Cutting Edge: A/WySnJ Transitional B Cells Overexpress the Chromosome 15 Proapoptotic Blk Gene and Succumb to Premature Apoptosis

Ian J. Amanna, Karen Clise-Dwyer, Faye E. Nashold, Kathleen A. Hoag and Colleen E. Hayes

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Better knowledge of peripheral B lymphocyte homeostasis is needed to address the human hypogammaglobulinemia diseases. A defect in the Bcmd gene shortens the B cell life span and causes B cell deficiency in A/WySnJ mice. Previous genetic mapping placed Bcmd near Sreb2 on chromosome 15. Inspection of the human chromosome 22 syntenic region identified the proapoptotic Bik gene as a candidate. Two mapping methods placed the homologous mouse gene, Bik, near Sreb2. The Bik genomic structure was highly homologous to Bik. Sequence analysis ruled out coding region mutations, but Bik transcripts were overly abundant in sorted A/WySnJ T1 B cells. Moreover, enriched transitional B cells showed a cell-autonomous defect leading to excessive apoptosis. Thus, Bcmd may be a direct mutation in Bik, or in a gene involved in Bik regulation, such that excess expression pushes the A/WySnJ transitional B cells past the apoptosis checkpoint to cell death. The Journal of Immunology, 2001, 167: 6069–6072.

Materials and Methods

Mice

The production and simple sequence-length polymorphism (SSLP) genotyping of (A/WySnJ × CAST/Ei)F1 × A/WySnJ mice for Chr 15 markers was described (7). The mice were housed in our pathogen-free mouse colony; protocols were approved by the Institutional Animal Care and Use Committee of the University of Wisconsin (Madison, WI).

Nucleic acid isolation and PCR

The DNA isolation, PCR, and electrophoresis were described (6). The expressed region of Bik was cloned and sequenced, and transcript abundance studies were performed with the PCR primers 5'-AGCTCAGCCTGGACAGAA CAC-3' and 5'-GGGGACAGTCAGAAAACACC-3'. The PCR primers for the intronic, Bcl-associated SSLP were 5'-TGTAGGACTTTCCAGTAGAG-3' and 5'-CCATGGAAACGGGACACACC-3'. The Bax and Bcl-2 specific primers were described (13).

RH mapping

The DNA from each T31 radiation hybrid (RH) cell line (Research Genetics, Huntsville, AL) and controls was amplified with Bik-specific PCR primers and analyzed for the unique 305-bp mouse gene product three times.

B cell enrichment, analysis, and culture

Flow-sorted splenic T1 B cells were obtained from adult mice 13 days after 500-rad irradiation (14). Splenocytes were stained following RBC lysis with biotin-goat Ab to mouse μ (Fab: Jackson ImmunoResearch Laboratories, West Grove, PA), and PE Ab to CD23 (clone B3B4), FITC Ab to CD21 (clone 7G6) and Cy5PE-streptavidin, all from BD PharMingen (San Francisco, CA).
Enriched transitional B cells were obtained from the spleens of 1-wk-old mice as described (15). A/J B cells, labeled with CellTracker Orange and A/WySnJ B cells labeled with CellTracker Green (Molecular Probes) were mixed in equal proportions and cultured. After culture, cells were analyzed for CellTracker dyes and propidium iodide (PI) exclusion. Some cell cultures also included splenic adherent cells (SAC). Splenocytes (106 cells in 1.0 ml/well; 48-well plates) were cultured overnight and nonadherent cells were removed before the purified B cells were added.

For apoptosis assays, RBC-depleted splenocytes (viability >95%) from neonatal mice were cultured in triplicate (2 × 106 cells in 1.0 ml/well; 48-well plates) in IMDM supplemented with 5% FBS, 50 μg/ml gentamicin, 100 μM penicillin, and 100 μg/ml streptomycin. Samples were analyzed by flow cytometry for apoptotic B cells after B220 staining, ethanol fixation, and PI staining.

Results

RH mapping of the Blk gene

To find the Chr 15 syntenic region, we searched the annotated human Chr 22 sequence (http://www.sanger.ac.uk/HGP/Chr22) for loci mapped in the human and the mouse (8). Sreb2 has been mapped in both species (8, 17) and is very close to D15 Mit118, the closest known SSLP to Bcmd (6). Near Sreb2 on human Chr 22 is the Bik locus (8, 9). The mouse Blk gene is believed to be the Bik homolog, because mouse BLK protein is 43% homologous to human BIK protein (18), but Blk has not been mapped.

We mapped Blk using the T31 RH mapping panel (Research Genetics). The mouse Blk gene retention pattern was analyzed by PCR and entered into a server at the Whitehead Center for Genome Research (Cambridge, MA) (http://www-genome.wi.mit.edu). The mouse Blk gene retention pattern was analyzed by PCR and entered into a server at the Whitehead Center for Genome Research (Cambridge, MA) (http://www-genome.wi.mit.edu). The RH mapping data were analyzed with Map Manager QT software (Whitehead Center for Genome Research (Cambridge, MA) (http://www-genome.wi.mit.edu)). The mouse Blk gene was mapped in both species (8, 17) and is very close to D15 Mit118 and D15 Mit33 at 48.6 cM on the Mouse Genome Database (MGI) (16) (http://www.informatics.jax.org/). The map positions in centimorgans were obtained from the 2000 Chromosome Committee Reports, Mouse Genome Database, Mouse Genome Informatics, The Jackson Laboratory (Bar Harbor, ME) (17) (http://www.informatics.jax.org/cctz).

Structure of the Blk gene and identification of a Blk gene SSLP

To confirm the RH mapping data, we sought a Blk-associated polymorphism between strains A/WySnJ and CAST/Ei that could be used to genotype our panel of Chr 15 recombinant (A/WySnJ × CAST/Ei)F1 × A/WySnJ mice (7). To this end we examined a Blk intron, anticipating polymorphisms between A/WySnJ and CAST/Ei due to less sequence conservation. To determine the genomic organization of Blk, basic local alignment search tool searches were done by comparing the Blk mRNA sequence to the publicly available High Throughput Genomic Sequence database (www.ncbi.nlm.nih.gov/genome/seq/MmHome.html). This search yielded a Chr 15 mouse genomic clone (National Center for Biotechnology Information LocusID AL583887) encompassing the Blk coding region. Comparing the mRNA sequence (18) to the genomic sequence, and following the gtag rule for intron/exon boundaries, yielded the gene structure (Fig. 1, B and C). The mouse Blk gene structure was similar to the human Blk gene, with five exons and four introns of similar size and functional organization. Inspection revealed a putative SSLP in intron 2. When genomic A/WySnJ and CAST/Ei DNA were amplified with PCR primer pairs designed to bracket this repeat, the two strains yielded distinct band sizes (CAST/Ei = 150 bp and A/WySnJ = 180 bp).

SSLP mapping of the Blk gene using Chr 15 recombinant strains

For fine resolution mapping of Bcmd, we assembled a panel of 17 (A/WySnJ × CAST/Ei)F1 × A/WySnJ N2 progeny with recombinations in the interval between D15 Mit144 and D15 Mit118, and measured splenic B cell phenotypes (7). The DNA from this recombinant panel was examined for the Blk-associated intronic SSLP. The genotyping results placed the Blk gene within the gray area shown in Fig. 2, consistent with the RH mapping data.

Overexpression of the Blk gene in A/WySnJ transitional B cells

We next sought evidence for a Blk coding sequence mutation. The cDNA from A/J and A/WySnJ B cells was PCR-amplified, cloned, and sequenced (data not shown). There were no sequence differences between these strains and the published Blk coding sequence (18). Therefore, we investigated the level of Blk transcript expression in sorted T1 B lymphocytes through the use of RT-PCR. Serially diluted cDNA was examined for expression of Blk and a
second proapoptotic gene, Bax, as well as the two antiapoptotic genes that interact with Blk, Bcl-2, and Bcl-x <sub>L</sub> (18) (Fig. 3). Blk transcripts were ~3-fold more abundant in the A/WySnJ T1 B cells than in the A/J B cells. This was in contrast to Bax and Bcl-2, which were not different, and Bcl-x <sub>L</sub>, which was increased ~2-fold.

**FIGURE 2.** Genetic mapping of the Blk-associated, intronic SSLP using the (A/WySnJ × CAST/Ei)F<sub>1</sub> × A/WySnJ recombinant N2 mapping panel. A Blk-associated SSLP was identified and the N2 mapping panel was genotyped for this SSLP. Interval mapping data analysis for this SSLP was accomplished by calculating the likelihood ratio statistic (16). The map positions are from the Mouse Genome Database (17).

**FIGURE 3.** Expression of proapoptotic and antiapoptotic transcripts in A/WySnJ and A/J T1 B cells. The T1 B cell total RNA was quantified, and equal amounts were reverse transcribed. A quantitative competitive PCR was done for G3PDH transcripts as described (24). The cDNA was normalized to G3PDH transcripts, serially diluted, amplified by PCR, and electrophoresed (A). The PCR products were imaged and quantified with TotalLab software, v. 1.10 (Nonlinear Dynamics, Durham, NC). The ratio of A/WySnJ:A/J transcript abundance was determined at each dilution and the mean for these ratios was calculated for one dilution series. This measurement was performed in triplicate on individual cDNA sample sets; a mean of means ± SEM was calculated from the triplicate measurements and graphed (B). The significance of the differences was calculated by a paired, two-tailed Student’s t test. For the Student t test all data points from the triplicate measurements of one cDNA sample were used to compare corresponding A/WySnJ and A/J band intensities. The Blk and Bcl-x <sub>L</sub> differences were significant (p < 0.05). The Bax and Bcl-2 differences were not significant. The values shown and the statistical analyses performed are from one representative experiment of two, each using separately derived T1 B cell samples.

**Premature apoptosis is a cell-autonomous trait of the A/WySnJ transitional B cells**

Increased Blk gene transcription in A/WySnJ transitional B cells suggested that these cells may have a defect leading to premature apoptosis. To test this hypothesis, splenocytes from neonatal mice were cultured and, at various times, collected and analyzed. At 1 wk of age the majority of splenic B cells are transitional B cells (19). Analysis of B220<sup>+</sup> cells with subdiploid amounts of DNA revealed a rapid B cell apoptosis rate for A/WySnJ compared with A/J cells (Fig. 4A). We next isolated the transitional B cells from 1-wk old mice as described (15). These B cells were cultured with or without SAC, recovered 1 day later, and analyzed by flow cytometry. Cell viability was determined by enumeration of B cells that excluded PI and appeared in the live lymphocyte scatter gate (Fig. 4B). The A/J B cells had more than twice the viability of the A/WySnJ B cells, in very good agreement with the apoptosis data. Adding A/J or A/WySnJ SAC had no effect on B cell survival for either A strain. Neither A/J nor A/WySnJ transitional B cells expressed Fas, ruling out Fas-mediated apoptosis as a mechanism for premature cell death (data not shown).

**Discussion**

The evidence presented in this work establishes that the Blk gene fits the Bcmd candidate gene profile by placement, expression polymorphism, and function. Two methods mapped Blk within the region with the highest probability of including Bcmd. Moreover, the A/WySnJ T1 B cells overexpressed Blk ~3-fold compared with A/J and showed a cell-autonomous defect leading to premature apoptosis, consistent with increased expression of a proapoptotic gene. The molecular basis for this expression polymorphism is not yet known.

The Blk gene encodes a death-inducing member of the BCL-2 protein family (10). The BCL-2 protein family members share one or more of the BCL-2 homology (BH) domains, BH1, BH2, BH3,
and BH4 (20). The Bcl-2 prosurvival gene was discovered at the characteristic chromosomal translocation breakpoint t(14;18) of follicular B cell lymphoma (21). The Bax pro-death gene was discovered due to interactions between Bcl-2-associated X protein (BAX) and BCL-2 (22). The BCL-2:BAX ratio determined a cell’s susceptibility to apoptosis, leading to the concept that the ratio of proapoptotic and antiapoptotic BCL-2 family proteins serves as a checkpoint or rheostat regulating apoptosis (20).

Bik was discovered due to interactions between Bik and prosurvival BCL-2 family members (10). Bik promoted cell death in transient transfection assays, and Bcl-2 and Bcl-xL suppressed this function. Thus, Bik is a proapoptotic protein and lacks all BH domains except BH3. The BH3-only proteins (BIK, BAD, BID, and HRK) are particularly potent death agonists (20). The mouse homolog of Bik, Bik, was discovered by searching GenBank for sequences encoding conserved BH3 domains (18). The BLK BH3 domain interacts directly with BCL-XL protein (18). In transfection experiments, Bik was a more potent death effector than Bik or Bax, and the apoptotic cell percentage doubled with each doubling of the BIK to Bcl-xL ratio, indicating that this ratio is a very sensitive apoptosis rheostat (18). In the present experiments, Bik was overexpressed 3-fold and Bcl-xL was overexpressed 2-fold, so the Bik:Bcl-xL ratio was higher in the A/WySnJ T1 B cells than in the control cells.

Little is known about regulation of Bik transcription (18). Jiang and Clark (11) showed that surface IgM but not surface IgD ligation initiated Bik transcription and apoptosis in B104 B lymphoma cells, a model for B cell Ag receptor (BCR)-mediated negative selection. We reported that excessive apoptosis occurs at the T1→T2 transition of B cell maturation in A/WySnJ mice (5). The T1 B cells are IgM+IgD− and retain their sensitivity to negative selection via a BCR-mediated apoptotic signal, whereas the T2 B cells are IgM+IgD+ and apparently insensitive to negative selection (23). Thus, our evidence is consistent with a model wherein the A/WySnJ B cells have difficulty making the T1→T2 transition because they express inappropriately high levels of a BCR-induced death gene, and excessive apoptosis is the result.

Intriguingly, Thompson et al. (12) very recently identified a new receptor for B cell activating factor belonging to the TNF family (BAFF), and showed that the gene encoding this receptor, provisionally termed Baffr, maps to human Chr 22q13.1, which is syntenic to the mouse Chr 15 Bcmd region. Also, they showed by Southern blotting that A/WySnJ and A/J genomic DNA are polymorphic at this locus, and that the A/WySnJ Baffr transcript was shortened and exon 3 was not intact. The exact nature of the Baffr mutation in A/WySnJ is not known, and whether the A/WySnJ BAFF receptor (BAFF-R) retains any signaling function is also unclear. Nevertheless, their studies together with ours and those of Jiang and Clark (11) suggest a very interesting model, wherein membrane IgM and BAFF-R may compete for control of Bik transcription and thus the maturation or apoptosis fate of the transitional B cells. The IgM may enhance Bik transcription and BAFF-R may inhibit it. The outcome of the competition might determine peripheral B cell homeostasis. The A/WySnJ T1 B cells may be unable to inhibit Bik transcription due to a BAFF-R defect, explaining their premature apoptosis phenotype. It is also interesting to note that Baffr and Bik are only ~1.3 Mb apart in the human genome, raising the possibility that chromatin remodeling during peripheral B cell development may lead to coordinated expression of these two genes, which may play critical roles in the life or death fate of the transitional B cell. Additional experiments will no doubt test these hypotheses and provide a detailed model of this most interesting B cell developmental transition.

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References


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