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Apoptosis of *bcl-x*-Deficient Telencephalic Cells *In Vitro*

Kevin A. Roth,[1,a] **Noboru Motoyama,**[2,a] **and Dennis Y. Loh**[2]

[1]*Departments of Pathology, and Molecular Biology and Pharmacology, and* [2]*Howard Hughes Medical Institute and Departments of Medicine, Genetics, and Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110*

bcl-x is a member of the *bcl-2* gene family, which is expressed at high levels in the embryonic brain. The targeted disruption of *bcl-x* results in massive cell death of immature neurons in the developing mouse brain (Motoyama et al., 1995). *bcl-x*-deficient mice die around embryonic day 13 (E13), probably secondary to their inability to produce mature red blood cells. To determine whether the death of immature neurons in the *bcl-x*-deficient brain is cell autonomous, we examined primary telencephalic cell cultures from E12.5 homozygous mutant (*bcl-x*$^{-/-}$), heterozygous mutant (*bcl-x*$^{+/-}$), and wild-type (*bcl-x*$^{+/+}$) mice. *bcl-x*$^{-/-}$ telencephalic cells cultured in 0.5 or 2.0% fetal calf serum (FCS)-containing medium for 48 hr showed increased apoptosis, defined by abnormal bisbenzamide staining and terminal-deoxytransferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL), and decreased numbers of microtubule-associated protein-2-immunoreactive neurons compared with *bcl-x*$^{+/-}$ and *bcl-x*$^{+/+}$ cultures. Cycloheximide treatment of *bcl-x*$^{-/-}$ telencephalic cell cultures failed to prevent the increased cell death observed in low FCS-containing medium, suggesting a protein synthesis-independent apoptosis. There were no significant differences among *bcl-x*$^{-/-}$, *bcl-x*$^{+/-}$, and *bcl-x*$^{+/+}$ telencephalic cells grown for 48 hr in 5% FCS-containing medium or in a chemically defined serum-free medium (ITS). *bcl-x*$^{-/-}$ neurons generated in ITS showed increased susceptibility to subsequent serum deprivation. These results indicate that *bcl-x* is important for both neuron maturation and survival.

Key words: apoptosis; programmed cell death; bcl-x; bcl-2; *neuronal development; neuroprotection*

bcl-x is a member of the *bcl-2* gene family, which is expressed at high levels in adult lymphoid tissues and the brain (Boise et al., 1993; González-García et al., 1994). *bcl-x* can be alternatively spliced to produce two major protein isoforms, Bcl-x_L and Bcl-x_S. Bcl-x_L, similar to Bcl-2, inhibits apoptosis, whereas Bcl-x_S inhibits the antiapoptotic action of Bcl-2. In the mouse, *bcl-*x_L is the predominant *bcl-x* mRNA and is widely expressed in embryonic tissues, where it appears to be more abundant than *bcl-2* (Fang et al., 1994; González-García et al., 1994; Frankowski et al., 1995). We have shown by *in situ* hybridization of embryonic day 12.5 (E12.5) mouse brain sections that *bcl-x* mRNA is low in rapidly proliferating ventricular zone cells and is highly expressed in cells in the more mature intermediate layer (Motoyama et al., 1995). Immunohistochemical and *in situ* hybridization studies of Bcl-x distribution in mouse and human tissues indicate that it is expressed in neurons of both the central and peripheral nervous systems but not in glia (Krajewski et al., 1994; Frankowski et al., 1995). The relatively high levels of Bcl-x_L in the adult brain contrast with the low level of Bcl-2 staining in most mature adult CNS neurons (Merry et al., 1994). Recent data indicate that, similar to Bcl-2, Bcl-x_L overexpression can block the *in vitro* nerve growth factor (NGF) deprivation-induced apoptotic death of sympathetic neurons (Martinou et al., 1994; Frankowski et al., 1995; González-García et al., 1995). In total, these observations suggest that Bcl-x_L may have an important neuroprotective role in the nervous system.

To elucidate the functional and developmental role of Bcl-x, we used homologous recombination in embryonic stem (ES) cells to generate *bcl-x*-deficient mice (Motoyama et al., 1995). Heterozygous mutant mice (*bcl-x*$^{+/-}$) were healthy and normal in size. Homozygous mutant mice (*bcl-x*$^{-/-}$) died around E13. Histological examination of E11.5 and 12.5 *bcl-x*$^{-/-}$, *bcl-x*$^{+/-}$, and wild-type (*bcl-x*$^{+/+}$) mice revealed a striking abnormality in the *bcl-x*$^{-/-}$ nervous system. In the nervous system, there were large areas containing pyknotic nuclei, karyorrhectic debris, and phagocytic cells with engulfed debris. Mutant mice had a dramatic increase in terminal-deoxytransferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) in these sites, and large numbers of bright, condensed, or fragmented bisbenzamide-labeled nuclei were observed, suggesting apoptotic death.

The lymphoid system was also found to be severely affected in *bcl-x*-deficient mice. Because of early embryonic lethality, lymphocytic cell function was studied in *bcl-x* double-knockout chimeric mice. Similar to immature neurons, *bcl-x* deficiency resulted in the decreased survival of immature lymphocytes. Red blood cell maturation was also affected and probably accounts for the embryonic lethality of *bcl-x*$^{-/-}$ mice (N. Motoyama, K. Roth, S. Senju, and D. Loh, unpublished data). To determine whether neuronal apoptosis in *bcl-x*-deficient mice was cell-autonomous or secondary to the hematopoietic defect, and to begin to define the mechanisms of Bcl-x action, we examined primary cell cultures from the

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[a]These authors contributed equally to this manuscript.

Correspondence should be addressed to Dr. Kevin A. Roth, Department of Pathology, Campus Box 8118, Washington University School of Medicine, St. Louis, MO 63110.

Dr. Motoyama's present address: Research Institute for Biological Science, Science University of Tokyo, 2669 Yamazaki, Noda-city, Chiba 278, Japan.

Dr. Loh's present address: Nippon Roche Research Center, 200 Kajiwara, Kamakura 247, Japan.

E12.5 telencephalon. The E12.5 telencephalon consists predominantly of immature neuroepithelial cells and shows only rare apoptotic cells in *bcl-x*$^{-/-}$ mice.

MATERIALS AND METHODS

Generation and detection of bcl-x*-deficient mice.* *bcl-x*-deficient mice were generated by homologous recombination in ES cells (Motoyama et al., 1995). The endogenous and disrupted *bcl-x* genes were detected by PCR analysis of tail DNA extracts.

Primary telencephalic cell cultures. Primary cell cultures were prepared as described previously (Flaris et al., 1995). Briefly, pregnant mice were killed on E12.5. The morning of the day that a vaginal plug was seen was designated as E0.5. The uterus was rapidly removed under sterile conditions, and the embryos were transferred to cold HBSS medium (Gibco, Grand Island, NY). The tails were harvested for DNA extraction and PCR analysis of genotype. The telencephalon was dissected away from the surrounding brain, and the pia and connective tissue were removed with forceps. The neuroepithelial cells were dissociated for 20 min in HBSS containing 0.01% trypsin, 0.004% EDTA, 0.04 mg/ml DNase I, and 0.1% bovine serum albumin (BSA). Trypsinization was stopped with 1 vol of 10% fetal calf serum (FCS). Cells were dissociated further by two rounds of trituration with a fire-polished Pasteur pipette. Dissociated cells were washed twice with HBSS and 0.1% BSA, resuspended in HBSS, and counted on a hemocytometer. Cells were plated at ~20,000 viable cells per 0.1 ml of medium in 48-well plates coated with poly-L-lysine and laminin. The serum-containing medium consisted of DMEM/F12 with 15 mM HEPES and 1.2 gm/l sodium bicarbonate, supplemented with 2 mM glutamine, 2.6 gm/l glucose, 0.66 pl/l 2-mercaptoethanol, and 0.5, 2.0, or 5.0% FCS (HyClone, Logan, UT). Chemically defined serum-free medium (ITS) was DMEM/F12 supplemented with 5 mg/ml insulin, 100 mg/ml transferrin, 100 μM putrescine, 30 nM selenium, and 20 mM progesterone. In some experiments, 30 μM 5′-bromo-2′-deoxyuridine (BrdU) was added to the culture medium immediately after plating. All reagents were purchased from Sigma (St. Louis, MO) unless indicated otherwise.

Immunohistochemical and TUNEL labeling. The immunohistochemical methods used in our laboratory have been described previously (Flaris et al., 1995; Motoyama et al., 1995). Briefly, cell culture wells were fixed in 4% paraformaldehyde for 30 min at room temperature and subsequently incubated overnight at 4°C in diluted primary antiserum. After several wash steps, immunostaining was detected with fluorescently labeled secondary antibodies (Jackson Immunoresearch, West Grove, PA). An antibody against microtubule-associated protein-2 (MAP2; Sigma) was used to detect neurons *in vitro*. A goat anti-BrdU serum was used to detect cells that had incorporated BrdU *in vitro* (a gift from S. Cohn, Washington University). A modified TUNEL protocol was used to detect DNA fragmentation (Gavrieli et al., 1992; Tornusciolo et al., 1996). Briefly, cells were permeabilized with 0.5% Triton X-100 in PBS and incubated with terminal-deoxytransferase- and indocarbocyanine (Cy3)-conjugated deoxyuridine triphosphate. Cells were then processed for immunohistochemical labeling and bisbenzamide staining (0.1 mg/ml for 30 min at room temperature).

The quantitation of immunoreactive and/or TUNEL-stained cells was performed by an investigator blind to the experimental conditions and the genotype of the cells being evaluated. In each experiment, cells from individual wells were quantitated from two to four representative 400× magnification microscopic fields. Statistical significance was determined by ANOVA, Student's *t* test, or χ^2 analysis.

RESULTS

Low FCS-containing medium increases apoptosis and decreases neuron generation in *bcl-x*-deficient telencephalic cells

We compared the effect of FCS concentration (0.5, 2.0, and 5.0%) on primary cultures of *bcl-x*$^{-/-}$, *bcl-x*$^{+/-}$, and *bcl-x*$^{+/+}$ E12.5 telencephalic cells. The number of cells, abnormal bisbenzamide-labeled nuclei, and MAP2-immunoreactive neurons was quantitated at 2, 24, and 48 hr after plating. In 0.5 and 2.0% FCS-containing medium, *bcl-x*-deficient cultures were equivalent to wild-type and heterozygote cultures at 2 hr but showed fewer total cells, fewer neurons, and increased numbers of abnormal nuclei at 24 and 48 hr (Fig. 1). *bcl-x*$^{+/-}$ telencephalic cell cultures were similar to *bcl-x*$^{+/+}$ cultures at all concentrations of FCS-containing medium tested.

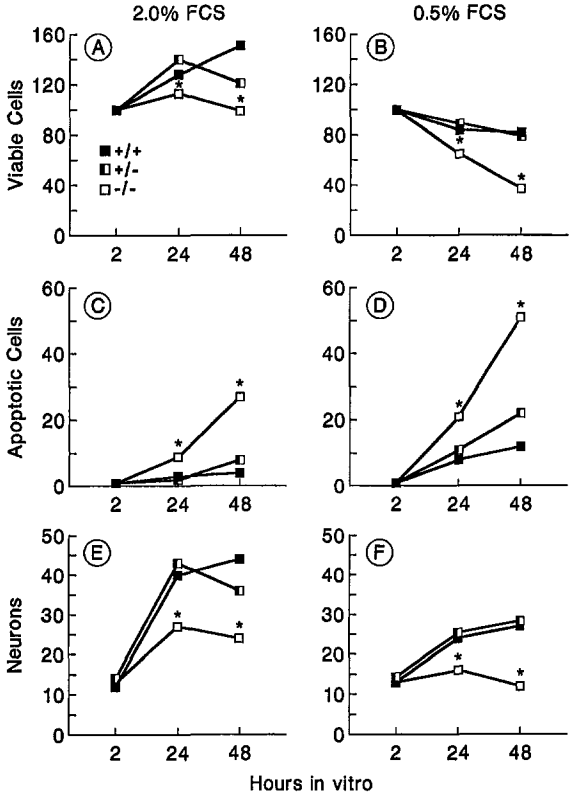

Figure 1. Effect of 2.0% (*A*, *C*, *E*) and 0.5% (*B*, *D*, *F*) FCS-containing medium on *bcl-x*$^{+/+}$, *bcl-x*$^{+/-}$, and *bcl-x*$^{-/-}$ E12.5 primary telencephalic cell cultures. Primary cells were fixed after 2, 24, or 48 hr in culture, and the number of cells, apoptotic cells, and neurons were quantitated after immunolabeling for MAP2 and bisbenzamide staining. All data are presented as a percentage of the viable cells present after 2 hr in culture. The number of viable cells (*A*, *B*) was determined by subtracting the number of abnormal bisbenzamide-stained nuclei from the total number of nuclei. Abnormal bisbenzamide-labeled nuclei were considered apoptotic (*C*, *D*), and MAP2 immunoreactivity was used as an indicator of neuronal differentiation (*E*, *F*). Data were obtained from 15 *bcl-x*$^{+/+}$, 15 *bcl-x*$^{+/-}$, and 9 *bcl-x*$^{-/-}$ individual telencephalic cell cultures, and the total number of cells counted at 2 hr in culture for each group was 1936, 2075, and 1274 in 2.0% FCS-containing medium and 2172, 2112, and 1194 in 0.5% FCS-containing medium, respectively (*$p < 0.01$ vs *bcl-x*$^{+/+}$).

Bisbenzamide staining of condensed, clumped, and/or fragmented nuclei has been used as an indicator of apoptotic death *in vitro* (Deckwerth and Johnson, 1993). To verify that abnormal bisbenzamide-stained nuclei actually represented apoptotic cells, we performed simultaneous TUNEL labeling and bisbenzamide staining on E12.5 *bcl-x*$^{-/-}$ telencephalic cell cultures after 48 hr in either 0.5 or 2.0% FCS-containing medium. Both TUNEL labeling and abnormal bisbenzamide staining revealed an approximately threefold increase in the number of apoptotic cells in 0.5 versus 2.0% FCS-containing medium (TUNEL-labeled nuclei: 39 ± 10 at 0.5% FCS, 14 ± 2 at 2.0% FCS; abnormal bisbenzamide-stained nuclei: 33 ± 10 at 0.5% FCS, 9 ± 3 at 2.0% FCS) ($n = 3$). Dual labeling also showed concordance of the two methods for detecting apoptotic cells (Fig. 2*A*,*B*).

In contrast to the effect of low FCS concentrations on *bcl-x*$^{-/-}$ cells, there were no statistical differences among *bcl-x*-deficient, heterozygote, and wild-type cultures grown in 5.0% FCS-containing medium. After 2 hr in culture medium, ~17% of the

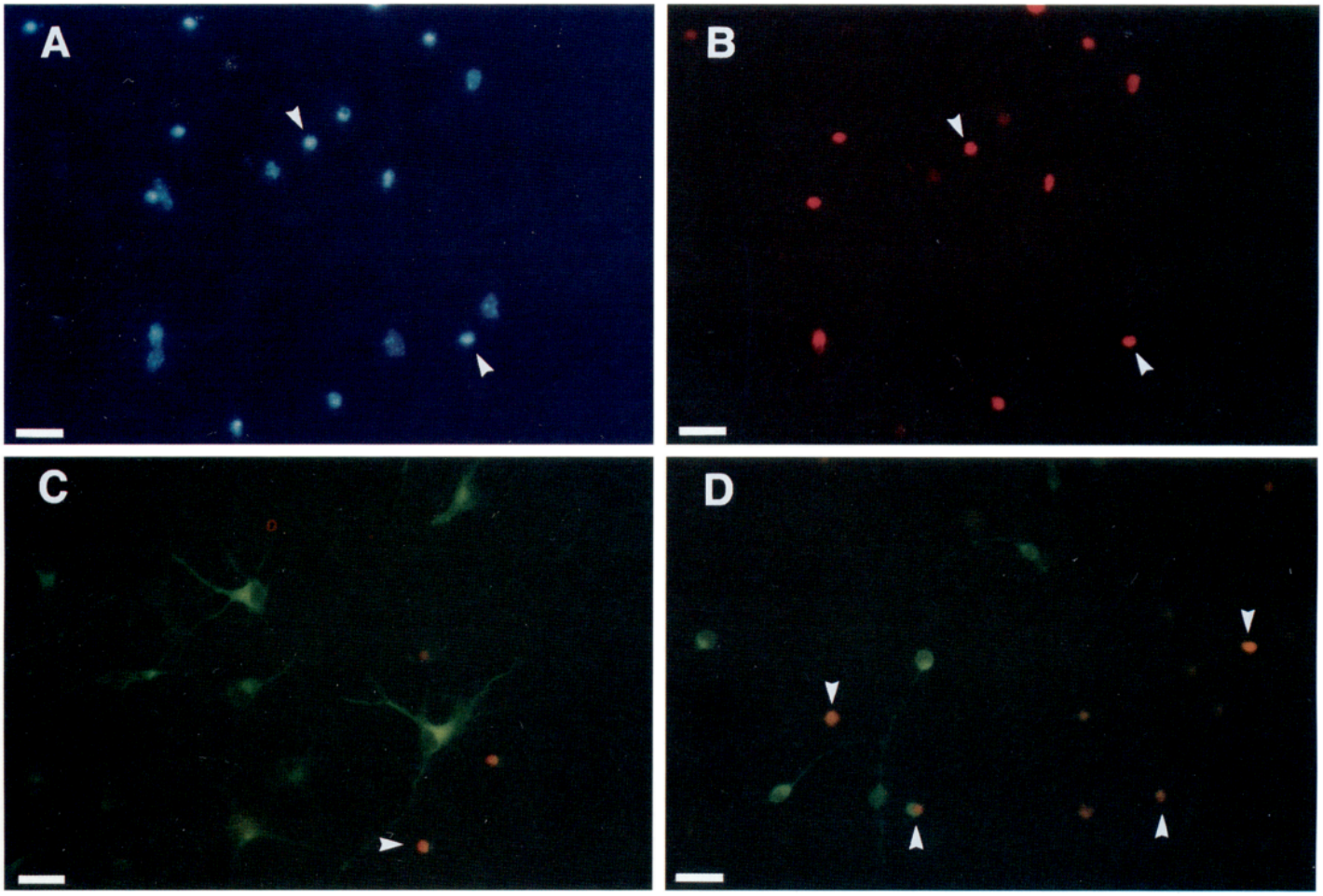

Figure 2. Apoptotic death of E12.5 *bcl-x*$^{-/-}$ telencephalic cells *in vitro*. Primary cell cultures of E12.5 *bcl-x*$^{-/-}$ telencephalic cells in 0.5% FCS-containing medium show markedly increased apoptosis as evidenced by a large percentage of condensed, bright bisbenzamide-stained nuclei (*A*) and TUNEL-positive nuclei (*B*). Sequential bisbenzamide and TUNEL labeling (*A*, *B*) reveals substantial overlap of the two signals in individual nuclei (examples indicated by *arrows*). E12.5 *bcl-x*$^{-/-}$ telencephalic cells grown in ITS medium for 48 hr (*C*) show strong MAP2 immunoreactivity (*green*) and, occasionally, TUNEL-positive nuclei (*red*). Switching the ITS medium after 48 hr to 0.5% FCS-containing medium (*D*) results in a marked reduction in MAP2-immunoreactive cells and increased numbers of TUNEL-labeled nuclei. (Examples of TUNEL-positive nuclei in *C* and *D* are indicated by *arrows*; scale bars, 25 μm.)

telencephalic cells were in S-phase of the cell cycle as determined by *in vitro* BrdU labeling (*bcl-x*$^{+/+}$: 141 BrdU-positive nuclei of 850 total nuclei counted, $n = 7$; *bcl-x*$^{+/-}$: 139/904, $n = 7$; *bcl-x*$^{-/-}$: 68/366, $n = 3$). By 24 hr, cumulative BrdU labeling revealed that ~60% of the cells had entered S-phase *in vitro* (*bcl-x*$^{+/+}$: 1316/2116, $n = 9$; *bcl-x*$^{+/-}$: 1444/2458, $n = 12$; *bcl-x*$^{-/-}$: 688/1098, $n = 6$), and by 48 hr ~75% of the cells were BrdU-labeled (*bcl-x*$^{+/+}$: 1918/2462, $n = 9$; *bcl-x*$^{+/-}$: 2404/3356, $n = 12$; *bcl-x*$^{-/-}$: 833/1157, $n = 6$). The percentage of cells with MAP2 immunoreactivity was similar among the three groups at 2, 24, and 48 hr *in vitro*. Approximately 13% of the cells in culture possessed MAP2 immunoreactivity at 2 hr (*bcl-x*$^{+/+}$: 258 MAP2 immunoreactive cells of 1916 total cells examined, $n = 14$; *bcl-x*$^{+/-}$: 308/2114, $n = 15$; *bcl-x*$^{-/-}$: 151/1230, $n = 9$), and this increased to ~25% of the cells at 24 and 48 hr (data not shown). Initially, <1% of the MAP2-immunoreactive cells contained cell processes, but this increased rapidly to ~15, 40, and >90% at 6, 12, and 24 hr, respectively (data not shown). Cumulative BrdU labeling indicated that ~10% of the MAP2-immunoreactive cells observed at 48 hr had been in S-phase during the culture period (data not shown). The percentage of abnormal bisbenzamide-labeled nuclei at 2, 6, 12, 24, and 48 hr did not differ significantly among the groups. For example, at 24 hr, <1% of the cell nuclei in each group (22/3102 wild-type cell nuclei, $n = 15$; 7/2906 heterozygote nuclei, $n = 15$; 7/1532 homozygous nuclei, $n = 9$) were considered abnormal. In total, these results indicate that *in vitro* telencephalic cell proliferation, differentiation, and death are equivalent in *bcl-x*-deficient, heterozygote, and wild-type cultures grown in 5.0% FCS-containing medium.

Cycloheximide does not block the increased apoptosis of *bcl-x*-deficient telencephalic cells in low FCS-containing medium

Because *bcl-x*$^{-/-}$ telencephalic cells showed increased apoptosis in 2.0% FCS-containing medium compared with wild-type and heterozygote cells, we tested the ability of cycloheximide, a protein synthesis inhibitor, to block the increased cell death. Cycloheximide, at 1 or 5 μg/ml, was added to the cultures at 0, 18, or 24 hr after plating. These doses were chosen based on their previously reported ability to block protein synthesis and to prevent apoptosis of NGF-deprived sympathetic neurons (Deckwerth and Johnson, 1993). Forty-eight hours after plating, the num-

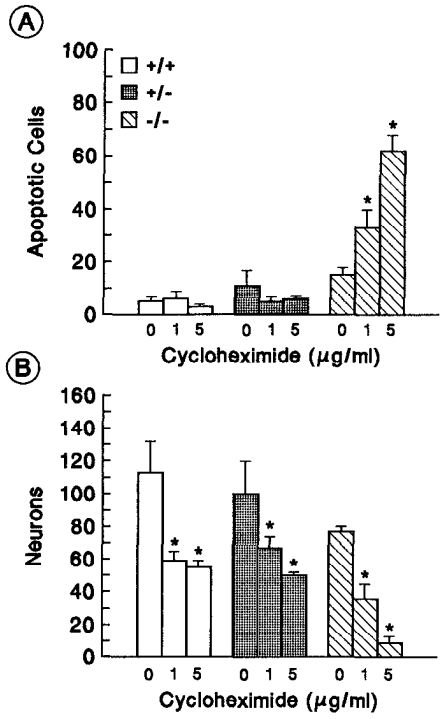

Figure 3. Effect of cycloheximide on E12.5 *bcl-x*$^{+/+}$, *bcl-x*$^{+/-}$, and *bcl-x*$^{-/-}$ primary cell cultures. Cells were plated at time 0 in 2.0% FCS-containing medium, and cycloheximide (0, 1, or 5 μg/ml) was added 24 hr later. After an additional 24 hr, the cells were fixed and the number of apoptotic cells (*A*) was determined by abnormal bisbenzamide staining, and the number of neurons (*B*) by MAP2 immunoreactivity. The data represent the mean ± SEM; n = 5, 4, and 3 for *bcl-x*$^{+/+}$, *bcl-x*$^{+/-}$, and *bcl-x*$^{-/-}$ cells, respectively. *$p < 0.05$ versus zero cycloheximide treatment.

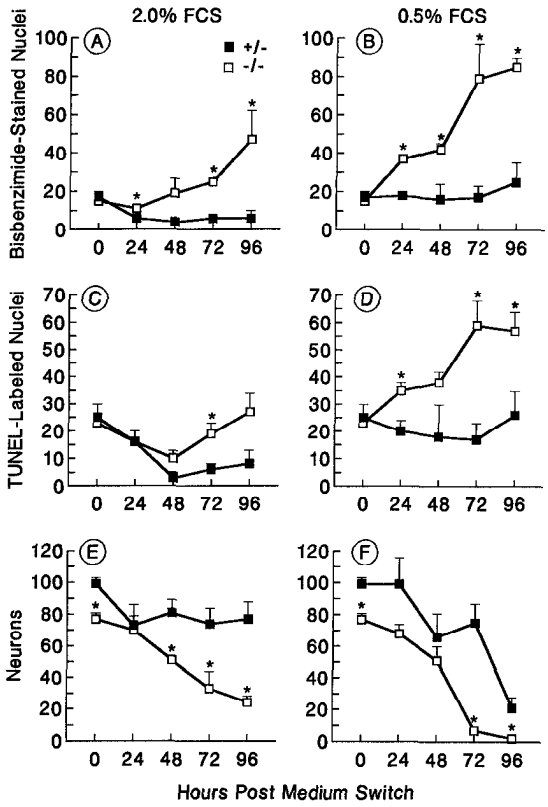

Figure 4. Effect of FCS-containing medium on E12.5 *bcl-x*$^{+/-}$ and *bcl-x*$^{-/-}$ telencephalic cells. Cells were grown in ITS medium for 48 hr and then switched to either 2.0% (*A*, *C*, *E*) or 0.5% (*B*, *D*, *F*) FCS-containing medium and fixed 24, 48, 72, or 96 hr later. Apoptotic cells were detected both by bisbenzamide staining (*A*, *B*) and TUNEL labeling (*C*, *D*). Neurons were identified by MAP2 immunolabeling (*E*, *F*). *bcl-x*$^{-/-}$ cells showed increased apoptosis and neuron loss at both FCS doses compared with *bcl-x*$^{+/-}$ cells. The data represent the mean ± SEM (*bcl-x*$^{+/-}$, n = 3; *bcl-x*$^{-/-}$, n = 3). *$p < 0.05$ versus *bcl-x*$^{+/-}$.

ber of cells, MAP2-immunoreactive neurons, and abnormal bisbenzamide-stained nuclei was assessed. Cycloheximide at both doses and at all incubation times tested decreased the total number of cells and MAP2-immunoreactive neurons at 48 hr in *bcl-x*$^{-/-}$, *bcl-x*$^{+/-}$, and *bcl-x*$^{+/+}$ telencephalic cell cultures (Fig. 3 and data not shown). Cycloheximide had no significant effect on the number of abnormal bisbenzamide-stained nuclei in control or heterozygote cell cultures. In *bcl-x*-deficient cell cultures, which at baseline had an approximately threefold increase in abnormal nuclei over wild-type cultures, cycloheximide addition exacerbated the effect of *bcl-x* deficiency (Fig. 3 and data not shown).

bcl-x$^{-/-}$, *bcl-x*$^{+/-}$, and *bcl-x*$^{+/+}$ telencephalic cells are similar in a chemically defined serum-free medium

bcl-x-deficient telencephalic cells were equivalent to wild-type cells in 5.0% FCS-containing medium. Because 5% FCS-containing medium promotes cell proliferation, and only ~25% of the cells in culture at 48 hr possess MAP2 immunoreactivity, we compared *bcl-x*$^{-/-}$, *bcl-x*$^{+/-}$, and *bcl-x*$^{+/+}$ primary telencephalic cells grown in ITS. *bcl-x*$^{-/-}$, *bcl-x*$^{+/-}$, and *bcl-x*$^{+/+}$ telencephalic cells appeared similar after 48 hr in ITS medium. Total cell numbers showed no significant increase between 2 and 48 hr in culture, and MAP2-immunoreactive cells represented >50% of the total cells at 48 hr (data not shown). The number of abnormal bisbenzamide-stained nuclei observed at 48 hr was increased in all groups over 5.0% FCS-containing cultures, and the *bcl-x*$^{-/-}$ and *bcl-x*$^{+/-}$ cells showed an increase in abnormal bisbenzamide-labeled nuclei (*bcl-x*$^{+/+}$: 2.7%, 36 abnormal nuclei in 1292 cells, n = 8; *bcl-x*$^{+/-}$: 5.7%, 105/1851, n = 12; *bcl-x*$^{-/-}$: 6.2%, 83/1331, n = 8) and in TUNEL-positive cells (*bcl-x*$^{+/+}$: 12%, 27 TUNEL-positive nuclei in 232 cells, n = 2; *bcl-x*$^{+/-}$: 16%, 177/1093, n = 8; *bcl-x*$^{-/-}$: 18%, 96/533, n = 4). Culturing of E12.5 telencephalic cells in ITS medium for an additional 48 or 96 hr resulted in a progressive increase in abnormal bisbenzamide-labeled nuclei and, ultimately, a decline in the number of MAP2-immunoreactive neurons in all groups (data not shown).

Serum deprivation of *bcl-x*-deficient neurons leads to rapid neuronal apoptosis

bcl-x$^{-/-}$, *bcl-x*$^{+/-}$, and *bcl-x*$^{+/+}$ E12.5 telencephalic cell cultures were roughly equivalent after 48 hr in ITS. To determine whether *bcl-x* deficiency increased the vulnerability of neurons that had matured for 48 hr in ITS, we switched the cells to low FCS (0.5, 1.0, or 2.0%)-containing medium and quantitated the number of cells, abnormal bisbenzamide-stained nuclei, and MAP2-immunoreactive neurons 24–96 hr later. For wild-type cells, there was no significant change in the number of neurons or apoptotic cells when the cells were switched to 2.0% FCS for up to 96 hr (data not shown). Switching cells to 0.5% FCS resulted in a significant loss of neurons and increased apoptosis at 96 hr but not 48 hr after switch (data not shown). Examination of *bcl-x*$^{+/-}$ telencephalic cells revealed an FCS dose dependence and time course similar to *bcl-x*$^{+/+}$ cells (Fig. 4). In contrast, *bcl-x*-deficient cells exhibited a greater serum sensitivity and a more rapid time

course of neuron loss and apoptosis than *bcl-x*$^{+/-}$ cells (Fig. 4). We performed TUNEL labeling on *bcl-x*$^{+/-}$ and *bcl-x*$^{-/-}$ telencephalic cells to verify that the abnormal bisbenzamide-stained cells were apoptotic (Fig. 2*C,D*). Twenty-four hours after the switch to 0.5% FCS-containing medium, there were almost twice as many TUNEL-positive nuclei in *bcl-x*$^{-/-}$ cultures than in *bcl-x*$^{+/-}$ cultures, and by 72 hr the difference was threefold (Fig. 4). Similar but less dramatic changes were observed in 2.0% FCS-containing medium.

DISCUSSION

Our studies indicate that *bcl-x*-deficient telencephalic cells are highly susceptible to apoptotic death *in vitro*. These findings suggest several interesting conclusions. First, the apoptotic death of neurons in the embryonic *bcl-x*$^{-/-}$ nervous system is probably not attributable to hematopoietic dysfunction because it can be recapitulated in primary neuronal cell cultures. Immunohistochemical studies indicate that Bcl-x is not expressed in glia; therefore, the apoptosis of *bcl-x*-deficient neurons is probably a cell autonomous process. Second, the death of immature *bcl-x*-deficient neurons can be prevented *in vitro* by 5.0% FCS or by ITS. This indicates that *bcl-x*-independent antiapoptotic pathways exist in immature neurons, and that these pathways can overcome the deleterious effect of *bcl-x* deficiency. Although extensive apoptosis of immature neurons is seen in the E12.5 *bcl-x*$^{-/-}$ nervous system, mature neurons are also present. It is unclear whether these neurons survive *in vivo* because they are truly *bcl-x*-independent or whether there are sufficient survival factors present in the developing nervous system to activate *bcl-x*-independent, antiapoptotic pathways and to rescue a subpopulation of immature neurons from *bcl-x* deficiency. An examination of the nervous system of double-knockout chimeric mice would permit an assessment of *bcl-x*-dependent and -independent neuronal subpopulations. Third, the inability of cycloheximide to block cell death suggests that new protein synthesis is not required for triggering apoptosis in *bcl-x*-deficient immature neurons. Protein- and RNA synthesis-independent neuronal apoptosis has been described in the nervous system (Galli et al., 1995). Because cycloheximide actually increased cell death in *bcl-x*-deficient neuronal cultures but not in heterozygote or wild-type cultures, this suggests that the survival of *bcl-x*-deficient cells requires ongoing protein synthesis and, thus, may be actively regulated. Fourth, *bcl-x*-deficient neurons that had differentiated *in vitro* were more susceptible to serum deprivation than *bcl-x*$^{+/-}$ and *bcl-x*$^{+/+}$ neurons, suggesting that *bcl-x* may play a significant neuroprotective role in mature neurons. In contrast to *bcl-2*, *bcl-x* is highly expressed in neurons throughout the adult CNS (Frankowski et al., 1995), suggesting an important role for *bcl-x* in regulating mature neuron survival. In the adult nervous system, neuronal apoptosis occurs in several pathophysiological states (Dickson, 1995). Recent data suggest that a significant portion of the neurons that are lost after a stroke (hypoxic/ischemic insult) dies via apoptosis (Linnik et al., 1993; Nitatori, 1995). Bcl-x_L can prevent hypoxia-induced cell death in PC12 cell lines, although the mechanisms of its antiapoptotic action are unclear (Shimizu et al., 1995). Neurodegenerative diseases, including Alzheimer's and Huntington's diseases, may also produce the apoptotic death of neurons (Lassmann et al., 1995; Portera-Cailliau et al., 1995). Because *bcl-x* expression appears to be important for the survival of mature neurons and because several neuropathological processes cause neuron death via apoptotic pathways, *bcl-x* may play a significant role in protecting neurons in the adult brain from neurodegenerative diseases and hypoxic/ischemic insults.

Our *in vivo* and *in vitro* results indicate that Bcl-x is a critical regulator of neuronal apoptosis during nervous system development. Apoptotic neuronal death occurs under a variety of circumstances in the developing nervous system (Oppenheim, 1991; Pittman, 1994; Raff et al., 1995). These include after terminal mitotic division but before the formation of synaptic contacts, during the competition for target-derived trophic factors, after synaptic contacts have been made, and after the loss of presynaptic inputs. Neuron death before synapse formation and before neurotrophic factor dependence has received relatively scant attention; however, it has been described in the retina, spinal cord, sensory ganglia, and telencephalon (Maruyama and D'Agostino, 1967; Lance-Jones, 1982; Oppenheim, 1991; Acklin and van der Kooy, 1993; Homma et al., 1994). The extent of cell death during this presynaptogenic period is difficult to determine precisely, in large part because of the rapidity with which dead cells are phagocytized (Ferrer et al., 1991, 1992). An interesting analysis performed by Acklin and van der Kooy (1993) on the late embryonic rat brain suggests that extensive presynaptogenic cell death occurs in telencephalic precursor cell lineages. Individual precursor cells in the E17–E19 rat telencephalon were labeled with a retroviral "tag," and the fates of their daughter cells were analyzed by [^{3}H]thymidine labeling. They found that ~80% of the precursors analyzed gave rise to at least one daughter cell that died within 48 hr of initial labeling. This report confirmed earlier observations of degenerative profiles in the immature mouse embryonic telencephalon (Smart and McSherry, 1982). Acklin and van der Kooy (1993) could not determine the phenotype of the dying cells but, based on the known developmental sequence of neurogenesis and gliogenesis in the rodent telencephalon (Angevine et al., 1970; Takahashi et al., 1992, 1995), it is likely that the dead cells represent neuronal progenitors and/or early postmitotic neurons. These authors suggested that this form of cell death might regulate the numbers and types of cells generated in any given cortical region, perhaps via local factors secreted by more mature cells.

In total, our work and these previous observations on immature neuronal death suggest that regulated expression of *bcl-x* may underlie early selection events in the developing nervous system. The cellular and molecular signals that regulate *bcl-x* expression in immature neurons require additional investigation.

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