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Analysis of Early Initiating Event(s) in Radiation-induced Thymic Lymphomagenesis

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Since the T cell receptor rearrangement is a sequential process and unique to the progeny of each clone, we investigated the early initiating events in radiation-induced thymic lymphomagenesis by comparing the oncogenic alterations with the pattern of γ T cell receptor (TCR) rearrangements. We reported previously that after leukemogenic irradiation, preneoplastic cells developed, albeit infrequently, from thymic leukemia antigen-2$^+$ (TL-2$^+$) thymocytes. Limited numbers of TL-2$^+$ cells from individual irradiated B10.Thy 1.1 mice were injected into B10.Thy 1.2 mice intrathymically, and the common genetic changes among the donor-type T cell lymphomas were investigated with regard to *p53* gene and chromosome aberrations. The results indicated that some mutations in the *p53* gene had taken place in these lymphomas, but there was no common mutation among the donor-type lymphomas from individual irradiated mice, suggesting that these mutations were late-occurring events in the process of oncogenesis. On the other hand, there were common chromosome aberrations or translocations such as trisomy 15, t(7F;10C), t(1A;13D) or t(6A;XB) among the donor-type lymphomas derived from half of the individual irradiated mice. This indicated that the aberrations/translocations, which occurred in single progenitor cells at the early T cell differentiation either just before or after γ T cell receptor rearrangements, might be important candidates for initiating events. In the donor-type lymphomas from the other half of the individual irradiated mice, microgenetic changes were suggested to be initial events and also might take place in single progenitor cells just before or right after γ TCR rearrangements.

Key words: Initiation — *p53* — Chromosome aberration — T cell receptor rearrangement — Clonality

Exposure of B10 mice to fractionated irradiation (four exposures of 1.61 Gy at 8-day intervals) induces a high incidence (>95%) of thymic lymphomas after an average latency of about 200 days.[1-3] The cellular processes leading to the development of preneoplastic cells are complex, involving interactions between the target cells for neoplastic transformation, the thymic microenvironment and the bone marrow.[2-7]

With the combined use of cell separation by cell sorter and intrathymic injection assay, we also found that the main target cells for radiation-induced thymic lymphomagenesis are CD4$^-$CD8$^-$, CD4$^-$CD8$^+$ and CD4$^+$CD8$^+$ cycling immature J11d$^+$ thymocytes. After leukemogenic irradiation, thymocytes expressing thymic leukemia antigen-2 (TL-2), which is not expressed on normal thymocytes of B10 mice, develop from these immature cycling thymocytes, and prelymphoma cells develop from these TL-2$^+$ thymocytes.[8]

During the last decade, although many oncogenes and tumor suppressor genes or chromosome aberrations/translocations in neoplasia have been identified and the functions of these related genes have been investigated extensively, it is still a difficult problem to determine exactly the early initiating event(s) in the process of radiation-induced lymphomagenesis. Since the rearranged T cell receptor (TCR) gene is a useful indicator of clonal origin and the TCR rearrangement is a sequential process and unique to the progeny of each clone, one of the approaches for examining the oncogenic process is to analyze both the alterations of the genes or chromosomes from lymphomas derived from a single donor by intrathymic (i.t.) injection of a limited number of prelymphoma cells and the patterns of TCR rearrangements of these lymphomas.

In this report, we first describe the kinetics of early development and clonality of preneoplastic (prelymphoma) cells. Using this experimental system, we analyzed the early initiating events in radiation-induced thymic lymphomagenesis. The results indicated that some mutations in the *p53* gene existed in these lymphomas, but there was no common mutation among the donor-type lymphomas from individual irradiated mice, suggesting that these mutations were late-occurring events in the process of oncogenesis. On the other hand, there were common aberrations or translocations among

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some donor-type lymphomas from individual irradiated mice. This suggested that these common chromosome aberrations/translocations could be considered as potential initiating events for radiation-induced lymphomagenesis. The contribution of specific microgenetic changes to the initiating pre-neoplastic phenotype was also suggested in other donor-type lymphomas derived from individual irradiated mice.

MATERIALS AND METHODS

Mice and irradiation Female B10f/Sn (Thy-1.2, H-2^b) (hereafter called B10.Thy-1.2) and B10.NRH-Thy-1^a (Thy-1.1, H-2^b) (hereafter called B10.Thy-1.1) mice were used. They were irradiated at 8-day intervals with four doses of 1.61 Gy beginning at the age of 33 ± 3 days (fractionated whole-body X-irradiation; FX).

i.t. injection and Thy-1 typing Thymocytes were recovered from individual B10.Thy 1.1 mice on day 28 after the last irradiation and the percentage of TL-2^+ cells was measured by staining with fluorescence isothiocyanate (FITC)-labeled anti-TL-2 monoclonal antibody (mAb) (Meiji Nyugyo Ltd., Tokyo). Various numbers of TL-2^+ cells were injected i.t. into 6-week-old B10.Thy 1.2 mice which had been irradiated with 3.78 Gy immediately before injection (groups A, B, C, D, E out of 11 groups in Table I; the other six groups did not develop donor-type lymphomas) or were without irradiation (groups F, G, H out of 4 groups in Table I; the other group did not develop donor-type lymphoma). Between 4 and 5 months after transplantation, the recipient mice were killed when they appeared moribund or developed clinical evidence of disseminated lymphoma. Each lymphoma was confirmed to have a donor-type origin by staining with FITC-labeled anti-Thy-1.1 and anti-Thy-1.2 antibodies as described previously.[6] Thy-1.1 donor-type lymphoma cells amounted to 98.2–99.7%, whereas Thy-1.2 recipient-type cells were undetectable. The ability to develop donor-type lymphomas in the non-irradiated recipients (3/4) was not lower than in the 3.78 Gy-irradiated recipients (5/11). No typical differences were found with regard to tumor phenotype or clonality between these groups.

Chromosome analysis Tumors excised from mice that had been transplanted with primary donor-type lymphomas were teased gently in RPMI 1640 supplemented with 10% fetal calf serum (FCS) to prepare cell suspensions. The cells (10^6/ml) were cultured in RPMI 1640 medium supplemented with 10% FCS, 5×10^{-5} *M* 2-mercaptoethanol, 10 ng/ml PMA (phorbol 12-myristate 13-acetate, Sigma, St. Louis, MO) and 250 ng/ml Ionomycine (Calbiochem Co., San Diego, CA) in Linbro tissue culture plates (Flow Lab. Inc., McLean, Virginia, CT). Cultures were maintained at 37°C in a humid atmosphere of 5% CO_2 and 95% air for 48 h. The cultured cells were then treated with 30 ng/ml of TN-16 for 2 h and harvested. The cells were expanded in a solution of 0.2% KCl and 0.2% NaCl, fixed 3 times in a methanol-acetic acid (3:1) mixture, and dropped onto clean slides. After 1–2 weeks at room temperature, the slides were treated with 31% H_2O_2 and 0.025% trypsin, washed in running water and stained with 3% Giemsa solution, pH 6.8. At least 50 cells of each sample were analyzed, and banded karyotypes were prepared according to the recommendation of the Committee on Standardized Genetic Nomenclature for mice.[9, 10] A minimum of 4 metaphase-spread ideograms were made for each lymphoma. Common abnormal karyotypes were identified when large proportions of the cells analyzed showed the same chromosomal aberrations.

DNA preparation from thymic lymphomas Tumors were excised from the implantation sites, frozen in liquid nitrogen and powdered with a cooled stainless steel crusher. High-molecular-weight DNA was extracted according to established procedures[11, 12] from frozen tissue powder added to 10 volumes of lysis solution (0.5 *M* EDTA, 100 μg/ml proteinase K and 0.5% Sarkosyl, pH 8.0).

Southern blot analysis Southern blot hybridization was performed by a modification of the method of Maniatis *et al.*, and Vγ4, Jγ1 probes were prepared as described.[11–13] pp53-176, spanning nucleotides −157 to 1188 of a mouse *p53* cDNA,[14] was obtained from Japanese Cancer Research Resources Bank.

PCR-SSCP (single strand conformation polymorphism of polymerase chain reaction products) analysis Polymerase chain reaction (PCR) amplifications were performed with 200 ng of genomic DNA in 20 μl volumes as described.[13] The primers used to amplify exons 4–9 of the *p53* gene were synthesized as described[15] except for exon 4 (sense): 5′-CCATCCACAGCCATCACCTC-3′, exon 4 (antisense): 5′-GCCCACTCACCGTGCACATA-3′, and exon 8 (sense): 5′-TCCCGGATAGTGGGAACCTT-3′. Thirty cycles were used for amplification, consisting of 1 min at 94°C, 2 min at 61–63°C, and 2 min at 72°C. Denatured samples (2 μl) were loaded onto 6% non-denaturing polyacrylamide gels containing 5% glycerol and run at 20°C for 120 min at 1500 V.[16] p-5 DNA from mouse carcinoma, which has been shown to have a mutation in exon 5, was provided by Dr. R. Kominami, Niigata University.

DNA sequence analysis PCR products of Vγ4–Jγ1 junctions were prepared by using primer PC2 (Vγ4): 5′-CGGGATCCAATATATTCCTTGGAGGAAG-3′ and PC6(Jγ1): 5′-CGGAATTCTGTTCTTTTCATCACTGGAA-3′. The DNA sequences of PCR products from A1–A7, E7–E11, and F1–F4 were determined by PCR direct sequencing (The ds DNA Cycle Sequencing

System, GIBCO BRL, Gaithersburg, MD) using primer Jγ1.seq: 5′-CAGAGGGAATTACTATGAGC-3′. Since the PCR products from other lymphomas were mixtures of different DNA fragments, the fragments were digested with *Eco*R I and *Bam*H I, and then ligated in the *Bam*H I-*Eco*R I site of the vector M13mp18 and sequenced using the dideoxy chain-termination method[17] with an automatic DNA sequencer (model 373A, Applied Biosystems).[18] The PCR products of *p53* exon 5 were ligated in the vector M13mp18 and the nucleotide sequences were also determined.

RESULTS

Kinetics of early development of preneoplastic (prelymphoma) cells Although we found that preneoplastic (prelymphoma) cells developed from immature TL-2$^+$ thymocytes in the thymus of irradiated mice, it was not clear whether all TL-2$^+$ cells undergo neoplastic initiation or whether preneoplastic cells only develop infrequently from TL-2$^+$ cells.

To answer this question and to evaluate the clonality of prelymphoma cells, we stained thymocytes from individual B10.Thy 1.1 mice with anti-TL-2 mAb on day 28 after irradiation and determined the TL-2$^+$ cell contents. Graded amounts of TL-2$^+$ cells from thymocytes of individual mice were injected into the thymus of 3.78 Gy-irradiated or non-irradiated B10.Thy 1.2 mice, and 4 to 6 months thereafter the recipient mice were killed when moribund. The incidence of donor-derived T cell lymphomas is shown in Table I. Although various numbers of TL-2$^+$ cells appeared in the thymus of individual mice on day 28 after irradiation, the donor-type T cell lymphomas developed when 10^2-10^5 TL-2$^+$ cells from 8 individuals (A–H, Table I) out of 15 mice were injected into the recipient mice. On the other hand, injection of TL-2$^+$ cells from the other mice (No. 9–15, Table I) did not develop donor-type T cell lymphomas even though TL-2$^+$ cells appeared in the thymus. These results indicate that TL-2$^+$ cells did not always undergo neoplastic initiation, and that prelymphoma cells in fact develop from TL-2$^+$ cells at very low frequency.

Clonality of prelymphoma cells developed during lymphomagenesis The next question is whether single neoplasms arise from single or multiple ancestral progenitor cells. To resolve this, high-molecular-weight DNAs were isolated from the above donor-derived T cell lymphomas and the rearrangement of the T cell receptors was examined by Southern blot analysis.

In developing fetal thymocytes, the expression of the γ TCR gene is observed earliest, followed by β, δ and α TCR genes.[19-21] Fig. 1 shows the genomic organization of γ T cell receptor and the representative Southern blot analysis of γ TCR gene rearrangements of donor-derived

Table I. Incidence of Donor-type T Cell Lymphomas Developed in Recipient Thymuses

Irradiated individual B10.Thy 1.1 mice	No. of TL-2$^+$ cells inoculated[a]				Frequency of prelymphoma cells in TL-2$^+$ cells
	10^2	10^3	10^4	10^5	
A	ND[b]	ND	3/4[c] (A5–A7)	4/4 (A1–A4)	1.4×10^{-4}
B	ND	ND	0/4	3/4 (B8–B10)	1.4×10^{-5}
C	0/4	0/4	2/4 (C11–C12)	ND	6.8×10^{-5}
D	0/4	2/4 (D5–D6)	4/4 (D1–D4)	ND	6.8×10^{-4}
E	ND	ND	3/4 (E10–E11)	4/4 (E7–E9)	1.4×10^{-4}
F	ND	ND	4/4 (F3–F4)	4/4 (F1–F2)	$>6.8\times10^{-4}$
G	ND	ND	2/4 (G9–G10)	4/4 (G5–G8)	6.8×10^{-5}
H	ND	ND	0/4	2/4 (H11–H12)	6.8×10^{-6}
No. 9–No. 15	ND	0/4	0/4	0/4	$<6.8\times10^{-7}$

a) Thymocytes were taken from individual B10.Thy 1.1 mice 28 days after irradiation and stained with anti-TL-2 mAb. The TL-2$^+$ cells (10^2–10^5) from individual mice were injected intrathymically into 3.78 Gy-irradiated or non-irradiated B10.Thy 1.2 mice as described in "Materials and Methods."
b) ND, not determined.
c) Incidence of donor-type T cell lymphomas was examined in groups of 4 recipient mice each.

lymphomas from individual F, G and H irradiated mice, revealing Vγ4–Jγ1 (1.4 kb, F, G and H group) and Vγ2–Jγ2 (3.6 kb, G and H group) rearrangements[13] (Fig. 1B). All other donor-derived T cell lymphomas (A1–A7, B8–B10, C11–C12, F1–F4, G5–G10, H11–H12) also showed the Vγ4–Jγ1 rearrangement (R1.4 in Table II), which is preferentially utilized by adult murine thymocytes[13, 22] (Table II).

To determine whether these lymphomas each originated from individual prelymphoma cells, the nucleotide sequences of the V–J junctions of Vγ4–Jγ1 in these lymphomas were examined. The results indicated that the nucleotide sequences of Vγ4–Jγ1 junctions of the donor-type lymphomas derived from irradiated A, F and E individual mice were the same in each group, but that three kinds of nucleotide sequences were observed in the D group (Table II). Different nucleotide sequences of Vγ4–Jγ1 junctions were also found in each donor-type lymphoma derived from G, B, C and H individual irradiated mice.

Thus, when we used the nucleotide sequences of Vγ4–Jγ1 junctions as a marker of clonality, we were able to conclude that the lymphomas derived from A, F and E individual irradiated mice arose from single ancestral progenitor cells, because the DNA sequences of the V–J junctions were the same. In the G group lymphomas, there were two kinds of nucleotide sequences of Vγ4–Jγ1 junctions. Since the germ line band was not shown in these lymphomas (Fig. 1, Table II), this suggested that γ TCR gene rearrangements had occurred in both alleles of each cell and the lymphomas which developed from G individual irradiated mouse were derived from single progenitor cells. However, in the lymphomas derived from D, B, C and H individual irradiated mice, because some different DNA sequences of Vγ4–Jγ1 junctions in the donor-type lymphomas of each group were found, we could not finally determine whether these lymphomas arose from multiple ancestral progenitor cells, or whether a single pre-neoplastic cell first developed and then the γ TCR gene became rearranged during the process of proliferation.

Experimental system for analysis of early initiating event(s) Since the TCR rearrangement is a sequential process and unique to the progeny of each clone, one of the strategies for investigating the oncogenic process is to compare TCR rearrangement patterns with the onco-

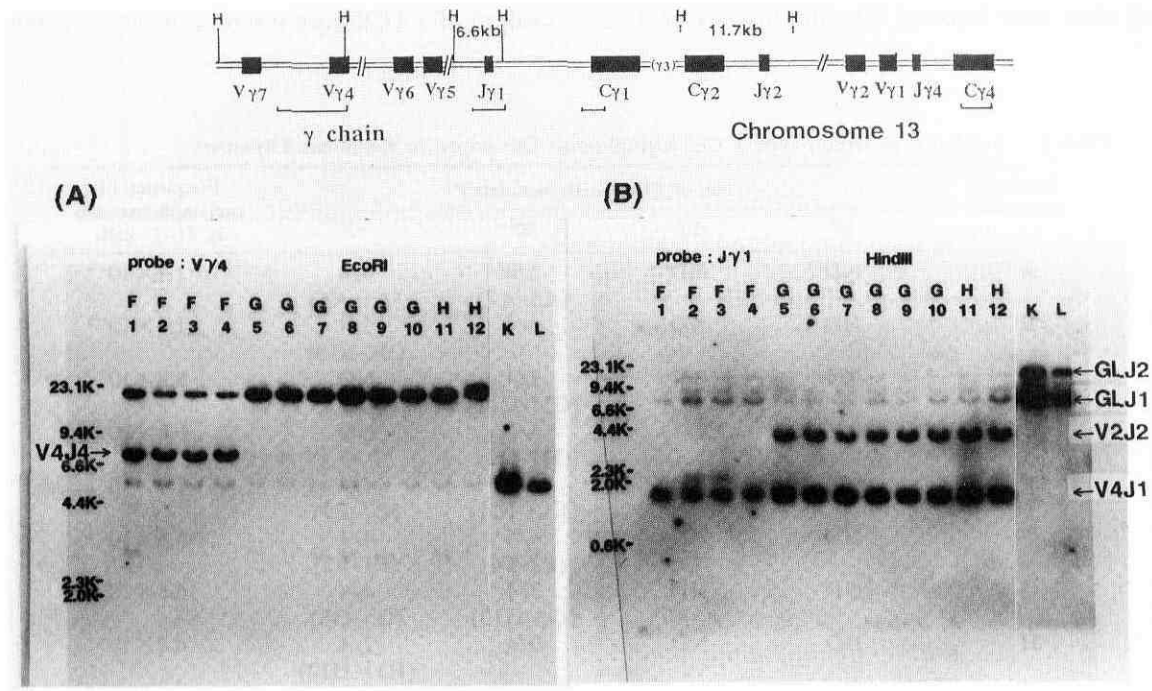

Fig. 1. The γ TCR genomic organization of B10 mice and Southern blot analysis of γ TCR gene rearrangements. Genomic DNAs from the liver (L), kidney (K), or from F1–F4, G5–G10, H11–H12 lymphomas were digested with *Eco*R I (A) or *Hin*d III (B), subjected to electrophoresis, and hybridized with (A) probe Vγ4 (pAA21) or (B) Jγ1 (pTG2.5). Because the Jγ1 and Jγ2 segments cross-hybridize, two Jγ1-hybridizing fragments of 6.6 kb (GLJ1) and 11.7 kb (GLJ2), deduced from the *Hin*d III sites, are expected from the Southern blots of *Hin*d III-digested germline DNA samples.[12] G5–G10, H11–H12 lymphomas shared the same pattern of rearrangement bands of TCR γ Vγ4Jγ1 and Vγ2Jγ2.[13]

Table II. Summary of the Southern Blot Analysis and Nucleotide Sequences of Vγ4–Jγ1 Coding Junctions of DNA from Donor-type T Cell Lymphomas Derived from Individual Irradiated Mice

Donor-type lymphomas	*Hind* III[d] Probe: Jγ1(2)	*Eco*R I[d] Probe: Vγ4	(Vγ4) TTCCTACGGCTAAGCACAGCA / CACTGTGATAGCTCAGGTTTT (Jγ1)	No. of clones
A1[a]	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1), R7.2(V4J4)	---------- GGAA ----------	
A2	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1), R7.2(V4J4)	---------- GGAA ----------	
A3	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1), R7.2(V4J4)	---------- GGAA ----------	
A4	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1), R7.2(V4J4)	---------- GGAA ----------	
A5	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1), R7.2(V4J4)	---------- GGAA ----------	
A6	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1), R7.2(V4J4)	---------- GGAA ----------	
A7	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1), R7.2(V4J4)	---------- GGAA ----------	
F1[a]	R1.4(V4J1)	R16.7(V4J1), R7.2(V4J4)	---------- AGGG ----------	
F2	R1.4(V4J1)	R16.7(V4J1), R7.2(V4J4)	---------- AGGG ----------	
F3	R1.4(V4J1)	R16.7(V4J1), R7.2(V4J4)	---------- AGGG ----------	
F4	R1.4(V4J1)	R16.7(V4J1), R7.2(V4J4)	---------- AGGG ----------	
E7[a]	R1.4(V4J1), G6.8	R16.7(V4J1), G5.2	---------- GAT ----------	
E8	R1.4(V4J1), G6.8	R16.7(V4J1), G5.2	---------- GAT ----------	
E9	R1.4(V4J1), G6.8	R16.7(V4J1)	---------- GAT ----------	
E10	R1.4(V4J1)	R16.7(V4J1)	---------- GAT ----------	
E11	R1.4(V4J1), G6.8	R16.7(V4J1), G5.2	---------- GAT ----------	
D1[b]	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1)	--- ------- TT ----------	3
D2	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1)	--- CCACTA ----------	3
D3	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1)	--- ------- GGAT ----------	3
D4	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1)	--- ------- TT ----------	5
D5	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1)	--- ------- TT ----------	3
D6	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1)	--- ------- TT ----------	3
G5[c]	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1)	---------- CAT ----------	3
G6	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1)	---------- CAT ----------	2
G6			---------- ATATGGT ----------	1
G7	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1)	---------- CAT ----------	2
G8	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1)	---------- CAT ----------	3
G9	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1)	---------- CAT ----------	1
G9			---------- ATATGGT ----------	2
G10	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1)	---------- ATATGGT ----------	1
G10			---------- CAT ----------	2
B8[c]	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1)	---------- TAG ----------	2
B8			---------- TAACC ----------	1
B9	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1)	---------- TAAAAG -C----------	3
B9			------ CGAA ----------	3
B10	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1)	---------- ACCAT ----------	4
B10			---------- AAGGGAG ----------	3
C11[c]	R1.4(V4J1)	R16.7(V4J1)	---------- AAAAAGTAA ----------	4
C11			---- CCAA ----------	2
C12	R1.4(V4J1)	R16.7(V4J1)	---------- CCAT ----------	2
C12			---------- TCGAT ----------	2
H11[c]	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1)	---------- TATTT ----------	1
H11			---------- CC ----------	1
H12	R1.4(V4J1), R3.6(V2J2)	R16.7(V4J1)	---------- TATGT ----------	1

a) The DNA sequences from A1–E11 were determined by PCR direct sequencing.
b) Data from Ref. 13).
c) DNA fragments of PCR products were ligated in the M13mp18 vector as described in "Materials and Methods."
d) DNAs were digested with *Hind* III or *Eco*R I and analyzed by Southern blot analysis. G, germ line band; R, rearranged band.

Table III. *p53* Gene Point Mutations Detected in PCR-amplified Fragments

Lymphoma	Mutation[a)]	Exon	Codon	Amino acid change	No of clones /total
B10	TAC→TGC	5	160	Tyr→Cys	9/11
E7	TTC→TTA	5	131	Phe→Leu	2/10
	TAC→CAC	5	160	Tyr→His	2/10
	ATC→GTC	5	159	Ile→Val	2/10

a) DNA sequence analysis confirmed the presence of wild-type and mutated *p53* alleles in B10 and E7 lymphomas.

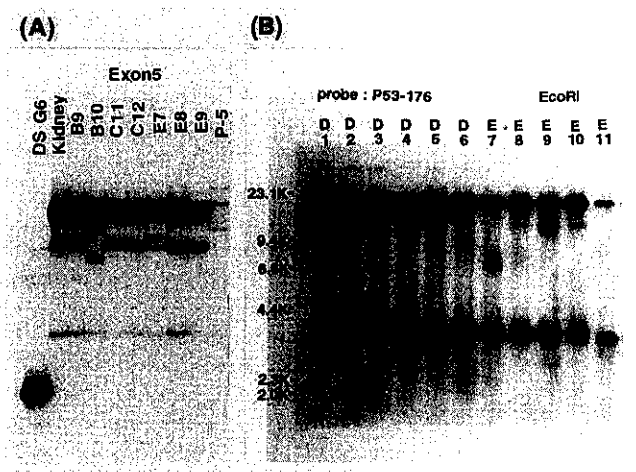

Fig. 2. (A) PCR-SSCP analysis of *p53* mutations in the T cell lymphomas. Genomic DNA was amplified by PCR in the presence of [α-^{32}P]dCTP for exons 4–9 of the *p53* gene. Representative samples are shown for exon 5. (B) Southern blot analysis of *p53* gene alterations. DNAs from D1–D6 and E7–E11 T cell lymphomas were digested with *Eco*R I and analyzed by hybridization with a ^{32}P-labelled probe, p53–176, specific for *p53*. DS G6: Double strand DNA of G6.

genic alterations. Thymocytes were taken from individual B10.Thy 1.1 mice 28 days after fractionated irradiation and were stained with anti TL-2 mAb. Graded amounts of TL-2$^+$ cells from thymocytes of individual B10.Thy 1.1 mice were injected into the thymus of 3.78 Gy-irradiated or non-irradiated B10.Thy 1.2 mice (Table I). The preneoplastic (prelymphoma) cells developing in the thymus of individual irradiated B10.Thy 1.1 mice within 28 days after irradiation can proliferate and may convert independently to malignant lymphomas in the thymic microenvironment of B10.Thy 1.2 mice, namely, through "*in vivo* culture."

If the same oncogenic alteration occurred in these lymphomas, this genetic change might be an early initiating event in oncogenesis. On the other hand, if different genetic changes were observed in each of the cells, they may represent a later event in the process of oncogenesis.

PCR-SSCP analysis of *p53* mutations in the donor-derived T cell lymphomas PCR-amplified genomic DNA fragments generated from exons 4–9 of the mouse *p53* gene were analyzed for possible sequence changes by the SSCP (single-strand conformation polymorphisms) method. Fig. 2A shows representative results for genomic DNA fragments of exon 5 of the *p53* gene, indicating that the separated single-strand DNA of B10 and E7 lymphoma cells shows different mobilities from those of normal kidney cell DNA. p-5 DNA, which is known to have a mutation at exon 5, was used as a positive control.

The mutations detected by PCR/SSCP were confirmed by DNA sequencing of the PCR products of *p53* exon 5, which were ligated into the vector M13mp18. Each of four mutations corresponded to a missense mutation which resulted in an amino acid substitution in the p53 protein (Table III). The mutations detected in codon 160 in B10 lymphoma or codons 159 and 160 in E7 lymphoma involve G-to-A or T-to-C transitions (Table III). E7 lymphoma also had C-to-A transversion at the third position of codon 131. The presence of both the wild-type and the mutated *p53* alleles was shown in both lymphomas.

DNAs from donor-type lymphomas were digested with *Eco*R I and hybridized with a *p53*-specific probe derived from cDNA clone pp53-176.[14)] The normal *p53* gene was detected as a 16-kb fragment, and the pseudogene as a 3.3-kb fragment[23)] (Fig. 2B). A summary of the PCR-SSCP analysis of the *p53* gene from various T cell lymphomas is shown in Table IV. As shown in this table, some mutations are observed in these lymphomas, but there is no common mutation of the *p53* gene in the respective groups. Therefore, the results suggest that these mutations are late-occurring events in the process of oncogenesis.

Chromosome aberrations/translocations in the donor-derived T cell lymphomas Many numerical as well as structural aberrations were observed in the donor-derived T cell lymphomas. Moreover, there were common aberrations/translocations in some donor-derived T cell lymphomas.

Table IV. Summary of PCR-SSCP and Southern Blot Analysis of *p53* Gene Alterations in the Donor-type Lymphomas

Irradiated B10.Thy 1.1 mice	Donor-type lymphomas (Thy 1.1)	Gene mutation *p53*[a]	*Eco*R I restriction pattern[b]
A	A1	—	normal
	A2	—	normal
	A3	—	normal
	A4	—	normal
	A5	—	normal
	A6	—	normal
	A7	—	normal
B	B8	—	normal
	B9	—	normal
	B10	+ (Exon 5)	6.6
C	C11	—	normal
	C12	—	normal
D	D1	—	normal
	D2	—	11.8, 10
	D3	—	normal
	D4	—	11.8, 10
	D5	—	11.8
	D6	+ (Exon 7)	normal
E	E7	+ (Exon 5)	6.6
	E8	—	11.8
	E9	—	11.8, 10
	E10	—	11.8
	E11	—	normal
F	F1	—	11.8
	F2	—	normal
	F3	—	normal
	F4	—	normal
G	G5	+ (Exon 8)	normal
	G6	—	normal
	G7	—	11.8
	G8	—	11.8
	G9	—	11.8
	G10	—	normal
H	H11	—	11.8
	H12	—	normal

a) The presence of mutated *p53* (exons 4–9) was determined by SSCP analysis. + indicates *p53* mutation.
b) Abnormal sizes (kb) of *Eco*R I restriction fragments are indicated; normal: only restriction fragments of normal sizes (16 kb, 3.3 kb) were detected.

(a) A1–A7 lymphomas: A common aberration of trisomy of chromosome 15 was found in A1, A2, A3, A5, A6 and A7 lymphomas (A4 cell line was lost), and 41% of the cells analyzed had 41 chromosomes, except for A3, which was mostly multiploid (86%) (Fig. 3, Table V).

(b) B8–B10 lymphomas: 15 and 18 trisomies were observed in B9 lymphoma (68%), but B8 and B10 lymphomas showed normal karyotype (Table V).

(c) C11–C12 lymphomas: There was a translocation between 12E and 15A in approximately 36% of C11 lymphoma cells, but a different translocation between 13D and 15B, and trisomy 15 were found in C12 lymphoma cells (80%) (Table V).

(d) D1–D6 lymphomas: D1, D3, D4, D5, D6, but not D2, had a common translocation between chromosomes 11A and 12E with a frequency of at least 68%. The basic number of chromosomes was 39, and D5 lymphoma had trisomy 12, one of which had the same translocation. Fifty-two percent of D6 lymphoma was tetraploid. D2 had a unique aberration in chromosome 12E (Fig. 3, Table V).

(e) E7–E11 lymphomas: E7–E10 lymphomas had a frequent translocation between chromosomes 12E and 15A. This aberration was observed in about 88% of cell idiograms. There were no uniform numbers of chromosomes in this group. Most of the E7 cells were tetraploid (64%). E8 and E10 cells had many aneuploids between diploid and triploid (47–53 chromosomes). The same translocation was not observed in E11 lymphoma (Fig. 3, Table V).

(f) F1–F4 lymphomas: There was a common translocation between chromosomes 7F and 10C, as well as trisomy 15 and monosomy X (82% of analyzed cells) in this group (Fig. 3, Table V).

(g) G5–G10 lymphomas: The same apparent karyotype of translocation between chromosomes 1A and 13D was found in G5–G10 lymphomas with a frequency of 79% (Fig. 3, Table V).

(h) H11–H12 lymphomas: There were about 87% cells in this group with a common translocation between chromosomes 6B and XB (Table V).

A summary of the chromosome analysis is shown in Table V.

DISCUSSION

In radiation-induced carcinogenesis, radiation is assumed to act as an initiator by damaging a specific cellular target directly or indirectly in a stable and irreversible fashion. Specific genetic changes in target genes may be initiating events for neoplasia and it may be expected that many neoplasia-initiating events will, in appropriate *in vivo* circumstances, provide target cells with some degree of proliferative or selective advantage.

It can be argued that in combination-oncogene activation events, the loss of regulatory gene functions, and epigenetic changes affecting gene activity may produce a cascade of inappropriate gene expression, thus generating the initiation of oncogenesis and subsequent neoplastic conversion. These changes may be brought about by a variety of different changes to DNA, point mutation, chromosome translocation/insertion, intragenic deletion, chromosomal deletion and non-mutational but stable changes to genes, such as DNA methylation.

To analyze the initial events in radiation-induced lymphomagenesis, we constructed an experimental system

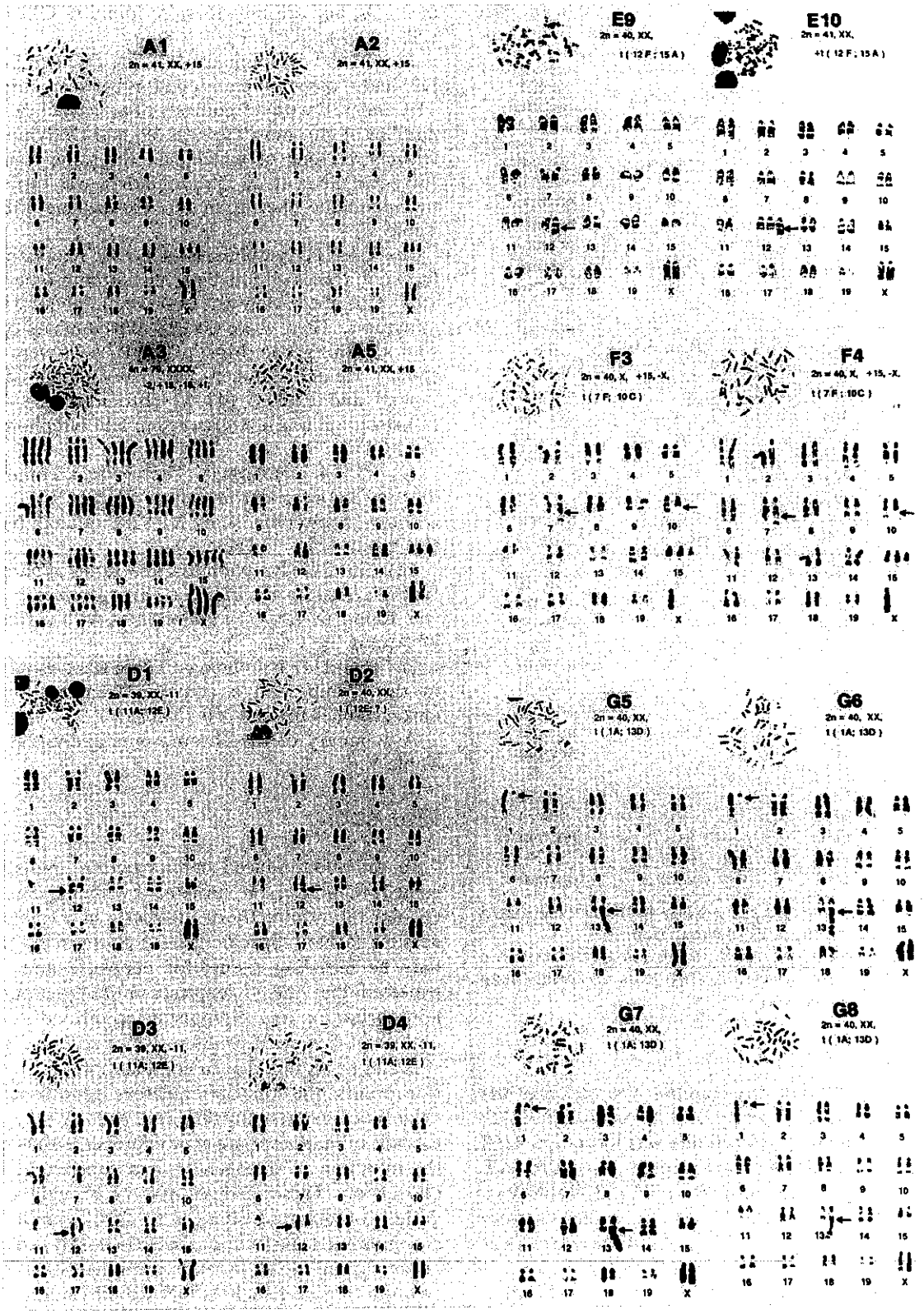

Fig. 3. Representative G-banded karyotype of chromosomes prepared from A1–A5, D1–D4, E9–E10, F3–F4 and G5–G8 T cell lymphomas.

Table V. Common Chromosome Aberrations and Frequency of Lymphoma Cells in Each Group

Group	Lymphoma cells	Basic karyotype	Common aberration	Frequency[b] (%)
A	A1, A2, A3[a], A5, A6, A7	2n=41, XX, +15	Trisomy 15	41 (245/600)
B	B9,	2n=42, XX, +15, +18	Trisomies 15,18	68 (34/50)
	B8, B10	2n=40, XX	—	
C	C11,	2n=40, XX, t	t(12F;15A)	36 (18/50)
	C12	2n=41, XX, +15, t	Trisomy 15 and t(13D;15B)	80 (40/50)
D	D1, D3, D4, D5, D6[a],	2n=39, XX, −11, t	t(11A;12E)	68 (15/22)
	D2	2n=40, XX, t	t(12E; ?)	
E	E7[a], E8, E9, E10,	2n=40, XX, t	t(12F;15A)	88 (14/16)
	E11	2n=40, XX	—	
F	F1, F2, F3, F4	2n=40, X, +15, −X, t	Trisomy15, Monosomy X, t(7F;10C)	82 (14/17)
G	G5, G6, G7, G8, G9, G10	2n=40, XX, t	t(1A;13D)	79 (237/300)
H	H11, H12	2n=40, XX, t	t(6A;XB)	87 (7/8)

a) Most of the cells are tetraploid.
b) Aberrated cells/total cells.

for comparing TCR rearrangement patterns with oncogenic alterations. Using this system, we examined the donor-type T cell lymphomas derived from individual irradiated mice (A–H) for the presence of mutations in the *p53* tumor suppressor gene by screening PCR products from *p53* exons 4–9 by SSCP analysis or Southern blot analysis. Although some mutations were observed in these lymphomas, there was no common mutation of the *p53* gene. Therefore, these results suggest that these mutations take place relatively late in the process of oncogenesis, perhaps during 1–2 months after the end of the radiation. We also looked for mutations in the 12th, 13th, or 61st codons of the N-*ras*, H-*ras* and K-*ras* protooncogenes by PCR-SSCP analysis. However, no point mutations in these codons of the *ras* protooncogenes were detected in these lymphomas (data not shown).

On the other hand, we found that there were common chromosome aberrations/translocations in some donor-type T cell lymphomas from individual irradiated mice. Therefore, we estimated the time of occurrence of these common chromosome aberrations/translocations based on a comparison with the DNA sequences of the V–J junctional sites of TCR γ gene rearrangements and classified into three types (Fig. 4).

The first type (type (I)) is that of chromosome aberration developing soon after the γ TCR rearrangement occurred. Since the donor-type lymphomas derived from A, F or G irradiated mice show the same DNA sequences of the Vγ4–Jγ1 junction and the same chromosome aberration, these lymphomas are belong to type (I) (Fig. 4).

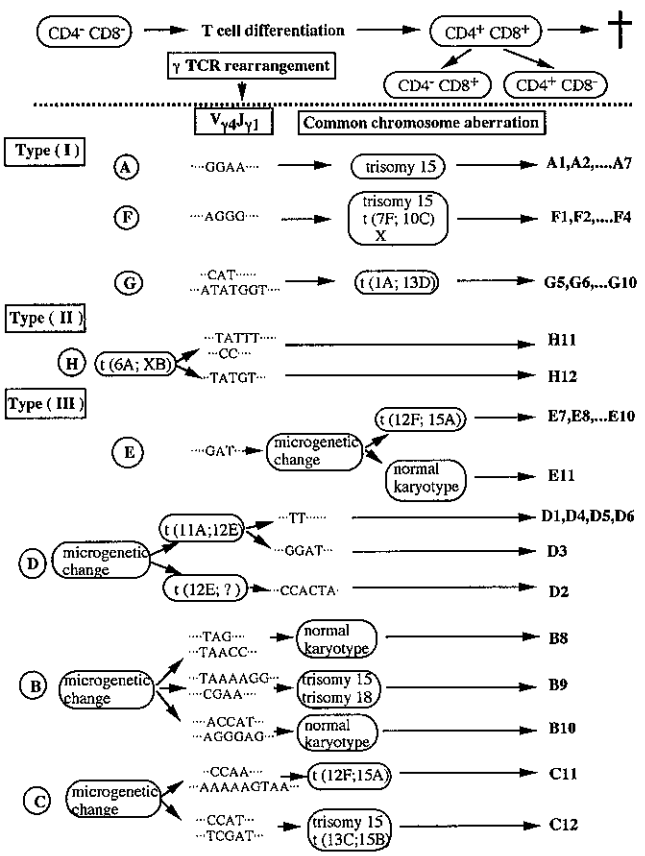

Fig. 4. Possible steps of change of the characteristic cytogenetic features and TCR γ gene rearrangements in the donor-type T cell lymphomas derived from irradiated mice.

The second type is that of chromosome aberration taking place just before TCR γ genes are rearranged. Since H11 and H12 lymphomas derived from the irradiated mouse H were shown to have a common chromosome translocation between 6A and XB, but the DNA sequences of the Vγ4–Jγ1 junction were different from each other, this may be explained if translocation between 6A and XB first took place, and after i.t. injection, the TCR γ genes became rearranged in these lymphomas (Fig. 4).

The third type is that of some mutations, small deletions, or non-mutational but stable changes to genes such as DNA methylation, namely "microgenetic changes," occuring in specific target genes just before or immediately after the γ TCR rearrangement take place. The lymphomas derived from the irradiated mouse E showed the same DNA sequences of the Vγ4–Jγ1 junction and E7–E10 lymphomas were observed to have a common chromosome translocation between 12F and 15A, but E11 lymphoma indicated an apparently normal karyotype. This can be understood if γ TCR genes were first rearranged and then some microgenetic changes occurred, although an apparently normal karyotype was shown, and in the next phase, translocation between 12F and 15A took place in E7–E10 lymphomas (Fig. 4). In the case of D group of lymphomas, if the microgenetic change first developed and then chromosome translocation t(11A; 12E) in D1, D3–D6 or t(12E; ?) in D2 occurred before the TCR γ gene was rearranged, the results obtained could be reasonably interpreted, as shown in Fig. 4. Since B8–B10 and C11–C12 lymphomas derived from the irradiated mice B and C were not shown to have any common chromosome aberration and the DNA sequences of the Vγ4–Jγ1 junction were also different from each other, if the microgenetic changes first occurred in specific target genes, and then γ TCR rearrangement and chromosome aberrations/translocations took place in these recipients independently, these changes would seem to be reasonable (Fig. 4). However, in these cases it can not be ruled out that multiple preneoplastic cells might have developed in the thymus of the irradiated mice B and C.

As shown in type (I) and (II) (Fig. 4), the chromosome aberrations/translocations such as trisomy 15, t(7F, 10C), t(1A, 13D) or t(6A, XB) among the donor-type lymphomas derived from half of the individual irradiated mice might be considered as potential initiating events for radiation-induced thymic lymphomagenesis. Trisomy 15 is known to be a frequent finding in mouse T cell lymphomas arising spontaneously or following induction by irradiation or chemical carcinogens.[24–28] With regard to t(6A, XB), for example, the β T cell receptor is known to lie in the region of chromosome 6A, and the IL-2 receptor gene is also located in chromosome XB. Chromosome translocations may thus identify possible sites of oncogene involvement, but detailed molecular characterization will be required for confirmation of such events. In addition, in other lymphomas from the other half of the individual irradiated mice, although an apparently normal karyotype was seen, it was suggested that some microgenetic changes such as mutations, small deletions, or non-mutational DNA methylation first occurred in specific target genes and then chromosome aberrations/translocations took place in a later promotion or progression phase.

If the microgenetic changes first occurred at the G1 and S phases of cell cycle in the target cells during the development of type (I) and (II) lymphomas (Fig. 4), and if these genetic changes induced the chromosome aberrations/translocations during the later process of G2 and M phases, the genetic changes as shown in type (I) and (II) can also be explained. In this case, the microgenetic change(s) might be the most important initiating event(s) for radiation-induced thymic lymphomagenesis.

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