NF-κB Family Proteins Participate in Multiple Steps of Hematopoiesis through Elimination of Reactive Oxygen Species

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To examine the roles for NF-κB family proteins in hematopoiesis, we first expressed dominant negative Rel/NF-κB (IκBSR) in a factor-dependent cell line, Ba/F3. Although IκBSR neither affected thrombopoietin-independent nor gp130-mediated growth, it suppressed interleukin-3- and erythropoietin-dependent growth at low concentrations. In addition, IκBSR enhanced factor-deprived apoptosis through the accumulation of reactive oxygen species (ROS). When expressed in normal hematopoietic stem/progenitor cells, IκBSR induced apoptosis even in the presence of appropriate cytokines by accumulating ROS. We also expressed IκBSR in an inducible fashion at various stages of hematopoiesis using the OP9 system, in which hematopoietic cells are induced to develop from embryonic stem cells. When IκBSR was expressed at the stage of Flk-1+ cells (putative hemangioblasts), IκBSR inhibited the development of primitive hematopoietic progenitor cells by inducing apoptosis through the ROS accumulation. Furthermore, when IκBSR was expressed after the development of hematopoietic progenitor cells, it inhibited their terminal differentiation toward erythrocytes, megakaryocytes, and granulocytes by inducing apoptosis through the ROS accumulation. These results indicate that NF-κB is required for preventing apoptosis at multiple steps of hematopoiesis by eliminating ROS.

The Rel/NF-κB family consists of five members (c-Rel, p65 (RelA), RelB, p50 (NF-B1), and p52 (NF-B2)) (1, 2). Each subunit shares a conserved N-terminal domain that encompasses sequences required for DNA binding, dimerization, and nuclear localization that is called the Rel homology domain. c-Rel, p65, and RelB each possess distinct transactivation domains in the C terminus, whereas p50 and p52, consisting of the only N-terminal domain, lack these intrinsic transactivation domains. Although these members form homodimers or heterodimers in various combinations, a heterodimer, p50/p52, is the most common form in mammalian cells and is specifically called NF-κB. Under the unstimulated condition, the major proportion of Rel/NF-κB is retained in cytoplasm by its inhibitor IκB proteins as an inactive complex. Various stimuli such as pro-inflammatory cytokines and viruses activate the IκB kinase complex and phosphorylate IκB. Upon phosphorylation, IκB is degraded by the ubiquitin/proteasome pathway. The released Rel/NF-κB enters the nucleus, binds to target DNA elements, and initiates transcription. Until now, Rel/NF-κB has been reported to promote the expression of over 150 target genes that regulate immune response, stress response, cell growth, or survival.

Mice homozygous for null mutations in genes encoding Rel/NF-κB subunits have revealed the unique roles of each family member protein (3, 4). Although p65 is not essential for the generation of mature hematopoietic cells, p65−/− mice are embryonic lethal at embryonic day 15 because of the massive apoptosis in the liver (5). Also, the cells from p65−/− mice are susceptible to apoptosis induced by various reagents. In contrast, c-Rel, RelB, p50, and p52 are dispensable for embryogenesis. c-Rel is specifically expressed in lymphocytes, monocytes, granulocytes, and erythroid cells. Although the number of hematopoietic cells is normal in c-Rel−/− mice, both T- and B-lymphocytes have defects in proliferative responses to various mitogens, isotype switching, and cytokine production (6–9). Also, mutant mice lacking RelB, which is specifically expressed in dendritic cells and B-lymphocytes, exhibit defects in acquired and innate immunity (10, 11). Although p50 is ubiquitously expressed, p50 is not essential for hematopoiesis. However, p50−/− mice reveal multiple defects in the immune system (12). Mature quiescent p50−/− B-lymphocytes are susceptible to apoptosis and show poor proliferative responses to the CD40 ligand and lipopolysaccharide (8, 12). Also, knockout mice for p52, of which expression is restricted to the epithelium of the stomach and selected areas of hematopoietic organs such as the thymic medulla and the marginal zone of the spleen, show abnormalities in splenic and lymph node structure (13, 14). These lines of evidence suggest that Rel/NF-κB family proteins are solely important for immune responses mediated by B- and T-lymphocytes. However, the redundancy among these family members was supposed to veil certain phenotypes in the single mutant mice. This hypothesis was supported by the subsequent findings that mice lacking two Rel/NF-κB subunits exhibit novel phenotypes or exaggerated versions of those seen in the single mutants. Examples include the blockage of lymphocyte development in p50−/− p65−/− mice (15), greater severity of the inflammatory disease in p50−/− RelB−/− mice than observed in RelB−/− mice (16), and impaired osteoclast and B-cell development in p50−/− p52−/− mice (14, 17). Furthermore, the combined loss of c-Rel and p65 was shown to result in impaired erythropoiesis and deregulated expansion of granulocytes by transplantation experiments, indicating that, besides lymphopoiesis, Rel/NF-κB is also required for normal hematopoiesis in the other lineages (18). However, at present, the precise roles of NF-κB in hematopoiesis in the respective lineage remain undetermined. Also, molecular mechanism through which Rel/NF-κB family proteins regulate hematopoiesis is still unknown.

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Reactive oxygen species (ROS)1 including superoxide anions (O2·−), organic peroxides, and hydroxyl radicals are by-products of oxidative phosphorylation and constantly generated in all aerobic cells during normal metabolism (19, 20). Although ROS are required for the physiologic function of the cells, excessive ROS cause apoptosis through several mechanisms as activation of c-Jun N-terminal kinase, disruption of mitochondrial membrane potential (ΔΨm), and/or direct activation of caspase cascades (21). Under normal circumstances, ROS are eliminated by antioxidant enzymes. The scavenger enzyme, superoxide dismutase (SOD) (MnSOD or Cu/ZnSOD), converts superoxide anions to H2O2. H2O2 is subsequently detoxified by catalase or glutathione peroxidase (19, 20). This redox regulation is essential for protecting cells from apoptosis.

In the present study, we expressed IκBζ, which can inhibit the function of Rel/NF-κB family proteins as a dominant negative mutant, in IL-3-dependent cell line Ba/F3 and normal hematopoietic stem/progenitor cells and assessed its effects on cytokine-dependent growth and survival. We also evaluated the roles for NF-κB by expressing IκBζ in an inducible manner at various stage of hematopoiesis using the OP9 system, in which hematopoietic cells are induced to develop from embryonic stem (ES) cells. We found here that Rel/NF-κB family proteins play critical roles in preventing apoptosis at multiple steps of hematopoiesis by eliminating ROS.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The antibodies against IκBζ, C-21 and B-9, were purchased from Santa Cruz Biotechnology. Mn(III) tetrakis (4-benzoic acid) porphyrin chloride; NAC, and thioredoxin X (TRX) were purchased from Calbiochem-Behring Corp. The antibodies against IκBζ and NF-κBp65 (Becton Dickinson) (23). Surface phenotype of the cells was analyzed by FACS (Becton Dickinson). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from K. Scharffetter-Kochanek (University of Cologne, Hamburg, Germany), and TRX was from Dr. J. Yodoi (Kyoto University, Kyoto, Japan).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assays—3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide rapid colorimetric assays were performed as previously described (22).

Flow Cytometric Analyses—DNA content of cultured cells was evaluated by retaining DNA-poorloid isolated and analyzed on FACScan (Becton Dickinson). Surface phenotype of the cells was analyzed by the direct immunofluorescence method. Total ROS were detected with RedSensorTM Red CC-1 (R14060); O2 by dihydroethidium (D-1168), and H2O2 with CM-H2DCFDA (C-6827) (Molecular Probes) as described previously (24). ΔΨm was detected with DePhealerTM (Trevigen) according to the manufacturer's instructions.

Northern Blot and Reverse Transcription-PCR—Northern blot and reverse transcriptase-PCR analyses were performed as described previously (25). The sequences of primer sets (a sense primer/antisense primer) were as follows: Bel-2, 5′-CTTGGAAAATACAGACATCGGCCGA- G3′/5′-GGATGGTCTTGTACCTTGGG-3′; Bel-XL, 5′-CAGTGTGGTGGGAAGGCTCTGA-3′/5′-AAGGTGCTGGACCCG-3′; A1, 5′-GAGGCTTGATGTAATCGG-3′/5′-GACGTTTGTGCACGTCCTATGAG- GC3′; glutathione peroxidase, 5′-TCGAACCTGAATACAAAGGCTCAG-3′/5′-CACCATTGCAAGGAACCCAG-3′; TRX, 5′-ATCATTTGGCAAGGTTCACCA-3′/5′-CAAGCAGGTTTCTTGACAGG-3′; xanthine oxidase, 5′- TTATGTCCCTACTCGCCACA-3′/5′-TGTTGGTCTTGGACATATAA-3′; Pdx1, 5′-GAGCAGCCAGAAGACTCTTGTG-3′/5′-AGAGAATGGTCTGCCAAAAC-3′; and β-actin, 5′-CATCACTATGGCAAGCAGAGC-3′/5′-ACCGGAGTCTGAAAGTGCT-3′. Immunoblot Analysis—Immunoblot analysis was performed by the chemiluminescence system, and immunoblotting were performed as described previously (26). Immunoreactive proteins were visualized with the chemiluminescence detection system (PerkinElmer Life Sciences).

Measurement of the Intracellular Glutathione—The amount of intracellular glutathione was measured using the glutathione assay kit (Cayman Chemical) according to the manufacturer’s instruction.

Stable and Inducible Expression of IκBζ in Ba/F3 Cells—A murine IL-3-dependent cell line Ba/F3 was cultured in RPMI1640 supplemented with 10% fetal bovine serum and 1 mg/ml of murine IL-3. To stably express IκBζ in Ba/F3 cells, we transfected 30 μg of pcDNA3.1 (Invitrogen) containing IκBζ by electroporation (300 V, 960 microfarads). After the culture with 1.0 mg/ml of G418, several single clones and a mixed clone were subjected to further analyses. To inducibly express IκBζ, we used a LacSwitch™ II inducible expression system (Stratagene) as described previously (27). In short, we initially transfected an expression vector for Lac repressor into Ba/F3 cells. After the culture with hygromycin, we selected one clone in which Lac repressor was most intensively expressed. We further transfected pOPRSVI containing IκBζ into this clone and cultured with G418. In this system, the expression of the target cDNA is initiated when isopropyl-β-D-thiogalactopyranoside (IPTG) is added to the cultured cells. We selected several clones in which IκBζ was effectively induced by IPTG and performed further experiments. We further introduce the EPO receptor, TPO receptor, and G-CSF/pgp130 consisting of the extracellular domain of G-CSFR and cytoklaytomic domain of gp130 (28) into a Lac-inducible IκBζ clone using the puromycin (Puro)-resistant plasmid.

OP9 System to Develop Hematopoietic Cells from ES Cells—E14tg2a ES cells and OP9 stromal cells were maintained as described previously (29, 30). To induce differentiation toward hematopoietic cells, ES cells were deprived of leukemia inhibitory factor and seeded onto confluent OP9 cells on six-well plates at a density of 104 cells/well in α-minimum essential medium supplemented with 20% fetal bovine serum. After 4.5 days, the cells were harvested by 0.25% trypsin-EDTA, and Flk-1+ cells were purified by fluorescence-activated cell sorter sorting. The sorted cells were replated onto OP9 cells at a density of 105 cells/well of 6-well plate or 7–8 × 103 cells/10-cm dish and cultured under the indicated conditions.

Tetracycline-Inducible Regulated Expression of IκBζ in ES Cells—To inducibly express IκBζ in ES cells, we utilized a Tet-off system as reported previously (31), in which transcription of the target mRNA is initiated in response to the removal of Tet. Briefly, we initially introduced pCMV-CAG20-1×T and pUHD10–3-puro carrying pUHD10–3-puro (800 V, 3 microfarads). pCAG20-1×T stably expresses the Tet-regulated transactivator under the control of the modified chicken β-actin promoter, and pUHD10–3-puro contains the Puro-resistant gene downstream of the Tet-off-cytogamolovirus promoter. Thus, if the Tet-off system efficiently works in the transfected cells, these cells are expected to be Puro-resistant in the presence of the medium and Puromycin (Puro) in the absence of Tet. According to this method, we selected one ES clone designated E14 by the culture with 1 μg/ml of Puro and/or 1 μg/ml of Tet, in which the Tet-regulatory system works most effectively. We further transfected pUHD10–3-IκBζ-IRES-GFP, which can inducibly express IκBζ and GFP as a single mRNA through internal ribosome entry site in response to the removal of Tet, together with the neomycin-resistant plasmid pBSR1-neo. After the culture with 0.2 mg/ml of G418 in the Tet+ medium, we selected several clones that can inducibly express GFP response to the Tet deprivation. Subsequently, we examined the Tet-regulated expression of IκBζ in the Tet+ and Tet− medium in these clones, and one clone was subjected to further analyses.

Preparation of Conditioned Media Containing High Titer Retrovirus Particles—The conditioned media containing high titer retrovirus particles were prepared as described previously (25). Briefly, pMSCV-IκBζ-neo or an empty pMSCV-neo (Clontech) was transfected into an ectopic packaging cell line Plat-E. After 12 h, the cells were washed and cultured for 48 h. Then the supernatant containing virus particles was collected, centrifuged, and concentrated by 50-fold in volume.

Retrovirus Transfection into Murine Hematopoietic Stem/Progenitor Cells—Lin− bone marrow cells harvested from C57BL/6 mice pretreated with 150 mg/kg of 5-fluorouracil for 2 days. Lin− Sca-1− cells were isolated with MACS™ (Miltenyi Biotec) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in the presence of murine (m) IL-3 (10 ng/ml), mSCF (50 ng/ml), mFLT3 ligand (L) (10 ng/ml), and human (h) IL-6 (10 ng/ml) for 48 h. Then the cells were cultured with 10% volume of conditioned media containing high titer retrovirus and 8 mg/ml of Polybrene. After 48 h.

1 The abbreviations used are: ROS, reactive oxygen species; TPO, thrombopoietin; EPO, erythropoietin; SCF, stem cell factor; Tet, Tetra cyclic; h, human; m, murine; IL, interleukin; ES, embryonic stem; SOD, superoxide dismutase; TRX, thioredoxin X; MnSOD, Mn(III) tetrakis (4-benzoic acid) porphyrin chloride; NAC, N-acetyl l-cysteine; MCI, 3-methyl-1-phenyl-2-pyrazolin-5-one; IPTG, isopropyl-β-D-thiogalactopyranoside; Puro, puromycin; GFP, green fluorescent protein; GFP-PF, GFP-positive fraction; G-CSF, granulocyte colony-stimulating factor receptor; SCF, colony forming unit.
The expression of IκBSR in Ba/F3 cells was examined in two single clones (Cl1 and Cl2) and a mixed clone (Mix) by Northern blot analysis. Also, we prepared several Lac-inducible clones from Ba/F3, in which the expression of IκBSR was induced by the IPTG treatment. The expression of IκBSR was examined in two clones (Cl1 and Cl2) before and after 2-h IPTG treatment by Northern blot analysis. The expression of IκBSR was intensively expressed in Ba/F3/Cl1 and Ba/F3/IκBSRCl1 cells, which were deprived of IL-3, cultured, and subjected to flow cytometric and Northern blot analyses at the indicated times. The expression of IκBSR was induced by the IPTG treatment in Lac-inducible Ba/F3 Cl1 cells. The cells of each clone were seeded at a cell density 100/μl, cultured under the indicated conditions for 48 h, and then subjected to 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide assays. The results are shown as the means ± S.D. of triplicate cultures.

To examine the effects of IκBSR on cytokine-dependent growth of Ba/F3 cells, we further introduced EPO receptor, TPO receptor, and G-CSFR/gp130. As shown in Fig. 1B, these clones dose-dependently proliferated in response to IL-3, EPO, TPO, and the gp130 ligand (gp130L) (we here denote G-CSF as gp130L when it acts on G-CSFR/gp130), respectively. Although IPTG-induced IκBSR did not affect TPO- or gp130L-dependent growth, it significantly inhibited IL-3-dependent growth at low concentrations (from 0.001 to 0.03 ng/ml) with a statistical significance (p < 0.05). Also, IκBSR suppressed EPO-dependent growth at low concentrations (from 0.005 to 0.15 unit/ml) with a statistical significance (p < 0.05) (Fig. 1B).

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**RESULTS**

**IκBSR Partially Suppressed Cytokine-dependent Growth of Ba/F3 Cells**—To examine the roles for Rel/NF-κB in the growth of hematopoietic cells, we stably expressed DN-NF-κB (IκBSR) in a murine IL-3-dependent cell line, Ba/F3; we designated two single clones as Ba/F3/IκBSRC11 and Ba/F3/IκBSRC12 and a mixed clone as Ba/F3/IκBSRMix, respectively. We also prepared several Lac-inducible clones from Ba/F3 cells, in which IκBSR was inducibly expressed by the IPTG treatment. As shown in Fig. 1A (left panel), IκBSR was intensively expressed in Ba/F3/IκBSRC11, Ba/F3/IκBSRC12, and Ba/F3/IκBSRMix, but not in Ba/F3/Mock. Also, the expression of IκBSR was effectively induced by the IPTG treatment in Lac-inducible clones (Fig. 1A, right panel). To examine the effects of IκBSR on cytokine-dependent growth of Ba/F3 cells, we further introduced EPO receptor, TPO receptor, and G-CSFR/gp130. As shown in Fig. 1B, these clones dose-dependently proliferated in response to IL-3, EPO, TPO, and the gp130 ligand (gp130L) (we here denote G-CSF as gp130L when it acts on G-CSFR/gp130), respectively. Although IPTG-induced IκBSR did not affect TPO- or gp130L-dependent growth, it significantly inhibited IL-3-dependent growth at low concentrations (from 0.001 to 0.03 ng/ml) with a statistical significance (p < 0.05). Also, IκBSR suppressed EPO-dependent growth at low concentrations (from 0.005 to 0.15 unit/ml) with a statistical significance (p < 0.05) (Fig. 1B).

Next, we examined the effects of IκBSR on factor-deprived apoptosis in Ba/F3 cells. In DNA content analysis, a subdiploid fraction formed from apoptotic cells was hardly detectable in Ba/F3/Mock cells under IL-3-deprived conditions up to 24 h (Fig. 1C). In contrast, 55.2% of Ba/F3/IκBSRMix cells led to apoptosis after 24-h IL-3 deprivation, indicating that IκBSR enhanced fac-
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Fig. 2. Roles for ROS in IxBSR-enhanced apoptosis. A, total ROS were detected with Red CC-1, O$_2^-$ with dihydroethidium, and H$_2$O$_2$ with CM-H$_2$DCFDA in Ba/F3/Mock and Ba/F3/BSR cells before and after IL-3 depletion by flow cytometry. Thin black line, Ba/F3/Mock at 0 h; thick black line, Ba/F3/Mock at 12 h; thin red line, Ba/F3/BSR at 0 h; thick red line, Ba/F3/BSR at 12 h. B, Ba/F3/IxBSRC11 was further transduced with a Mock vector, MnSOD, and TRX, respectively. These clones were deprived of IL-3, cultured for the indicated times, and subjected to DNA content analysis. The percentage of apoptotic (Apo) cells is indicated.

Roles for ROS in IxBSR-enhanced apoptosis. We also analyzed the changes of $\Delta\Psi_m$ during these cultures by flow cytometric analysis, in which the cells with the decreased $\Delta\Psi_m$ are detected as those with the increased green fluorescent intensity and decreased red fluorescent intensity in the lower right area (Fig. 1D). After 18 h of IL-3 depletion, $\Delta\Psi_m$ decreased in only 6.2% of Ba/F3/Mock, whereas it decreased in a substantial fraction (31.9%) of Ba/F3/IxBSRClone. As for this mechanism, we found that the expression of Bcl-2 and Bcl-XL mRNA declined to an undetectable level in both Ba/F3/Mock and Ba/F3/IxBSR after IL-3 deprivation (Fig. 1E). However, we did not detect an apparent difference in their expression levels between these clones that can explain IxBSR-enhanced apoptosis.

IxBSR Accumulated ROS in Ba/F3 Cells—Because Rel/NF-κB is involved in the metabolism of ROS (24), we examined the state of ROS accumulation in Ba/F3/Mock and Ba/F3/IxBSR during IL-3 deprivation. We detected total ROS with a fluorescent sensor Red CC-1, O$_2^-$ with dihydroethidium, and H$_2$O$_2$ with CM-H$_2$DCFDA by flow cytometry. As shown in Fig. 2A, a small amount of total ROS, O$_2^-$, and H$_2$O$_2$ accumulated in Ba/F3/Mock cells after 12 h of IL-3 starvation. As compared with Ba/F3/Mock cells, a far more increased amount of total ROS, O$_2^-$, and H$_2$O$_2$ were already accumulated in Ba/F3/IxBSRClone cells even in the presence of IL-3, which did not further increase after IL-3 depletion. To access the roles of ROS in IxBSR-enhanced apoptosis, we overexpressed ROS scavenger enzymes MnSOD and TRX in Ba/F3/IxBSRC11, respectively. Although Mock-transfected cells led to severe apoptosis after IL-3 depletion, MnSOD and TRX almost completely canceled this apoptosis (Fig. 2B), suggesting that ROS may be involved in the IxBSR-enhanced apoptosis.

IxBSR Inhibited Cytokine-dependent Survival of Normal Hematopoietic Cells—We next analyzed the effects of IxBSR on the growth and survival of normal hematopoietic cells. We introduced IxBSR into murine Lin$^-$/Sca-1$^-$ cells with the retrovirus system. After the selection with G418, we further cultured these cells in the presence of IL-3, SCF, IL-6, and FLT3L for 7 days. Although only 11.9% of Mock-transfected cells led to apoptosis, 75.9% of IxBSR-transfected cells underwent apoptosis (Fig. 3A). We also examined the effects of IxBSR on hematopoietic stem/progenitor cells with colony assays. As shown in Fig. 3B, IxBSR drastically reduced the number of CFU-Mix, BFU-E, and CFU-GM. These results indicated that although IxBSR showed little effect on survival of Ba/F3 cells cultured with an appropriate cytokine, it disrupted cytokine-dependent survival of normal hematopoietic cells. As for this mechanism, we found that although $\Delta\Psi_m$ decreased in only 18.7% of Mock-transfected cells, $\Delta\Psi_m$ was disrupted in 85.7% of IxBSR-transfected cells after 5 days (Fig. 3C). Furthermore, flow cytometric analysis showed that total ROS, O$_2^-$, and H$_2$O$_2$ accumulated in IxBSR-transfected cells under the culture with SCF, IL-3, IL-6, and FLT3L (Fig. 3D, upper panel). Similarly, IxBSR induced the ROS accumulation under the cultures with different cytokine combinations (Fig. 3D, lower panel). Regarding the mechanisms of IxBSR-induced ROS accumulation, we found that the intracellular glutathione was reduced in IxBSR-transfected cells (Fig. 3E). Also, the semiquantitative reverse transcriptase-PCR analysis showed that the expression of several ROS scavenger enzymes was suppressed in IxBSR-transfected cells: MnSOD, about 80% suppression at 26–35 cycles; glutathione peroxidase, about 30% suppression at 26–32 cycles; and TRX, about 25% suppression at 26–35 cycles (Fig.
In contrast, there was a slight difference in the expression of the ROS-regulating enzymes Prdx1 and xanthine oxidase. In addition, the expression of several anti-apoptotic Bcl-2 family members was also inhibited by Ix/H9260 BSR: Bcl-2, about 60% suppression at 26–35 cycles; and Bcl-XL and A1, about 30% suppression at 26–35 cycles. These results indicated that Ix/H9260 BSR disrupted two major anti-apoptotic systems: ROS scavenger cascades and the Bcl-2 family members. Next, we assessed the roles for ROS in Ix/H9260 BSR-induced apoptosis by adding several ROS scavenger enzymes into the culture medium. As shown in Fig. 3G, MCI, NAC, and TRX efficiently protected normal hematopoietic cells from Ix/H9260 BSR-induced apoptosis, whereas MnTBAP was hardly effective. Because MCI mainly acts on NO− and NAC, TRX on H2O2, and MnTBAP on O2−, NO− and H2O2 were supposed to be more toxic than O2− in normal hematopoietic cells.

IxBSR Inhibited the Development of Hematopoietic Cells from ES Cells—To examine the roles for Rel/NF-κB in the development of hematopoietic cells, we utilized the OP9 system (29). In this system, ES cells, which are deprived of leukemia inhibitory factor and cultured on OP9 cells, develop into Flk-1+(so-called hemangioblasts that have an ability to differentiate into both endothelial cells and hematopoietic cells) after 4.5-day cultures (Fig. 4A). To develop hematopoietic cells with high purity, we sorted Flk-1+ cells by fluorescence-activated cell sorter and further cultured on OP9 cells (Fig. 4A). In this system, we inducibly expressed IxBSR with the Tet-off system, in which the expression of IxBSR is induced by the Tet removal from the culture medium (31). In addition, because our Tet-off vector is a bicistronic vector, it can express IxBSR and GFP through internal ribosome entry site as a single mRNA in response to the Tet removal. We cultured ES clones each transfected with IxBSR and a Mock vector (designated as E14/IxBSR and E14/Mock, respectively) without leukemia inhibitory factor on OP9 cells for 4.5 days. At this point, about 35% of the cultured cells were Flk-1+(data not shown), and we sorted these cells and cultured on OP9 cells with or without Tet for 48 h. As shown in Fig. 4B, the expression of GFP was induced in most of E14/IxBSR cells under the culture without Tet. Also, immunoblot analysis showed that IxBSR protein was effectively induced by Tet deprivation in E14/IxBSR cells.

After the culture with Tet for 14 days, 29.2% of E14/IxBSR
cells became to be positive for an erythroid marker Ter119, 30.5% for a macrophage marker Mac1, and 18.7% for a B-lymphoid marker B220 (Fig. 4C, left panel), indicating that our OP9 system could effectively yield erythroid cells, macrophages and B-lymphocytes. In contrast, when Tet was washed out from the culture medium, Ter119^+^, Mac^+^, and B220^+^ cells scarcely developed from E14/IxBBSR cells (Fig. 4C, right panel). In addition, we found that 74.7% of E14/IxBBSR cells led to apoptosis after 12-day culture without Tet, whereas only 19.7% of the cells underwent apoptosis in the presence of Tet (Fig. 4D). Also, we found that total ROS accumulated under the culture without Tet at days 6 and 10 (Fig. 4E). These results suggested that IxBBSR might inhibit the development of hematopoietic cells by inducing apoptosis through the ROS accumulation.

**Rel/NF-κB Was Required for the Development of Primitive Hematopoietic Progenitors**—In an attempt to determine at which step IxBBSR inhibited the development of hematopoietic cells, we initially analyzed the effect of IxBBSR on the development of primitive hematopoietic progenitors. We cultured Flk-1^+^ cells in the presence or absence of Tet for 4 days and performed flow cytometric analysis at day 8.5 (Fig. 5A). After the culture with Tet, 66.4% of E14/IxBBSR cells were positive for CD45, 17.9% were positive for CD34, and 28.8% were positive for c-Kit (Fig. 5B), indicating that a substantial fraction of the cultured cells developed into primitive hematopoietic progenitors. Next, we cultured E14/IxBBSR cells without Tet in the presence or absence of MCI and evaluated the effects of IxBBSR in the GFP-positive fraction (GFP-PF), because IxBBSR was supposed to be effectively induced in this fraction. In the absence of MCI, CD45^+^, CD34^+^, and c-Kit^+^ fractions were severely reduced in the GFP-PF as compared with those after the culture with Tet; the relative percentages in the GFP-PF were: CD45^+^, 17.1%; CD34^+^, 7.6%; and c-Kit^+^, 14.3% (shown in parentheses in Fig. 5B). However, when MCI was added, MCI significantly restored these fractions in the GFP-PF: CD45^+^, 54.1%; CD34^+^, 28.6%; and c-Kit^+^, 21.8%. Regarding this mechanism, we found that although only 5.39% of the cultured cells were annexin V^+^ after the culture with Tet, 57.1% of the cells were Annexin V^+^ in the GFP-PF after the culture without Tet and MCI, which was reduced to 12.0% by MCI. These results indicated that IxBBSR inhibited the development of primitive hematopoietic progenitors by inducing apoptosis, largely through the ROS accumulation.

**Rel/NF-κB Was Also Required for the Subsequent Maturation of Hematopoietic Cells from Primitive Hematopoietic Progenitors**—Next, we examined the roles for Rel/NF-κB in the subsequent maturation stage of hematopoiesis. For this purpose, we cultured Flk-1^+^ cells with Tet for 4.5 days and confirmed the development of primitive hematopoietic progenitors from E14/IxBBSR cells by flow cytometry: CD45^+^, 51.6%; CD34^+^, 10.1%; and c-Kit^+^, 27.2% (flow cytometric data not shown). Then we further cultured these E14/IxBBSR cells under the indicated conditions up to day 12 (Fig. 6A). As shown in Fig. 6B, substantial fractions of E14/IxBBSR cells cultured with Tet developed into Gr-1^+^ (myeloid), Ter119^+^ (erythroid), and CD41^+^ (megakaryocytic) cells after the respective cultures: the relative proportion of Gr-1^+^ cells in the GFP-negative fraction, Ter119^+^ cells cultured with Tet without MCI, and CD41^+^ cells after the culture with Tet; the relative percentages in the GFP-PF were: CD45^+^, 37.2%; CD34^+^, 14.3%; and c-Kit^+^, 29.8% (shown in parentheses in Fig. 6B). In contrast, when E14/IxBBSR cells were cultured without Tet and MCI, the relative proportions of these mature cells severely decreased in the GFP-PF: Gr-1^+^, 23.7%; Ter119^+^, 14.1%; CD41^+^, 29.8% (shown in parentheses). However, MCI almost completely restored these fractions: Gr-1^+^, 43.8%; Ter119^+^, 52.4%; and CD41^+^, 75.6% (shown in parentheses). As for this reason, we found that IxBBSR induced apoptosis in 51.8% of E14/IxBBSR cells cultured without Tet and that this apoptotic fraction was reduced to 14.1% by MCI (Fig. 6B). These results indicated that Rel/NF-κB was also necessary for hematopoietic cells to undergo terminal differentiation toward various lineages.

**DISCUSSION**

Rel/NF-κB family proteins play important roles in various biologic phenomena such as immune responses, stress responses, and inflammation. Also, knockout mice for each subunit revealed that these factors crucially control cell growth and survival of B- and T-lymphocytes (8, 9, 12). In addition, recent studies have demonstrated that these factors are constitutively activated in various types of hematologic malignancies, including lymphomas (Hodgkin’s disease, adult T-cell leukemia/lym-
phoma, Burkitt's lymphoma, and anaplastic lymphoma), multiple myeloma, acute myeloid leukemia, and acute lymphoblastic leukemia, thereby causing these diseases and/or affecting their pathophysiologic aspects (32). These results suggest that the appropriate regulation of NF-κB activity is required for normal hematopoiesis.

In a previous study, IL-3 and granulocyte-macrophage colony-stimulating factor were reported to activate NF-κB through the activation of Jak2 (34). Also, EPO was shown to activate NF-κB through the activation of Jak2 (34). In these studies, NF-κB was considered to mainly mediate anti-apoptotic functions of these growth factors. On the other hand, NF-κB has been shown to regulate cell growth through the induction of c-myc and cyclin D1 in other cell types (35, 36). Supporting these results, B- and/or T-lymphocytes obtained from c-Rel−/− or p50−/− mice showed the decreased proliferative response to various mitogens (8, 9, 12). Moreover, constitutively activated NF-κB was reported to play a crucial role in regulating the growth of Hodgkin's lymphoma cells (37, 38). However, in the present study, we found that IκBSR neither influenced TPO- nor gp130L-dependent growth and showed only a limited degree of growth inhibitory effect on IL-3- and EPO-dependent growth of Ba/F3 cells. Also, when we prevented IκBSR-induced apoptosis by MCI, cytokine-dependent growth of normal hematopoietic cells was hardly suppressed by IκBSR (data not shown). Therefore, it was speculated that, although NF-κB is important for the growth regulation in some aspects of hematopoiesis such as

**Fig. 5. Effects of IκBSR on the development of hematopoietic stem cells.** A, experimental design 2. Sorted Flk-1+ cell were replated onto OP9 cells, cultured with IL-3, EPO, and SCF in the presence or absence of Tet for 4 days and subjected to flow cytometric analysis. In some experiments, MCI was added to Tet+cultures as indicated. B, the expression of CD45, CD34, and c-Kit was examined by the direct immunofluorescence method. Also, the intensity of GFP and reactivity to phycoerythrin (PE)-labeled annexin V were analyzed by flow cytometry. The percentage of each fraction is indicated. The relative frequency of the upper right panel in the GFP-PF is shown in parentheses.
inflammatory responses, immune responses, and oncogenesis, it may be dispensable for cytokine-dependent growth of normal hematopoietic cells.

As for the mechanisms of Rel/NF-κB-mediated cell survival, Rel/NF-κB has been shown to transcriptionally regulate the expression of anti-apoptotic Bel-2 family members Bel-2, Bcl-xl, and Bcl-xL in various cell types (1, 2). Also, Rel/NF-κB promotes the expression of the Bcl-2 in EB virus-transformed B lymphocytes, probably through the indirect mechanism (39). In addition, NF-κB has been reported to prevent apoptosis by reducing the oxidative stress through the induction of the ROS scavenger enzyme, MnSOD (24, 40). In agreement with these findings, we found here that IκBα-, Bcl-2 and caspase inhibitors IκBα-1 and IκBα-2 in various media. Furthermore, regarding the mechanism of the ROS accumulation in IκBα-introduced cells, we found that the amount of the intracellular glutathione and the expression of glutathione peroxidase and TRX were reduced by IκBα-introduced cells as well as that of MnSOD, suggesting that NF-κB would regulate the expression of these molecules. In addition, our experiments showed that catalytic antioxidants efficiently cancelled IκBα-enhanced apoptosis. This result suggests that the antioxidant activity of NF-κB would be more important to protect hematopoietic cells from apoptosis, whereas we should be aware that Bcl-2 family members also play a certain role in NF-κB-mediated cell survival.

In this study, MCI, NAC, and TRX but not MnTBAP efficiently cancelled IκBα-enhanced apoptosis, suggesting that NO⁻ and H₂O₂ were more toxic to normal hematopoietic stem/progenitors cells than O₂⁻. However, based on the fact that MnSOD⁻/⁻ mice exhibited severe anemia caused by ineffective erythropoiesis, O₂⁻ was also assumed to be toxic to erythroid progenitor cells (41, 42). Thus, further studies are required to assign the roles of O₂⁻ in erythroid progenitor cells, especially in both normal and pathologic states of erythropoiesis.

As for the roles of Rel/NF-κB in hematopoietic stem cells, Pyatt et al. (33) showed that Rel/NF-κB was latent under physiologic conditions and that p65, c-Rel, and p50 but not p52 or RelB were activated and bound to the target DNA in 12-O-tetradecanoylphorbol-13-acetate-stimulated human CD34⁺CD19⁻ cells using gel shift assays. In addition, they demonstrated that anti-apoptotic activity of Rel/NF-κB was dispensable for the colony formation from CD34⁺ cells using the membrane-permeable peptide that can inhibit Rel/NF-κB activity. Furthermore, we found that Rel/NF-κB plays a crucial role in the development of primitive hematopoietic progenitors by preventing apoptosis through ROS elimination. Together, these results suggest that Rel/NF-κB activity is indispensable for both development and maintenance of hematopoietic stem/progenitors. With regard to the function of Rel/NF-κB in hematopoietic progenitor cells, Grossman et al. (18) previously reported that the number of hematopoietic progenitor cells that can generate CFU-spleen in the recipient mice was reduced in the fetal liver of c-Rel⁻/⁻/p65⁻/⁻ mice. However, in their analysis, fetal liver cells obtained from c-Rel⁻/⁻/p65⁻/⁻ mice yielded the same number, size, and appearance of various colonies (CFU-granulocyte, CFU-granulocyte macrophage, CFU-macrophage, CFU-erythroid, CFU-E/Meg, and CFU-Mix) to control cells in colony assays. This result seems to be inconsistent with our result that IκBα has drastically reduced the number of CFU-Mix, BFU-E, and CFU-granulocyte macrophage. Thus, further studies are required to define the roles of NF-κB in clonogenic growth and survival of hematopoietic progenitor cells.

Regarding the roles for Rel/NF-κB in terminal differentiation of hematopoietic cells, Zhang et al. (43) previously showed that p50, p52, and p65 were highly expressed in erythroid progenitors and bound to the target DNA in the promoters of c-myc and c-myb genes, suggesting a possibility that Rel/NF-κB would play some role in erythropoiesis. Also, Grossman et al. (18) demonstrated that mice transplanted with c-Rel⁻/⁻/p65⁻/⁻ hematopoietic cells revealed severe anemia and granulocytosis as compared with those transplanted with normal cells, suggesting that Rel/NF-κB is a positive regulator of erythropoiesis and a negative regulator of granulopoiesis. However, we found here that Rel/NF-κB activity was required for terminal differentiation of erythrocytes, granulocytes, and megakaryocytes. Although our result on the function of NF-κB in granulopoiesis was at variance with their finding, we speculate that these conflicting data may originate from the difference in the experimental systems. That is, our result was obtained from the in vitro experiments using the OP9 system, whereas their finding was from the in vivo transplantation assays. Alternatively, it is also possible that functional roles for Rel/NF-κB in granulopoiesis in vivo might be essentially different according to the state of hematopoiesis, such as the steady state of hematopoiesis, a recovery period after transplantation, and the acute phase of inflammation.

In summary, we demonstrated here that NF-κB family proteins play crucial roles at multiple stages in hematopoiesis by preventing apoptosis through the elimination of ROS. Further studies focusing on the regulation of cell survival and death by NF-κB/ROS system would undoubtedly provide useful information to understand both normal and ineffective hematopoiesis.
and to construct useful therapeutic strategies against hematologic malignancies.

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