muscle fibers were isolated from the hind limb muscles of W/W$^v$ mice or lethally irradiated mice transplanted, and single fibers were cultured onto matrigel-coated plates. After 14 days, most of the satellite cells had migrated from the fibers, proliferated, and formed new muscle fibers, which were confirmed to be myosin-positive by immunohistochemical staining (Fig. 7D, E). Since the GFP signal in vitro was too weak to be detected by fluorescent microscope, we determined the presence of GFP$^+$ cells by means of immunohistochemical staining using the anti-GFP antibody or by GFP DNA amplification with the aid of PCR. Staining with the anti-GFP antibody proved the presence of GFP$^+$ myofibers (Fig. 7F), which were also myosin-positive. In addition, GFP DNA was also confirmed by PCR to be present in samples extracted from culture dishes of single-muscle fiber (Fig. 7G). No GFP$^+$ fibers were generated in the single-fiber culture in the early phase of transplantation in irradiated mice (data not shown). In brief, GFP$^+$ KSL cell that engrafted muscle tissues could repair damaged muscle but not produce muscle fibers in vitro in the early phase. In the long term, however, they acquired the potential to differentiate into muscle fibers like satellite cells also in vitro. Since the single-fiber culture is a functional assay of the presence of satellite cells, it can be said that GFP$^+$ KSL cells can give rise to satellite cells with myogenic potential in the long term after transplantation.

## Discussion

In this study, we showed early repair of damaged muscles by KSL cells and first demonstrated that KSL cells could differentiate into satellite-like cells in skeletal muscle in the long term after transplantation (Fig. 8).

The thoroughly studied and identified characteristics of surface markers for HSCs showed that our KSL cells contained a dominant stem cell population. A single CD34$^{-/low}$ c-Kit$^+$Sca-1$^+$ Lin$^-$ cell (CD34$^-$ KSL cell) has been demonstrated to be capable of reconstituting long-term hematopoiesis in about 30% of lethally irradiated adult mice [25]. Our $1 \times 10^3$ of KSL cells contained about 5%–10% of CD34$^-$ cells, which was enough to reconstitute BM of the irradiated mice, and hardly any CD45$^-$ cells (less than 0.1%; Fig. 1). And we cultured 1 to $5 \times 10^3$ of KSL cells for 14 days and obtained no adherent cells. It can therefore be safely assumed that the $1 \times 10^3$ of KSL cells were originally all hematopoietic and did not contain any mescenchymal cells, which do not express CD45 antigen, demonstrating that CD45$^+$ KSL cells can differentiate into muscle fibers. So far, unfractionated BM cells have been used to demonstrate transdifferentiation to muscle fibers [12, 29]. LaBarge and Blau [30] reported recently on the biological progression from adult BM cells to muscle stem cells to muscle fiber, and their findings support our hypothesis. Their study used unfractionated BM cells, which contained both HSCs and MSCs. In contrast, we used CD45$^+$ KSL cells as the enriched HSC fraction, and we showed that KSL cells could contribute to muscle regeneration in both the early and late phases after transplantation. Very recently, it has been reported that a single HSC generated skeletal muscle [31, 32]. These reports support our results. BM SP cells are the same as HSCs and are reported to be involved in muscle regeneration after transplantation, but no satellite cells derived from BM SP cells have been documented [15, 31]. Our study, by analyzing single fibers, is the first to demonstrate that CD45$^+$ KSL cells can give rise to satellite cells both in vivo and in vitro. Since BM SP cells and KSL cells are still heterogeneous populations, further classification of HSCs may lead to the identification of a subpopulation specific to muscle regeneration.

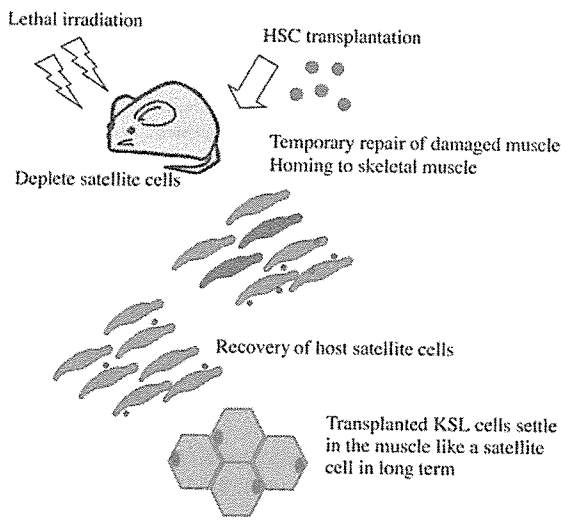


**Figure 8.** Schematic representation of hypothesized behavior of transplanted KSL cells in muscle tissues. See Discussion for a detailed explanation. Abbreviation: HSC, hematopoietic stem cell.

It has been reported that mononuclear cells harvested from murine skeletal muscle are capable of hematopoietic reconstitution in lethally irradiated mice and that these muscle-derived hematopoietic progenitor cells are originally derived from BM [20, 33]. However, determination of the time when these hematopoietic cells settle in muscles after transplantation, as well as the histological identification of their location in muscle tissues, has not yet been accomplished. Our observations demonstrate that GFP$^+$ mononuclear cells gather in damaged muscles before BM has been fully replaced. Nearly all of these GFP$^+$ mononuclear cells consist of CD45$^+$ in muscle fibers. Interestingly, a few CD45$^{low}$ myogenin$^+$GFP$^+$ cells were also detected, and this implies that transplanted KSL cells might have an important and direct role in regeneration in the very early phase after transplantation.

Thirty days after transplantation, single fibers from irradiated mice were not stained by anti-PAX7, anti-MyoD, or anti-Myf5 antibody, and they did not grow in vitro in culture, whereas myofibers could develop in vitro in single-fiber culture from age-matched nontreated mice. Because it is said that irradiation eliminates satellite cells [26, 34], these phenomena might mean that satellite cells had been damaged by irradiation at this

time point. We speculated that, after 30 days, host satellite cells recovered from radiation damage, producing new muscle fibers, which replaced GFP$^+$ fibers gradually. We assume that GFP$^+$ fibers were diluted, fusing with host-derived muscle fibers. Simultaneously, GFP$^+$ cells remain beneath the basal lamina as satellite cells in muscle tissue and acquire the potential to differentiate into muscle fibers in the long term after transplantation. In this late phase, muscle regeneration can be obtained from GFP$^+$ satellite-like cells, as well as from endogenous satellite cells, as was confirmed by single-fiber culture. We therefore propose two different roles of KSL cells in muscle regeneration: repair of muscles damaged by irradiation in the early phase, and generating satellite cells in the late phase.

In the case of transplantation into W/W$^v$ neonates, GFP$^+$ cells engrafted into muscle tissues as early as 30 days after transplantation without irradiation. Interestingly, we could detect satellite cell markers MyoD, PAX7, and Myf5 on GFP$^+$ cells on muscle fibers as early as 1 or 2 months after transplantation. GFP$^+$ single fibers can develop in vitro at the same time (data not shown). This means that KSL cells can migrate into muscle fibers and become satellite-like cells without muscle damage. We therefore speculate that KSL cells might be part of a physiological satellite cell source. Neonatal environment might give some effect to KSL cells. And it is not yet clear why hematopoietic cells engraft into undamaged muscles. The fact that CD45$^+$ hematopoietic cells engraft into skeletal muscles suggests the existence of some muscle-specific molecular mechanism for HSC engraftment. During the processes of HSC homing and engraftment into BM, the contribution of some adhesion molecules such as VCAM-1, which is a ligand for $\alpha$4 integrins, has been reported [35, 36]. HSCs are reported to have $\alpha$4 integrins [37]. Furthermore, laminin, which is a ligand for $\alpha$4 integrin, which in turn is a component of muscle basement membrane, may be important for the maintenance of hematopoietic cells in skeletal muscles.

About 1,000 KSL cells, which proved to be enough to reconstitute BM of irradiated mice, are also sufficient to distribute throughout all the muscles in the body and regenerate all damaged muscles such as intercostals, diaphragm, and limb muscles. This is an important finding in terms of potential for clinical application. Patients with muscular dystrophy suffer from respiratory distress due to respiratory muscle weakness. Our findings of powerful engraftment of transplanted KSL cells in the entire body, including respiratory muscles, may be the first step on the way to the establishment of therapeutic strategies using HSCs. Further studies are needed, however, to establish efficient engraftment of HSCs to muscle tissues and to identify responsible molecules.

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# 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-dependent 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, p65/p50, 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 dendric 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 ($O_2^-$), $H_2O_2$, 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 such as activation of c-Jun N-terminal kinase, disruption of mitochondrial membrane potential ($\Delta\Psi_m$), 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 $H_2O_2$. $H_2O_2$ 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 IκBSR, 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 IκBSR 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 IκBα, C-21 and B-9, were purchased from Santa Cruz Biotechnology. Mn(III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), *N*-acetyl L-cysteine (NAC), and thioredoxin X (TRX) were purchased from Calbiochem-Novabiochem Corp. MCI-186 (3-methyl-1-phenyl-2-pyrazolin-5-one) (MCI) was kindly provided by Mitsubishi Pharma Co.(Osaka, Japan).

*Plasmids*—The expression vector for IκBSR was a gift from Dr. D. W. Ballard (Vanderbilt University School of Medicine, Nashville, TN); Mn-SOD 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 staining with propidium iodide and analyzed on FACSort (Becton Dickinson) (23). Surface phenotype of the cells was analyzed by the direct immunofluorescence method. Total ROS were detected with RedoxSensor™ Red CC-1 (R14060), $O_2^-$ with dihydroethidium (D-1168), and $H_2O_2$ with CM-$H_2$DCFDA (C-6827) (Molecular Probes) as described previously (24). $\Delta\Psi_m$ was detected with DePsiher™ (Trevigen) according to the manufacturer's instructions.

*Northern Blot and Reverse Transcriptase-PCR*—Northern blot and reverse transcriptase-PCR analyses were performed as described previously (25). The sequences of primer sets (a sense primer/an antisense primer) were as follows: Bcl-2, 5′-CTTAGAAAATACAGCATTGCGGAG-3′/5′-GGATGTGCTTTGCATTCTTGG-3′; Bcl-XL, 5′-CACTGTGCGTGGAAAGCGTA-3′/5′-AAAGTGTCCCAGCCGCC-3′; A1, 5′-GATGGCTGAGTCTGAGCTCA-3′/5′-GGCAATCTGCTCTTGTGGAA-3′; MnSOD, 5′-GACCTGCCTTACGACTATGG-3′/5′-GACCTTGCTCCTTATTGAAGC-3′; glutathione peroxidase, 5′-TCGAACCTGACATAGAAACCCT-3′/5′-CACCATCATGGAAGAACCG-3′; TRX, 5′-ATCATTTTGCAAGGTCCACA-3′/5′-CAAGGAAGCTTTTCAGGAGG-3′; xanthine oxidase, 5′-TTATGTCCCTACTCGCCACA-3′/5′-TGGTGGCTTGGCAGATATAA-3′; Prdx1, 5′-GAGCAGCCAGAAGAAACTCTTG-3′/5′-AGAAGATTGGTCTGCCCAAAA-3′; and β-actin, 5′-CATCACTATTGGCAACGAGC-3′/5′-ACGCAGCTCAGTAACAGTCC-3′.

*Immunoblot Analysis*—Isolation of total cellular lysate, electrophoresis, 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 IκBSR 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 ng/ml of murine IL-3. To stably express IκBSR in Ba/F3 cells, we transfected 30 μg of pcDNA3.1 (Invitrogen) containing IκBSR 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 IκBSR, 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 IκBSR 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 culture medium. We selected several clones in which IκBSR was effectively induced by IPTG and performed further experiments. We further introduce the EPO receptor, TPO receptor, and G-CSFR/gp130 consisting of the extracellular domain of G-CSFR and cytoplasmic domain of gp130 (28) into a Lac-inducible IκBSR 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 $10^4$ 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 $10^5$ cells/well of 6-well plate or $7–8 \times 10^5$ cells/10-cm dish and cultured under the indicated conditions.

*Tetracycline-regulated Inducible Expression of IκBSR in ES Cells*—To inducibly express IκBSR 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 pCAG20–1-tTA and pUHD10–3-puro by electroporation (800 V, 3 microfarads). pCAG20–1-tTA 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-cytomegalovirus promoter. Thus, if the Tet-off system efficiently works in the transfected cells, these cells are expected to be Puro-resistant in the Tet$^-$ medium and Puro-sensitive in the Tet$^+$ medium. 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-IκBSR-IRES-GFP, which can inducibly express IκBSR and GFP as a single mRNA through internal ribosome entry site in response to the removal of Tet, together with the neomycin-resistant plasmid pcDNA3.1-neo. After the culture with 0.2 mg/ml of G418 in the Tet$^+$ medium, we selected several clones that can inducibly express GFP in response to the Tet deprivation. Subsequently, we examined the Tet-regulated expression of IκBSR 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-IκBSR-neo or an empty pMSCV-neo (Clontech) was transfected into an ecotropic 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*—Bone marrow cells were harvested from 9–12-week-old Balb/c 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

[1] The abbreviations used are: ROS, reactive oxygen species; TPO, thrombopoietin; EPO, erythropoietin; SCF, stem cell factor; Tet, Tetracycline; h, human; m, murine; IL, interleukin; ES, embryonic stem; SOD, superoxide dismutase; TRX, thioredoxin X; MnTBAP, 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-CSFR, granulocyte colony-stimulating factor receptor; CFU, colony forming unit.

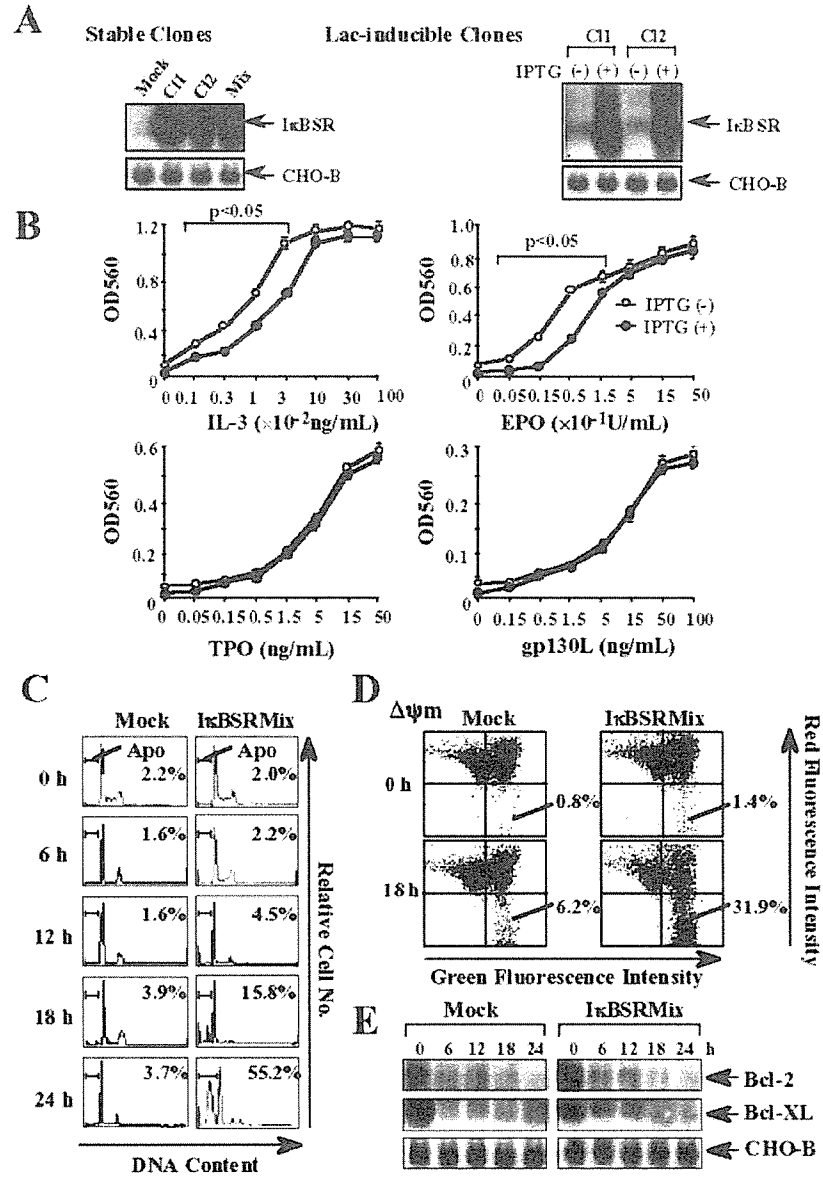


FIG. 1. **Effects of IκBSR on the growth and survival of Ba/F3 cells.** *A*, IκBSR was stably expressed in Ba/F3. The expression of IκBSR was examined in two single clones (*Cl1* and *Cl2*) and a mixed clone (*Mix*) by Northern blot analysis. Also, we prepared several Lac-inducible clones from Ba/F3, 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. *B*, EPO receptor, TPO receptor, and G-CSFR/gp130 were individually introduced into Lac-inducible Ba/F3 Cl1. 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-yl)-2,5-diphenyltetrazolium bromide assays. The results are shown as the means ± S.D. of triplicate cultures. *C–E*, Ba/F3/Mock and Ba/F3/IκBSRCl1 cells were deprived of IL-3, cultured, and subjected to flow cytometric and Northern blot analyses at the indicated times. *C*, DNA content of the cultured cells was analyzed by propidium iodide staining. The percentage of the subdiploid fraction formed from apoptotic (*Apo*) cells is indicated. *D*, changes in $\Delta\Psi_m$ were examined by flow cytometric analysis. In this method, the cells with the disrupted $\Delta\Psi_m$ are detected as those with the decreased red fluorescent intensity and the increased green fluorescent intensity in the *lower right panel*. The percentage of the cells in this area is indicated. *E*, the expression of Bcl-2 and Bcl-XL was examined by Northern blot analysis. The expression of CHO-B was examined as a loading control.

24 h, the cells were washed and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, the above described cytokines, and 1 mg/ml of G418 for 48 h.

*Colony Assays*—Retrovirus-transduced $1 \times 10^5$ cells were cultured in the methylcellulose medium (Stem Cell Technologies) containing 1 mg/ml of G418 and cytokines (mSCF 50 ng/ml, mIL-3 10 ng/ml, hIL-6 10 ng/ml, and EPO 3 units/ml) in 35-mm dishes. The number of colonies was counted after 16 days.

*Statistical Analysis*—Statistical analysis was performed with Student's *t* test.

## 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/IκBSRCl1 and Ba/F3/IκBSRCl2 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. 1*A* (*left panel*), IκBSR was intensively expressed in Ba/F3/IκBSRCl1, Ba/F3/IκBSRCl2, 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. 1*A*, *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. 1*B*, 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. 1*B*).

*IκBSR Enhanced Factor-deprived Apoptosis in Ba/F3 Cells*—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. 1*C*). In contrast, 55.2% of Ba/F3/IκBSRMix cells led to apoptosis after 24-h IL-3 deprivation, indicating that IκBSR enhanced fac-

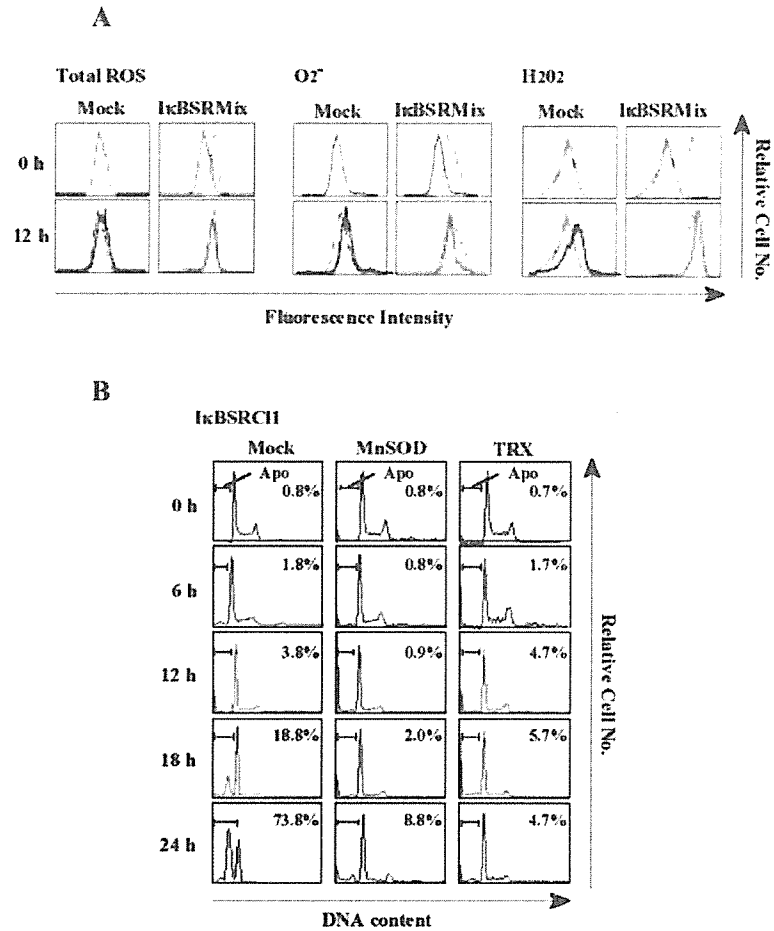


FIG. 2. **Roles for ROS in IκBSR-enhanced apoptosis.** *A*, total ROS were detected with Red CC-1, $O_2^-$ with dihydroethidium, and $H_2O_2$ with CM-$H_2$DCFDA in Ba/F3/Mock and Ba/F3/IκBSR cells before and after IL-3 depletion by flow cytometry. *Thin black line*, Ba/F3/Mock at 0 h; *thick black line*, Ba/F3/Mock at 12 h; *thin red line*, Ba/F3/IκBSR at 0 h; *thick red line*, Ba/F3/IκBSR at 12 h. *B*, Ba/F3/IκBSRCl1 was further transfected 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.

tor-deprived apoptosis in Ba/F3 cells. 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. 1*D*). 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/IκBSRMix. 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/IκBSR after IL-3 deprivation (Fig. 1*E*). However, we did not detect an apparent difference in their expression levels between these clones that can explain IκBSR-enhanced apoptosis.

*IκBSR 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/IκBSR during IL-3 deprivation. We detected total ROS with a fluorescent sensor Red CC-1, $O_2^-$ with dihydroethidium, and $H_2O_2$ with CM-$H_2$DCFDA by flow cytometry. As shown in Fig. 2*A*, a small amount of total ROS, $O_2^-$, and $H_2O_2$ 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 $H_2O_2$ were already accumulated in Ba/F3/IκBSRMix cells even in the presence of IL-3, which did not further increase after IL-3 depletion. To access the roles of ROS in IκBSR-enhanced apoptosis, we overexpressed ROS scavenger enzymes MnSOD and TRX in Ba/F3/IκBSRCl1, respectively. Although Mock-transfected cells led to severe apoptosis after IL-3 depletion, MnSOD and TRX almost completely canceled this apoptosis (Fig. 2*B*), suggesting that ROS may be involved in the IκBSR-enhanced apoptosis.

*IκBSR Inhibited Cytokine-dependent Survival of Normal Hematopoietic Cells*—We next analyzed the effects of IκBSR on the growth and survival of normal hematopoietic cells. We introduced IκBSR into murine $Lin^-Sca\text{-}1^+$ 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 IκBSR-transfected cells underwent apoptosis (Fig. 3*A*). We also examined the effects of IκBSR on hematopoietic stem/progenitor cells with colony assays. As shown in Fig. 3*B*, IκBSR drastically reduced the number of CFU-Mix, BFU-E, and CFU-GM. These results indicated that although IκBSR 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 IκBSR-transfected cells after 5 days (Fig. 3*C*). Furthermore, flow cytometric analysis showed that total ROS, $O_2^-$, and $H_2O_2$ accumulated in IκBSR-transfected cells under the culture with SCF, IL-3, IL-6, and FLT3L (Fig. 3*D*, *upper panel*). Similarly, IκBSR induced the ROS accumulation under the cultures with different cytokine combinations (Fig. 3*D*, *lower panel*). Regarding the mechanisms of IκBSR-induced ROS accumulation, we found that the intracellular glutathione was reduced in IκBSR-transfected cells (Fig. 3*E*). Also, the semiquantitative reverse transcriptase-PCR analysis showed that the expression of several ROS scavenger enzymes was suppressed in IκBSR-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.

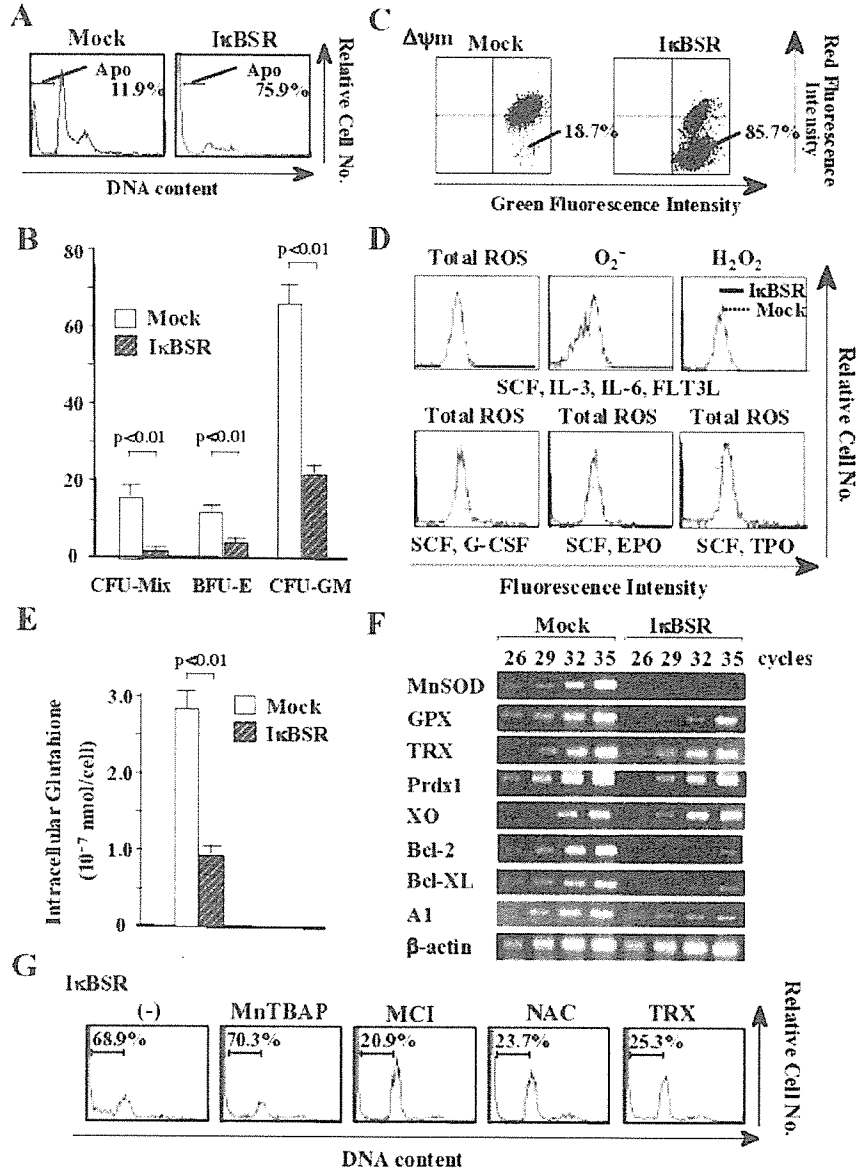


FIG. 3. **Effects of IκBSR on cytokine-dependent survival of normal hematopoietic cells.** IκBSR was retrovirally introduced into murine marrow Lin Sca-1$^+$ cells. After the selection with G418, the cells were subjected to further liquid cultures or colony assays. *A*, DNA content analysis was performed after a 7-day culture with IL-3, SCF, IL-6, and FLT3L. The proportion of apoptotic cells is indicated. *B*, total number of the colonies was counted after 14 days. The results are shown as the means ± S.D. of triplicate cultures. *C*, changes in $\Delta\Psi_m$ were analyzed after 5-day cultures with IL-3, SCF, IL-6, and FLT3L by flow cytometry. *D*, after 5-day cultures with the indicated cytokine combination, the cells were subjected to flow cytometric analysis. Total ROS were detected with Red CC-1, $O_2^-$ with dihydroethidium, and $H_2O_2$ with CM-$H_2$DCFDA. *E*, the amount of the intracellular glutathione was measured after 5-day cultures with IL-3, SCF, IL-6, and FLT3L. The results are shown as the means ± S.D. of triplicate cultures. *F*, semiquantitative reverse transcriptase-PCR analysis was performed after 5-day cultures with IL-3, SCF, IL-6, and FLT3L. *G*, IκBSR- or Mock-transfected cells were cultured with IL-3, SCF, IL-6, and FLT3L in the presence or absence of the indicated reagent: MnTBAP, MCI, NAC, or TRX. After 7 days, the cultured cells were subjected to DNA content analysis. The proportion of apoptotic (*Apo*) cells is indicated.

3*F*). 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 IκBSR: 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 IκBSR disrupted two major anti-apoptotic systems: ROS scavenger cascades and the Bcl-2 family members. Next, we assessed the roles for ROS in IκBSR-induced apoptosis by adding several ROS scavenger enzymes into the culture medium. As shown in Fig. 3*G*, MCI, NAC, and TRX efficiently protected normal hematopoietic cells from IκBSR-induced apoptosis, whereas MnTBAP was hardly effective. Because MCI mainly act on $NO^-$ and NAC, TRX on $H_2O_2$, and MnTBAP on $O_2^-$, $NO^-$ and $H_2O_2$ were supposed to be more toxic than $O_2^-$ in normal hematopoietic cells.

*IκBSR 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$^+$ cells (so-called hemangioblasts that have an ability to differentiate into both endothelial cells and hematopoietic cells) after 4.5-day cultures (Fig. 4*A*). To develop hematopoietic cells with high purity, we sorted Flk-1$^+$ cells by fluorescence-activated cell sorter and further cultured on OP9 cells (Fig. 4*A*). In this system, we inducibly expressed IκBSR with the Tet-off system, in which the expression of IκBSR 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 IκBSR and GFP through internal ribosome entry site as a single mRNA in response to the Tet removal. We cultured ES clones each transfected with IκBSR and a Mock vector (designated as E14/IκBSR 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. 4*B*, the expression of GFP was induced in most of E14/IκBSR cells under the culture without Tet. Also, immunoblot analysis showed that IκBSR protein was effectively induced by Tet deprivation in E14/IκBSR cells.

After the culture with Tet for 14 days, 29.2% of E14/IκBSR

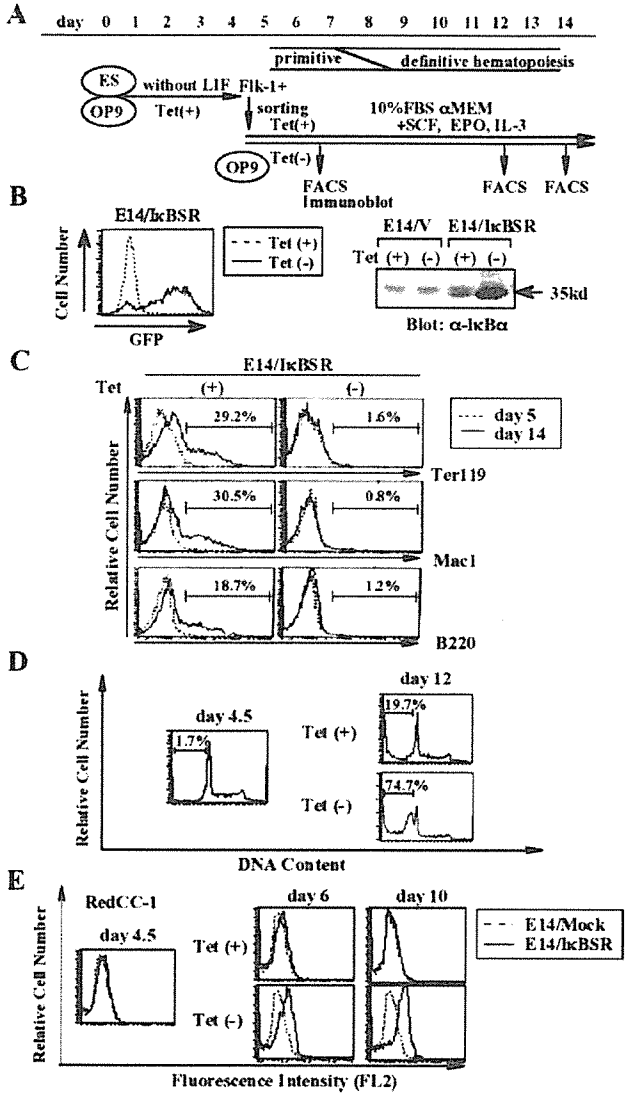


FIG. 4. **Effects of IκBSR on the development of hematopoietic cells.** *A*, experimental design 1 using the OP9 system. ES cells were deprived of leukemia inhibitory factor and cultured on OP9 cells for 4.5 days. Then Flk-1$^+$ cell were sorted, replated onto OP9 cells, and cultured with IL-3, EPO, and SCF with or without Tet for the time indicated. *B*, Flk-1$^+$ cell were cultured for 48 h. Then the expression of GFP was examined by flow cytometric analysis, and the induction of IκBSR was examined by immunoblot analysis in the indicated clones. *C*, after 14-day cultures with or without Tet, the expression of Ter119, Mac1, and B220 was examined by flow cytometric analysis. The percentage of the positive fraction is indicated. *D*, DNA content of the cells was analyzed by propidium iodide staining at days 4.5 and 12. The percentage of apoptotic cells is indicated. *E*, total ROS were detected with RedCC-1 by flow cytometric analysis at days 4.5, 6, and 10.

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. 4*C*, *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/IκBSR cells (Fig. 4*C*, *right panel*). In addition, we found that 74.7% of E14/IκBSR 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. 4*D*). Also, we found that total ROS accumulated under the culture without Tet at days 6 and 10 (Fig. 4*E*). These results suggested that IκBSR 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 IκBSR inhibited the development of hematopoietic cells, we initially analyzed the effect of IκBSR 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. 5*A*). After the culture with Tet, 66.4% of E14/IκBSR cells were positive for CD45, 17.9% were positive for CD34, and 28.8% were positive for c-Kit (Fig. 5*B*), indicating that a substantial fraction of the cultured cells developed into primitive hematopoietic progenitors. Next, we cultured E14/IκBSR cells without Tet in the presence or absence of MCI and evaluated the effects of IκBSR in the GFP-positive fraction (GFP-PF), because IκBSR 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. 5*B*). 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 IκBSR 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/IκBSR 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/IκBSR cells under the indicated conditions up to day 12 (Fig. 6*A*). As shown in Fig. 6*B*, substantial fractions of E14/IκBSR 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, where IκBSR was not expressed, 37.2%; Ter119$^+$, 49.2%; and CD41$^+$, 77.6% (shown in parentheses). In contrast, when E14/IκBSR 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 IκBSR induced apoptosis in 51.8% of E14/IκBSR cells cultured without Tet and that this apoptotic fraction was reduced to 14.1% by MCI (Fig. 6*B*). 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-

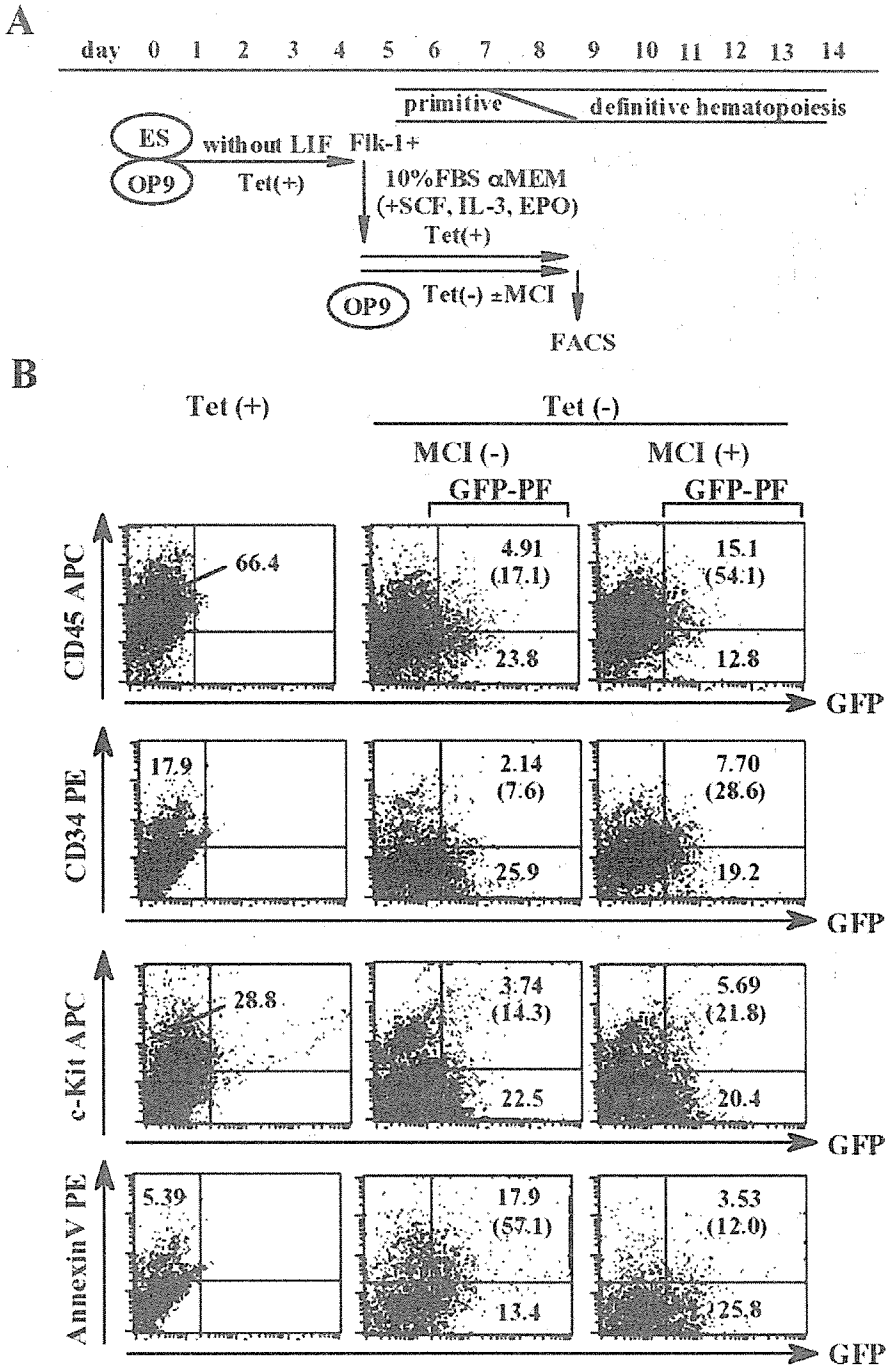


FIG. 5. **Effects of IκBSR on the development of hematopoietic stem cells.** *A*, experimental design 2. Sorted Flk-1$^+$ cell were replated onto OP9 cells, cultured with IL-3, EPO, and SCF in the presence or absence of Tet for 4 days and subjected to flow cytometric analysis. In some experiments, MCI 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 the each fraction is indicated. The relative frequency of the *upper right panel* in the GFP-PF is shown in *parentheses*.

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-macrophate colony-stimulating factor were reported to activate NF-κB in hematopoietic cells (33). 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 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 plays 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

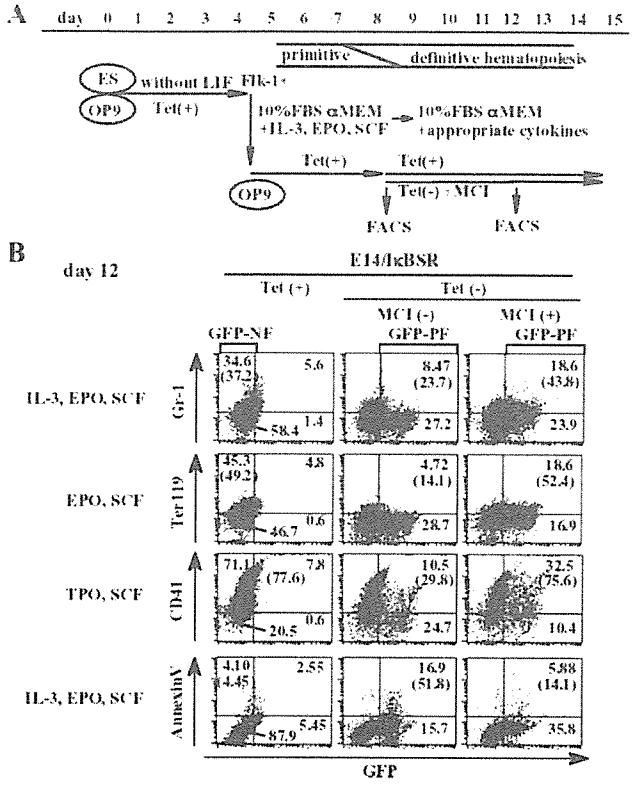


FIG. 6. **Effects of IκBSR on terminal differentiation of hematopoietic cells.** *A*, experimental design 3. Sorted Flk-1$^+$ cells were cultured with IL-3, EPO, and SCF in the presence of Tet on OP9 cells for 4 days. Then the cells were washed, replated onto OP9 cells, and cultured under the indicated conditions up to day 12. *B*, after 12-day cultures under the indicated conditions, the cells were subjected to flow cytometric analysis on the expression Gr-1, Ter119, and CD41, respectively. Also, the intensity of GFP and reactivity to phycoerythrin-labeled annexin V were analyzed by flow cytometry. The percentage of the each fraction is indicated. The relative frequency of the *upper panel* in the GFP-negative fraction (*GFP-NF*) or 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 Bcl-2 family members Bcl-XL and A1/Bfl1 and caspase inhibitors c-IAP1 and c-IAP2 in various cell types (1, 2). Also, Rel/NF-κB promotes the expression of 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 IκBSR down-regulated the expression of Bcl-XL, A1, Bcl-2, and MnSOD in normal hematopoietic cells. Furthermore, regarding the mechanism of the ROS accumulation in IκBSR-introduced cells, we found that the amount of the intracellular glutathione and the expression of glutathione peroxidase and TRX were reduced by IκBSR in these 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 IκBSR-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 still 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 IκBSR-enhanced apoptosis, suggesting that $NO^-$ and $H_2O_2$ were more toxic to normal hematopoietic stem/progenitors cells than $O_2^-$. However, based on the fact that MnSOD$^{-/-}$ mice exhibited severe anemia caused by ineffective erythropoiesis, $O_2^-$ was also assumed to be toxic to erythroid progenitor cells (41, 42). Thus, further studies are required to assign the roles of $O_2^-$ in erythroid progenitor cells, especially in both normal and pathologic states of erythropoiesis.

As for the roles for 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 indispensable 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 similar number, size, and appearance of various colonies (CFU-granulocyte, CFU-granulocyte macrophage, CFU-macrophage, CFU-eosinphil, CFU-megakaryocyte, CFU-erythroid, CFU-E/Meg, and CFU-Mix) to control cells in colony assays. This result seems to be inconsistent with our result that IκBSR 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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特集 臍帯血移植

# 臍帯血造血幹細胞の体外増幅*

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**Key Words**：cord blood, hematopoietic stem/progenitor cells, *ex vivo* expansion, translational research

## はじめに

造血幹細胞は自己複製能と多分化能とを併せもつ細胞であり，その増殖，分化の過程は骨髄ストローマ細胞や種々のサイトカインなどの外的因子および転写因子や細胞内シグナル伝達分子などの内的因子により厳密に制御されていることが知られている．近年造血幹細胞のもつ自己複製能を利用して*ex vivo*で造血幹細胞を増幅し，移植医療に応用しようとする研究が盛んに行われ，海外では臨床研究も進められている．本研究の成果は造血幹細胞移植への展望を開くだけでなく，造血幹細胞の遺伝子治療，さらにはその可塑性を利用した再生医療など多方面への応用が期待されている．本稿では，臍帯血造血幹細胞の*ex vivo*増幅の現状と課題，およびわれわれが先端医療センターにおいて取り組んでいる*ex vivo*増幅臍帯血の臨床応用について概説する．

## 造血幹細胞増幅の臨床的意義

臍帯血移植の長所としては，まずドナーへの直接的負担がないことに加え，ドナー検索から移植までの期間が短いことがあげられる．また移植後，急性GVHDの程度が軽く重症GVHDの頻度が低いこと，HLA 1および2抗原不一致移植が可能であることなどが知られている．一方短所として，臍帯血移植では細胞数の問題から体重の重い患者には適応とならない場合が多いこと，また移植後の造血回復，とくに血小板の生着が他の造血幹細胞移植と比べ顕著に遅延することが知られている[1]．

造血回復の関連因子として，Migliaccioらは，臍帯血移植施行例204例において移植細胞数と造血回復能，および移植関連合併症との関連について検討したところ，移植細胞数が多くなるほど好中球数および血小板数の回復が早くなる傾向にあり，総有核細胞数は$25\times10^6$/kg以上，コロニー形成細胞数は$50\times10^3$/kg以上で有意に移植関連合併症が減少したと報告している[2]．またWagnerらはCD34陽性細胞数で$1.7\times10^5$/kg以上，Laughlinらは$1.2\times10^5$/kg以上移植した場合，それ以下の細胞数を移植した群に比べて有意に生存率が高いと報告している[3][4]．すなわち，移植細胞数とくにCD34陽性細胞数が多いほど造血回復能が高く，予後も良好と考えられることから，*ex vivo*増幅した造血幹/前駆細胞が移植に応用可能となれば，従来臍帯血移植の対象とならなかった体重の重い患者も対象となるばかりでなく，生着不全の減少や生着日数の短縮が期待される．

* *Ex vivo* expansion of human umbilical cord blood hematopoietic stem/progenitor cells.

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同様の考え方で，十分な細胞数を確保することを目的として，複数の臍帯血を同時に移植する試みがミネソタ大学を中心に行われており，安全に移植が施行できるだけでなく，複数の臍帯血を用いることでの何らかの免疫学的効果により，これまでに十分な造血回復に必要とされている細胞数に満たない臍帯血を移植した場合でも，早期から生着が得られる可能性のあることが報告されている[5]．

## 造血幹細胞の体外増幅研究の現状

造血幹細胞の体外増幅方法は，培地へのサイトカイン添加や支持細胞との共培養などによる外的因子操作法，および遺伝子や蛋白導入などによる内的因子操作法に大きく分類される[6]．

### 1．外的因子操作による造血幹細胞の増幅

これまで臍帯血から分離したCD34陽性造血幹/前駆細胞の増幅効果はさまざまなサイトカインの組合わせにより検討されたが，現時点ではIL-6/sIL-6R, SCF, TPO, Flk2/Flt-3 ligand(FL)の組合わせがもっとも効率よく増幅できることが明らかとなっている[7]．これらのサイトカインの組合わせで1週間培養することによりSRC(SCID repopulating cells)は4倍以上増幅され，移植後6か月以上経過してもマウス末梢血中にある一定の割合でヒト細胞が認められたことから，この系を用いることにより比較的長期の骨髄再構築能を維持した造血幹細胞が増幅可能であると考えられている[8]．

造血幹細胞は骨髄中のnicheと呼ばれる特殊な場所に存在し，幹細胞としての性質を維持していると考えられている．近年nicheを構成する細胞群やnicheに発現する造血幹細胞の自己複製に関与する分子が明らかにされつつあり[9]，これら細胞との共培養や分子の固相化などの加工培養容器を用いることにより効率的な造血幹細胞の体外増幅法の開発が期待されている．さらに増幅のみならず生着促進効果や長期骨髄再構築能の維持を考慮した場合，造血幹細胞を生体内に近い状態で培養することが望ましいと考えられる．最近，採取した臍帯血と同じ臍帯より得た臍静脈上皮細胞，human umbilical vein endothelial cell(HUVEC)と共培養することにより，サイトカインのみで培養した場合よりも効率的な造血幹細胞の増幅が可能であることが報告されており[10]，今後生着に及ぼす効果などのさらなる検討が期待される．

### 2．内的因子操作による造血幹細胞の増幅

これまでの検討から外的因子操作による増幅効率には限界があり，より効率よく造血幹細胞を増幅するためには，転写因子操作などによる内的因子操作が必要であると考えられている．内的因子操作は，*HOX*, *Wnt*, *Notch*, *Bmi-1*などの転写因子群のほか，*p21*や*p27*などの細胞周期制御因子など，造血幹/前駆細胞の増幅への関与が明らかにされている遺伝子群をレトロウイルスを用いて導入する方法が中心である．それらの中でもっとも効率的なものが*HOXB4*を導入する方法で，マウス骨髄細胞への過剰発現により白血病を起こすことなく，造血幹細胞を約1,000倍に増やすことが可能であることが報告されている[11]．ただしレトロウイルスを用いた遺伝子導入方法は，安全性の問題から現時点での臨床応用は難しいと考えられる．一方で細胞透過型の可溶性*HOXB4*蛋白(TAT-*HOXB4*)を導入することにより，造血幹細胞での*HOXB4*蛋白レベルを上昇させ，効率的な増幅が可能であるとの報告があり[12]，今後このような蛋白導入による内的因子操作と種々外的因子操作を併用した形での増幅方法が有力かつ実用的な方法になると考えられる．

## *Ex vivo*増幅造血幹/前駆細胞を用いた移植成績

*Ex vivo*増幅した臍帯血を用いた臨床試験は1997年に2つのグループが開始しており，いずれも増幅しない臍帯血と増幅した臍帯血を混合して移植する方法がとられている．McNieceらは，37名(成人25名，小児12名)の血液腫瘍および乳がん患者を対象として，保存された臍帯血の40%あるいは60%の画分からCD34陽性細胞を分離後，SCF, G-CSF, およびmegakaryocyte growth and differentiation factor(MGDF)添加培地で10日間培養した細胞と，増幅しなかった残りの画分の細胞とを合わせて移植した[13]．一方Kurtzbergらは，27名(成人2名，小児25名)の血液腫瘍患者に対して培地還流培養システムAstrom Replicell™ Systemを

表1　*Ex vivo*増幅臍帯血の培養成績と臨床成績

| グループ | McNiece[13] | Kurtzberg[14] |
|---|---|---|
| 使用サイトカイン | SCF＋G-CSF＋MGDF | PIXY 321＋FL＋EPO |
| 培養日数 | 10日間 | 12日間 |
| 総細胞増幅率* | 56倍(1.3～278) | 2.05倍(0.06～10.19) |
| CD34陽性細胞増幅率* | 4倍(0.1～20) | 0.5倍(0.09～2.45) |
| 症例数 | 37 | 27 |
| 移植総細胞数(×$10^7$/kg)* | 0.99(0.28～8.5) | 2.4(1.0～8.5) |
| 移植CD34陽性細胞数(×$10^5$/kg)* | 1.04(0.97～31.1) | 0.22(0.001～2.59) |
| 生着までの日数* | | |
| 好中球＞500/μl | 28日(15～49) | 22日(13～40) |
| 血小板＞20,000/μl | 106日(28～345) | 94日(41～370) |
| 生着不全 | 0/30例 | 3/24例 |
| 急性GVHD(≧Ⅲ度) | 40% | 36% |

* 中央値，PIXY321：IL-3/GM-CSF融合蛋白．

用いて臍帯血を12日間培養し，未処理臍帯血を移植後12日目に増幅臍帯血を移植した[14]．培養成績および移植成績を表1に示す．2つの臨床試験の結果から明らかになったことは，*ex vivo*増幅臍帯血は安全に移植可能であり，生着不全率は低率であったが，期待されたような造血回復の促進効果は得られなかったということで，この結果は決して満足できるものではない．ただし両者の培養成績をみてみると，有核細胞はある程度増幅されているが，CD34陽性細胞の増幅率はMcNiece法で約4倍と低率で，Kurtzberg法においては逆に半分に減少していることから，両グループによる方法では自己複製能を有すると考えられる未熟な造血幹/前駆細胞が十分量移植されておらず，*ex vivo*増幅することの本来の目的である“より多くの造血幹/前駆細胞を移植した場合”の効果が評価されていないと考えられる．最近，Shpallらは銅キレート剤であるTEPA(tetraethylenepentamine)をサイトカインカクテルと組み合わせ，効率的に*ex vivo*増幅させた臍帯血を用いた臨床研究を進めており，症例数は少ないもののより多くの増幅CD34陽性細胞を移植することにより，好中球，血小板生着までの期間が有意に短縮することを報告している[15]．また，彼らは複数臍帯血移植において，一方を増幅した場合としなかった場合の比較試験も進めており，その結果が待たれる．

## 先端医療センターでの取組み

生体外で培養や加工した細胞を用いた臨床応用を行うためには，細胞の機能，品質およびその安全性が科学的に保証されていなければならない．細胞治療の普及に伴い2001年米国の食品医薬品局(FDA)から「ヒト細胞治療におけるGTP(good tissue practice)ガイドライン」が施行された．これを受けわが国でも，2003年7月に薬事法の改正が行われ「細胞治療用生物製剤の取り扱い」に関しての基準が明確にされており，細胞療法を行うにあたってはこれら基準に合致した細胞の加工が必要である．具体的には，伝染物質の混入などを防止するための閉鎖系培養や無血清培養法の確立，無菌的な細胞操作のためのCPC(cell processing center)の整備，標準作業手順書(SOP)の作成，および品質管理体制の確立などが必要である．さらにGCP(good clinical practice)に準拠した臨床研究実施計画書(臨床プロトコル)の作成，高い水準の診療体制，および倫理審査体制など総合的な基盤を確立してはじめて質の高いトランスレーショナルリサーチが可能になると考えられる．われわれは先端医療センターでの*ex vivo*増幅臍帯血を用いた臨床研究を行うにあたり，2002年度から京都大学，大阪大学，東京医科歯科大学，および企業との共同研究でハード面，ソフト面の総合的な整備を進めてきた．

1．GMP(good manufacturing practice)に準拠した培養法の確立

われわれが開発した培養法は，凍結臍帯血からCD34陽性細胞を純化し，IL-6/sIL-6R, SCF, TPO, FL添加無血清培地で12日間培養するもの

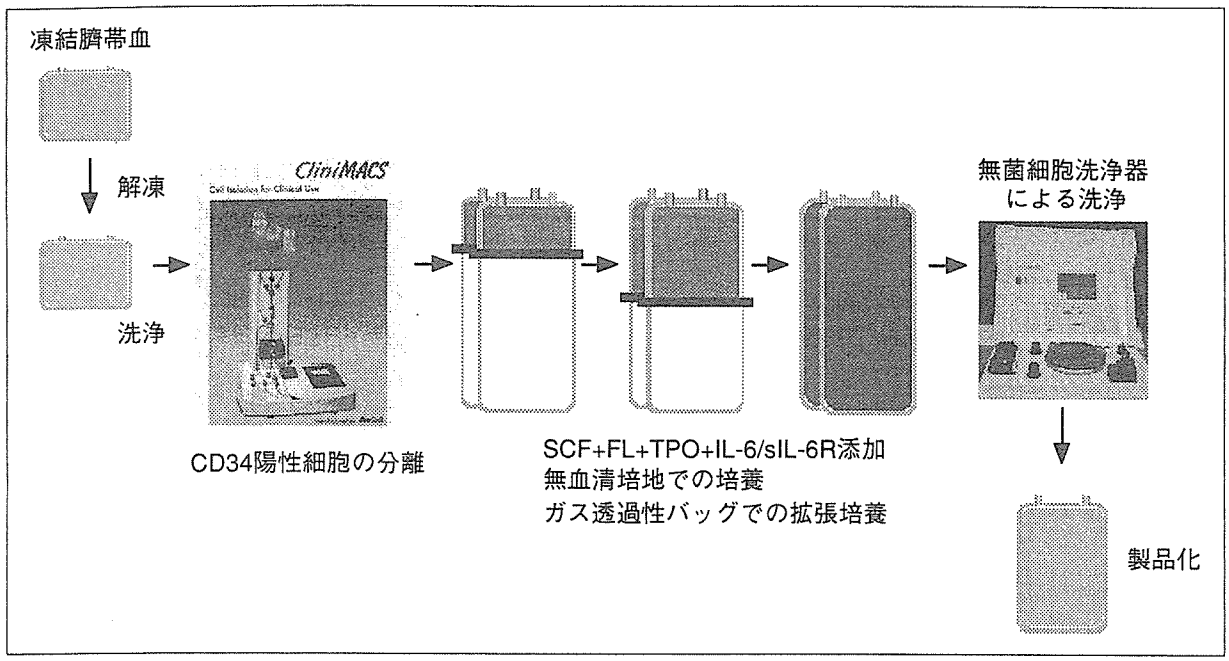


図1　GMPに準拠した*ex vivo*増幅臍帯血の製造工程

で，テフロン製のガス透過性バッグを用いることにより，分離から培養，洗浄および最終製品出荷までのすべての工程を閉鎖系で行うことが可能である(図1)．またこの方法は，前述したこれまで臨床研究が行われている方法と比較して，有意に高い総細胞増幅率，CD34増幅率が得られる培養系であり，総細胞で約150倍，CD34陽性細胞で約40倍の増幅が可能である．

2．GMPに準拠した培養システムの開発，基盤整備

ハード面では先端医療センター内にCPCを整備し，取違いを防止する電磁ロック付きインキュベーターや無菌細胞洗浄装置などのデバイス開発を進めている．またソフト面では，標準作業手順書，環境衛生基準書，品質管理基準書，製品概要書などの文書体系を作成し，安全な製造法確立のための整備を進めている．

3．*Ex vivo*増幅した臍帯血幹細胞の品質管理法の確立

われわれは*ex vivo*増幅臍帯血の安全性および性能について「ヒト又は動物由来成分を原料として製造される医薬品等の品質及び安全性確保について」(医薬発第1314号，厚労省医薬安全局長通知)に示されている概要に従う形で，より具体化した項目をそれぞれに設け，増幅臍帯血の形態，表面抗原，サイトカイン産生能，染色体数の異常，転座などの構造異常，およびコロニー形成能などの評価を行っている．また，NOD/SCIDマウスへの移植実験を前臨床試験と位置づけ，生着能の評価のほか，体内分布，炎症，変性，および腫瘍化の有無などの評価を行うことで*ex vivo*増幅臍帯血の安全性および性能を確認している．製造ごとに行う規格試験については，可能なかぎり最終製品の安全性，品質を担保すべく，細胞生存率，無菌試験，エンドトキシン定量，マイコプラズマ試験，およびウイルス試験を製造工程に併せ行う予定にしている．

4．GCPに準拠した臨床試験実施体制の確立

われわれはこれまでの*ex vivo*増幅臍帯血の効能，および安全性に関する基礎研究や基盤整備を背景として，2005年9月から*ex vivo*増幅臍帯血を用いた臨床試験を計画している．本試験では日本造血細胞移植学会「造血幹細胞移植のガイドライン」の移植適応に合致し，骨髄移植および末梢血幹細胞移植において適切なドナーを得ることができない急性骨髄性白血病の患者を対象としている．図2に示すように凍結臍帯血を解凍後に分割し，$2.0\times10^7$/kg相当の臍帯血を未処理のまま移植，もう一方はCD34陽性細胞に純化後，上述した培養法で12日間増幅し移植するも

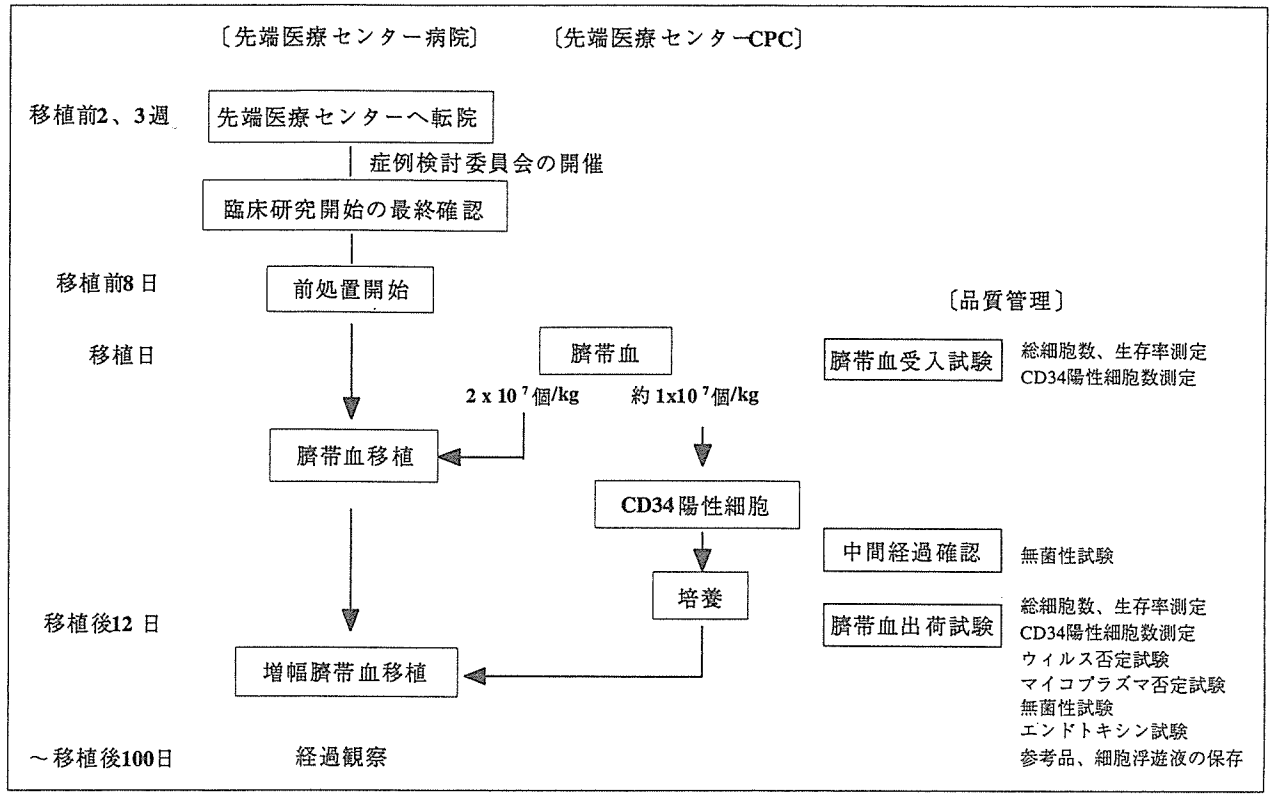


図2　GCPに準拠した*ex vivo*増幅臍帯血の臨床応用

のであり，主要エンドポイントとしてその安全性さらには増幅培養したCD34陽性細胞の輸注細胞数と生着率の相関を検討することを予定している．

また，具体的な再生医療実践のための指針として2004年4月に発行された「トランスレーショナルリサーチ実施にあたっての共通倫理審査指針」[16]はトランスレーショナルリサーチの定義，理念およびその倫理審査体制についてまとめられたものであり，われわれは現在このガイドラインに従った形での臨床試験のデザイン構築を進めている．

## 今後の展望

造血幹細胞移植は多様化しており，本研究も含めいまだ実験段階にある移植法がいくつかある．今後質の高いトランスレーショナルリサーチを行うことにより，これら治療法の安全性，効果および実施可能性を証明し，患者にとって最適な移植が選択できるよう努めていく必要があろう．

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# Ⅶ. （財）ヒューマンサイエンス振興財団

「平成 17 年度ヒトゲノム・再生医療等研究推進事業」
に基づく研究班事業報告並びに研究実績報告書

［ 研究支援者活用事業 ］
（ヒトゲノム・再生医療等研究推進事業）

# 研究業務実施報告書

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3. 研究支援期間
   平成 17 年 10 月 1 日 ～ 平成 18 年 3 月 31 日

4. 研究課題
   *Ex vivo* 増幅臍帯血の製造法の確立に関する研究。

5. 研究業務実施の概要
「サイトカインによる増幅培養臍帯血による臍帯血移植の臨床試験 (平成 17 年度厚生労働科学研究費補助金ヒトゲノム再生医療等研究事業) 」では、*ex vivo* 増幅した臍帯血を臍帯血移植及び DLI(Donor Lymphocyte Infusion; ドナーリンパ球輸注)へ臨床応用し、その有効性及び安全性を証明すること、さらにはそれらを新たな治療法として確立することを目的としている。

本分担研究では、トランスレーショナルリサーチに不可欠である「GMP(Good Manufacuturing Practice)に準拠した細胞プロセッシングのための基盤整備」を目的として、CPC (cell processing center) の整備、全作業工程の文書体系の整備を中心に行い、製造バリデーションにより臍帯血の *ex vivo* 増幅法の作業行程を検証し、さらには臨床試験における製造担当者としての業務を遂行すると同時に、整備した基盤を新たな研究へと発展させていく予定である。