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Tracing the Pre-B to Immature B Cell Transition in Human Leukemia Cells Reveals a Coordinated Sequence of Primary and Secondary IGK Gene Rearrangement, IGK Deletion, and IGL Gene Rearrangement

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The BCR-ABL1 kinase expressed in acute lymphoblastic leukemia (ALL) drives malignant transformation of pre-B cells and prevents further development. We studied whether inhibition of BCR-ABL1 kinase activity using STI571 can relieve this differentiation block. STI571 treatment of leukemia patients induced expression of the Ig L chain-associated transcription factors IRF4 and SPIB, up-regulation of RAG1 and RAG2, Cκ and CA germline transcription, and rearrangement of Ig κ L chain (IGK) and Ig λ L chain (IGL) genes. However, STI571-treated pre-B ALL cells expressed λ L, but almost no κ L chains. This could be explained by STI571-induced rearrangement of the κ-deleting element (KDE), which can delete productively rearranged Vκ-Jκ joints. Amplifying double-strand breaks at recombination signal sequences within the IGK, KDE, and IGL loci revealed a coordinated sequence of rearrangement events induced by STI571: recombination of IGK gene segments was already initiated within 1 h after STI571 treatment, followed by KDE-mediated deletion of Vκ-Jκ joints 6 h later and, ultimately, IGL gene rearrangement after 12 h. Consistently, up-regulation of Cκ and CA germline transcripts, indicating opening of IGK and IGL loci, was detected after 1 and 6 h for IGK and IGL, respectively. Continued activity of the recombination machinery induced secondary IGK gene rearrangements, which shifted preferential usage of upstream located Jκ- to downstream Jκ-gene segments. Thus, inhibition of BCR-ABL1 in pre-B ALL cells 1) recapitulates early B cell development, 2) directly shows that IGK, KDE, and IGL genes are rearranged in sequential order, and 3) provides a model for Ig L chain gene regulation in the human. The Journal of Immunology, 2005, 174: 367–375.
in addition to productive Vκ-Jκ gene rearrangements. These findings argue for sequential rearrangement of IGK and IGL genes. However, these studies do not formally demonstrate that rearrangement of IGK genes in λ-expressing B cells do necessarily precede successful Vλ-Jλ gene recombination. In λ-expressing B cells, for instance, IGL genes may well be rearranged first, followed by, needless Vκ-Jκ gene rearrangements, which then would subsequently be inactivated by rearrangement of the κ-deleting element (KDE). The Kκ gene may reorganize to signal sequence (RSS) flanking germline Vκ gene segments or an RSS within the intron between the Jκ cluster and the Cκ gene, which leads to inactivation of a pre-existing Vκ-Jκ joint in either case (15). Also the existence of B cells expressing κ L chains with λ L chains (16) suggests that recombination events in the IGK and IGL loci are not mutually exclusive in all instances. Instead of κ L or λ L chains, human PBC express surrogate L chains composed of VpreB and A5.

In the vast majority of acute lymphoblastic leukemia (ALL), the malignant clone represents the outgrowth of a transformed PBC. In many cases, pre-B ALL clones carry specific oncogenic gene rearrangements defining both biological and clinical subentities (17). Among these translocation events, the (t;9;22)(q34;q11) results in fusion of the BCR and ABL1 genes, which codes for a constitutive active variant of the ABL1 tyrosine kinase (18) and plays a role in the most frequent recurrent aberration leading to ALL in adults (19). ABL1 represents the human homologue of the transforming gene of the Abelson murine leukemia virus, v-abl. Although v-abl has been used for many years to transform murine PBC, recent work showed that v-abl also confers a differentiation block at the PBC stage of development and, hence, prevents rearrangement of IGK or IGL L chain genes (20, 21). Recently, a specifically designed inhibitor of BCR-ABL1, termed STI571, has become available and is now widely used as an anti-leukemia drug for BCR-ABL1+ leukemias (22). Therefore, we investigated whether and in which way inhibition of BCR-ABL1 kinase activity by STI571 might induce differentiation including IGK and IGL gene rearrangement in pre-B ALL cells.

Materials and Methods

Patient samples, primary cells, and cell lines

BCR-ABL1+ pre-B ALL cells from five patients were analyzed. From three of these patients, leukemia cells were available before and during treatment with STI571 (cases III, IV, and V, Refs. 23 and 24). Samples of two cases of bone marrow-derived pre-B ALL carrying a BCR-ABL1 gene rearrangement (cases I and II), CD19+CD27- NBC, CD20+CD77+ GCB, CD19+CD27+ MBC, and CD19+CD138+ PC (see Fig. 1A).

All SAGE libraries were normalized to 100,000 tags. SAGE data were graphically visualized using the Cluster and Treeview software (http://rana.lbl.gov/) and sorted according to the ratio between SAGE-tag counts in PBC and in BCR-ABL1+ ALL cases (26–28). In these SAGE profiles, 30 genes were identified which have been implicated in the regulation of Ig L chain expression. These genes were searched in the literature, as well as in Unigenes (<www.ncbi.nlm.nih.gov/Unigene>) and OMIM (<www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = OMIM>) databases.

RT-PCR analysis of IRF4, SPIB, and RAG1 and RAG2 expression and germline Cκ and Cα transcripts

mRNA levels of the Ig L chain-associated transcription factors IRF4 and SPIB were measured in BCR-ABL1+ leukemia samples from three patients (cases III, IV, and V, Ref. 24) before and during treatment with STI571 (see Fig. 1). From patient samples, total RNA was isolated and transcribed into cDNA as previously described (26–28). Amounts of cDNA were normalized by OD measurement and semi-quantitative RT-PCR using 5′-TT AGACCCCCGCTGGAACG-3′ and 5′-CTTACTCTTGTGGGCACGAT-3′ for amplification of a cDNA fragment of GAPDH. For semi-quantitative RT-PCR analysis of IRF4 the oligonucleotides 5′-CAAGGACAAATGACTCTTGAG-3′ and 5′-TGGGACATTGGTACGGGATT-3′ were used and for SPIB 5′-AAAGACTTACGTTGACGAGC-3′ and 5′-CTTGGAGGAAGACCTTGGAA-3′.

To determine L chain locus germline transcription by RT-PCR, RNA was isolated from BV173, NALM1, and SUP-B15 cell lines with or without STI571 incubation, reverse-transcribed into cDNA, and normalized as described above. For amplification of Cκ gene transcripts, 5′-TCTCAA CAGGGGAGAGTTGTTAGAG-3′ and 5′-ATGGCCCTTGAAGGACTCATA-3′ primers were used, and 5′-TGTCTGATCAGTACCTTACCC-3′ and 5′-CTGTAGGCTGTTGGGACATC-3′ primers were used for Cα germ-line transcripts. RT-PCR for RAG1 and RAG2 was conducted using the oligonucleotides 5′-ATAGGAAAGACGAACTAAACGAAC-3′ and 5′-ATA CCGAGCTATCCTGGAAGA-3′ for RAG1 and 5′-ATACGAAAGGCT TACACTACCC-3′ and 5′-AAAAATCAGATCGAAATACCTC-3′ for RAG2 (see Fig. 2A).

Analysis of nuclear RAG1 expression

BCR-ABL1+ pre-B ALL cells (BV173 and SUP-B15) were cultured in the presence or absence of STI571 for 1, 2, or 4 h and subsequently stained with a Cy5-labeled Ab against CD19. After treatment with methanol and 4% paraformaldehyde, cells were stained with a mouse anti-human IgG1 (BD Biosciences) together with a Cy2-labeled goat anti-mouse IgG Ab. Expression of CD19 and RAG1 was visualized by immunofluorescence (see Fig. 2B).

Flow cytometry

Surface expression of IGK or IGL on BV173, NALM1, and SUP-B15 pre-B ALL cells in the presence or absence of STI571 was monitored using Abs against IGK or IGL (BD Biosciences) after the incubation times indicated (see Fig. 3). Apoptotic or dead cells were identified by staining with FITC-labeled annexin V and propidium iodide (BD Biosciences) and excluded from analysis. Normal B cells were enriched from umbilical cord blood by MACs using immunomagnetic beads against CD19 (Miltenyi Biotech) and were used as a positive control for κ and λ staining (see Fig. 3).

Amplification of double-strand RSS breaks by ligation-mediated PCR

From ~2.5 × 10⁶ untreated BV173, NALM1, and SUP-B15 cells or after treatment with 10 μM STI571 after the times indicated, genomic DNA was extracted and ligated-end linkers (Invitrogen Life Technologies) at 14°C overnight. The linker was constructed by annealing the oligonucleotides 5′-TTTCCGTGCCTGATC AAGCTTCTAACCAGTACGGGGACAGT-3′ and 3′ amino (C7)-GAC GAGCCTTCAAGTTGCAAGATGGTACGTACCCCT-5′ and protruding 3′ overhangs were removed by 3′-5′ exonuclease activity of the Klenow fragment of Escherichia coli DNA polymerase I (Invitrogen Life Technologies). Ligation-mediated PCR (LM-PCR; Ref. 29) was conducted with modifications as previously described (28). In two seminested rounds of amplification (35 and 45 PCR cycles at an annealing temperature of 59°C), RSS intermediates with a DNA double-strand break at the 5′ heptamer of Jκ gene segments were amplified (see Figs. 4 and 5A) using 5′-GTAAT TAACAATCTGATCTACCTC-3′ as external forward and 5′-TACAT TCAGTCTCCTCTTCAAA-3′ as internal forward primers together with 5′-TCCCCTCACTACGTGTTAG-3′ as reverse primer specific for DNA-ligated linker molecules. To amplify RSS intermediates with a DNA-double-strand break at the 5′ heptamer of Jκ gene segments, 5′-TTCATC
A mRNA levels of Ig L chain-associated genes in BCR-ABL1+ pre-B ALL cells. A, Focusing on 30 genes implicated in the regulation of Ig L chain expression, genome-wide SAGE profiles of two BCR-ABL1+ pre-B ALL cases (ALL1 and ALL2) were compared with SAGE profiles of normal CD34+CD38low HSC, CD15+ CMP, CD7+CD10+ TLP, CD10+CD19+ PBC, CD19+CD27+ NBC, CD20+CD77+ GCB, CD19+CD27+ MBC, and CD19lowCD138+ PC. SAGE-tag counts are visualized by colors, red indicating high and green depicting low levels or no expression, respectively. SAGE data were sorted according to the ratio of SAGE-tag counts in normal B cell subsets to leukemia samples. B, From three patients with BCR-ABL1+ pre-B ALL, leukemia samples were available before and during treatment with the BCR-ABL1 kinase inhibitor STI571. In these matched leukemia sample pairs, mRNA levels of IRF4 and SPIB were compared by semiquantitative RT-PCR. cDNA amounts were normalized by OD measurements and amplification of a specific fragment of the housekeeping gene GAPDH.
TTCTTCCATGGTAC-3' and 5'-ACTTCTTCCATGGTACAGTCTC-3' were used in two rounds of PCR amplification as described above (see Fig. 6). Accordingly, 5'-TCTTCTCTGAGCTGCTCCCTTGAATAGTCC-3' were used to amplify RSS intermediates with a DNA double-strand break at the 5' heptamer of the KDE. LM-PCR products were cloned and five randomly picked clones were sequenced showing that indeed Jκ RSS-linker ligation products were amplified.

 Amplification of Vκ-Jκ and Vλ-Jλ gene rearrangements

Genomic DNA from 5 × 10^6 STI571-treated (24 h) or untreated BV173, NALM1, and SUP-B15 cells was isolated and subjected to two rounds of seminested PCR (35 and 45 cycles) using six Vκ and Vλ family-specific primers together with Jκ- and Jλ-specific primers, respectively. Primers and PCR conditions were used as previously described (30).

Analysis of the genomic configuration of the KDE

Three primers (KDE germline: 5'-CTCATCGGCGCTCCCTTGAATAGTCC-3', Jκ intron: 5'-CCGGCGGTCTTCTCGATGTGC-3', KDE external reverse: 5'-CTCTTCTGGGAGCGGACAG-3') were added to Vκ family-specific primers for amplification of Vκ-KDE rearrangements ([Vκ primers and KDE external reverse primer]. One-microliter aliquots of the first rounds were used in separate second rounds for the six family-specific Vκ together with 5'-Jκ primers and the internal reverse KDE primer (5'-AGACAGGTCTGAGCAGCAG-3'), and one second round with Jκ intron and KDE internal reverse and another with KDE germline and internal reverse KDE (see Fig. 5). Vκ family-specific primers used are 5'-GAGATCGGCGCTCCCTTGAATAGTCC-3', for Vκ1, 5'-GAGATCGGCGCTCCCTTGAATAGTCC-3', for Vκ3, 5'-GAGATCGGCGCTCCCTTGAATAGTCC-3', for Vκ6, 5'-CATACCTCGGCGCTCCCTTGAATAGTCC-3', for Vκ8, and 5'-TTCTCTGAGTCTCAGCAGCAG-3' for Vκ5 and 5'-TTCTCTGAGTCTCAGCAGCAG-3' for Vκ6. Jκ-specific primers used are 5'-TTGATGCTCTTGGC-3', for Jκ1 and Jκ2, 5'-TTGATGCCACGTCCCACTTGGC-3', for Jκ3, 5'-TTGATGCCACGTCCCACTTGGC-3', for Jκ4, and 5'-TTATGCTCAGTCCCACTTGGC-3', for Jκ5.

**FIGURE 2.** Inhibition of BCR-ABL1 by STI571 results in Cκ and CA germline transcription and rapid up-regulation of RAG1 and RAG2. Three BCR-ABL1+ pre-B ALL cell lines were incubated in the presence or absence of 10 μmol/L STI571 for the times indicated and analyzed for the expression of Cκ and CA germline transcription, RAG1, RAG2, and GAPDH (control) by RT-PCR (A). Nuclear RAG1 expression in individual leukemia cells was measured by staining for CD19 (green) and intracellular RAG1 (red; B).

Analysis of secondary IGK gene rearrangement

To analyze potential secondary IGK recombination events, we tested whether rearrangement of individual Jκ-gene segments occurs sequentially. Genomic DNA were isolated from untreated BCR-ABL1+ pre-B ALL cells or treated with STI571 for the times indicated and subjected to two rounds of seminested PCR. 5'-GTTCTAGCTCCCTTGGC-3' and 5'-GACATCCGCGCTCCCTTGGC-3' were used as primers specific for the leader and framework region I, respectively, of Vκ1 gene segments. 5'-TTATGCTCAGTCCCACTTGGC-3' was used for amplification of Jκ1 and Jκ2 gene rearrangements and 5'-TTATGCTCAGTCCCACTTGGC-3' for Jκ5.

Results and Discussion

Regulation of genes implicated in Ig L chain expression in BCR-ABL1+ leukemia cells

Comparing genome-wide gene expression profiles from normal bone marrow PBC with two cases of BCR-ABL1+ pre-B ALL generated by the SAGE technique, we identified 16,786 individual transcripts (unique tags) in PBC (26) and each ~9,200 in the pre-B ALL cases, respectively. To elucidate transcriptional regulation during the pre-B to immature B cell transition, we identified 182 genes for which a specific role in early B cell development is known (26). Among these genes, transcription factors such as PAX5, E2A, and EBF and genes coding for (pre-) B cell receptor signaling molecules (e.g., LYN, BTK, SYK, FYN) are silenced in...
the leukemia cells (24). Furthermore, we focused on a subset of 30 genes which are specifically implicated in Ig L chain regulation (Fig. 1). Here, transcription factors that positively regulate Ig L chain gene transcription (IRF4, SPIB, OBF1, IRF8, E2A, AP4, PU.1, CREM, MEF2, ATF1, JUN) are down-regulated in the leukemia cells compared with normal PBC, which argues for a differentiation block at the PBC to immature B cell transition. However, besides genes implicated in Ig L chain gene rearrangement (pre-B to immature B cell transition), also genes coding for components of the pre-B cell receptor (μ-chain: 565 tags in PBC and 48 and 148 tags in leukemia cells (two cases in Fig. 1); VpreB: 278 tags in PBC and 4 and 12 tags in leukemia cells; λ5: 1,932 tags in PBC and 12 and 20 tags in leukemia cells) at the pro-B to pre-B cell transition are down-regulated in the leukemia cells. This may suggest that in the leukemia cells, expression and function of the pre-B cell receptor is impaired in general, including its specific function at the PBC to immature B cell checkpoint (2). We next investigated whether down-regulation of the Ig L chain-associated transcription factors IRF4 and SPIB is indeed linked to BCR-ABL1 kinase activity. To this end, we analyzed primary BCR-ABL1+ leukemia samples, which were derived from three patients before and during treatment with the BCR-ABL1-inhibitor STI571 (cases III, IV, V, Ref. 24, Fig. 1B). Comparing matched pairs of patient-derived leukemia cells, IRF4 and SPIB mRNA levels were increased when BCR-ABL1 kinase activity was blocked by STI571.

Before rearrangement of Ig L chain genes can be initiated, IGK and IGL loci have to be opened to become accessible to the recombination machinery. Therefore, Cκ and Cλ germline transcription indicating transcriptional activation of Ig L chain loci was analyzed by RT-PCR in BCR-ABL1+ cell lines treated with or without STI571 (Fig. 2A). Upon inhibition of BCR-ABL1, germline transcription of Cκ was up-regulated already after 1 h, followed by Cλ germline transcription after 6 h. However, low germline transcription activity at both loci could also be detected in the absence of STI571. These data suggest that IGK and IGL L chain loci become in sequential order accessible to Ig-specific transcription factors and the recombination machinery.

As constituents of the recombination machinery, the expression of recombination activation genes, RAG1 and RAG2, was analyzed by RT-PCR (Fig. 2A). mRNA levels for both RAG genes were significantly up-regulated after 1 h of STI571 treatment. To investigate RAG1 expression in individual cells, RAG1 protein was stained by immunofluorescence in a leukemia cell line (SUP-B15), from which we previously amplified a productive VH3.53-DH2.8-JH6 Ig H chain gene rearrangement (24), in the presence or absence of the BCR-ABL1-inhibitor STI571. Although some cells already exhibit nuclear RAG1 expression before STI571 treatment, RAG1 protein levels were visibly up-regulated after 2 h in some and after 4 h in almost all cells (Fig. 2B).

To determine whether up-regulation of Ig-L chain-associated transcription factors IRF4 and SPIB increased expression of RAG1...
Extensive KDE rearrangement results in preferential λ L chain expression on STI571-surviving leukemia cells

Using three different PCR strategies (Fig. 4), we investigated at which level the expression of κ L chains may have been impaired. By LM-PCR (Fig. 4A), we first showed that inhibition of BCR-ABL1 kinase activity induces DNA double-strand breaks at RSS within the IGK locus after 8 h (Fig. 5A). For validation of the LM-PCR assay, PCR products were cloned and five randomly chosen clones were sequenced. Sequence analysis confirmed Jκ RSS-linker ligation products (not shown). Such RSS-specific DNA strand breaks represent an initial step within the recombination process of IGK genes and demonstrate that the IGK locus is indeed targeted by the recombination machinery upon treatment with STI571. Because Jκ-RSS-specific DNA strand breaks represent a precondition for Vκ-Jκ gene rearrangements, we next amplified Vκ-Jκ rearrangements from genomic DNA of B cell precursor leukemia cells treated with or without STI571 (Fig. 4B). Although sporadic Vκ-Jκ and Vκ-λA joints were already detectable under control conditions, V-J rearrangements at both IGK and IGL loci were clearly inducible by STI571 (Fig. 5B). As a specificity control, genomic DNA of BCR-ABL1− chronic myeloid leukemia cells was subjected to amplification of Vκ-Jκ gene rearrangements and Vκ-λA rearrangements but no product was obtained. Integrity of DNA isolated from chronic myeloid leukemia cells was verified by amplification of a Cβ1 germline fragment of the TCRβ locus (Fig. 5B). In agreement with a previous study on the effect of STI571 on v-abl-transformed murine pre-B cell lines (21), we observed a polyclonal pattern of Vκ and Vλ gene rearrangement upon STI571 treatment. STI571-induced V-J gene rearrangements involved all six IGK and all nine IGL V gene families. Whether IGK and IGL genes were also rearranged at similar frequencies in response to STI571 treatment remains unclear, because the PCR approach used here was not quantitative.

Rearrangement of the KDE may lead to inactivation of productively recombined Vκ-Jκ joints and hence represents another level at which Ig L chain expression may be impaired (15). To assess whether pre-existing functional Vκ-Jκ joints may have been inactivated by rearrangement of the KDE, we analyzed both types of KDE recombination within the IGK locus (Fig. 4C). The KDE can rearrange either to an RSS site within the intron region between the cluster of Jκ gene segments and the κ intronic enhancer or to an RSS site immediately flanking a Vκ gene segment (Fig. 4C). KDE rearrangement leads to deletion of both κ enhancers and the Cκ gene in the former (Jκ intron RSS-KDE) or to deletion of a Vκ-Jκ joint in the latter case (Vκ RSS-KDE). To investigate whether KDE rearrangement may contribute to reduced κ L chain usage in STI571-treated leukemia cells, we amplified specific DNA fragments for KDE-germline configuration, Jκ intron RSS-KDE rearrangement, and Vκ RSS-KDE rearrangement from three BCR-ABL1− leukemia cell lines (BV173, NALM1, SUP-B15) in the absence or presence of STI571 (Fig. 5C). Although KDE was found in germline configuration in untreated leukemia cells, STI571-mediated inhibition of BCR-ABL1 induced deletion of IGK alleles by rearrangement of the KDE to Jκ intron RSS sites and to Vκ RSS sites. Targeting of the IGK locus by the recombination machinery and generation of Vκ-Jκ joints appears to be normal, but overrepresentation of λ L chains can be attributed to extensive KDE rearrangement observed here (Fig. 5C). In the

FIGURE 4. Molecular analysis of recombination events within the IGK locus. A, The rearrangement of Vκ to Jκ segments is initiated by DNA double-strand breaks at the heptamer RSS sequence (RSS) immediately flanking the Jκ segment. DNA double-strand breaks result in hairpin formation at the Jκ-break and 5′ phosphorylated blunt ends at the RSS break, which can be ligated to a blunt-ended linker molecule (Linker). Using PCR primers specific for sequences upstream of the RSS and the ligated linker sequence, broken-ended Jκ-RSS DNA intermediates can be amplified. B, During primary recombination events, a Vκ gene segment rearranges to a (mostly upstream located) Jκ gene segment. In secondary rearrangements, pre-existing Vκ-Jκ joints are deleted by juxtaposition of more upstream Vκ to a more downstream Jκ element. C, A preformed Vκ-Jκ joint (top) can be inactivated by two types of rearrangement of the KDE. Using an upstream Vκ-RSS of an unarranged Vκ gene segment, rearrangement of the KDE results in a large deletion within the κ locus including downstream unarranged Vκ gene segments, the pre-existing Vκ-Jκ joint, unarranged Jκ gene segments, and the Cκ gene (bottom, left). In alternative, the KDE can rearrange to an intronic RSS between the cluster of Jκ-gene segments and the Cκ gene. In this case, KDE rearrangement results in a deletion of the Cκ gene and both κ enhancers (bottom, right).

and RAG2 and whether Cκ and Cλ germline transcription is followed by the expression of conventional L chains, we analyzed three BCR-ABL1− pre-B ALL cell lines for κ and λ L chain expression in the presence or absence of STI571 by flow cytometry (Fig. 3). After 48 h of STI571 treatment, >50% of the cells had already undergone apoptosis and were excluded from the analysis. Apoptotic or dead cells were identified by annexin V membrane expression and uptake of propidium iodide. Among the surviving cells, ~10% exhibit de novo Ig L chain expression (Fig. 3). Unexpectedly, BCR-ABL1 inhibition resulted in a pattern of L chain expression heavily biased for λ L chains (Fig. 3). Only a few κ L chain-producing leukemia cells were detectable, while the anti-κ Ab used clearly identified normal κ-expressing B cells (Fig. 3). For control stainings, B cells from umbilical cord blood were used that were enriched by MACS for CD19 expression (Fig. 3).
absence of BCR-ABL1 kinase activity, differentiating leukemia cells seem to undergo multiple rounds of rearrangement targeting both \( \kappa \) and \( \lambda \) L chain loci, which ultimately leads to preferential \( \lambda \) L chain expression due to KDE-mediated deletion of potentially productive \( \kappa \)-Jk joints.

Rearrangements at the IGK and IGL loci occur in sequential order

For this reason, we considered STI571-induced Ig L chain gene rearrangement as a model for the pre-B to immature B cell transition in normal B cell development and investigated whether IGK, KDE, and IGL gene rearrangements follow a defined sequence of
events. Sequential rearrangement of IGK, KDE, and IGL genes would be predicted by the ordered model of L chain gene recombination. To the contrary, according to the stochastic model of L chain gene rearrangement, IGK, KDE, and IGL genes would be recombined independently at each locus. Therefore, we incubated the three BCR-ABL1+ pre-B ALL cell lines in the presence or absence of STI571 and analyzed genomic DNA for RSS-specific double-strand breaks within the IGK, KDE, and IGL loci at different time points (Fig. 6). In the three cell lines, RSS-specific DNA double-strand breaks were already detectable at the IGK locus within 1 h after STI571 treatment. RSS breaks flanking the KDE followed after 6 h of STI571 treatment and RSS-DNA breaks were found at the IGL locus only after 12 h (Fig. 6). These findings are consistent with a sequential order of rearrangement events: ablation of BCR-ABL1 kinase activity almost immediately initiates IGK gene rearrangements, many of which are subsequently deleted by KDE- and ultimately replaced by IGL-gene rearrangements (Fig. 6). Of note, up-regulation of CA germline transcripts did not precede the onset of KDE RSS breaks (Figs. 2A and 6). Unlike normal B cell development, only very few STI571-treated leukemia cells seem to stay at a γ stage, while the vast majority of cells continue to rearrange, first KDE and ultimately IGL genes. It should be noted that between 12 and 24 h of STI571 incubation, RSS breaks could be detected consecutively in IGK, KDE, and IGL loci (Fig. 6). Therefore, we cannot exclude that during this period of time, IGL gene rearrangements may have occurred before recombination within the IGK locus. Stability of RSS-strand break intermediates is cell cycle-dependent and mainly found in G1 and G0 phases (29). Indeed, STI571 treatment induces cell cycle arrest in BCR-ABL1+ B cell precursor leukemia cells (25). Thus, the detection of Jκ-RSS breaks for an extended period of time (1–24 h), partially overlapping with KDE-RSS and even Jλ-RSS breaks, can be explained by cell cycle arrest and increased stability of RSS breaks induced by STI571.

**Indication for secondary IGK gene rearrangements**

In contrast, the relatively wide time frame during which IGK gene rearrangements can occur might reflect that the leukemia cells are not all synchronized with respect to STI571-induced IGK gene rearrangement. This is consistent with a rather heterogeneous pattern of RAG1 up-regulation following treatment with STI571 (Fig. 2). Moreover, RSS-specific DNA-strand breaks within the IGK locus do not necessarily reflect a single definitive Vk-Jκ gene rearrangement and would also arise from any secondary recombination event during L chain revision (Fig. 4B). To determine whether ongoing κ L chain editing may extend the time window for RSS-specific DNA-strand breaks, we searched for traces of secondary rearrangements within the IGK locus. A pre-existing Vk-Jκ joint can only be replaced by a rearrangement which uses an upstream-located Vk-κ together with a downstream-located Jκ segment (Fig. 4B). Individual Vk segments are not arranged in the order of Vk gene families within the IGK locus. Therefore, we only compared the usage of upstream Jκ1 and Jκ2 segments to downstream Jκ5 elements (see Fig. 4B) at different time points after STI571-induced initiation of L chain gene rearrangement. Consistent with a shift from upstream to downstream Jκ elements, Jκ1 and Jκ2 gene rearrangements were detected slightly earlier than rearrangements involving the Jκ5 gene segment (Fig. 7). Given that the two round PCR approach used here for analysis of Vk-Jκ gene rearrangements is not quantitative, the relative amount of PCR products does not necessarily reflect the usage of individual Jκ segments.

**Concluding remarks**

We conclude that inhibition of BCR-ABL1 initiates a coordinated sequence of Ig L chain gene rearrangement events: almost immediately after ablation of BCR-ABL1 kinase activity by STI571, the IGK locus opens and is targeted by the recombination machinery. The continuous generation of new Jκ RSS-specific DNA strand breaks even after 24 h together with a shift from upstream Jκ1 and Jκ2 elements to downstream Jκ5 segments suggests that initial Vk-Jκ rearrangements are followed by at least one, perhaps multiple, round(s) of secondary rearrangements. A possible initiation

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**FIGURE 6.** Sequentially ordered recombination events within the IGK, KDE, and IGL loci. BCR-ABL1+ BV173, NALM1, and SUP-B15 cells were cultured in the presence or absence of 10 μmol/L STI571 for the times indicated. Genomic DNA was isolated from 5 × 106 cells. Broken-ended DNA strand breaks were ligated to linker molecules and subjected to two rounds of PCR amplification using primers specific for breaks at Jκ-RSS, KDE-RSS, and JA-RSS sites.

**FIGURE 7.** Shifting the preferential Jκ usage from upstream to downstream located Jκ gene segments indicates secondary IGK gene rearrangements. Vκ1 gene rearrangements were amplified from three BCR-ABL1+ pre-B ALL cell lines using Vκ1 leader- and Vκ1 framework region 1-specific primers together with either primers matching to Jκ1 and Jκ2 or primers matching to Jκ5. As controls, PCRs were performed to which water was added instead of DNA.
signal for subsequent KDE rearrangement could be exhaustion of the recombination potential of IGK alleles in multiple rounds of Vk-Jk rearrangement. Recombination of the KDE deletes Vk-Jk rearrangements from the chromosome and may initiate VA-Ja gene rearrangements, which can first be detected after 12 h. According to these observations, it is not surprising that the vast majority of leukemia subclones that have been primed to differentiate, ultimately express λ L chains on their surface (Fig. 3). Obviously, the leukemia cells, unlike normal PBC, are lacking a feedback mechanism which prompts them to halt the recombination machinery in the presence of a productively rearranged IGK allele. Upon inhibition of BCR-ABL1, the leukemia cells seem to enter a pre-determined (ordered) sequence of rearrangement events, in which κ-expressing cells would only exist as a transition stage, inevitably leading to the expression of λ L chains.

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