Involvement of Illegitimate V(D)J Recombination or Microhomology-Mediated Nonhomologous End-Joining in the Formation of Intragenic Deletions of the Notch1 Gene in Mouse Thymic Lymphomas

Hideo Tsuji, Hiroko Ishii-Ohba, Takanori Katsube, Hideki Ukai, Shiro Aizawa, Masahiro Doi, Kyoji Hioki, and Toshiaki Ogiu

Abstract

Deregulated V(D)J recombination-mediated chromosomal rearrangements are implicated in the etiology of B- and T-cell lymphomagenesis. We describe three pathways for the formation of 5'-deletions of the Notch1 gene in thymic lymphomas of wild-type or V(D)J recombination-defective severe combined immune deficiency (scid) mice. A pair of recombination signal sequence-like sequences composed of heptamer- and nonamer-like motifs separated by 12- or 23-bp spacers (12- and 23-recombination signal sequence) were present in the vicinity of the deletion breakpoints in wild-type thymic lymphomas, accompanied by palindromic or nontemplated nucleotides at the junctions. In scid thymic lymphomas, the deletions at the recombination signal sequence-like sequences occurred at a significantly lower frequency than in wild-type mice, whereas the deletions did not occur in Rag2-/- thymocytes. These results show that the 5'-deletions are formed by Rag-mediated V(D)J recombination machinery at cryptic recombination signal sequences in the Notch1 locus. In contrast, one third of the deletions in radiation-induced scid thymic lymphomas had microhomology at both ends, indicating that in the absence of DNA-dependent protein kinase-dependent nonhomologous end-joining, the microhomology-mediated nonhomologous end-joining pathway functions as the main mechanism to produce deletions. Furthermore, the deletions were induced via a coupled pathway between Rag-mediated cleavage at a cryptic recombination signal sequence and microhomology-mediated end-joining in radiation-induced scid thymic lymphomas. As the deletions at cryptic recombination signal sequences occur spontaneously, microhomology-mediated pathways might participate mainly in radiation-induced lymphomagenesis. Recombination signal sequence-mediated deletions were present clonally in the thymocyte population, suggesting that thymocytes with a 5'-deletion of the Notch1 gene have a growth advantage and are involved in lymphomagenesis.

Introduction

Chromosomal rearrangements such as translocation or deletion are frequently observed in T-cell and B-cell neoplasms and are implicated in the pathogenesis of these tumors through the activation of proto-oncogenes (1-4). Chromosomal translocations are often produced between immunoglobulin/T-cell receptor genes and proto-oncogene loci (1-6). On the basis of the presence of a cryptic recombination signal sequence near the translocation breakpoints of the partner proto-oncogene, illegitimate V(D)J recombination is proposed to be involved in the process. Recent experiments to directly assess the formation of rearrangements with the proto-oncogene substrates bearing cryptic recombination signal sequences confirmed the presence of illegitimate V(D)J recombination and its role in lymphomagenesis (7-9). Illegitimate V(D)J recombination-mediated deletions also occur in T-cell lymphomas (9-11). These findings indicate an extensive involvement of illegitimate V(D)J recombination in lymphomagenesis.

Received 4/28/03; revised 8/18/04; accepted 10/20/04.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Hideo Tsuji, Low Dose Radiation Effects Research Project Group, National Institute of Radiological Sciences, 4-9-1, Anagawa, Inage-ku, Chiba 263-8555, Japan. Phone: 81-43-206-3165; Fax: 81-43-251-4268; E-mail: tsujihid@nirs.go.jp.
©2004 American Association for Cancer Research.

8882

Downloaded from cancerres.aacrjournals.org on April 20, 2017. © 2004 American Association for Cancer Research.
rearrangements. There were three rearrangement regions: the 5'-end, the juxtamembrane extracellular, and the 3'-end regions. The rearrangements in the 5'-region were the most abundant and were all deletions. Identification of the pathways involved in the 5'-deletion of the Notch1 gene might help to clarify the mechanism(s) underlying radiation-induced lymphomagenesis. We analyzed the sequence specificity of 5'-deletion junctions in the Notch1 gene in wild-type or scid thymic lymphomas and discovered recombination signal sequence-like sequences in the vicinity of the deletion junctions in wild-type mice and microhomology at both ends in scid mice. These results suggest that in the presence of DNA-PKcs, deletions are formed by illegitimate V(D)J recombination, whereas in the absence of DNA-PKcs, deletions are produced by microhomology-mediated nonhomologous end-joining. The 5'-deletions formed via illegitimate V(D)J recombination occurred spontaneously in wild-type thymocytes and were found clonally, suggesting that thymocytes with a 5'-deletion have a growth advantage and spontaneous 5'-deletions might be involved in lymphomagenesis.

MATERIALS AND METHODS

Mice, Irradiation, and Tissue Collection. The STS, C.B-17/scid (C.B-17), C.B-17/scid (scid), B10.Sn (B10; Thy1.2), B10.NRHT- Thy1.1 (B10. Thy1.1), and BALB/c-Rag2−/− mice were bred in the animal facility of our institute. B10. B10.Thy1.1, scid, and Rag2−/− mice were fed under specific pathogen-free conditions. Other mice were maintained in a microbiologically clean conventional animal facility. Mice were handled according to the Guide lines for Animal Experiments compiled by the Committee on the Safety and Handling Regulations for Laboratory Animal Experiments at our institute. STS mice (5-week-old) were exposed to four consecutive whole-body X-irradiation sessions (200 keV, 20 mA, with 0.5 mm of Cu and 0.5 mm of Al filters at a dose rate of 0.5 Gy/min) at a dose of 2.4 Gy at 1-week intervals. C.B-17 mice (5-week-old) were irradiated with 137Cs γ-rays (0.662 MeV at 0.5 Gy/min) at a dose of 1.6 Gy four times at 1-week intervals. B10 mice (8 to 10-week-old) were exposed to a lethal dose (10 Gy) of 137Cs γ-rays and reconstituted 24 hours later with bone marrow cells of B10 Thy1.1. Four weeks later, the transplanted B10 mice were additionally irradiated with 137Cs γ-rays at a dose of 4 Gy. Scid mice were treated with a single irradiation with 137Cs γ-rays at doses of 0.1 to 2 Gy or X-rays at a dose of 2 Gy. Mice exhibiting signs of distress and becoming moribund were killed for autopsy. Thymic lymphomas were confirmed by histologic examination and cell surface marker expression by staining with phycoerythrin-labeled anti-CD4 and fluorescein-labeled anti-CD8 (BD Biosciences, San Jose, CA). The stained cells were analyzed on a FACScan with CELLQuest software (BD Biosciences). Most of the thymic lymphomas were CD4 and CD8 positive.

To examine deletion formation in the thymocytes, C.B-17, scid, and Rag2−/− mice were irradiated with γ-rays one or four times at 1-week intervals. Thymus genomic DNA was isolated from unirradiated or irradiated 8-week-old mice 7 or 14 days after the final irradiation with the standard phenol-chloroform extraction method.

Cell Culture. Thymic lymphoma cell lines were established as described previously (38). The cell lines were cultured in ES medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% inactivated fetal bovine serum at 37°C in a 5% CO2 incubator at an atmosphere of 95% humidity in air.

Southern Blot Hybridization. Southern blot hybridization was conducted as described previously (38). Three probes used for hybridization, which contained exon 1a, exon 1b + 1b', and exons 1 and 2 of the Notch1 gene (38), respectively, were obtained by PCR with C.B-17 genomic DNA.

PCR. The rearranged DNA fragments in thymic lymphomas were obtained by PCR with the Long Template PCR System (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Genomic DNA was amplified in 30 cycles of 94°C for 10 seconds, 61°C for 30 seconds, and 72°C for 1 minute. Second-step PCR was done with primer pairs NF3 (5'-CTCTCGCTGTCCTGAGTCCACCTTCC-CAA-3') and NR3 (5'-TTCCCTCATGAGTAGTGGTAGTCCTCTGTG-3') and 1 μL of first-step PCR product in a 30-μL reaction. PCR was done in 30 cycles of 94°C for 10 seconds, 66°C for 30 seconds, and 72°C for 1 minute. For a positive control, genomic DNA of thymic lymphoma C69 possessing a heterozygous deletion between the hot spots was used. First-step PCR was done with 0.5 μg of kidney DNA of C.B-17 mouse plus 2.5, 5, or 10 pg of C69 genomic DNA. Some cases of nested PCR were conducted with different primer pairs. First-step PCR was done with the primer pair of NF21 (5'-CAGAGGGCCCTGACAGACACCCCTTCTCTA-3') and NR1 (5'-TAGGGTGTGACAGAGTGCTACAGGCTCAAAG-3') at 65°C for annealing. Second-step PCR was done with the NF22 and NR3 primer pair at 64°C for annealing.

DNA Cloning and Sequencing. After PCR and agarose gel electrophoresis, rearranged fragments were purified with a Gene Clean II kit (BIO 101, Carlsbad, CA). The DNA was sequenced with the dye-termination method using a Prism 7100 sequencer (Applied Biosystems, Foster, CA) directly or after cloning with the pIT blue vector (Invitrogen Corp., Carlsbad, CA).

Identification of Recombination Signal Sequence-like Sequences. A DNA element is composed of a heptamer-like motif in which at least the first three nucleotides CAC (39–41) were conserved, and a nonamer-like motif in which at least one of the fifth (40), sixth (39, 41), or seventh (42) residues was conserved and, with a 12- or 23-bp spacer, was regarded to be an
RESULTS

Incidence of Radiation-Induced Thymic Lymphomas and Occurrence of the Notch1 Deletion. Mice irradiated with X-rays or γ-rays with different protocols were examined for the induction of thymic lymphomas. STS mice resistant to the induction of thymic lymphomas by radiation exhibited a 23% incidence of thymic lymphoma after split-dose irradiation, whereas C.B-17 mice displayed a higher frequency (36%) of spontaneous thymic lymphomas by radiation exhibited a 23% incidence of thymic lymphoma after split-dose irradiation (Table 1). The spontaneous frequency of thymic lymphomas in C.B-17 mice was 1.6 Gy × 4, B10 4 Gy, STS 2.4 Gy × 4, m70

Breakpoints at hotspots, 8/46 (17.4%).

Sequence position (nt)

Fig. 2. Distribution of breakpoints in the 5'-deletions of the Notch1 gene. The first nucleotide of exon 1a is regarded as position 1. The breakpoints located 5' of exon 1 are the 5'-breakpoints and the breakpoints 3' of the exon are the 3'-breakpoints. The thick bars indicate breakpoint hot spots, and thin bars indicate random breakpoint sites. The number of breakpoints analyzed and percentage of breakpoints located at hot spots are shown on the top right of each figure. A, distribution of breakpoints in radiation-induced wild-type thymic lymphomas. Four deletions in STS mice, 7 in B10 mice, and 24 in C.B-17 mice were combined. B, distribution of breakpoints in spontaneous scid thymic lymphomas. C, distribution of breakpoints in scid thymic lymphomas induced by 2 Gy of γ-rays.

breakpoints, rearranged regions in wild-type thymic lymphomas were amplified by PCR, cloned, and sequenced (Fig. 1). Recombination signal sequence-like elements were present in the vicinity of the breakpoints clustered around positions 4926 and 8191 on the 5'-side of the deletion and position 16674 on the 3'-side. They were composed of canonical heptamer or heptamer- and nonamer-like elements separated by 12-bp (12-recombination signal sequence) or 23-bp (23-recombination signal sequence) spacers. At least the first three nucleotides of the heptamer-like elements were conserved. The sixth and following several nucleotides were conserved in the nonamer-like elements. Recombination signal sequence-like elements were also present at position 22145 in C53 thymic lymphomas. A pair of recombination signal sequence-like elements, 12-recombination signal sequence and 23-recombination signal sequence, was present in the deletion junctions with added nontemplated (N)-nucleotides and/or palindromic (P) nucleotides. On the other hand, recombination signal sequence-like elements were not present in a minor fraction of deletions in radiation-induced wild-type thymic lymphomas (see below).

Breakpoint Distribution. To characterize differences in the nature of deletions in thymic lymphomas of wild-type and scid mice, the breakpoint distribution was examined. Fig. 2A shows two major breakpoint hot spots and one minor hot spot in radiation-induced wild-type thymic lymphomas, as suggested from the results of Fig. 1. Most, 74.3% (52 of 70), of the breakpoints were located at the hot spots, and the remainder were randomly distributed. In contrast, in spontaneous scid thymic lymphomas (Fig. 2B), the frequency of the breakpoints at the hot spots (17.4%) was much lower than that of wild-type mice, and the breakpoints were more broadly distributed. In radiation-induced scid thymic lymphomas (Fig. 2C), there was a broad distribution of breakpoints, similar to that of spontaneous scid thymic lymphomas. Thus, most of the deletions in wild-type mice occurred at the breakpoint hot spots, whereas those in scid mice occurred randomly.

Deletion Characteristics in scid Thymic Lymphomas. To elucidate the role of DNA-PKcs in deletion formation, sequence specificities of deletions in radiation-induced scid thymic lymphomas were examined (Fig. 3). The rearranged regions ranged from exon 1b + 1b' to exon 2, with the minimum deletion regions including exon 1, similar to those in wild-type thymic lymphomas. Sequence specificities were different from those of wild-type mice: a considerable proportion of deletions occurred at sites with microhomology (1 to 6 bp; data not shown) at both ends. In the TL35 and TL43 thymic lymphomas, 14-bp and 1.9-kb templated nucleotides were inserted in

<table>
<thead>
<tr>
<th>Strain</th>
<th>Irradiation (Gy)</th>
<th>No. of mice examined (sex)</th>
<th>No. of thymic lymphomas (%)</th>
<th>No. of thymic lymphomas analyzed (sex)</th>
<th>Thymic lymphomas with 5'-deletion of Notch1 gene (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STS</td>
<td>2.4 × 4 †</td>
<td>125 (F)</td>
<td>29 (23)</td>
<td>5 (F)</td>
<td>4 (80)</td>
</tr>
<tr>
<td>B10</td>
<td>4 ‡</td>
<td>100 (F)</td>
<td>19 (M)</td>
<td>7 (37)</td>
<td></td>
</tr>
<tr>
<td>C.B-17</td>
<td>1.6 × 4 ¶</td>
<td>205 (F + M)</td>
<td>75 (F + M)</td>
<td>22 (29)</td>
<td></td>
</tr>
<tr>
<td>scid</td>
<td>0</td>
<td>211 (F + M)</td>
<td>75 (M + S)</td>
<td>23 (29)</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td></td>
<td>49 (F + M)</td>
<td>16 (33)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td></td>
<td>50 (F + M)</td>
<td>23 (46)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>60 (F + M)</td>
<td>18 (30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>63 (F + M)</td>
<td>18 (29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>115 (F + M)</td>
<td>20 (34)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* F, female; M, male.
† Mice were irradiated with 2.4 Gy X-rays four times at 1-week intervals.
‡ Mice were irradiated with 4 Gy γ-rays 4 weeks after bone marrow transplantation.
§ Not determined.
¶ Mice were irradiated with 1.6 Gy γ-rays four times at 1-week intervals.
‖ Mice were irradiated with γ-rays at the indicated doses.
sequences on one side were paired with several recombination signal sequence-like sequences on another side. The cases were most notable in radiation-induced scid thymic lymphomas. Most of the deletions possessed a pair of recombination signal sequence-like sequences, whereas a small number of deletions carried only one recombination signal sequence-like sequence at either side. The recombination signal sequence-like sequence composed of only a heptamer-like sequence did not contribute very much to breakpoint formation, even if the sequence was identical to the canonical sequence as shown in the 6028 sequence. This suggests that the nonamer is important for recombination signal sequence function. Among the 3′-recombination signal sequence-like sequences possessing a canonical heptamer, the 16673 sequence functioned efficiently as a recombination signal sequence, whereas the 20095 sequence did not. Because sequence conservation of the nonamer was similar between the two sequences, the difference might be attributable to the partner recombination signal sequence-like sequence: the efficient 5′-recombination signal sequence-like sequence at positions 4927 and 8192 both possessed a 12-bp spacer, so the 20095 sequence with a 12-bp spacer would not function as an recombination signal sequence to these sequences.

Deletion Formation via Recombination Signal Sequence-Mediated and Microhomology-Mediated Pathways. To better evaluate the involvement of the recombination signal sequence-mediated pathway and other pathways in deletion formation, we summarized the results by delineating the events with or without recombination signal sequence-like sequences at breakpoints (Table 3). Most of the deletions in wild-type thymic lymphomas were accompanied by recombination signal sequence-like sequences at both ends with nucleotide additions in deletion junctions, whereas a minor fraction of deletions was accompanied by microhomology sequences at both ends. Deletions associated with an recombination signal sequence-like sequence at only one end were rarely observed. In contrast, in scid thymic lymphomas, deletions with an recombination signal sequence at both ends occurred at significantly lower frequencies than in wild-type lymphomas. The frequency of deletions without recombination signal sequence was significantly higher in scid thymic lymphomas than in wild-type lymphomas. Among them, the deletions with microhomology sequences were most abundant, and these deletions were more remarkably induced in radiation-induced scid thymic lymphomas than in wild-type lymphomas. In radiation-induced scid thymic lymphomas, the deletions associated with recombination signal sequence at only one end were formed at a higher frequency than in wild-type lymphomas. This was largely due to an increase in the frequency of deletions associated with microhomology. Thus, in the presence of DNA-PKcs, deletions were formed mainly via the recombination signal sequence-mediated pathway. In the absence of DNA-PKcs, the microhomology-mediated pathway functioned as the major pathway.

Rag-Mediated Spontaneous Deletions in the 5′-Region of the Notch1 Gene. To more clearly show the involvement of the Rag-mediated pathway in the generation of 5′-deletions and to elucidate the role of radiation in deletion induction, we examined the deletion formation between breakpoint hot spots (positions 4926 and 16674) in thymuses of unirradiated and γ-ray–irradiated mice with nested PCR (Table 4). Using genomic DNA of thymic lymphoma C69 heterozygous to the deletion between the hot spots, we checked the validity of the nested PCR to detect deletions. When 2.5, 5, or 10 pg of C69 DNA, including ~0.5, 1, or 2 copies of deletions, respectively, were mixed with 0.5 µg of C.B-17 kidney DNA, a deletion frequency of 0.86 per cell was obtained based on the assumption that the copy number in a reaction is under Poisson distribution. The result indicates that the presence of approximately one copy of a deletion in a reaction is sufficient to detect the deletion. Deletions were not detected in

the deletion regions, respectively. In TL31 and TL45, recombination signal sequence-like elements were present at only one end (data not shown). Similar sequence specificities of deletions were observed in spontaneous scid thymic lymphomas (data not shown).

Characteristics of Recombination Signal Sequence-Like Sequences Adjacent to Breakpoints. We identified the recombination signal sequence-like sequences composed of heptamer- and nonamer-like sequences, and those composed of only the heptamer-like sequence according to the criteria described in the Materials and Methods. Of the 266 breakpoints identified in thymic lymphomas, 8 had only the first 3 nucleotides or those 3 plus 1 other nucleotide of the heptamer motif. We did not regard those sequences as heptamer-like sequences to avoid ambiguity between a genuine heptamer-like sequence and a sequence derived merely by chance. The recombination signal sequence-like sequences present in the vicinity of breakpoints are shown in Table 2. In addition to the nucleotides used for identification, other nucleotides were also conserved. Specifically, the fourth nucleotide (18 of 30 cases) of the heptamer-like sequence and the fifth, sixth, and seventh nucleotides (13 of 30, 15 of 30, and 18 of 30, respectively) of the nonamer-like sequence were well conserved. The consensus sequences of 30 recombination signal sequence-like sequences identified were CACAGAC for the heptamer and ACAAAAAA/GC for the nonamer. Thus, the first five nucleotides of the heptamer and eight nucleotides of the nonamer were canonical nucleotides. Hot spot recombination signal sequence-like sequences (the 4927, 8192, and 16673 recombination signal sequences) were observed frequently. The hot spot recombination signal sequence-like sequences were AB104448 to AB104458, AB100879, and AB104459.
C.B-17 kidney DNA, indicating that the deletions do not occur in kidney. In thymuses of unirradiated C.B-17 mice, deletions occurred spontaneously at a frequency of $7.1 \times 10^{-7}$. When C.B-17 mice were treated with split-dose irradiation, the deletion frequency was significantly decreased in irradiated mice compared with unirradiated mice after 7 days. There was an individual difference in the deletion frequency on the 14th day: two mice exhibited no deletions, whereas two displayed a higher frequency than the spontaneous deletion ($1.6 \times 10^{-6}$ and $2.4 \times 10^{-6}$, respectively). The deletion frequency remained low 14 days after irradiation with 6.5 Gy. In scid mice, spontaneous deletions occurred at a 9-times lower frequency than in wild-type mice, suggesting that the DNA-PKcs is relevant to deletion formation. Seven or 14 days after irradiation, deletions were not detected in scid mice. In Rag2$^{-/-}$ mice, deletions were formed neither spontaneously nor by radiation, indicating that the Rag2-mediated pathway is essential for deletion formation.

We analyzed nucleotide sequences of deletions in the representative wild-type mice (Table 5). Although many types of deletions were identified, some types were detected at a high frequency: the sequences of 4926/TA/16674 and 4925/AGGGGGACC/16676 in the unirradiated CB11 mouse were observed at 35 and 31%, respectively. There were similar findings in the unirradiated CB12 and CB15 mice.

### Table 2: RSS-like sequences located in the vicinity of breakpoints

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of sites</th>
<th>5' RSS-like sequence* Position and sequence</th>
<th>3' RSS-like sequence* Position and sequence</th>
<th>No. of sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type n=31</td>
<td>23</td>
<td>4927 CACCTCA12ACCTGAAGG</td>
<td>16673 CACACGTG23GCTATTAG</td>
<td>25</td>
</tr>
<tr>
<td>scid 0 Gy n=10</td>
<td>3</td>
<td>4927 CACCTCA12ACCTGAAGG</td>
<td>16673 CACACGTG23GCTATTAG</td>
<td>3</td>
</tr>
<tr>
<td>scid 0.1-2 Gy n=42</td>
<td>18</td>
<td>4927 CACCTCA12ACCTGAAGG</td>
<td>16673 CACACGTG23GCTATTAG</td>
<td>16</td>
</tr>
</tbody>
</table>

* The position of the first nucleotide of the heptamer is shown. Nucleotides identical to the canonical sequence are underlined. Pairs of RSS-like sequences are linked by lines. The long bars indicate the absence of an RSS-like sequence.

Abbreviation: RSS, recombination signal sequence.
If each deletion was independently formed, the number of cells with the same deletion would be very low. The large number of cells harboring identical deletions could be attributable to some form of selection pressure. A high frequency of the deletion 4926/16676 occurred in the irradiated CB1.6–7 mouse 14 days after irradiation. The finding was similar in the irradiated CB1.6–6 mouse. If these deletions are radiation induced, there would be a large variety of deletions with each deletion accounting for only a small percentage of the total deletions. Rather, it is attributable to clonal expansion during the recovery time (14 days) of deletion-bearing cells present at the time of irradiation. Thus, it appears that radiation does not induce Rag-mediated deletions.

**DISCUSSION**

Involvement of Illegitimate V(D)J Recombination and Microhomology-Mediated Nonhomologous End-Joining in the 5′-Deletions of the Notch1 Gene. Sequence analysis of the deletion breakpoints showed that there are at least two distinct types of elements at the breakpoints: one is a recombinogenic signal-sequence-like element and the other is a microhomology sequence. The presence of recombination signal-sequence-like elements suggests that the cryptic recombination signal-sequence-like elements in the Notch1 gene functions as a genuine recombination signal sequence recognizable by Rag proteins, and deletions are formed via Rag-mediated cleavage of DNA sequences adjacent to recombination signal-sequence-like elements, followed by end-processing and ligation by V(D)J recombination machinery (Fig. 4). This view is supported by the following results. (1) The cryptic recombination signal-sequence-like elements identified preserve essential residues for recombination signal sequence function: e.g., the first three residues CAC in the heptamer, which are key determinants of joining signal function (39–41). Among the nucleotides of the nonamer, the fifth, sixth, or seventh A residues, which function primarily in the initial binding of Rag proteins to the recombination signal sequence element and strengthen recombination signal sequence function (39–42), are also conserved. We obtained the consensus sequence of the recombination signal sequence-like element (CACAGAC for the heptamer and AAAAAAA/GC for the nonamer; the canonical nucleotides are italic). The obtained consensus sequence matches well with that of previously identified cryptic recombination signal sequences (42). (2) A pair of 12- and 23-recombination signal sequence is present near the breakpoints, which is consistent with the 12/23 rule in V(D)J recombination (44). (3) N-Nucleotides and P-nucleotides that are unique to V(D)J recombination are observed in the deletion junctions. The presence of P-nucleotides suggests that the Rag-cleaved DNA ends are hair-pinned in the early process of deletion formation. (4) In scid mice defective in V(D)J recombination, the frequencies of recombination signal sequence-mediated deletions were remarkably lower than in wild-type

**Table 3** Deletions with or without RSS-like elements at the breakpoint junctions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dose (Gy)</th>
<th>No. of deletions analyzed</th>
<th>Presence of RSS-like sequence*</th>
<th>Absence of RSS-like sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>At both ends</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ N or P † (%) + MH ‡ (%) None (%) Total (%)</td>
<td>At one end</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ N or P † (%) + MH ‡ (%) None (%) Total (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+ MH ‡ (%) + N § (%) None (%) Total (%)</td>
</tr>
<tr>
<td>STS</td>
<td>2.4 × 4</td>
<td>4</td>
<td>3 (75%) 0 (0) 0 (0) 3 (75%)</td>
<td>1 (25%) 0 (0) 0 (0) 1 (25%)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7</td>
<td>0 (0) 0 (0) 0 (0) 0 (0)</td>
<td>0 (0) 0 (0) 0 (0) 0 (0)</td>
</tr>
<tr>
<td>B10</td>
<td>1.6 × 4</td>
<td>24</td>
<td>15 (63%) 2 (18%) 1 (4%) 18 (75%)</td>
<td>0 (0) 0 (0) 0 (0) 0 (0)</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>25 (71%) 2 (6%) 1 (3%) 28 (80%)</td>
<td>1 (3) 2 (6%) 0 (0) 3 (9)</td>
<td></td>
</tr>
<tr>
<td>scid</td>
<td>0</td>
<td>23</td>
<td>3 (13%) 2 (9%) 0 (0) 5 (22%)</td>
<td>6 (26) 0 (0) 0 (0) 6 (26)</td>
</tr>
<tr>
<td>scid</td>
<td>0.1–2 ‡</td>
<td>74</td>
<td>13 (18%) 4 (5%) 1 (1) 18 (24%)</td>
<td>24 (32) 6 (8) 1 (1) 31 (42)</td>
</tr>
</tbody>
</table>

* RSS-like sequences composed of a heptamer-like motif and a nonamer-like motif with 12-bp or 23-bp spacers were present in the junctions.
† MH was present at both ends of the breakpoints.
‡ Nucleotide addition in the junctions.
§ The number of deletions included 1 induced by 0.1 Gy of irradiation, 20 by 0.25 Gy, 8 by 0.5 Gy, 17 by 1 Gy, and 28 by 2 Gy.

**Table 4** Frequencies of illegitimate V(D)J recombination in unirradiated or irradiated mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Tissue</th>
<th>Irradiation (Gy)</th>
<th>Days after irradiation</th>
<th>No. of mice examined</th>
<th>1 µg (10 pg) Range</th>
<th>0.5 µg (5 pg) Range</th>
<th>0.1 µg (2.5 pg) Range</th>
<th>No. of positive reactions/No. of reactions *</th>
<th>Frequency of deletions/cell †</th>
</tr>
</thead>
<tbody>
<tr>
<td>C59</td>
<td>Thymic</td>
<td>1.6 × 4</td>
<td>3/40</td>
<td>1/40</td>
<td>0.86</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C.B-17</td>
<td>Kidney</td>
<td>0</td>
<td>0/100</td>
<td>0/100</td>
<td>0</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>0.5</td>
<td>0/50</td>
<td>0/50</td>
<td>0</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>1.6 × 4</td>
<td>7/50</td>
<td>7/50</td>
<td>1.1 × 10^{-7}</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>1.6 × 4</td>
<td>14/90</td>
<td>14/90</td>
<td>7.1 × 10^{-9}</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>scid</td>
<td>Thymus</td>
<td>6.5</td>
<td>4/150</td>
<td>4/150</td>
<td>20.2 × 10^{-3}</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>10</td>
<td>2/100</td>
<td>2/100</td>
<td>1.3 × 10^{-3}</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>1</td>
<td>0/50</td>
<td>0/50</td>
<td>0.8 × 10^{-3}</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rag2‡−</td>
<td>Thymus</td>
<td>0</td>
<td>0/50</td>
<td>0/50</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>1.4 × 4</td>
<td>0/50</td>
<td>0/50</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Ten or 20 reactions were performed in each mouse with 0.1, 0.5, or 1 µg genomic DNA per reaction. In the positive control, 2.5, 5, or 10 pg of genomic DNA of the thymic lymphoma C59 were reacted with 0.5 µg kidney DNA of C.B-17 mouse.
† Deletion frequencies were calculated based on the assumption that 5 pg of genomic DNA is equivalent to the DNA content of a G1-phase cell, and one abnormal copy present in a reaction is sufficient to detect deletions by nested PCR.
‡ Significantly different from that of unirradiated wild-type mice by the χ² test at P < 0.001 level.
§ Two mice exhibited no deletions while two other mice exhibited a significantly higher frequency of deletions than that in unirradiated mice.
Abbreviation: ND, not determined.
The MH and subsequent end processing and ligation. Deletions are formed by strand pairing at the site of processed, and single-strand portions are created. DSBs are induced double-strand breaks (DSBs) are homologous end-joining (MNHEJ) pathway. The radiation-induced double-strand breaks (17, 20). These data support the notion that the microhomology-mediated nonhomologous end-joining repair pathway works as a backup system, and in the absence of the classic nonhomologous end-joining, the microhomology-mediated nonhomologous end-joining pathway functions as the major pathway, thereby contributing to deletion formation. In this scenario, it is likely that radiation-induced double-strand breaks are efficiently repaired by the classic nonhomologous end-joining pathway in wild-type mice so that the microhomology-mediated nonhomologous end-joining pathway does not contribute effectively to deletion formation. In scid mice, radiation-induced double-strand breaks might be repaired by the microhomology-mediated nonhomologous end-joining pathway in the absence of the classic nonhomologous end-joining pathway. Therefore, there are many 5'-deletions mediated by the microhomology-mediated nonhomologous end-joining in scid mice.

The present study showed that in radiation-induced scid thymic lymphomas, deletions associated with a recombination signal sequence-like elements in thymocytes. Because the deletions are generated at hot spots in the C.B-17, B10, and STS strains, occurrence of the illegitimate V(D)J recombination in the 5'-region of the Notch1 gene is a general phenomenon in thymuses of wild-type mice. These deletions occur spontaneously, and radiation does not appear to induce illegitimate V(D)J recombination, although stimulation of V(D)J recombination does occur in irradiated scid mice (45).

Nonhomologous end-joining is the major pathway for double-strand break repair in mammalian cells (34, 35). There are at least two different nonhomologous end-joining pathways: one is DNA-PK dependent and joins DNA ends accurately (16, 17), and the other is DNA-PK independent and generates deletions whose breakpoints display microhomology (Fig. 4; refs. 12, 14, 16, 17). In the absence of Ku, there was an increased frequency of deletions associated with microhomology in yeast (15) and mammalian cells (12, 14, 16, 17, 46), which are created by the Ku-independent microhomology-mediated nonhomologous end-joining pathway. In DNA-PKcs–defective cells, abnormally large deletions arise in the late nonhomologous end-joining process of V(D)J recombination (27–30, 33). Furthermore, analysis of double-strand break repair with DNA-PKcs–deficient cell extract showed the inaccurate rejoining of double-strand break (17, 20). These data support the notion that the microhomology-mediated nonhomologous end-joining repair pathway works as a backup system, and in the absence of the classic nonhomologous end-joining, the microhomology-mediated nonhomologous end-joining pathway functions as the major pathway, thereby contributing to deletion formation. In this scenario, it is likely that radiation-induced double-strand breaks are efficiently repaired by the classic nonhomologous end-joining pathway in wild-type mice so that the microhomology-mediated nonhomologous end-joining pathway does not contribute effectively to deletion formation. In scid mice, radiation-induced double-strand breaks might be repaired by the microhomology-mediated nonhomologous end-joining pathway in the absence of the classic nonhomologous end-joining pathway. Therefore, there are many 5'-deletions mediated by the microhomology-mediated nonhomologous end-joining in scid mice.

The present study showed that in radiation-induced scid thymic lymphomas, deletions associated with a recombination signal sequence-like elements in thymocytes.
sequence at only one end and with a microhomology sequence at both ends occur frequently. The process for the formation of such deletions can be delineated as follows (Fig. 4). In scid mice, the Rag-mediated hairpins at cryptic recombination signal sequence sites could persist for a long time as they do in antigen receptor genes (28, 30, 35), and double-strand breaks are induced by radiation at different 

Notch1 sites. The persistent hairpin end would be cleaved by the residual hairpin-opening activity of Artemis (31) or by other potential hairpin-opening activities (33, 47). The protruding single-strand portion of the processed hairpin would base pair with the single-strand portion of the processed double-strand break via a microhomology sequence. The coupled pathway of Rag-mediated cleavage and microhomology-mediated nonhomologous end-joining occurs in the formation of rearrangements between IgH and c-myc loci in pro-B lymphomas in Xrc64−/−, p53−/− or LigIV−/−, p53−/− double mutant mice (25). A transient V(D)J recombination substrate assay showed that the coding joint formation in the V(D)J recombination proceeds through the microhomology-mediated nonhomologous end-joining process in the absence of DNA-PKcs (33), which is similar to the present results. We report here that in the deficient condition of both the classic microhomologous end-joining repair and the hairpin-opening process of V(D)J recombination, deletion via the coupled process is induced by radiation in thymus.

Role of Notch1 Deletion in Lymphomagenesis. We identified frequent deletions in the 5′-region of the Notch1 gene in mouse thymic lymphomas. The 5′-deletions result in the formation of abnormal mRNA encoding truncated proteins composed of only the intracellular domain (38). These results indicate that the Notch1 deletion is a major pathway in the development of thymic lymphomas and that illegitimate V(D)J recombination and the microhomology-mediated nonhomologous end-joining pathway are major determinants for the 5′-deletion and hence important contributors to lymphomagenesis. Because the illegitimate V(D)J recombination occurs spontaneously, the microhomology-mediated nonhomologous end-joining pathway might be a main contributor to the radiation-induced thymic lymphomas. Our notion is consistent with the fact that double-strand breaks are repaired by the microhomology-mediated nonhomologous end-joining pathway (12–16). Indeed, our results indicate a major contribution of the microhomology-mediated nonhomologous end-joining pathway to the generation of the 5′-deletion of the Notch1 gene in radiation-induced scid thymic lymphomas. We analyzed deletions in other Notch1 regions, the extracellular juxtamembrane and the 3′-end regions, and found that the Notch1 deletions induced by radiation in these regions are mediated mainly via the microhomology-mediated nonhomologous end-joining, but not via the illegitimate V(D)J recombination, in both wild-type and scid thymic lymphomas. As a result, the majority of deletions induced by radiation in the Notch1 gene are microhomology-mediated nonhomologous end-joining-mediated deletions. Thus, the microhomology-mediated nonhomologous end-joining pathway might cause the alteration in other oncogenes and tumor suppressor genes in response to radiation and thereby contribute to radiation-induced lymphomagenesis.

Thymocytes rarely develop into thymic lymphomas in unirradiated wild-type mice, irrespective of the possession of a spontaneous 5′-deletion in the Notch1 gene (Tables 1 and 4), indicating that the 5′-deletion alone is not sufficient to induce the development of thymic lymphoma. Radiation probably induces conditions in which deletion-bearing thymocytes become more malignant. Spontaneous deletions formed by illegitimate V(D)J recombination were present clonally in the thymocyte population (Tables 4 and 5). This suggests that deletion-bearing thymocytes have a growth advantage to expand or cells with aberrant rearrangements at various loci, including the Notch1 locus merely expand without a growth advantage as cells replicate and expand after T-cell receptor rearrangement. Although the latter possibility is not excluded, the former is likely to be the case because the frequency of deletion-bearing cells was increased, and clonality was more pronounced after irradiation (Tables 4 and 5). Radiation has direct and indirect effects on the development of thymic lymphomas: a direct effect on the induction of mutations or chromosomal aberrations and indirect effects through changes in the thymic microenvironment (48, 49). As evidence for indirect effects, transplantation of unirradiated thymus into thymectomized, irradiated mouse induces thymic lymphomas from unirradiated donor thymocytes (48, 49). Radiation-induced changes in the microenvironment might promote tumorigenesis and act as promoters apparently by creating a favorable microenvironment through alterations of signal transduction and gene expression such as growth factors and cytokines in thymus (50).

Alternatively, radiation might cause (epi)genetic changes in an oncogene or tumor suppressor gene in surviving thymocytes with a Notch1 deletion during forced proliferation after irradiation. Such a situation would be created by thymocyte depletion because of thymocyte death in the thymus and a radiation-induced shortage in the supply of pre-T cells to the thymus (51). Transplantation of the thymocytes surviving after irradiation into thymuses of lethally irradiated mice revealed the presence and the induction of prelymphoma T cells in the irradiated thymocyte population, which can proliferate abnormally to additionally evolve into fully autonomous thymic lymphomas in thymus (51–53). Prelymphoma cells compose 5.2 × 10−6% of thymocytes on the 14th day after irradiation (52). It is conceivable that deletion-bearing thymocytes with a (epi)genetic change in tumor-related genes would convert into prelymphoma cells 14 days after irradiation. Whether these thymocytes have characteristics of prelymphoma cells, including the capacity to develop overt thymic lymphoma, and the role of deletions in thymic lymphomagenesis remains to be clarified.

The formation of spontaneous intragenic deletions of the Rit1/Bcl11b tumor suppressor gene in thymocytes via illegitimate V(D)J recombination has been reported in which there is no clonality of deletion-bearing cells in thymus (54). For comparison with the Notch1 gene, we examined spontaneous deletions mediated by illegitimate V(D)J recombination in the Rit1/Bcl11b locus and found that the spontaneous frequency of the Rit1 deletions is 5.6 × 10−6, 8-fold higher than that of Notch1.7 We examined the clonality of deletions in three unirradiated mice and found no clonality in Rit1 deletions. It is probable that in a tumor suppressor gene, both alleles are not altered simultaneously in a cell so that cells bearing alterations in only one allele would not exhibit clonality unless haploinsufficiency occurs. In contrast, in an oncogene, deletions would directly lead to a change in gene function. Therefore, formation of deletions in an oncogene could lead to the clonal expansion of deletion-bearing cells. Additional analyses are needed to clarify the significance of clonality of deletion-bearing cells on lymphomagenesis.

ACKNOWLEDGMENTS

We thank Dr. Frederick W. Alt for providing the Rag2−/− mice, Harumi Osada and Keiko Yamada for care of the mice, Shizue Sasaki for preparation of tissue slides, and Dr. Yoshiya Shimada and Mayumi Nishimura for their assistance in preparing thymic lymphoma tissue samples for the analysis.

---

6 Unpublished results.

7 Unpublished result.
REFERENCES

Involvement of Illegitimate V(D)J Recombination or Microhomology-Mediated Nonhomologous End-Joining in the Formation of Intragenic Deletions of the Notch1 Gene in Mouse Thymic Lymphomas

Hideo Tsuji, Hiroko Ishii-Ohba, Takanori Katsube, et al.