

# Role of *kit*-Ligand in Proliferation and Suppression of Apoptosis in Mast Cells: Basis for Radiosensitivity of *White Spotting* and *Steel* Mutant Mice

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## Summary

The receptor tyrosine kinase Kit and its cognate ligand KL/steel factor are encoded at the *white spotting* (*W*) and *Steel* (*Sl*) loci of the mouse, respectively. Mutations at both the *W* and the *Sl* loci affect hematopoiesis including the stem cell hierarchy, erythropoiesis, and mast cells, as well as gametogenesis and melanogenesis. In addition, mutant mice display an increased sensitivity to lethal doses of irradiation. The role of KL/*c-kit* in cell proliferation and survival under conditions of growth factor-deprivation and  $\gamma$ -irradiation was studied by using bone marrow-derived mast cells (BMMC) as a model. Whereas apoptosis induced by growth factor deprivation in BMMC is a stochastic process and follows zero order kinetics,  $\gamma$ -irradiation-induced apoptosis is an inductive process and follows higher order kinetics. In agreement with these results,  $\gamma$ -irradiation-induced apoptosis in BMMC was shown to be dependent on p53 whereas apoptosis induced by deprivation is partly dependent on p53, implying that there are other mechanisms mediating apoptosis in KL-deprived BMMC. In the presence and in the absence of serum, KL stimulated proliferation by promoting cell cycle progression. The presence of KL was required only during the early part of the G<sub>1</sub> phase for entry into the S phase. At concentrations lower than those required for proliferation, KL suppressed apoptosis induced by both growth factor-deprivation and  $\gamma$ -irradiation, and internucleosomal DNA fragmentation characteristic of apoptosis. The ability of KL to suppress apoptosis was independent of the phase of the cell cycle in which the cells were irradiated and suppression of apoptosis was a prerequisite for subsequent cell cycle progression. Moreover, addition of KL to  $\gamma$ -irradiated and growth factor-deprived cells could be delayed for up to 1 h after irradiation or removal of growth factors when cells became irreversibly committed to apoptosis. KL and IL-3 induce suppression of apoptosis in mast cells by different mechanisms based on the observations of induction of *bcl-2* gene expression by IL-3 but not by KL. It is proposed that the increased sensitivity of *W* and *Sl* mutant mice to lethal irradiation results from paucity of the apoptosis suppressing and proliferative effects of KL.

During development and in the adult, maintenance of homeostasis of cell systems is achieved by a balance between the rate of cellular proliferation, differentiation, and cell loss. Under physiological conditions, cell death most often occurs by apoptosis, an active, inherently programmed process (1). Cells undergoing apoptosis display morphological changes and internucleosomal cleavage of chromatin DNA by specific endonucleases. External stimuli that initiate apoptosis are quite diverse and include deprivation of trophic factors (2) and exposure to ionizing radiation (3–6). Intracellular inducers of apoptosis include the tumor suppressor p53 (5–7), the protooncogene *c-myc* and *bax* (8). Multiple mechanisms of active suppression of apoptosis are known including a large number of trophic growth factors. The best known intracellular suppressor of apoptosis is the protooncogene *bcl-2* and

recently identified relatives of *bcl-2* (9, 10). The ability of growth factors to promote cell survival by suppressing apoptosis is fundamental for their ability to promote cell proliferation and differentiation. Since suppression of apoptosis, proliferation, and differentiation take place concurrently, it has been difficult to study these processes separately and to determine how they affect each other, though recent studies have shed some light on these questions (11).

The pleiotropic effects of growth factors are mediated by the cognate receptors which may possess intrinsic protein tyrosine kinase activity essential for triggering distinct signal transduction pathways. The protooncogene *c-kit* encodes a receptor tyrosine kinase related to the platelet-derived growth factor receptor subfamily (12, 13). The ligand of the *c-kit* receptor, KL also called steel factor, encodes two transmembrane pro-

teins, generated by alternative splicing, which may be proteolytically cleaved to produce soluble or cell-associated forms of KL (14). The *c-kit* and KL genes, respectively, are allelic with the *white spotting (W)*<sup>1</sup> and *steel (Sl)* loci of the mouse (14–16). Mutations at the *W* and *Sl* loci generate deficiencies in three cell systems during embryogenesis and in the post-natal animal: in the hematopoietic system including the stem cell hierarchy, erythropoiesis, and mast cells; in the pigmentary system, and in germ cells (17). *W* and *Sl* mutant mice suffer from macrocytic anemia, lack tissue mast cells, are sterile and devoid of coat pigment. In addition, *W* and *Sl* mutant mice exhibit an increased sensitivity to the lethal effects of x-irradiation (18, 19) and this had been proposed to be associated with delay in regeneration of erythroid tissue. However, the recent finding of a radioprotective effect of KL *in vivo* suggests a critical role for KL during the administration of radiation much rather than during the recovery period (20). The defects of *W* and *Sl* mutant mice strongly suggest a role of the *c-kit* receptor system in mediating proliferation, survival, radioresistance, migration, and differentiation of precursor cells as well as enhancing functions of more differentiated cells (21).

*In vitro* studies in the hematopoietic system have addressed the role KL may have in promoting the proliferation and differentiation of progenitor cell populations and mature hematopoietic cells (for reviews see 22). In bone marrow and fetal liver-derived mast cells as well as connective tissue-type peritoneal mast cells KL facilitates proliferation and differentiation (23, 24). To facilitate the elucidation of the mechanisms governing the pleiotropic responses of KL, we studied the role of the KL/*c-kit* receptor system in promoting cell proliferation and survival as well as resistance to the effects of lethal irradiation in bone marrow-derived mast cells (BMMC). We employed BMMC as the model cell system, since KL and *c-kit* play essential roles in the development/maturation of mast cells (25), and homogeneous populations of BMMC can be cultured readily *in vitro*, for biological assays and biochemical characterization. Moreover, the results obtained from studies in mast cells may be extrapolated to other cell systems, particularly hematopoietic stem cells which are rare cells and therefore difficult to obtain as homogeneous cell populations for *in vitro* analysis. The role of KL as a proliferation and survival factor was studied under serum-free conditions by determining the effect of KL on apoptosis induced by growth factor-deprivation and  $\gamma$ -irradiation as a function of the cell cycle phase. In addition, the mechanism by which KL suppressed apoptosis was explored by examining gene products implicated in apoptosis. Our results indicate that suppression of apoptosis is a prerequisite for cell cycle progression and DNA damage-induced cell division delay/arrest.

<sup>1</sup> Abbreviations used in this paper: BMMC, bone marrow-derived mast cells; BrdU, bromodeoxyuridine; CHX, cycloheximide; PI, propidium iodide; rm, recombinant murine; *Sl*, *steel*; *W*, *white spotting*.

## Materials and Methods

**Mice, Mast Cell Culture, and Materials.** C57Bl/6J +/+, *W*/+, and *W*<sup>42</sup>/+, and *W*<sup>v</sup>/+ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and p53<sup>-/-</sup> (TSG-p53) mice and normal littermates from GenPharm International (Mountain View, CA). Primary BMMC were cultured as described (26). Recombinant murine KL (rmKL) expressed in *Escherichia coli* was produced as described (26). rmIL-3 and rmIL-4 were obtained from Genzyme Corp. (Cambridge, MA).  $\alpha$ -[<sup>32</sup>P]dCTP (3,000 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Cycloheximide (CHX) (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO and stored at -20°C. For irradiation of BMMC, a <sup>137</sup>Cs  $\gamma$  source was used at a preset rate of 82.1 rad/min.

**Proliferation Assay.** BMMC were starved by incubating in RPMI complete medium (26) with 10-fold reduced Wehi-3 conditioned medium for 18 h and then washed three times with RPMI medium containing 0.1% BSA or serum prior to the experiment. BMMC were seeded at 6 × 10<sup>4</sup> cells/well in 96-well plates and incubated with growth factors at 37°C. Cell viability was assessed by 0.3% trypan blue exclusion. Serum-free medium contained RPMI 1640 medium, 1% BSA (fraction V), iron-saturated human transferrin (300  $\mu$ g/ml), cholesterol (96  $\mu$ g/ml), and soybean lecithin (160  $\mu$ g/ml) (all from Sigma Chemical Co.).

**Flow Cytometric Analysis of Cell Cycle and Apoptosis.** BMMC (5 × 10<sup>5</sup> cells/ml) were starved in RPMI complete medium for 12–18 h. After incubation with growth factors, cells were collected by centrifugation, resuspended in 0.25 ml of ice-cold PBS and 0.5 ml of ice-cold methanol, and fixed at 4°C overnight. Samples were collected by centrifugation, resuspended in 0.2 ml of propidium iodide (PI; Calbiochem Corp., La Jolla, CA) solution (50  $\mu$ g/ml in 0.1% sodium citrate/0.1% Triton X-100) and incubated with RNase A (0.5  $\mu$ g/ml) for 30 min at room temperature. For cell cycle analysis, the relative DNA contents were analyzed by FACScan<sup>®</sup> (Becton Dickinson & Co., Mountain View, CA). Dead cells were gated out on the basis of forward and 90° light scatter. The percentage of cells in G<sub>1</sub>, S, and G<sub>2</sub>/M phases was determined with 10,000 cells by using the CELLFIT program (Becton Dickinson & Co.). To determine the proportion of apoptosis, the cells were fixed and stained with PI as above; the proportion of cells that had hypodiploid DNA content representing the apoptotic cells was determined by the LYSIS II program (Becton Dickinson & Co.) as the mean percent apoptosis of 20,000 cells. For bromodeoxyuridine (BrdU; Sigma Chemical Co.) labeling, BrdU was added to a final concentration of 10  $\mu$ M, the cells were fixed and incubated with FITC conjugated anti-BrdU antibody conjugated with FITC (Becton Dickinson & Co.) followed by PI according to the manufacturer's instruction, and the nuclei were analyzed by FACS<sup>®</sup> with dead cells gated out as above.

**DNA Fragmentation Analysis.** DNA samples were extracted from 2 × 10<sup>6</sup> BMMC using standard protocol. DNA (5  $\mu$ g) was fractionated by agarose gel electrophoresis (1% agarose) in 0.04 M Tris-acetate 0.001 M EDTA (TAE) buffer containing 0.5  $\mu$ g/ml of ethidium bromide. The DNA fragments were visualized under ultraviolet light.

**RNA Analysis.** Total RNA was extracted by using RNeasy<sup>™</sup> (Qiagen, Crawley, UK). RNA samples (10  $\mu$ g) were fractionated by agarose-formaldehyde gel electrophoresis, transferred to nitrocellulose blot (BA-S 85 from Schleicher & Schuell, Inc., Keene, NH), and hybridized with <sup>32</sup>P-labeled DNA probes as described (14). The *bcl-2* probe was prepared by the PCR amplification of the first exon (0.6 kb) of the murine *bcl-2* locus (27) using the sense primer 5' CATAAGCTTATGGCGCAAGCCGGGAGAACA-GGGTATGAT 3' and the antisense primer 5' AACCTCGAGTTC-

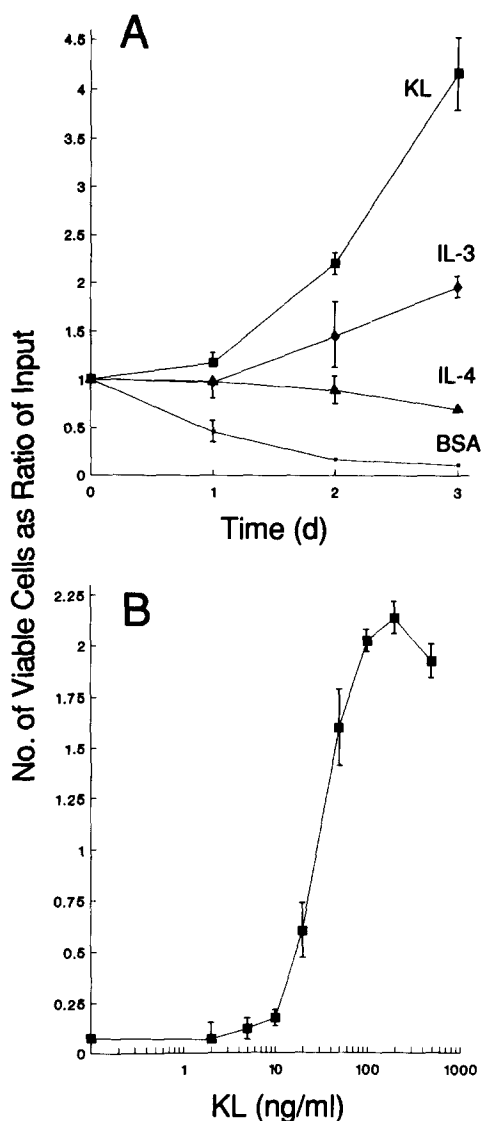
AACCAGACATGCACCTACCCAGCCTCC 3'. The reaction was performed in the DNA Thermal Cycler using the protocol and reagents from Perkin-Elmer Cetus (Norwalk, CT). The PCR product was electrophoresed in a 1% agarose gel and purified using GENECLEAN kit (Bio 101 Inc., Vista, CA).

## Results

**KL-induced Proliferation of Mast Cells and Requirement of Serum.** The mitogenic effect of *E. coli*-expressed KL (rmKL) was characterized by determining the time course and dose-response relationship for KL-induced cell proliferation (Fig. 1, A and B). The number of viable BMDC doubled within the initial 48 h of incubation, whereas the second doubling occurred within the next 24 h (Fig. 1 A). In comparison with KL, addition of saturating concentration of IL-3 to BMDC produced a doubling of cell number within 3 d, while the number of viable cells remained unaltered in the presence of IL-4 for the initial 2 d. The dose-response analysis shown in Fig. 1 B indicates a half-maximal cell proliferation response at 50 ng/ml KL whereas a maximal response is obtained at 100–500 ng/ml. The serum requirement of KL-induced cell proliferation was determined next. KL alone stimulated DNA synthesis and this activity was enhanced by serum (not shown). However, by proliferation assay no increase in cell number was seen in the absence of serum, but the cell viability was partially maintained under this condition. Addition of BSA, holo-transferrin, cholesterol, and soybean lecithin to RPMI 1640 medium restored the mitogenic properties of KL (data not shown). Therefore, KL exerts its activities as mitogen and survival factor in the absence of other growth factors.

**KL-mediated Suppression of Apoptosis Induced by Growth Factor-deprivation and  $\gamma$ -Irradiation.** The role of KL as a survival factor in BMDC was investigated in serum-free condition. BMDC used in these experiments had been grown with KL as the sole mitogen for 22 h. Upon removal of KL, the cells initiated apoptosis within 1–2 h after removal of KL, and 95% of the cell population was apoptotic after 30 h of KL-deprivation (Fig. 2 A). By contrast,  $\gamma$ -irradiation (2,500 rad) induced apoptosis with relatively rapid kinetics such that >90% of the irradiated BMDC became apoptotic within 12 h (Fig. 2 B). Since nonsynchronized BMDC were used in this experiment, these results represented an average response. By starving the cells for 12 h in RPMI 1640 complete medium, a synchronized population (>95%) arrested in the early G<sub>1</sub> phase of the cell cycle was obtained.  $\gamma$ -Irradiation of synchronized cells essentially gave similar results to those obtained with randomly growing cells (data not shown). KL suppressed apoptosis induced by deprivation and irradiation in a dose-dependent manner and with similar saturating concentrations (50 ng/ml) (Fig. 2, C and D).

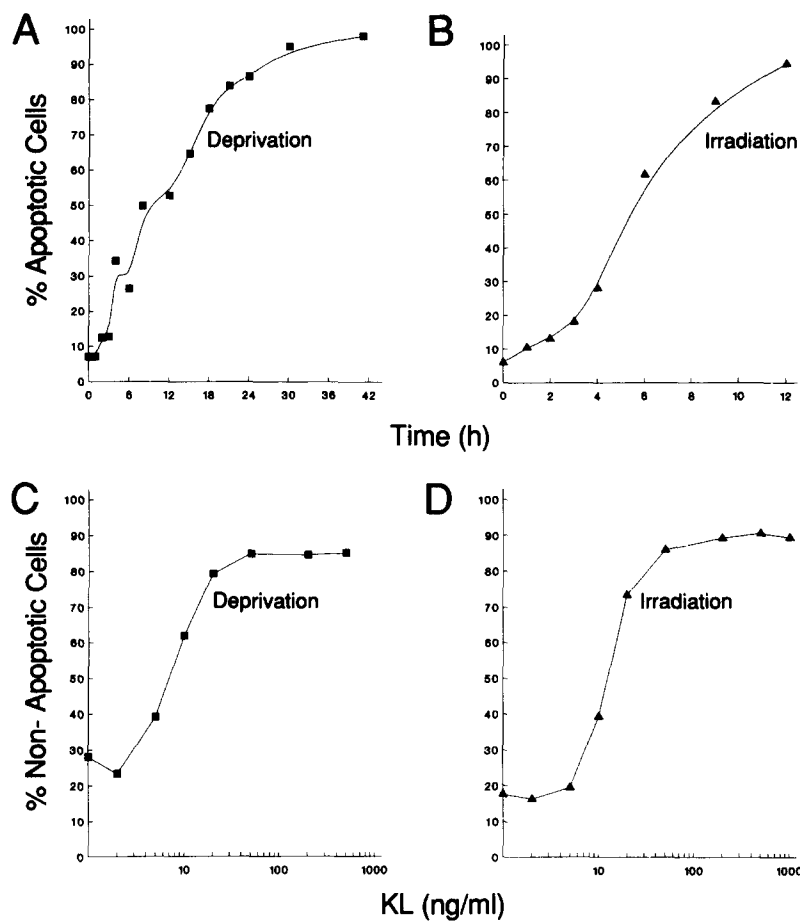
Apoptosis in BMDC was confirmed by examining DNA integrity. While in apoptotic cells the characteristic pattern of DNA fragmentation was observed (Fig. 3 A), KL suppressed DNA fragmentation in a dose-dependent manner (Fig. 3 B). The effect of KL on irradiation-induced apoptosis was also supported by the demonstration of suppression of internucleosomal DNA fragmentation (Fig. 3 C). Therefore,



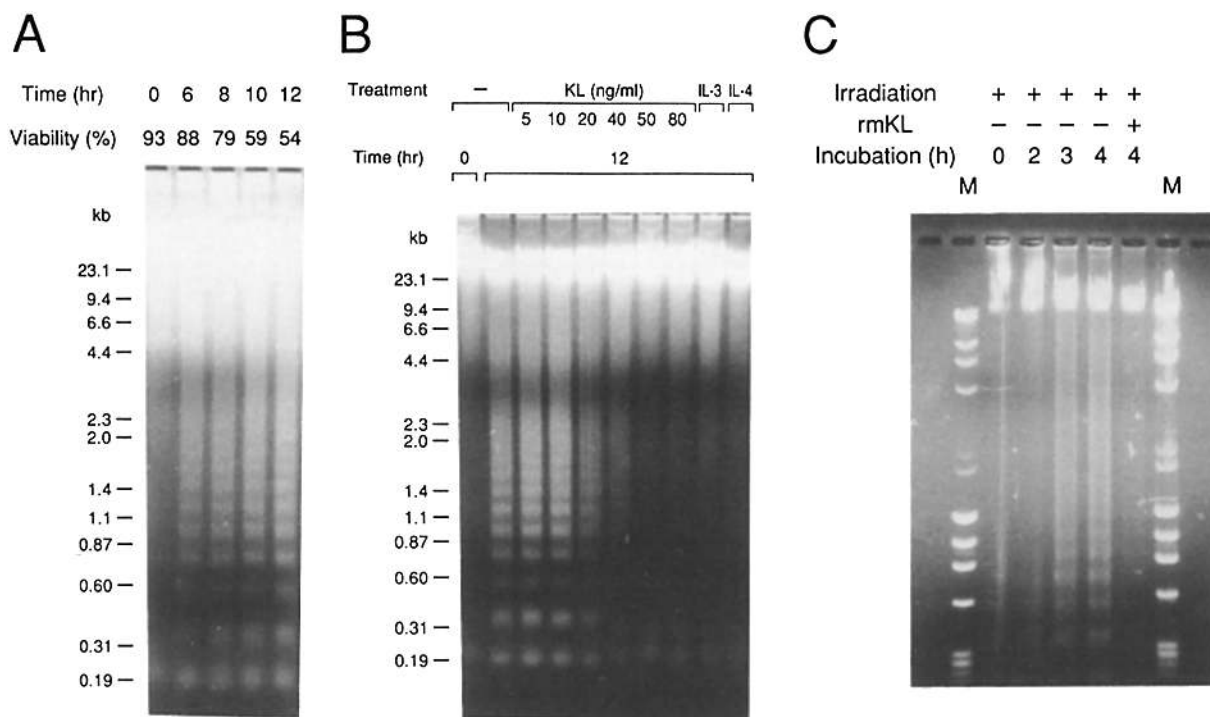
**Figure 1.** Mitogenic effect of KL. Starved BMDC were incubated in RPMI complete medium at 37°C with (A) 200 ng/ml KL, 100 U/ml IL-3, or 50 ng/ml IL-4 for up to 72 h, or (B) with various concentrations of KL for 48 h. The number of viable cells (excluding trypan blue) was determined. Each point represents the mean ( $\pm$ SD) of triplicate measurements and the results are representative of three independent experiments with triplicate samples each.

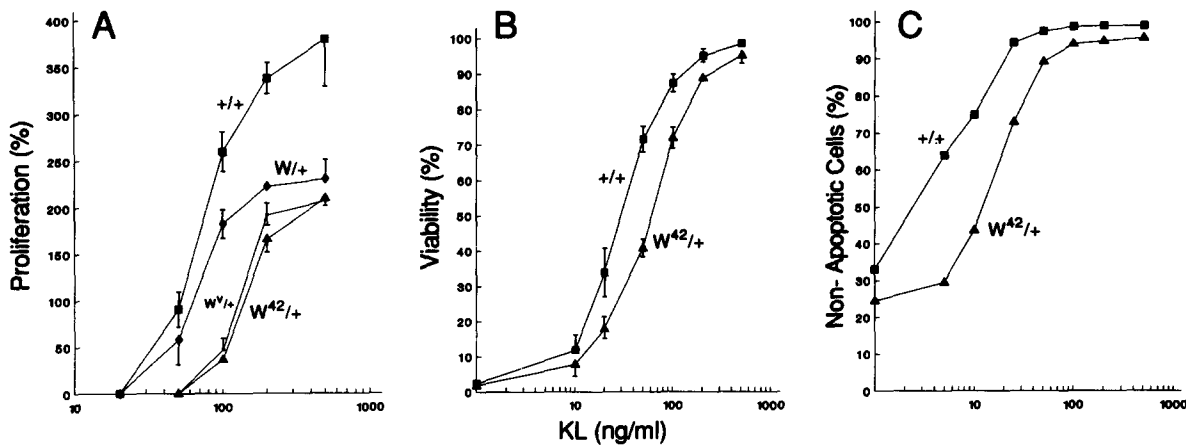
BMDC rapidly undergo apoptosis and exhibit internucleosomal DNA fragmentation when deprived of growth factors and serum as well as after treatment with  $\gamma$ -irradiation. Without requiring any additional factors, KL is capable of inhibiting apoptosis and the associated DNA fragmentation, and these effects are specifically mediated by the *c-kit* receptor. However the possibility that KL is inducing other factors in BMDC that alone or with KL are responsible for inhibition of apoptosis cannot be excluded.

**KL and *c-kit* Receptor Are Limiting in Determining Cellular Responsiveness.** Regulation of proliferation and suppression of apoptosis by KL was dose-dependent, such that at suboptimal concentrations, KL became a limiting factor in deter-



**Figure 2.** KL-mediated suppression of apoptosis induced by KL-deprivation and/or  $\gamma$ -irradiation. (A) BMMC were incubated in serum-free medium with KL for 22 h at 37°C, and then incubation was continued in the presence or absence of KL for up to 41 h. At each time point, cells were harvested for determining apoptosis by FACS<sup>®</sup>. (B) BMMC were incubated in serum-free medium with KL for 22 h and then treated with or without  $\gamma$ -irradiation (2,500 rad), and incubation was continued in serum-free medium in the presence or absence of KL for up to 12 h. At each time point, the cells were harvested for determining apoptosis. (C and D) Growth factor-deprived BMMC (C) and BMMC irradiated with 2,500 rad (D) were incubated with KL (2–1,000 ng/ml) for 30 and 10 h, respectively, and then analyzed for apoptosis.





**Figure 4.** Titration of KL and *c-kit* receptor for proliferation and suppression of apoptosis. BMMC derived from +/+, *W*/+, *W<sup>v</sup>*/+, and *W<sup>42</sup>*/+ mice were (A and B) stimulated with KL for 4 d at 37°C, and the percentages of proliferation and viability were determined and expressed as the mean (± SD) of triplicate samples, and (C)  $\gamma$ -irradiated (2,500 rad) in the presence of KL, incubated for 4 h, and then analyzed for apoptosis by FACS<sup>®</sup>. The same results were obtained in a repeated trial.

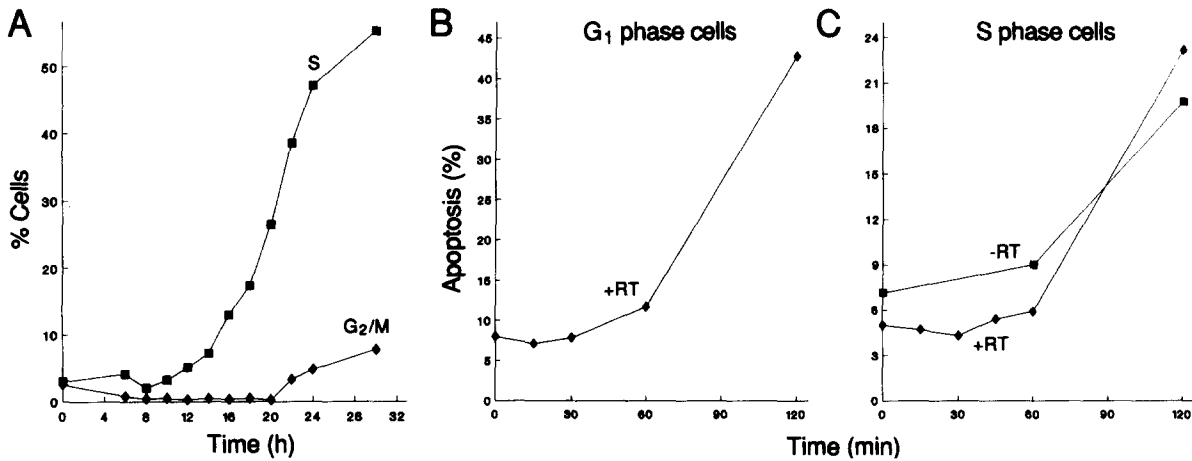
mining the responses elicited (Figs. 1 B and 2, C and D). Similarly, the number of functional cell surface *c-kit* receptors could be important in controlling the cellular responsiveness in BMMC. Many mutations are known at the *c-kit*/*W* locus and several alleles have been characterized at the molecular level. Whereas *W* is a null allele that produces a nonfunctional receptor that is not expressed at the cell surface, *W<sup>v</sup>* and *W<sup>42</sup>* contain missense mutations that inactivate the *c-kit* kinase to differing degrees and have dominant negative characteristics (28, 29). Therefore, BMMC derived from +/+, *W*/+, *W<sup>v</sup>*/+, and *W<sup>42</sup>*/+ mutant mice contain decreasing numbers of functional *c-kit* receptors on their cell surface. We used +/+, *W*/+, *W<sup>v</sup>*/+, and *W<sup>42</sup>*/+ BMMC for assaying proliferation and suppression of apoptosis induced by growth factor-deprivation and irradiation in response to various concentrations of KL to evaluate whether the number of functional cell surface *c-kit* receptors is limiting. The concentrations of KL for half-maximal proliferation were similar for +/+ and *W*/+ (75 ng/ml and 65 ng/ml, respectively) and approximately double for *W<sup>v</sup>*/+ and *W<sup>42</sup>*/+ (140 ng/ml and 150 ng/ml). At a saturating concentration of 500 ng/ml of KL, *W*/+ BMMC exhibited 60% of the maximal proliferative response of +/+, and *W<sup>v</sup>*/+ and *W<sup>42</sup>*/+, 55% (Fig. 4 A). Next, KL-mediated suppression of apoptosis induced by deprivation and  $\gamma$ -irradiation in +/+ and *W<sup>42</sup>*/+ BMMC was investigated (Fig. 4, B and C). The concentrations of KL for half-maximal suppression of apoptosis induced by deprivation and irradiation were relatively higher for *W<sup>42</sup>*/+ mast cells compared to +/+ mast cells. However, at saturating concentration of KL, apoptosis induced

by both deprivation and irradiation was completely inhibited in both cell types. Therefore, the number of functional *c-kit* receptors is limiting in the proliferative response to KL but not in its ability to suppress apoptosis, provided KL is present at saturating concentrations. Furthermore, the dominant negative effects of *c-kit<sup>W<sup>v</sup></sup>* and *c-kit<sup>W<sup>42</sup></sup>* were evident at suboptimal levels of KL.

**Requirement of KL for Cell Cycle Progression and Suppression of Apoptosis Induced by Deprivation of Growth Factors and  $\gamma$ -Irradiation.** BMMC were starved from IL-3 (Wehi-3 CM) for 18 h resulting in accumulation of >95% of cells in G<sub>1</sub> phase (data not shown). Analysis of the cell cycle kinetics using PI by FACS<sup>®</sup> indicated that KL stimulated BMMC to enter S phase with a lag period of 12–14 h, and completion of the cycle and return to the G<sub>1</sub> phase occurred after 48 h for the first round and after 72 h for the second round (not shown). These results are in agreement with the time course of cell proliferation shown in Fig. 1 A. A maximum of 65% of the cells at any given time had entered the cell cycle and were in S phase and G<sub>2</sub>/M. Incorporation of BrdU indicated that within 42 h of incubation of G<sub>1</sub> phase BMMC with KL, >95% of the cells were cycling (not shown) and this is in agreement with the doubling time of 44–48 h (Fig. 1 A). Taken together, in vitro-derived BMMC provide a homogeneous cell population which can be arrested in the G<sub>1</sub> phase of the cell cycle and stimulated by KL alone to progress synchronously through the cell cycle.

To further understand the role of KL in cell cycle progression and suppression of apoptosis, we determined the time periods in which KL is required during these events. First,

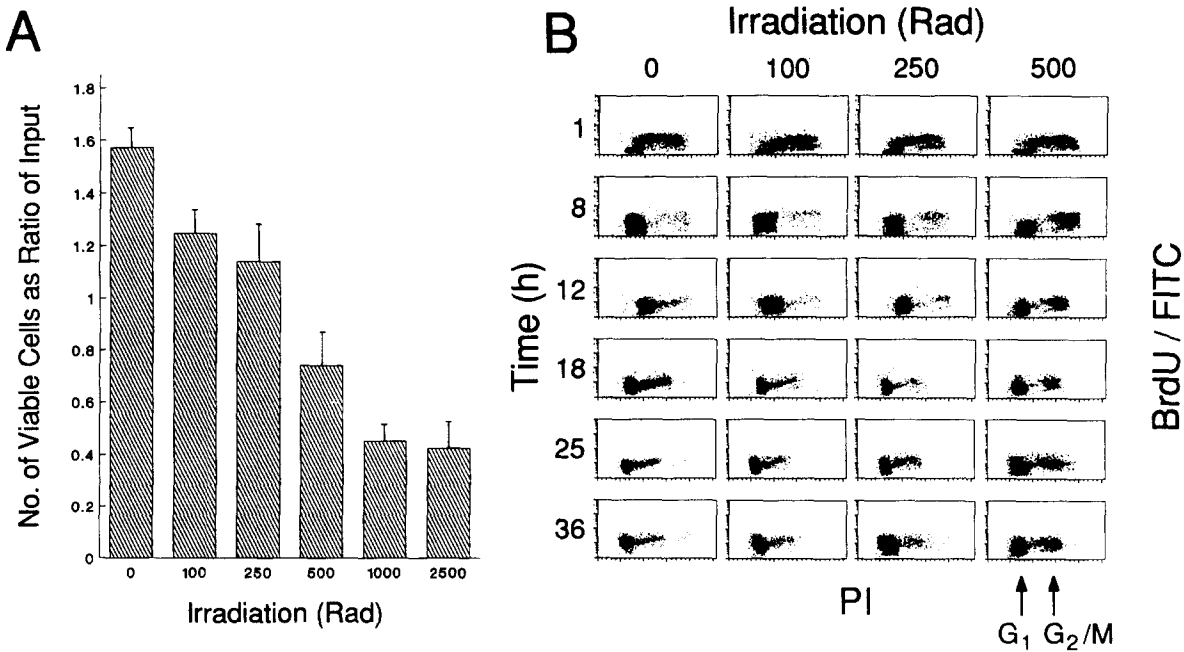
**Figure 3.** KL-mediated suppression of DNA fragmentation induced by growth factor deprivation and/or  $\gamma$ -irradiation. BMMC were washed free of growth factors and incubated in serum-free medium for up to 12 h at 37°C in the absence of KL (A), or in the presence of various concentrations of KL, IL-3 (100 U/ml), or IL-4 (50 ng/ml) (B). DNA was extracted and assayed for fragmentation. Migration of HindIII  $\lambda$  phage DNA fragments is indicated in kilobases. (C) BMMC were  $\gamma$ -irradiated (2,500 rad) and then incubated for up to 4 h with or without KL. DNA fragments were analyzed by gel electrophoresis and ethidium bromide staining. HindIII  $\lambda$  phage DNA was used for molecular size markers (M).



**Figure 5.** Temporal requirements of KL for cell cycle progression and suppression of apoptosis. (A) To determine the requirements for cell cycle progression starved BMMC were treated with KL for various time intervals. KL was removed at the indicated times and incubation continued for up to 31 h. The DNA contents of each cell sample were analyzed by FACS<sup>®</sup>. (B) To determine the temporal requirements of KL for suppression of apoptosis starved BMMC (G<sub>1</sub> phase cells) were irradiated (2,500 rad) and then incubated for 4 h at 37°C. During the postirradiation incubation period KL was added at the indicated times and cells were analyzed for apoptosis by FACS<sup>®</sup>. (C) BMMC cultured for 22 h in KL-containing serum-free medium were washed free of KL and incubation was continued in serum-free medium. KL was readded at the indicated times and incubation was continued for up to 12 h (-RT). Alternatively, BMMC were  $\gamma$ -irradiated and incubated for 4 h and KL was added at the indicated time points (+RT). The cells were analyzed for apoptosis by FACS<sup>®</sup>.

the requirement of KL in cell cycle progression was investigated. Synchronized BMMC cultures were incubated with KL for increasing time periods after which they were washed and incubation continued in the absence of KL. After a total incubation period of 31 h cells were harvested and analyzed for DNA content. In addition, control cell samples were col-

lected at the time of KL removal for determination of DNA content by FACS<sup>®</sup> analysis. While 12–14 h are required for cells to traverse G<sub>1</sub>, KL was required only during the initial 8–10 h after which the presence of KL was no longer necessary for cells to enter S phase (Fig. 5 A). Nevertheless, presence of KL for 20 h was necessary for cells to enter G<sub>2</sub>/M,



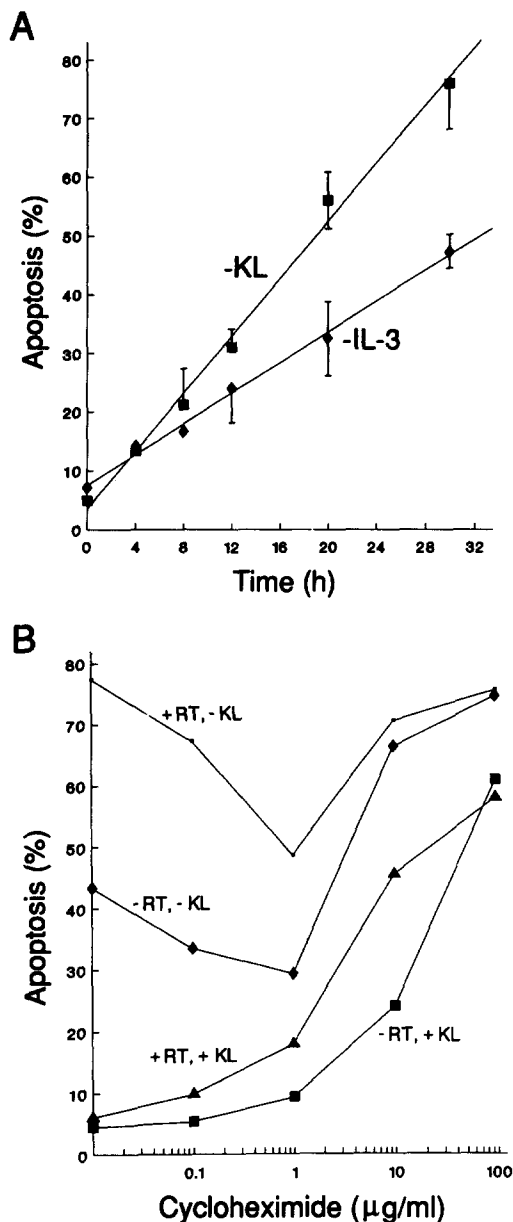
**Figure 6.** KL-promoted proliferation following low dose  $\gamma$ -irradiation. (A) BMMC were  $\gamma$ -irradiated in the presence of KL added both before and immediately after irradiation, incubated for 48 h at 37°C, and counted. The results are expressed as the mean ( $\pm$  SD) of two experiments with triplicate determinations each. (B) BMMC were pretreated with KL for 18 h,  $\gamma$ -irradiated at various doses (100–500 rad), and then incubated in the presence of KL and BrdU. Cells were collected at the indicated time intervals which refers to the time after addition of BrdU, and analyzed for DNA contents by FACS<sup>®</sup>.

suggesting that once cells have entered S, KL is still required for survival to ensure that cells can complete the cell cycle.

Next, we determined the temporal requirements of KL for suppression of apoptosis by delayed addition of KL to BMMC following growth factor-deprivation and  $\gamma$ -irradiation. Addition of KL to cells irradiated during G<sub>1</sub> phase could be delayed for up to 1 h before a significant fraction of the cells irreversibly initiated apoptosis (Fig. 5 B). However, interpretation of the results of suppression of apoptosis of irradiated G<sub>1</sub> cells is complicated by the fact that the effects of KL on cell cycle progression and suppression of apoptosis are difficult to dissociate. Since KL is not required for cell cycle progression after the "restriction point," i.e., after the initial 8–10 h, we determined if KL could suppress radiation-induced apoptosis in S-phase cells. Thus, G<sub>1</sub> phase BMMC were incubated for 18–22 h with KL until they had entered S phase (> 60% as indicated by FACS<sup>®</sup>) where KL was no longer required for completion of the cell cycle. BMMC were washed, irradiated and KL was added at different time points after irradiation. In S-phase cells, similar to G<sub>1</sub>-cells, the addition of KL could be delayed for up to 1 h after irradiation and/or KL-deprivation before a significant fraction of the cells irreversibly initiated apoptosis (Fig. 5 C).

The effects of ionizing radiation include the induction of apoptosis as well as cell division delay which is temporary at low doses of radiation and the length of the delay is proportional to the dose delivered. Thus, we determined the proliferative capacity of BMMC incubated with KL for 48 h after treatment of  $\gamma$ -irradiation. Although KL effectively suppresses apoptosis induced by irradiation, we observed a dose-dependent decrease of cell proliferation (Fig. 6 A). FACS<sup>®</sup> analysis of BrdU/FITC-labeled cells after treatment with increasing radiation dosage (0–500 rad) in the presence of KL, revealed an increasing fraction of cells accumulated at the G<sub>2</sub>/M (8 h) transition and in G<sub>1</sub> phase (12 and 18 h) (Fig. 6 B). Irradiated control cultures in the absence of KL died in a dose-dependent manner within 8 h of treatment (data not shown). Therefore, KL promotes cell survival, however radiation-induced delay of cell cycle progression cannot be modulated by KL.

**Mechanism of KL-mediated Suppression of Apoptosis.** Upon deprivation from KL the proportion of BMMC undergoing apoptosis follows that of a "gamma distribution" characteristic of stochastic processes (Fig. 2 A). In agreement with this, apoptosis induced by deprivation from KL or IL-3 follows zero order kinetics but with different rates ( $2.4 \pm 0.2\% \text{ h}^{-1}$  vs.  $1.3 \pm 0.2\% \text{ h}^{-1}$ ,  $p < 0.005$ ) (Fig. 7 A). This implies that factor deprivation-induced apoptosis is a stochastic process and that there are cell intrinsic factors that modulate the apoptotic process. In contrast,  $\gamma$ -irradiation-induced apoptosis follows higher order kinetics (Fig. 2 B) and appears to be an inductive process. A requirement for new protein synthesis exists for induction of apoptosis in some instances but not in others. In both  $\gamma$ -irradiated and growth factor-deprived BMMC, CHX, a known inhibitor of protein synthesis, appears to suppress apoptosis with maximal inhibition at 1  $\mu\text{g/ml}$  of CHX (Fig. 7 B). The effect of CHX on apoptosis induced



**Figure 7.** Kinetics of apoptosis induced by deprivation of KL or IL-3, and requirement of protein synthesis for KL-mediated suppression of apoptosis. (A) BMMC were pretreated with KL or IL-3 for 22 h, washed extensively, and incubated in the absence of either growth factor for up to 30 h at 37°C. The cells were analyzed for apoptosis by FACS<sup>®</sup>. Each point represents the mean ( $\pm$  SD) of three independent experiments. Linear regression analysis revealed correlation coefficients of 0.99 and 0.97 for KL and IL-3 pretreated cells, respectively. (B) BMMC were treated with or without  $\gamma$ -irradiation (2,500 rad) and then incubated in the presence or absence of KL for 4 h at 37°C. CHX at various concentrations was present at all times. The cells were analyzed for apoptosis by FACS<sup>®</sup>.

by growth factor-deprivation was confirmed by the demonstration of suppression of DNA fragmentation and chromosomal degradation at 1  $\mu\text{g/ml}$  of CHX, whereas other concentrations of CHX did not suppress DNA fragmentation appreciably (data not shown). Therefore, continuous pro-

tein synthesis is at least in part required for inducing apoptosis by growth factor-deprivation or  $\gamma$ -irradiation.

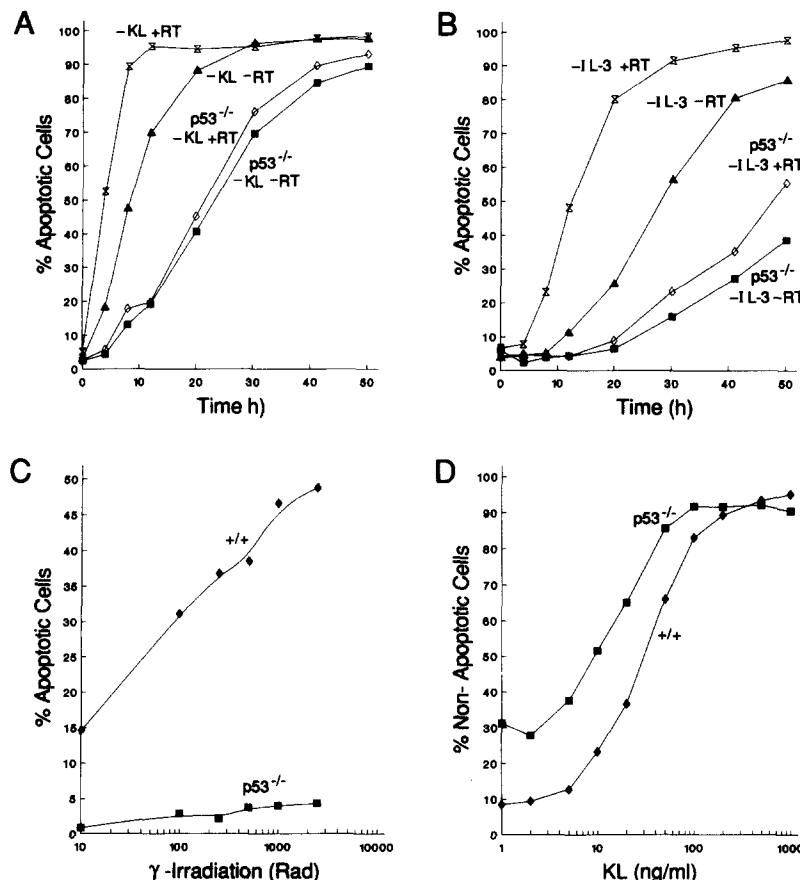
The dependence on protein synthesis for inducing apoptosis suggested the involvement of labile proteins. A requirement for p53 in radiation-induced apoptosis in thymocytes has recently been demonstrated (5, 6). Here we determined the possible role of p53 in growth factor deprivation-induced apoptosis in BMMC and in KL-mediated suppression of apoptosis. p53-deficient (p53<sup>-/-</sup>) BMMC were derived from the bone marrow of a p53<sup>-/-</sup> mouse, in which the p53 gene was disrupted by homologous recombination, and assayed for induction of apoptosis. The kinetics of apoptosis in  $\gamma$ -irradiated (500 rad) p53<sup>-/-</sup> BMMC resembled that in nonirradiated cells (Fig. 8 A), demonstrating that p53 is required for  $\gamma$ -irradiation-induced apoptosis in BMMC. The percentage of apoptotic p53<sup>-/-</sup> BMMC remained low even when treated with a saturating dose of  $\gamma$ -irradiation (2,500 rad). By contrast p53<sup>+/+</sup> BMMC were sensitive to as little as 100 rad of  $\gamma$ -irradiation (Fig. 8 C). However, upon deprivation from both KL or IL-3, p53<sup>-/-</sup> BMMC underwent apoptosis, but at a reduced rate compared with wild-type (Fig. 8, A and B), suggesting that apoptosis induced by deprivation from KL and IL-3 is only partly dependent on p53. Consequently, relatively low concentrations of KL are sufficient for suppression of deprivation-induced apoptosis in p53<sup>-/-</sup> BMMC (Fig. 8 D).

Next, we investigated the role of known inducible genes such as *bcl-2* and *bax* in the suppression of apoptosis mediated by KL and IL-3. By RNA blot analysis, there was no significant induction of *bcl-2* RNA above background level by KL under either serum-free or serum-containing conditions (Fig. 9). In contrast, however, *bcl-2* transcripts were clearly induced by IL-3 and the effect was enhanced by serum. On the other hand, *bax* RNAs were induced by both KL and IL-3, and this induction was particularly prominent for KL. Thus, *bcl-2* may not be the key regulator mediating the apoptosis-suppressing effects of KL.

## Discussion

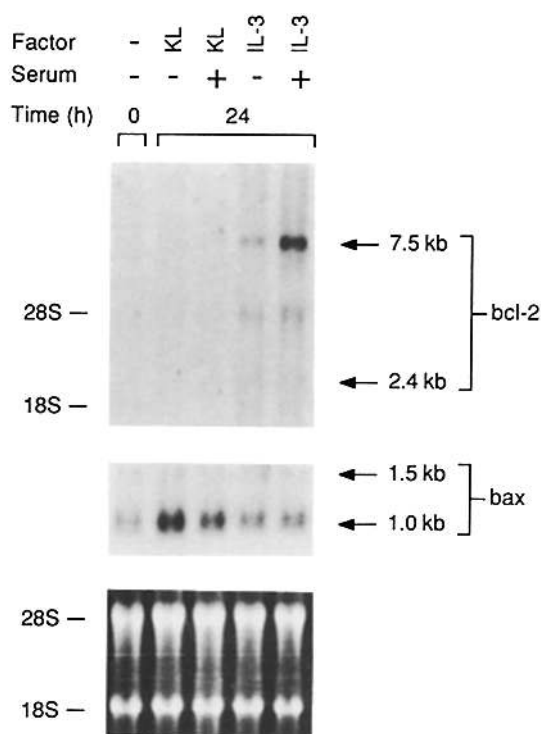
The deficiencies of mice with *W* and *S1* mutations in hematopoiesis, melanogenesis and gametogenesis and their increased radiosensitivity indicate a role for the *c-kit* receptor and its ligand in facilitating cell proliferation, survival, differentiation and conferring resistance to the lethal effects of irradiation. Here we studied the direct action of KL in promoting proliferation and survival from growth factor-deprivation and  $\gamma$ -irradiation by using suspension culture of BMMC under serum-free conditions.

We show that KL is a direct-acting mitogen for BMMC without requiring serum factors or accessory cells, although serum seems to enhance the mitogenic effect of KL. In previous studies the proliferative effect of KL had been determined



**Figure 8.** Role of p53 in deprivation or  $\gamma$ -irradiation-induced apoptosis and in apoptosis-suppressing effect of KL. (A and B) p53-deficient (p53<sup>-/-</sup>) BMMC and wild-type BMMC were incubated with (A) KL or (B) IL-3 for 22 h at 37°C in serum-free medium. The cells were then washed extensively and treated with 500 rad  $\gamma$ -irradiation (+RT) or without irradiation (-RT), incubation was continued at 37°C for various time periods, and cells were analyzed for apoptosis by FACS<sup>®</sup>. (C) P53<sup>-/-</sup> and +/+ BMMC pretreated with KL were washed, treated with various doses of  $\gamma$ -irradiation and incubation was continued for 4 h at 37°C before determination of apoptosis by FACS<sup>®</sup>. (D) KL-deprived p53<sup>-/-</sup> and +/+ BMMC were incubated with various concentrations of KL for 30 h at 37°C and apoptosis was determined by FACS<sup>®</sup>.





**Figure 9.** *bcl-2* and *bax* RNA in KL or IL-3-treated BMMC. Total RNA was extracted from BMMC treated with KL or IL-3 in the presence or absence of 10% fetal bovine serum for 24 h at 37°C and analyzed for *bcl-2* and *bax* expression. The sizes of *bcl-2 $\alpha$*  and *bcl-2 $\beta$*  are indicated as 7.5 and 2.4 kb, respectively, and *bax*  $\beta,\gamma$  and *bax*  $\alpha,\gamma$  as 1.5 and 1.0 kb, respectively. Ethidium bromide staining of ribosomal 28S and 18S RNA was shown to indicate equal loading of RNA.

by measuring the accumulated increase in cell number or colony formation or by measuring [ $^3\text{H}$ ]thymidine incorporation (23, 24, 30, 31). KL facilitates the progression of synchronized  $G_1$ -phase BMMC through the cell cycle. To promote entry into S phase KL was required only during the initial 8–10 h of the 12–14 h  $G_1$  phase. However, continuous presence of KL was necessary for supporting survival, thus enabling the cells to complete the cycle. Sustained activity of KL is required during most of the  $G_1$  phase for cells to enter S phase implying a requirement for a high level of *c-kit* receptors on the cell surface and persistent occupancy. In agreement with the observation that the concentration of KL for half-maximal proliferation is higher than that for half-maximal viability, the number of *c-kit* receptors was shown to be dose-limiting for the proliferative and not for the cell survival response of KL. Previously, CSF-1 at relatively low concentrations had been shown to support cell survival and not proliferation (32). Taken together, the extent of interaction between KL and its receptor Kit may determine differing cellular responses as a consequence of quantitative or qualitative differences in the downstream signaling pathways.

Our observation of zero order kinetics of apoptosis in growth factor-deprived cells is of interest and suggests a stochastic mechanism for apoptosis of mast cells similar to the stochastic model for the proliferation and differentiation of hematopoietic stem cells (33). By contrast,  $\gamma$ -irradiation-in-

duced apoptosis follows a more rapid and higher order kinetics and appears to be an inductive process. The intracellular mediator of apoptosis p53 is known to be induced in response to DNA damage (34) and genetic ablation of p53 abolishes radiation induced apoptosis in thymocytes (5, 6). Here we demonstrate an essential role for p53 in radiation induced apoptosis in BMMC. However, p53-deficient BMMC upon deprivation of KL continued to undergo apoptosis but at a lower rate than normal BMMC. These results imply that apoptosis induced by deprivation is partly dependent on p53, but that there are also other mechanisms mediating apoptosis in KL-deprived BMMC. KL suppresses radiation-induced and growth factor deprivation-induced apoptosis. After the initiation of deprivation and/or irradiation, addition of KL for rescue can be delayed for up to 1 h before cells are irreversibly committed to the apoptotic pathway. This implies and confirms that the level of labile proteins such as p53 (7, 34, this study) are involved in inducing apoptosis.

An increasing number of growth factors in diverse cell systems is recognized to have a role in suppressing apoptosis induced by growth factor deprivation. Several recent studies have documented a cell survival function for KL in primordial germ cells, melanoblasts, spermatogonia, erythroid progenitors, and mast cells (this study, 21, 22, 35, 36). Intracellular mediators of suppression of apoptosis are less well known. Members of the *bcl-2* gene family are the best studied normal cellular suppressors of apoptosis (9). Whereas both KL and IL-3 suppress apoptosis in BMMC, IL-3 significantly induced the expression of *bcl-2* in these cells in agreement with a role for *bcl-2* as an apoptosis suppressor; but no induction of *bcl-2* expression above background levels was observed with KL in serum-free and serum-containing condition. By contrast expression of the apoptosis inducer *bax* was induced with KL and to a lesser extent with IL-3. Interaction of Bcl-2 with Bax is thought to play a role in regulating apoptosis (8). Our results may suggest a role for Bax in mediating suppression of apoptosis induced by KL possibly through the interaction with as yet unknown proteins. Therefore, although KL and IL-3 share components of signal transduction pathways (37), they appear to facilitate cell survival by different mechanisms. The elucidation of the mechanisms by which KL is suppressing apoptosis remains to be determined.

*W* and *S1* mutant mice display an increased sensitivity toward the lethal effects of  $\gamma$ -radiation (18, 19). The radiation sensitivity may stem either from increased sensitivity of progenitors in mutant mice and/or from an effect on the regeneration of the hematopoietic tissue. The observations of KL-mediated suppression of radiation-induced apoptosis in mast cells are in good agreement with a role for KL as a radio-protective agent (18–20). The finding that significant apoptosis occurs if KL is not added within 1 h of radiation, is consistent with the recently reported failure of KL in vivo to exert a radio-protective effect when administered 4 h after irradiation (20). However, the deficit of KL activity in *W* and *S1* mutant mice quite likely also has an effect on the regeneration of hematopoietic tissue in irradiated animals and this is in agreement with the observation that postirradiation administration of KL increases survival (20).

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