Copyright © 2004 Tech Science Press MCB, vol.1, no.4, pp.233-243, 2004

The Effects of BclX_L and Bax Over-expression on Stretch-injury Induced Neural Cell Death

Bryan Pfister[1], George Oyler[2], Michael Betenbaugh[3] and Gang Bao[4]

Abstract: The Bcl-2 family of proteins has recently been implicated as a possible player in the complex cascade of neural cell death due to traumatic brain injuries. However, it is unclear if the Bcl-2 pathways are activated in mechanically injured neurons. Here we report the effects of BclX_L and Bax over-expression on stretch-induced neural cell death using an *in vitro* uniaxial stretch model of traumatic axonal injury. Specifically, YFP, YFP-tagged Bax and YFP-tagged BclX_L proteins were expressed in differentiated NG108-15 cells and stretch-injury assays were carried out at different strain and strain rate combinations. As a control, insults known to act within the Bcl-2 pathways were used to study cell viability and to compare with the results of cell death due to mechanical stretching. Surprisingly, under the stretch-injury conditions in this study, BclX_L did not provide protection against cell death. Further, translocation of Bax could not be identified after stretch-injury. The implications of these findings to cell death pathways in traumatic brain injury are discussed.

1 Introduction

It has been well established that cell death occurs through different pathways including apoptotic and necrotic pathways. Apoptosis is a natural and organized path to cell death, also known as programmed cell death whereas necrosis is an accidental cell death due to non-physiological incidents and injuries. However, it has been suggested that both physiological and non-physiological cellular events can trigger either apoptosis or necrosis depending on the sequence of events (Trump et al., 1997; Raff, 1998; Bredesen, 2000). Likewise, both necrosis and apoptosis can be triggered by external stimuli or stresses including chemical and mechanical insults to cells.

Traumatic brain injury (TBI) is due to a physical insult to the brain that results in a variety of focal and diffuse injuries (Smith and Meaney, 2000), leading to a progressive neuronal loss. It has been believed that this neural cell death is mainly a necrotic event (Raghupathi et al., 2000; Wang, 2000). This notion is supported by the observation of necrotic features in axonal pathology after TBI, including massive ionic shifts, mitochondrial swelling, cellular swelling, compromised cellular membrane, vacuolated cytoplasm, and nuclear pyknosis (Wang, 2000). Recently, however, there is evidence suggesting that the evolution of cell death in traumatic brain injuries is a combination of apoptosis and necrosis, and the relative degrees of each pathway depends on the specific injury mode and severity (Raghupathi et al., 2000). For example, mild forms of TBI may lead to more apoptotic cell death while more severe injuries to neurons may lead to necrosis.

The Bcl-2 family of proteins is involved in the intracellular control of apoptosis, including either suppressors of apoptosis such as Bcl-2 and Bcl-X_L or promoters of apoptosis such as Bax and Bid. The regulated expression and interaction of these proteins in promoting or protecting against neural cell death following TBI has received increasing attention in recent years (Ragupathi, 2004). The Bcl-2 protein expression level was shown to increase following experimental TBI in the rat and postmortem in humans, suggesting that apoptosis was occurring after injury (Clark et al., 1997; Clark et al., 1999; Clark et al., 2000). More recently, neuronal cell loss after TBI was found to correlate well with the Bax:Bcl-2 expression ratio (Ragupahti, 2003). There is also evidence to suggest that overexpression of Bcl-2 reduces cell death following

[1] Department of Neurosurgery, University of Pennsylvania, Philadelphia, PA 19104

[2] Department of Biology, University of Maryland at Baltimore County, Baltimore, MD 21210.

[3] Department of Chemical Engineering, The Johns Hopkins University, Baltimore, MD 21218.

[4] Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA 30332, gang.bao@bme.gatech.edu

TBI. However, it is unclear whether Bcl-2 is also protecting against necrotic cell death (Raghupathi et al. 1998; Nakamura et al. 1999). It is likely that the Bcl-2 family of proteins does not protect against all forms of cell death but only regulates certain cell death pathways (Graham et al., 2000). In contrast to Bcl-2, the expression level of Bcl-X_L does not appear to increase following TBI (Clark et al., 1999).

TBI is often a complex injury process comprised of several injury mechanisms such as focal mechanical damage, diffuse injury to axons, ischemia and hemorrhage. Accordingly, *in vivo* animal models of TBI are often associated with multiple injury mechanisms and competing neural death pathways. Thus, using an animal model, it can be exceedingly difficult to pinpoint the roles of the Bcl-2 family of proteins and the injury mechanism that triggers their expression. Of particular interest is the role of the Bcl-2 family of proteins in traumatic axonal injury (TAI), a diffuse mechanical damage to axons as a result of brain tissue deformation (Smith and Meaney, 2000). It is therefore advantageous to study neuronal cell death induced solely by a mechanical insult. To investigate the underlying mechanisms of TAI, many *in vitro* injury models have been developed to deliver a rapid stretch to neuronal cultures thereby significantly simplifying the analysis of injury mechanisms (Pfister et al., 2003, Geddes, 2001, Morrison, 1998).

In this study, we investigated the roles of Bax and BclX_L proteins in stretch-injury induced neural cell death using an *in vitro* injury model. In particular, YFP alone, YFP-tagged Bax and YFP-tagged BclX_L proteins were expressed in NG108-15 cells and stretch injury assays were carried out in their differentiated state. As a positive control, staurosporine, sindbis virus and hypoxia, which are known to act within the BclX_L and Bax pathways, were used to study cell viability and to compare with the results of cell death due to stretch. Surprisingly, under the stretch conditions in this study, BclX_L did not seem to provide protection against cell death following stretch injury. Further, the translocation of Bax could not be identified after injury. These results may have significant implications to cell death pathways in traumatic brain injury.

2 Materials and Methods

2.1 NG108-15 Cell Line Maintenance and Differentiation

The NG108-15 cell line used in this study is a somatic cell hybrid formed by a Sendai virus induced fusion of the neuroblastoma rat cell N18TG-2 and the rat glioma cell C6. (Klee and Nirenberg, 1974; Nirenberg et al., 1983) Cell cultures were maintained in Dubecco's Modified Eagles culture medium containing 4.5g/L glucose, 0.1mM hypoxanthine, 400nM aminopterin, 0.016mM thymidine (Lifetech, HAT 100X solution, Gaithersburg, MD), 10% FBS (not heat inactivated, Lifetech), and 1% penn/strep (Lifetech) and incubated at 37^{o}C. Subculturing was accomplished by rinsing with DPBS without Ca^{2+} followed by addition of 1mL 0.05% trypsin. Cells were immediately dislodged by striking flask with the palm, suspended with culture media and passed (1:10) to new flasks.

All NG108-15 cell lines were differentiated on custom made experimental elastic wells. The silicone substrates were coated with 5 μg/mL poly-D-lysine (Sigma, St. Louis, MO) in a borate buffer (77mM Boric acid, 6.7 mM Borax, pH8.4, sterile filtered) overnight. Following two rinses with sterile water, substrates were coated with 10 μg/mL laminin (Sigma) diluted in serum free medium and incubated at 37^{o}C with 5% CO_2 for a minimum of 1 hour. Substrates were then rinsed twice with media and cells were plated immediately in differentiation media containing 1% FBS, 1% penn/strep, and 1mM dibutyryl cyclic AMP (dbcAMP, Sigma) and incubated at 37% ^{o}C and 5% CO_2 for five to seven days.

2.2 Expression of YFP, YFP-Bax and YFP-BclX_L in NG108-15 Cells

To study the roles of Bax and BclX_L proteins in stretch injury of neural cells, pEYFP-BclX_L and pEYFP-BAX plasmids were created using the yellow fluorescent protein (YFP) construct pEYFP-C1 (Clontech Laboratories, Inc., Palo Alto, CA) by inserting the BclX_L and Bax genes into the multi-cloning site. This technique is well established and has been shown not to interfere with Bax or BclX_L function (Aokage 2004, Figueroa 2003). Undifferentiated NG108-15 cells were transfected with pEYFP-C1, pEYFP-BclX_L and pEYFP-BAX plasmids using Lipofectamine reagent (Lifetech, Gaithersburg, MD). Specifically, NG108-15 cells were plated at a den-

sity of 1x10^5 cells per well in a six well plate. For each well, 1μg of plasmid and 10μg Lipofectamine was prepared in 1 mL OptiMEM (Gibco, Gaithersburg, MD), vortexed gently for 30 seconds and held at room temperature for 30 minutes before placing on the cells. Following 4-5 hours of incubation on the cells, the transfection medium was removed and replaced with complete growth medium. Cells were grown to confluency and each well was passed into a T75 (1:7.5) flask for chemical selection in complete growth medium. However, transfected NG108-15 cells still seemed unusually sensitive to G418 treatment and a low dose was required for cell survival. Unfortunately chemical selection of stable transfectants was therefore not effective in creating a highly expressed cell population. Stable populations of each cell line (pEYFP-C1, pEYFP-BclX_L and pEYFP-Bax) were created by sorting each cell line using a fluorescently activated cell sorter (FACS). The overexpression of each protein in each cell line was verified by western blot and flow cytometry analysis, data not shown.

2.3 Induction of Apoptosis and Necrosis

Staurosporine (STS, Sigma, St. Louis, MO) was used as a model chemical insult to induce apoptosis in the undifferentiated and differentiated NG108-15 cell lines. Undifferentiated cells were plated with 2x10^5 cells per well in six-well plates and treated with complete growth medium supplemented with 1μM STS from a 100X stock prepared in DMSO. Differentiated cells were plated with 1.5x10^5 cells per well in a six-well plate and differentiated for 5 days. On day 5, cells were treated with medium supplemented with 1% FBS, 1% Penn/Strep, and 1μM STS. This treatment led to 100% cell death of control (native) NG108-15 cells within 24 hours of initial exposure.

The Sindbis virus was used as model system for biologically induced apoptosis of the modified NG108-15 cell lines. Sindbis is an alphavirus that can infect a variety of cell types especially immature neurons (Griffin and Hardwick, 1997; Griffin, 1998), and induce apoptosis in host cells. It has been shown that Bax may accelerate viral-induced apoptosis while Bcl-2 and BclX_L provide protection against it (Griffin and Hardwick, 1997). Both differentiated and undifferentiated cells were infected with the Sindbis virus (multiple of infection, MOI=2).

NG108-15 cells were also injured by chemical hypoxia which interrupts cellular respiration, resulting in ischemic injury as a model of necrosis (Jurkowitz-Alexander et al., 1992; Swanson, 1992; Borle and Barsic, 1995; Cargill and Thibault, 1996). For both differentiated and undifferentiated cells, the culture medium was replaced with a glucose-free medium with 10% FBS and 2 mM potassium cyanide. Trypan blue exclusion was used to determine viability of undifferentiated cells. Fluorescent dye exclusion was used to determine viability of differentiated neurons as described below.

2.4 Stretch Injury Assays

The uniaxial stretching device developed by Pfister et al. was used to perform *in vitro* assays of axonal injury (Pfister et al., 2003). Using displacement-control rather than force-control, this device is capable of achieving strains $> 70\%$ and strain rates up to 90 s^{-1}. It was demonstrated that, using this device, the pre-specified displacement profiles can be realized almost precisely, and the stretching deformation generated by the device is uniaxial, uniform, and highly reproducible. The range of strains and strain rates in the injury studies was chosen based on previous results (Pfister et al., 2003). Specifically, in the first set of experiments a strain of 70% with a strain rate of 70 s^{-1} was applied in order to produce over 50% cell death within 24 hours. Additional experiments were performed with strain and strain rate combinations of 50% - 50 s^{-1} (strain – strain rate) and 70% - 30 s^{-1}, respectively. In all the experiments, the effects of strain and strain rate on stretch-induced cell death were quantified at 0 (control) and 24 hours after injury.

2.5 Cell Viability Assays

A viability assay was used to measure the change in neural cell survival after stretch injury. Since differentiated neural cells could not be removed without damaging the neurons, stretch injured cells were examined in real time while still attached to the silicone substrate. To quantify viable and dead cells, viability assays were performed by treating cells with 5μg/mL acridine orange (AO, labeling all cells) and propidium iodide (PI, labeling dead cells) diluted in PBS with Ca^{2+} and Mg^{2+} for 5 minutes. To obtain a sufficient sample size, fluorescence images of the AO labeled and PI labeled cells were taken at ten different locations for each injured specimen. The labeled cells in each frame were counted and the number of live cells was calculated from the total and dead cell counts. Viability calculations included the disappearance

of dead neurons and were calculated by V/V_0 where V is the number of viable cells 24 hours after injury and V_0 is the average number of viable cells in the control (uninjured) specimens.

Injured cells were examined morphologically under light microscopy and by fluorescence microscopy to assess apoptotic or necrotic cell death. However, *in vitro* apoptosis and necrosis were sometimes difficult to be conclusively distinguished with light and fluorescent microscopy, since secondary necrosis (necrosis of apoptotic bodies) could resemble late necrotic cells. Evaluation of apoptosis vs. necrosis was also complicated by the large number of cells undergoing apoptosis during differentiation after which many apoptotic cells still remain loosely attached to the culture substrate.

An attempt was made to determine if both apoptosis and necrosis were present after stretch injury and to determine which one was more prominent. Using the abovementioned DNA dyes, the mode of death in some cells was determined. Apoptotic cells or apoptotic bodies appeared rounded with DNA staining easily visible in small spherical condensed nuclei. Necrotic cells, on the other hand, appeared as a "footprint" or a fragmented cell body where the healthy cell used to be; they also preserved their chromatin pattern and nucleus size as shown by acridine orange and propidium iodide staining where the nucleus and DNA have yet to be fragmented.

3 Results

3.1 Differentiation of transfected cell lines

For stretch-injury assays, the NG108-15 cell lines transfected with YFP, YFP-Bax, and YFP-BclX_L (henceforth NG108-YFP, NG108-Bax, and NG108-BclX_L respectively) must be able to differentiate properly. Each cell line was plated at the same density on silicone substrate specimens, differentiated for five days and the morphological development of neurons was examined. We found that NG108-YFP cells differentiated identically to native NG108-15 cells with rounded cell bodies and long interconnecting neurites, as shown in Figure 1A.

NG108-BclX_L cells also differentiated into morphologically correct neurons with very little cell death during differentiation (Fig. 1B). In contrast, NG108-Bax cells experienced significant cell death with clear signs of Bax translocation to the mitochondria; only a small number of viable cells developed limited neurite outgrowth (Fig. 1C). The percentage of viable cells after five days of differentiation for native NG108, NG108-YFP, NG108-Bax, and NG108-BclX_L cells is summarized in Figure 2.

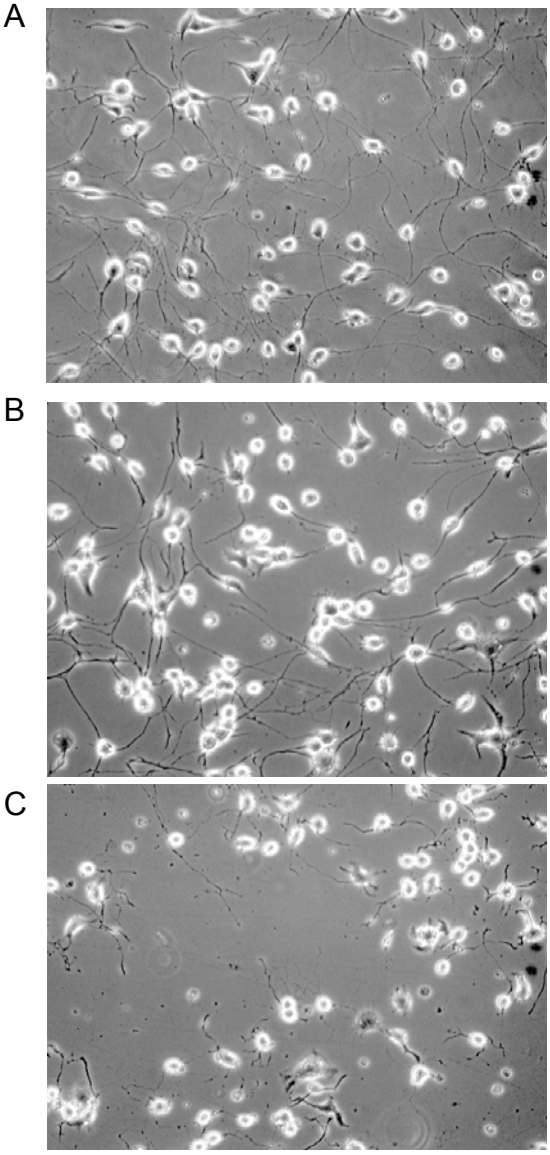

Figure 1 : NG108 cells differentialed for five days. (A) NG108-YFP and (B) NG108-BxlX$_L$ cells shown well developed neurite networks; (C) a much smaller number of NG108-Bax cells developed neurites.

As can be seen from Fig. 2, NG108-BclX_L cells differentiated with almost no related cell death while there was a high level of cell death associated with NG108-Bax differentiation.

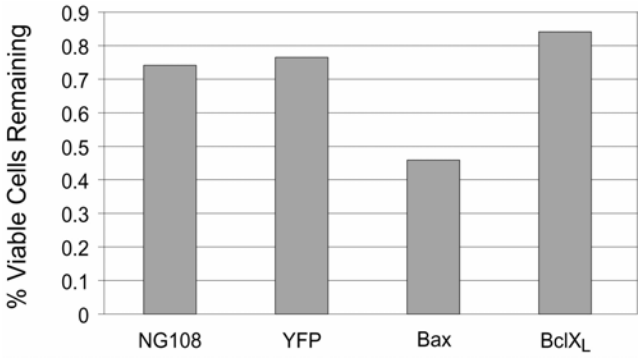

Figure 2 : The percentage of viable NG108 cells after five days of differentiation. Most native NG108 (NG108), NG108-YFP (YFP) and NG108-$BclX_L$ ($BclX_L$) cells survived, but a large amount of NG108-Bax (Bax) cells died during differentiation.

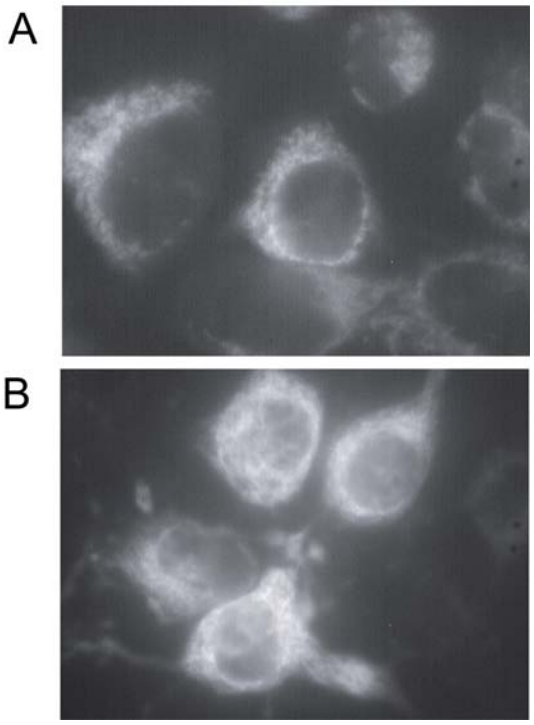

Figure 3 : Fluorescence images showing the expression of YFP-$BclX_L$ proteins in (A) undifferentiated and (B) differentialed NG108-$BclX_L$ cells.

We used fluorescence microscopy and flow cytometry analysis to confirm that YFP, YFP-Bax and YFP-$BclX_L$ proteins were expressed in respective NG108 cell lines NG108-YFP, NG108-Bax, and NG108-$BclX_L$ throughout differentiation. As demonstrated in Figures 3A and 3B, the expression of YFP-$BclX_L$ showed a distinctive pattern due to the association of $BclX_L$ with the membranes of mitochondria and other intracellular organelles, indicating that the YFP-$BclX_L$ proteins were intact and localized properly within the cell.

In contrast, cells expressing YFP-Bax showed a typical diffuse fluorescence image similar to that of YFP expression in differentiated cells, as demonstrated in Figures 4A and 4B. Expectedly, Bax translocation was identified in cells undergoing apoptotic cell death during differentiation. Flow cytometry analysis further confirmed that the expression of YFP, YFP-Bax and YFP-$BclX_L$ proteins was carried through the differentiation process (data not shown).

3.2 Stretch Injury and Necrotic Cell Death

Specimens with a population of each cell line (native NG108, NG108-YFP, NG108-Bax, and NG108-$BclX_L$) were injured under uniaxial stretching with strain and strain rate combinations of 70% - 30 s^{-1} (70% strain with 30 s^{-1} strain rate), 50% - 50 s^{-1} and 70% - 70 s^{-1}. Morphologically, all injured native NG-108 (non-transfected) cells produced swellings within 8 hours following injury. However, the major feature of these cells was the appearance of substantial neurite beading and cellular debris morphologically indicative of necrotic cell death, as shown in Figure 5.

Likewise, NG108-YFP and NG108-$BclX_L$ cells produced similar morphologies due to stretch injury. Unlike apoptotic cells with highly condensed nuclei, these cells preserve their chromatin pattern and nucleus size (another feature of necrosis) as shown by acridine orange and propidium iodide staining in Figures 6A and 6B.

The high level of necrotic morphology following stretch was clearly injury related. In contrast, dying cells in non-stretch injured controls exhibited morphological features of apoptosis rather than necrosis.

3.3 Bax Did Not Translocate Following Stretch Injury

Although the NG108-Bax cells were difficult to differentiate, experiments were performed in an attempt to identify if Bax translocation occurs as a result of stretch injury. Differentiated NG108-Bax cells were stretched at strain and strain rate combinations of 50%-50 s^{-1} and

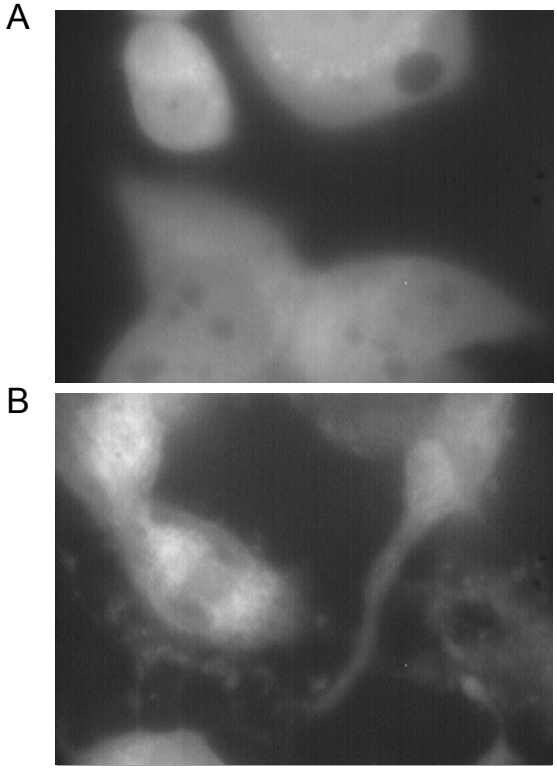

Figure 4 : Fluorescence images showing the expression of YFP-Bax proteins in (A) undifferentiated and (B) differentiated NG108-Bax cells.

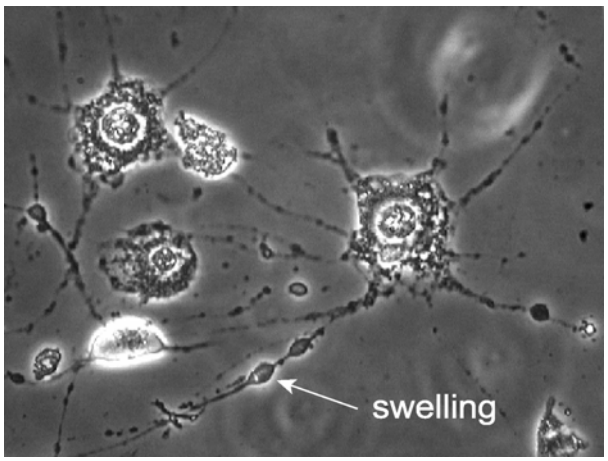

Figure 5 : Stretch-injured NG108-15 cells showing neurite beading and the footprint of necrosis.

70%-30 s^{-1} and 70%-70 s^{-1}; however, during the first 24 hours after injury, no cells were found to undergo Bax translocation as a result of stretch injury.

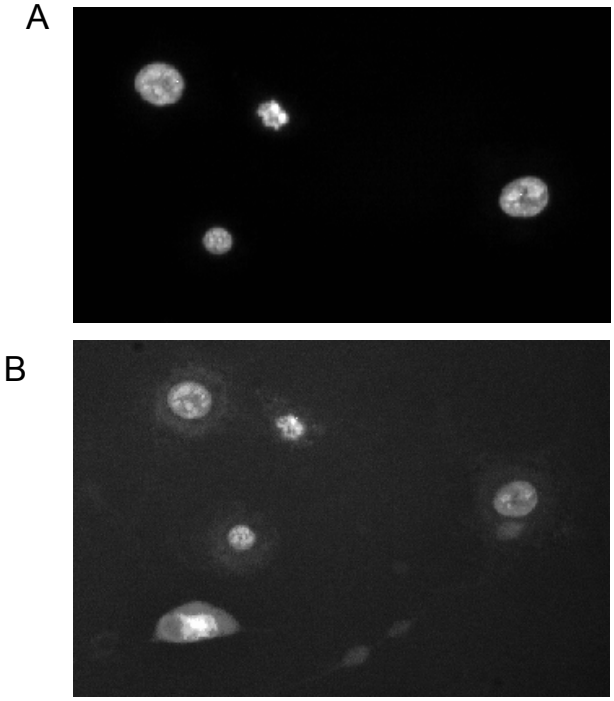

Figure 6 : Indications of necrosis of injured cells: (A) chromatin pattern and (B) preserved nuclear size.

3.4 $BclX_L$ Did Not Provide Protection from Stretch Injury

In a control study, we found that the overexpression of YFP-$BclX_L$ in NG108 cells could provide substantial protection in staurosporine-induced apoptosis, as demonstrated in Figure 7. Within 9 hours of 1 μM staurosporine treatment, over 50% of native NG108-15 and NG108-YFP cells were dead, while $BclX_L$ provided a significant protection to the NG108-$BclX_L$ cells throughout the 48 hours of testing. As expected, the overexpression of Bax increased staurosporine-induced cell death within 9 hours after treatment. In fact, within 1 hour of staurosporine treatment, Bax translocation, a well-known early event in apoptosis, could be identified.

Surprisingly, $BclX_L$ did not provide any protection from cell death due to stretch-injury. Rather, as shown in Figure 8, under different applied strain and strain rate combinations (70% - 30 s^{-1}, 50% - 50s^{-1} and 70% - 70 s^{-1}), the amount of viable NG108-$BclX_L$ cells 24 hours after stretch injury was even smaller compared with that

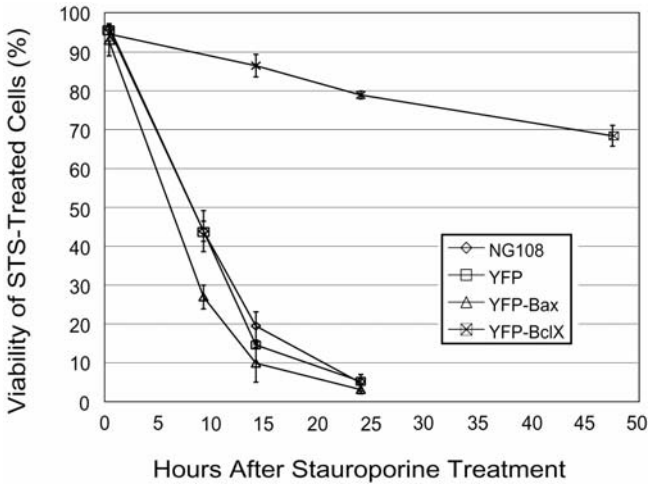

Figure 7 : Viability of undifferentiated NG108 cell lines after treatment of 1 μM Staurosporine (STS) indicated substantial protection of BclX$_L$ expression.

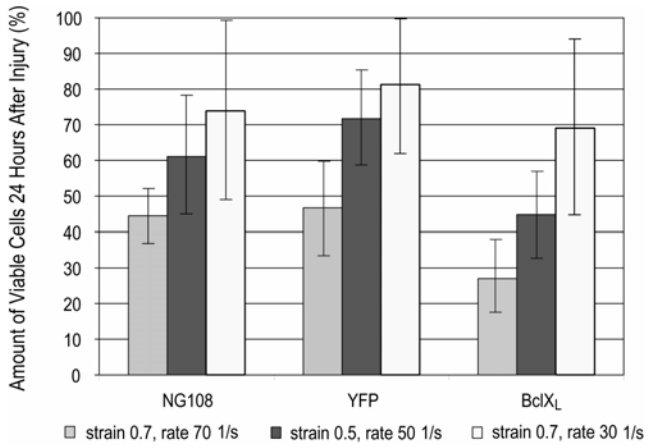

Figure 8 : The amount of viable NG108 cells, including native NG108, NG108-YFP and NG108-BclX$_L$ cells 24 hours after stretch injury.

of native NG108 cells and NG108-YFP cells, suggesting that the overexpression of BclX$_L$ proteins did not help prevent cell death, be it necrotic or apoptotic, due to stretch injury. NG108-BclX$_L$ cells developed necrotic morphology within 8 hours following stretching whereas the control (native NG108-15) cells developed necrotic morphology much later, at 24 hours after stretch-injury. Overall, there were more widespread necrotic features and extensive neurite beading in NG108-BclX$_L$ cells compared with the NG108-YFP and native NG108 cells, indicating that overexpression of BclX$_L$ assisted injury-induced cell death. It is not clear, however, why BclX$_L$ increased *and* accelerated cell death.

3.5 BclX$_L$ May Act on Different Cell Death Pathways

To further investigate how BclX$_L$ overexpression in NG108-15 cells can provide protection, two additional models of cell death were considered: Sindbis virus was used as a model of apoptosis and potassium cyanide plus glucose deprivation as a model of necrosis. It is known that Sindbis virus triggers apoptosis (Griffin et al., 1977; Griffin and Hardwick, 1997; Griffin, 1998), whereas potassium cyanide with glucose deprivation induces necrosis (Jurkowitz-Alexander et al., 1992; Swanson, 1992; Borle and Barsic, 1995). The results displayed some interesting features. Contrary to the staurosporine induced apoptosis, BclX$_L$ overexpression offered no protection to NG108-15 cells infected with the Sindbis virus, as shown in Figure 9. On the other hand, BclX$_L$ overexpression provided substantial protection in the necrotic cell death induced by potassium cyanide plus glucose deprivation, as demonstrated by Figure 10.

Taken together, our results suggest that BclX$_L$ proteins provide protection in specific apoptotic and necrotic cell death pathways but not the pathway triggered by stretch injury in NG108 cells.

4 Discussion

Different proteins in the Bcl-2 family play distinct roles in apoptosis and regulate distinct cell death pathways. For example, Bcl-2 and Bcl-X$_L$ proteins are suppressors of apoptosis, while Bax and Bid are promoters of apoptosis. In particular, Bcl-2 and Bcl-X$_L$ are typically localized on the membranes of mitochondria, nucleus and endoplasmic reticulum. These apoptosis suppressors are believed to stabilize the mitochondria membrane potential, modulate calcium influx and efflux, and prevent the release of apoptosis initiation factor (AIF) from the mitochondria (Raff, 1998; Bredesen, 2000; Graham et al., 2000). It has been shown that BclX$_L$ could protect many cell types from apoptosis including the neural precursor cell MN9D (Oh, O'Malley et al. 1997; Oh, Uhland-Smith et al. 1997; Kim et al., 1999). It has also been shown that Bax proteins can be a player in the apoptosis of undifferentiated neural precursor cells (Oh, O'Malley et al., 1997; McGinnis et al., 1999). However,

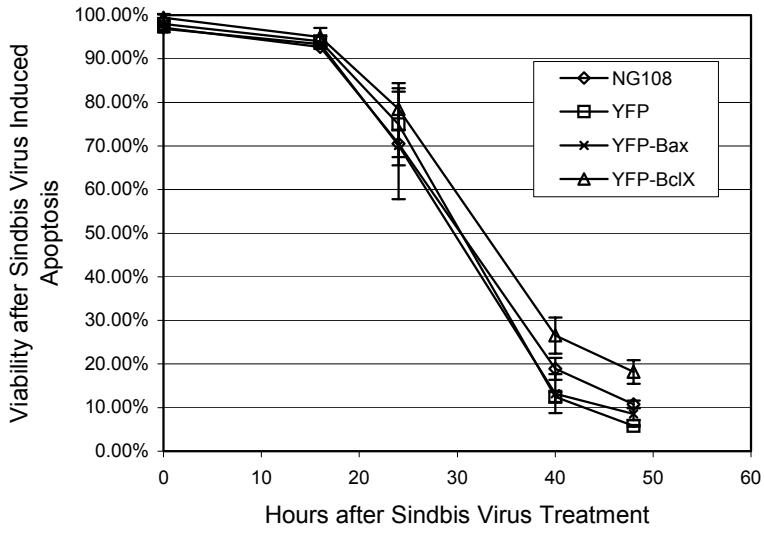

Figure 9 : Sindbis virus induced apoptosis of NG108-15 cells, indicating that YFP-BclX_L did not provide much protection.

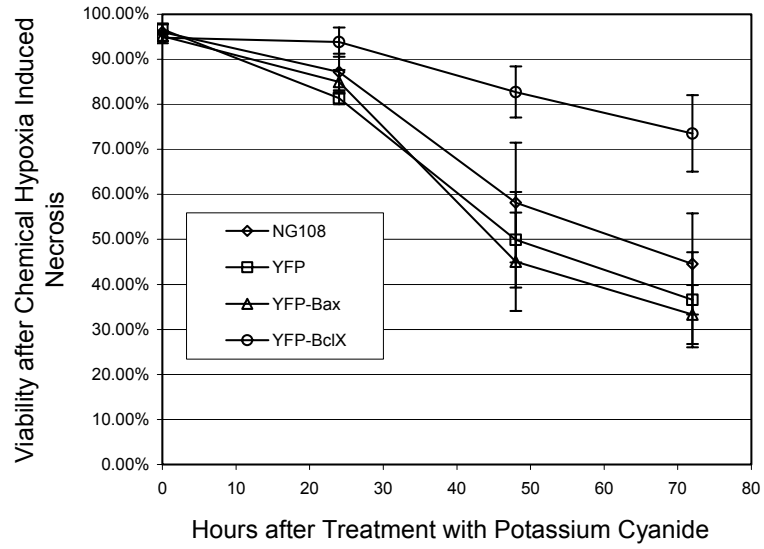

Figure 10 : 10 Chemical hypoxia induced NG108 cell death. YFP-BclX_L appeared to provide some protection against necrosis.

it is still unclear whether Bcl-2 and Bcl-X_L can protect against necrotic or apoptotic cell death associated with TBI (Raghupathi et al., 1998; Nakamura et al., 1999).

In this study an attempt was made to reveal the roles of Bax and Bcl-X_L proteins in stretch injury of differentiated neural cells. To date most cell culture studies involving overexpression of proteins in the Bcl-2 family was carried out only in the undifferentiated state; to our knowledge, no study has been conducted to determine the role of Bax or BclX_L in differentiated neural cell apoptosis and necrosis after stretch injury. To demonstrate the expression and functionality of Bax and BclX_L in neural cells after differentiation, we chose to tag these proteins with YFP. Specifically, YFP, YFP-tagged Bax and YFP-tagged BclX_L proteins were expressed in undifferentiated NG108-15 precursor cells through transfection. These cells were then induced to differentiate into neurons. We found that over five days of differen-

tiation, native NG108-15 cells, and NG108-15 cells expressing YFP and YFP-tagged BclX$_L$ proteins all differentiated into morphologically correct neurons with very limited cell death during differentiation. In particular, cells transfected with YFP-BclX$_L$ showed superior survivability and development during differentiation compared with native NG108-15 cells.

Although most NG108-BclX$_L$ cells survived differentiation and appeared very healthy, BclX$_L$ over-expression resulted in higher levels of cell death following stretch injury compared with control cells (native NG108-15). This is in contrast to the assumption that the over-expression of BclX$_L$ proteins protects against cell death. The neurite beading and cellular morphology of stretch-injured NG108-BclX$_L$ neurons suggested that stretch-injury induced cell death was mainly necrotic, a feature recognized in other *in vivo* and *in vitro* studies of axonal injury (Sengoku et al., 2000; Zhang et al., 2000). We hypothesized that under the stretch injury conditions, i.e., the applied strain and strain rate combinations used in this study, neural cell death was mainly due to necrosis, and the over-expression of BclX$_L$ proteins may protect neural cells against apoptosis only.

To examine this hypothesis, staurosporine, Sindbis virus infection and potassium cyanide poisoning were used as additional models of induced cell death in NG108-BclX$_L$ cells. Our results indicated that the overexpression of BclX$_L$ provided protection to necrosis induced by potassium cyanide and apoptosis induced by staurosporine, but not to apoptosis induced by Sindbis virus. It is therefore clear that BclX$_L$ over-expression can have very different effects on both apoptosis and necrosis depending on the specific cell death pathway activated. Accordingly, BclX$_L$ may or may not be involved in the cell death pathway activated by stretch injury of NG108-15 cells. However, the finding that BclX$_L$ may exacerbate neuronal death following stretch injury implies that it does play a role, but in a contrary fashion. BclX$_L$, for example, may be cleaved into pro-death fragments (Basanez, 2001, Clem, 1998) by a process initiated by the stretch injury. This warrants further investigation.

A large amount ($> 50\%$) of cell death occurred in the NG108-Bax cells during differentiation, which is likely due to the pro-apoptotic function of Bax. Overexpression of Bax in undifferentiated neural cell lines has shown to accelerate apoptosis (Oh, O'Malley et al. 1997), with a characteristic Bax translocation to the mitochondria (McGinnis et al., 1999; Putcha et al., 1999). For the NG108-Bax cells that survived the differentiation process, we found that they still expressed YFP-Bax proteins, as shown by Bax translocation in these cells upon further staurosporine treatment. Unfortunately, these viable NG108-Bax cells developed poor neurite extensions and did not connect with other nearby neurons as demonstrated in Figure 1C. Since axonal injury, i.e., damage along neural processes, is a main indicator of stretch induced injury to neurons (Smith and Meaney, 2000), the role of Bax may not be revealed properly using these differentiated YFP-Bax cells. Nevertheless, we performed a stretch-injury assay with differentiated NG108-Bax cells but Bax translocation in these cells could not be positively identified.

In conclusion, our results suggest that BclX$_L$ does not provide protection against cell death in stretch-injury; rather, it may even play a pro-death role. Furthermore, the presumed pro-apoptotic function of Bax in stretch-injury was not evident under the stretch conditions used in this study. It is likely that under severe stretch injury conditions, necrotic cell death is the dominant pathway to neural death. Accordingly, it is likely that the injury mechanisms revealed using *in vivo* models of TBI reflect competing cell death pathways where the expression of Bcl-2 protein is believed to protect against neuronal death and Bax to facilitate neuronal death. Further studies are warranted on roles of Bcl-2 and Bax in the apoptotic and necrosis pathways activated during stretch injury.

References

Aokage, T.; Ohsawa, I.; et al. (2004): Green fluorescent protein causes mitochondria to aggregate in the presence of the Bcl-2 family proteins. *Biochem. Biophys. Res. Comm.* 314: 711-16.

Basanez, G.; Zhang, J.; et al. (2001): Pro-apoptotic cleavage products of Bcl-xL from cytochrome c-conducting pores in pure lipid membranes. *J. Biol. Chem.* 276: 31083-31091.

Borle, A. B.; Barsic, M. (1995): Chemical hypoxia increases cytosolic Ca2+ and oxygen free radical formation. *Cell Calcium* 17: 307-315.

Bredesen, D. E. (2000): Apoptosis: overview and signal transduction pathways. *J. Neurotrauma* 17: 801-810.

Cargill, R. S.; Thibault, L. E. (1996): Acute alterations in [Ca2+]i in NG108-15 cells subjected to high strain rate

deformation and chemical hypoxia: an in vitro model for neural trauma. *J. Neurotrauma* 13: 395-407.

Clark, R. S.; Chen, J.; et al. (1997): Apoptosis-suppressor gene bcl-2 expression after traumatic brain injury in rats. *J. Neurosci*. 17: 9172-982.

Clark, R. S.; Kochanek, P. M.; et al. (2000): Increases in bcl-2 protein in cerebrospinal fluid and evidence for programmed cell death in infants and children after severe traumatic brain injury. *J. Pediatr*. 137: 197-204.

Clark, R. S.; Kochanek, P. M.; et al. (1999): Increases in Bcl-2 and cleavage of caspase-1 and caspase-3 in human brain after head injury. *Faseb. J*. 13: 813-821.

Clem, R. J.; Cheng, E. H. Y.; et al. (1998): Modulation of cell death by Bcl-xL through caspase interaction. *Proc. Natl. Acad. Sci. USA* 95: 554-559.

Figueroa, B. Jr.; Sauerwald, T. M.; et al. (2003): A comparison of the properties of a Bcl-xL variant to the wild-type anti-apoptosis inhibitor in mammalian cell cultures. *Metab. Eng.* 5(4): 230-245.

Geddes, D. M.; Cargill, R. S. 2nd (2001): An in vitro model of neural trauma: device characterization and calcium response to mechanical stretch. *J. Biomech. Eng.* 123: 247-255.

Graham, S. H.; Chen, J.; et al. (2000): Bcl-2 family gene products in cerebral ischemia and traumatic brain injury. *J. Neurotrauma* 17: 831-841.

Griffin, D. E. (1998): A review of alphavirus replication in neurons. Neurosci. *Biobehav. Rev.* 22: 721-723.

Griffin, D. E.; Hardwick, J. M. (1997): Regulators of apoptosis on the road to persistent alphavirus infection. *Annu. Rev. Microbiol*. 51: 565-592.

Griffin, J. W.; Price, D. L.; et al. (1977): The pathogenesis of reactive axonal swellings: role of axonal transport. J. Neuropathol. *Exp. Neurol*. 36: 214-227.

Jurkowitz-Alexander, M. S.; Altschuld, R. A.; et al. (1992): Cell swelling, blebbing, and death are dependent on ATP depletion and independent of calcium during chemical hypoxia in a glial cell line (ROC-1). *J. Neurochem*. 59: 344-352.

Kim, J. E.; Oh, J. H.; et al. (1999): Sequential cleavage of poly(ADP-ribose)polymerase and appearance of a small Bax-immunoreactive protein are blocked by Bcl-X(L) and caspase inhibitors during staurosporine-induced dopaminergic neuronal apoptosis. *J. Neurochem*. 72: 2456-2463.

Klee, W. A.; Nirenberg, M. (1974): A neuroblastoma times glioma hybrid cell line with morphine receptors. *Proc. Natl. Acad. Sci. U.S.A*. 71: 3474-3477.

McGinnis, K. M.; Gnegy, M. E.; et al. (1999): Endogenous bax translocation in SH-SY5Y human neuroblastoma cells and cerebellar granule neurons undergoing apoptosis. *J. Neurochem.* 72: 1899-1906.

Morrison, B. 3rd; Meaney, D. F.; et al. (1998): Mechanical characterization of an in vitro device designed to quantitatively injure brain tissue. *Ann. Biomed. Eng.* 26: 381-390.

Nakamura, M.; Raghupathi, R.; et al. (1999): Overexpression of Bcl-2 is neuroprotective after experimental brain injury in transgenic mice. *J. Comp. Neurol*. 412: 681-692.

Nirenberg, M.; Wilson, S.; et al. (1983): Modulation of synapse formation by cyclic adenosine monophosphate. *Science* 222: 794-799.

Oh, J. H.; O'Malley, K. L.; et al. (1997): Bax accelerates staurosporine-induced but suppresses nigericin-induced neuronal cell death. *Neuroreport* 8: 1851-1856.

Oh, Y. J.; Uhland-Smith, A.; et al. (1997): Regions outside of the Bcl-2 homology domains, BH1 and BH2 protect a dopaminergic neuronal cell line from staurosporine-induced cell death. Brain Res. *Mol. Brain Res*. 51: 133-142.

Pfister, B. J.; Weihs, T. P.; et al. (2003): An in vitro uniaxial stretch model for axonal injury. *Ann. Biomed. Eng*. 31: 589-598.

Putcha, G. V.; Deshmukh, M.; et al. (1999): BAX translocation is a critical event in neuronal apoptosis: regulation by neuroprotectants, BCL-2, and caspases. *J Neurosci* 19: 7476-7485.

Raff, M. (1998): Cell suicide for beginners [news]. *Nature* 396: 119-122.

Raghupathi, R.; Fernandez, S. C.; et al. (1998): BCL-2 overexpression attenuates cortical cell loss after traumatic brain injury in transgenic mice. *J. Cereb. Blood Flow Metab*. 18: 1259-1269.

Raghupathi, R.; Graham, D. I.; et al. (2000): Apoptosis after traumatic brain injury. *J Neurotrauma* 17: 927-938.

Raghupathi, R.; Strauss, K. I.; et al. (2003): Temporal alterations in cellular bax:bcl-2 ratio following traumatic brain injurt in the rat. *J. Neurotrauma* 20: 421-435.

Raghupathi, R. (2004): Cell death mechanisms following traumatic brain injury. *Brain Pathol.* 14: 215-222.

Smith, D. H.; Meaney, D. F. (2000): Axonal Damage in Traumatic Brain Injury. *The Neuroscientist* 6: 483-495.

Swanson, R. A. (1992): Astrocyte glutamate uptake during chemical hypoxia in vitro. *Neurosci Lett.* 147: 143-146.

Trump, B. F.; Berezesky, I. K.; et al. (1997): The pathways of cell death: oncosis, apoptosis, and necrosis. *Toxicol. Pathol*. 25: 82-88.

Wang, K. K. (2000): Calpain and caspase: can you tell the difference? *Trends Neurosci*. 23: 20-26.