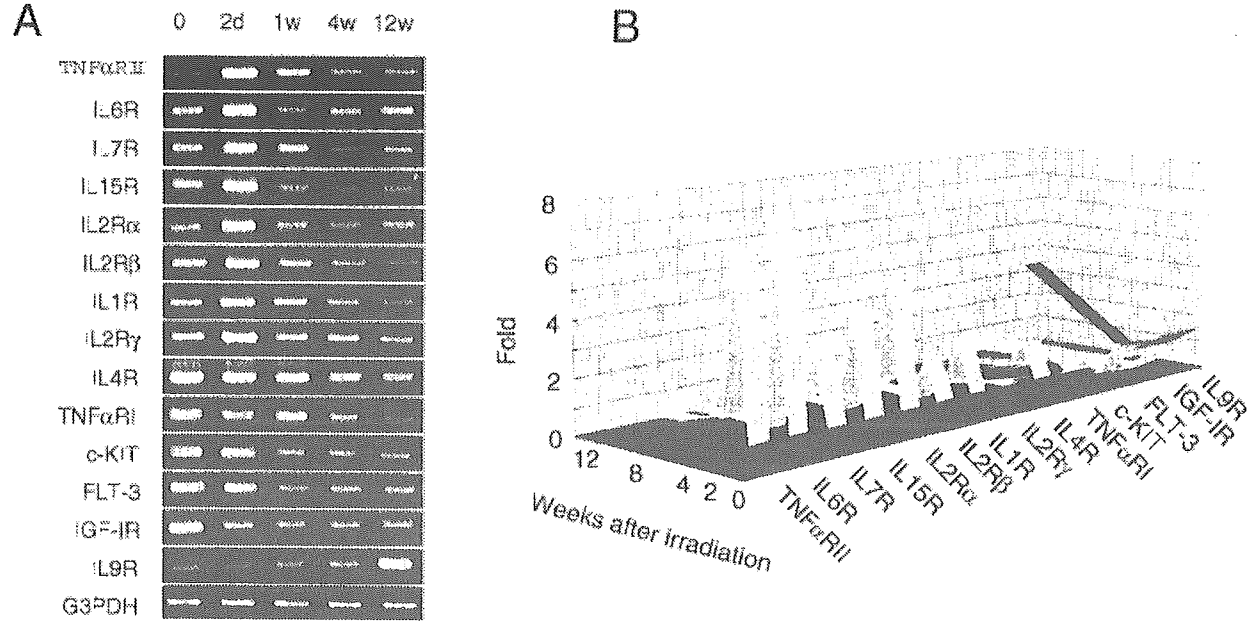

Fig. 2. Expression of cytokine receptor mRNAs in thymocytes after split-dose irradiation. Each experiment was performed using three mice. **A**: Representative results of RT-PCR analysis. Primer sequences and PCR conditions are indicated in Table 1. **B**: Data represent time-dependent changes in mRNA levels (mean ± SE) relative to *G3PDH* expression. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

levels did not change significantly up to 1 week post-irradiation but decreased thereafter. The expression of *TNFαRI* remained unchanged while that of *TNFαRII* increased immediately after irradiation, indicating that these two TNF receptors are regulated independently (Fig. 2A,B). The third group included *IGF1R* whose expression decreased significantly immediately after irradiation. *IL-9R* comprised a fourth group in that its expression increased substantially from 4 to 12 weeks after irradiation. Thus, increased *IL-9R* expression preceded the expansion of lymphomas in situ. *IL-9R* expression was very high in 94.1% (32/34) of thymic lymphomas implying that IL-9R-mediated signaling is important for cell proliferation during both early and late stages of lymphomagenesis (Fig. 3A). Thymus leukemia antigen (TL-2) is an unequivocal preleukemic/leukemic antigen of radiation-induced lymphomas in C57BL/6 or C57BL/Ka mice (Stockert and Old, 1977; Muto et al., 1990) but was scarcely detected in the thymocytes in B6C3F1 mice examined at 12 weeks post-irradiation (Fig. 3B).

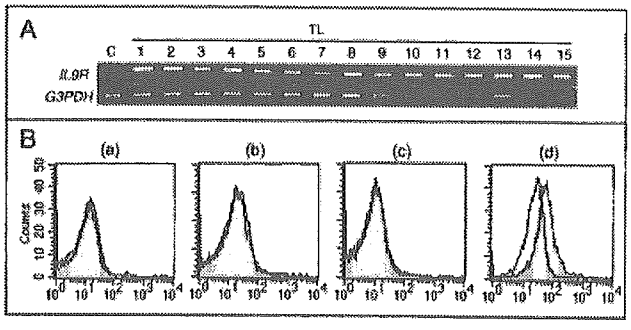

Fig. 3. Expression of *IL-9R* mRNA and TL-2 in thymic lymphoma cells. **A**: Expression of *IL9-R* in thymic lymphomas (TL) from 15 experimental animals. **B**: Flow cytometry analysis of TL-2 expression on control thymocytes (a) and thymocytes from selected preleukemic mice 12 weeks after irradiation (b–d).

Response of thymocytes to IL-9 and IL-7 before the onset of T cell lymphomas

As is shown in Figure 2, enhanced *IL-9R* expression occurred before the onset of lymphomic cell expansion, suggesting an important role for IL-9/IL9-R signaling in lymphomagenesis. Indeed, IL-9R mediates signaling that is essential for human fetal thymocyte proliferation and development (De Smedt et al., 2000). Thus, to determine the biological role of *IL-9R* expression prior to lymphomic cell expansion, we examined the proliferative response of the cells to IL-9 at this stage. The growth stimulators PHA and IL-7 were also examined, the latter of which is essential for both thymocyte development and leukemic progression.

[^{3}H]-thymidine uptake was measured in control thymocytes from non-irradiated mice and thymocytes from post-irradiation week 12 mice after 3 days of culture in the presence of IL-9, IL-7, or PHA, or combinations thereof. IL-9 alone did not induce thymocyte proliferation in non-irradiated control mice (Fig. 4A) as reported previously (Suda et al., 1990). Unexpectedly, IL-9 did not elicit a response in thymocytes from irradiated mice even though they expressed *IL-9R* (Fig. 2). In contrast, PHA induced [^{3}H]-thymidine uptake in thymocytes from irradiated mice. PHA-mediated enhancement varied from 1–27-fold among thymuses with an average of eightfold, but no effect was seen in control thymocytes. IL-7 increased uptake in irradiated mice by 2 to 501-fold with an average of

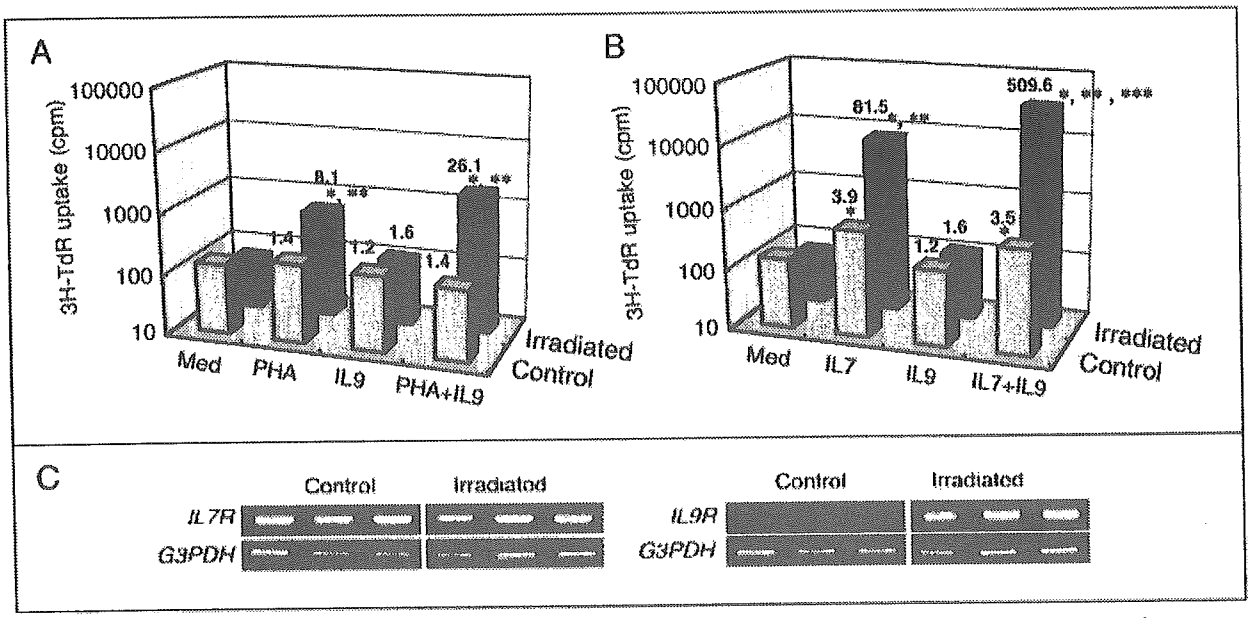

Fig. 4. Enhanced proliferation of thymocytes in response to PHA, IL-7, and IL-9 at 12 weeks post-irradiation. **A**: Thymocytes were stimulated by PHA or IL-9 (or combinations thereof) and average [^{3}H]-thymidine incorporation was measured after 3 days of culture. Mean counts (cpm) due to autoproliferation (without cytokines) of control thymocytes and those from irradiated mice were 135 and 69, respectively. Each column represents data averages of five control mice and seven irradiated mice. **B**: Thymocytes were stimulated by IL-7 or IL-9 (or combinations thereof) and average [^{3}H]-thymidine incorporation was measured after 3 days of culture. **C**: RT-PCR analysis of *IL-7R* and *IL-9R* expression in thymocytes from control and irradiated mice at 12 weeks post-irradiation. $^*P < 0.05$ compared to thymocytes without cytokines or PHA treatment (Med). $^{**}P < 0.05$ compared to thymocytes from non-irradiated control mice. $^{***}P < 0.05$ compared to thymocytes treated with PHA (A) or IL-7 alone (B).

82-fold (Fig. 4B), and also increased uptake fourfold in control thymocytes.

It has been reported that IL-9 enhances the cellular response to other cytokines (Suda et al., 1990). Hence, the effect of IL-9 on PHA- and IL-7-induced proliferation was examined. IL-9 enhanced the PHA-mediated response in thymocytes from irradiated mice (1–125-fold; 26-fold on average) but not in control cells (Fig. 4A). The combination of IL-7 with IL-9 did not enhance proliferation in control cells. However, in cells from irradiated thymuses, IL-7 and IL-9 elicited a 510-fold (3–1,680-fold) increase in [^{3}H]-thymidine uptake compared to an 82-fold increase for IL-7 alone, indicating substantial synergy (Fig. 4B). This enhanced stimulation appeared to be independent of *IL-7R* expression since its expression was comparable between control and irradiated mice (Fig. 4C).

Thymocyte survival

To date, one of the major functions of IL-9 and IL-7 is in anti-apoptosis (Hultner and Moeller, 1990; Renauld et al., 1995). To evaluate IL-9 or IL-7-mediated anti-apoptotic responsiveness in thymocytes from control and irradiated mice, cell survival and spontaneous cell death were examined after DEX treatment in the presence or absence of these cytokines. After 24 h in culture, ~60% of control thymocytes survived without DEX and no effect of cytokines was observed (Table 2). The cells from irradiated mice were much more prone to spontaneous cell death in that cell survival was only

TABLE 2. Effects of IL-7 and/or IL-9 on survival of thymocytes from non-irradiated control and irradiated mice after DEX exposure

		Survival of thymocytes after treatments (%)			
Mice	DEX	Medium	IL-7	IL-9	IL-7+IL-9
Non-irradiated (n = 6)	w/o	58.1 ± 1.4	60.1 ± 2.9	60.0 ± 1.8	62.6 ± 1.1
	w/	3.7 ± 0.3	9.6 ± 1.5	3.0 ± 0.3	11.5 ± 0.4
	Adjusted survival (%)[a]	6.6 ± 0.5	16.0 ± 2.4	5.0 ± 0.5	18.4 ± 0.8
Irradiated (n = 9)	w/o	29.7 ± 7.2	35.3 ± 4.3	30.4 ± 7.2	43.9 ± 5.0
	w/	2.1 ± 0.6	13.1 ± 2.1	2.0 ± 0.5	14.6 ± 1.6
	Adjusted survival (%)[a]	7.0 ± 0.6	35.3 ± 4.3[b]	7.8 ± 0.5	35.3 ± 1.6[b]

The results are expressed as mean ± SE of triplicate cultures. The thymocytes from non-irradiated and irradiated mice were incubated in the combinations of DEX (0.25 μM), IL-7, and IL-9, and evaluated for survival after 24 h.
[a]Adjusted survival (%); the survival rate adjusted for cells undergoing spontaneous cell death (w/o DEX treatment).
[b]The adjusted survivals (%) of thymocytes after DEX treatment in the presence of IL-7 with or without IL-9 were significantly higher in irradiated mice than those in non-irradiated control mice ($P < 0.05$).

~30% (Table 2). IL-7 marginally increased cell survival in thymocytes from both control and irradiated mice, but IL-9 had no effect. DEX substantially induced cell death in that only 3.7 and 2.1% of cells from control and irradiated mice survived, respectively (Table 2). In the presence of IL-7, DEX-treated cell survival was 9.6% (control) and 13.1% (irradiated). When adjusted for the fraction of cells that underwent spontaneous death, the survival of thymocytes from control and irradiated mice after DEX treatment was 16.0 and 35.3%, respectively ($P < 0.01$). This result implies that IL-7 prevented cell death more effectively in cells from irradiated mice than those from controls. IL-9 did not affect the survival of DEX-treated cells in either group, and the effect of IL-9 and IL-7 in combination was similar to IL-7 alone. These results indicate a lack of interaction/synergy between these cytokines with respect to cell survival in contrast to their co-stimulatory action on cell growth in culture (Fig. 4B).

DISCUSSION

The present study demonstrates that the early stage of radiation-induced lymphomagenesis in B6C3F1 mice consists of two phases as determined by cytokine receptor gene expression. The first phase occurs within one week post-irradiation and is characterized by an expansion of radioresistant T cell subsets expressing receptor genes for IL-1, IL-2, IL-6, IL-7, IL-15, and TNFα. The appearance of these T cell subsets corresponds to the induction of inflammatory cytokines such as IL-1, IL-6, TNFα, and IL-7 in the regenerating thymus after sublethal X-irradiation (Mizutani et al., 2002). In the second phase that begins 12 weeks after irradiation (just prior to the onset of lymphoma), the cells show an increase in *IL-9R* receptor expression and acquire IL-9 responsiveness as revealed by enhanced proliferation in combination with IL-7. The cells also exhibit an enhanced response to IL-7 and PHA, suggesting that growth advantaged cells are selected before lymphomic transformation.

IL-9 was originally identified as a T cell-derived cytokine with growth stimulating activity for mouse helper T cell clones and T cell lymphomas in vitro (Uyttenhove et al., 1988; Vink et al., 1993). Subsequent studies showed that induction of IL-9 expression in transgenic mice results in the development of spontaneous T cell lymphoma and a dramatic increase in susceptibility to *N*-methyl-*N*-nitrosourea (MNU) during the induction of lymphomas (Renauld et al., 1994). The involvement of IL-9 in human leukemia is further suggested by the discovery of an autocrine IL-9 loop in Hodgkin's lymphoma cells (Gruss et al., 1992). Thus, several lines of evidence implicate IL-9 in the initiation and progression of lymphoma.

Our results show that IL-9 alone is insufficient to stimulate growth in thymocytes from mice at 12 weeks post-irradiation. However, IL-9 enhances IL-7-induced cell proliferation in a synergistic manner, a phenomenon that may be ascribed to increased *IL-9R* receptor expression. Given that IL-9 alone is sufficient to promote the proliferation of lymphoma cells (Vink et al., 1993), we hypothesize that thymocytes expressing IL-9R reflect intermediate cells at the stage before complete neoplastic transformation. In support of this hypothesis, cultured helper T cell clones exhibit varied IL-9 responses during acquisition of autonomous growth, when they are repeatedly stimulated with antigens (Renauld et al., 1993). No response to IL-9 is detected in T cell clones during the early stage of transformation, but after additional weeks in culture, the cells proliferate in response to IL-9 but only in the presence of a synergistic factor, namely, IL-4 or IL-3. In the final stage, IL-9 alone induces significant proliferation. The progressive deregulation of IL-9 responsiveness is reminiscent of the multi-step process leading to neoplastic transformation in myelocytic cells (Metcalf, 1994). Thus, the acquisition of IL-9 responsiveness, which may result from increased *IL-9R* expression, could be considered as an early sign of transformation during multi-step leukemogenesis.

It has been suggested that aberrant expression of cytokine receptors is involved in neoplastic transformation. Receptors such as IL-2R, IL-6R, and IL-7R are overexpressed (or abruptly expressed) in primary leukemia and the expression levels correlate well with cytokine-induced proliferation and the prevention of apoptosis (Uchiyama et al., 1985; Dibirdik et al., 1991; Digel et al., 1991; Rawstron et al., 2000). Our results show that enhanced *IL-9R* expression precedes the appearance of lymphoma, a phenomenon for which there are several possible explanations. Firstly, these cells emerge from an immature T cell subset that expresses IL-9R; and indeed the fetal thymus contains immature thymocytes that can respond to IL-9 to form developing T cell subsets (De Smedt et al., 2000). However, such a cell population has not yet been sufficiently characterized. A second explanation is that *IL-9R* expression is induced by quantitative changes in the cytokine population within thymic microenvironments following split-dose irradiation. A previous study showed that increased IL-9R expression is observed after repeatedly stimulating freshly isolated human T cells with a combination of PHA, IL-2, and allogenic feeder cells (Houssiau et al., 1993). It is well documented that homologous and/or heterologous cytokines regulate the expression of other cytokine receptors such as IL-2R, IL-4R, and IL-8R (Ohara and Paul, 1988; Bonecchi et al., 2000; Alileche et al., 2001). Thus, the production of inflammatory cytokines in the thymus after split-dose irradiation deserves further investigation. Thirdly, irradiation of thymic stromal cells selects a subset of cells expressing receptors for IL-9. Greenberger et al. (1992a,b) have demonstrated that co-cultures of heavily irradiated bone marrow stromal cells selectively bind M-CSF receptor-positive hematopoietic progenitor cells which results in the selection of tumorigenic clones. Lastly, the integration of a virus-LTR into the *IL-9R* gene could result in high expression of *IL-9R*. Consistent with this possibility, human T cell leukemia virus type I (HTLV-I) was found to be integrated upstream of the *IL-9R* gene in the MT-2 cell line (which was established through transmission of HTLV-I) (Kubota et al., 1996).

Our data may highlight a selective advantage for cells that exhibit increased susceptibility to IL-7 and IL-9 during the evolution and expansion of cells before the onset of lymphomas and suggest the importance of acquiring a susceptibility to the growth-stimulating milieu. The growth characteristics of preneoplastic cells following carcinogen treatment have been well studied