Insulin-like Growth Factor-I Prevents Caspase-Mediated Apoptosis in Schwann Cells

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ABSTRACT: Both neurons and glia succumb to programmed cell death (PCD) when deprived of growth factors at critical periods in development or following injury. Insulin-like growth factor-I (IGF-I) prevents apoptosis in neurons in vitro. To investigate whether IGF-I can protect Schwann cells (SC) from apoptosis, SC were harvested from postnatal day 3 rats and maintained in serum-containing media until confluency. When cells were switched to serum-free defined media (DM) for 12–72 h, they underwent PCD. Addition of insulin or IGF-I prevented apoptosis. Bisbenzamide staining revealed nuclear condensation and formation of apoptotic bodies in SC grown in DM alone, but SC grown in DM plus IGF-I had normal nuclear morphology. The phosphatidylinositol 3-kinase (PI 3-K) inhibitor LY294002 blocked IGF-I-mediated protection. Caspase-3 activity was rapidly activated upon serum withdrawal in SC, and the caspase inhibitor BAF blocked apoptosis. These results suggest that IGF-I rescues SC from apoptosis via PI 3-K signaling which is upstream from caspase activation.

Programmed cell death (PCD) is an important mechanism for regulating cell numbers in the developing nervous system. Many more cells are produced than are needed in the adult organism. The overabundant cells are eliminated as they compete for a limited supply of trophic factor during critical periods of development (Oppenheim, 1991). Glial cell line–derived neurotrophic factor (GDNF) rescues spinal motor neurons from naturally occurring PCD, without which a greater proportion will die (Oppenheim et al., 1995). Similarly, platelet-derived growth factor (PDGF) promotes survival of developing oligodendrocytes (Barres et al., 1992). Astrocytes undergo PCD during cerebellar development, which may be regulated by a supply of some yet unknown growth factor (Krueger et al., 1995). Schwann cells (SC), the glia that form myelin in the peripheral nervous system (PNS), are susceptible to PCD during critical periods of development, a process that is likely controlled by growth factors (Grinspan et al., 1996; Nakao et al., 1997; Syroid et al., 1996).

A number of growth factors influence SC development and death, including neuregulins, (NRG), transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), PDGF, and insulin-like growth factor-I (IGF-I) (Cheng et al., 1999; Dong et al., 1995; Eccleston et al., 1993; Gavrilovic et al., 1995; Kopp et al., 1997; Marushige and Marushige, 1994; Shaw et al., 1997; Skoff et al., 1998; Syroid et al., 1996) (for review, see Scherer and Salzer, 1996). SC express receptors for these trophic factors, including the PDGF and NRG receptors, and the type-I IGF receptor (IGF-IR) (Carroll et al., 1997; Eccleston et al., 1993; Grinspan et al., 1996; Leventhal et al., 1995). SC express IGF-IR and make IGF-I during critical periods of development (Cheng et al., 1996; D’Ercole et al., 1996) when PCD is occurring. However, the mechanisms responsible for governing SC response to IGF-I are relatively poorly understood.

We have demonstrated the pluripotent actions of
IGF-I on SH-SY5Y neuroblastoma cells, dorsal root ganglia neurons, and SC in vitro. IGF-I promotes motility in SC, an important attribute for proper myelination of developing axons (Cheng et al., 1999). Similarly, IGF-I promotes motility in neurons (Kim and Feldman, 1998; Kim et al., 1997; Leventhal and Feldman, 1997; Leventhal et al., 1997) and serves as a protective agent against apoptosis in SH-SY5Y cells and sensory neurons (Cheng and Feldman, 1998; Matthews and Feldman, 1996; Matthews et al., 1997; Russell et al., 1998; Singleton et al., 1996a,b).

The aim of the current study was to examine whether and how IGF-I prevents apoptosis in SC. We report that SC grown in serum-free defined media undergo apoptosis. Insulin or especially IGF-I added to the defined media is protective. PI 3-kinase inhibitors block the protective effect of IGF-I. Caspase inhibitor either alone or in conjunction with PI 3-kinase inhibitor prevents apoptosis, suggesting that caspases function downstream from PI 3-kinase. Hence, IGF-I, signaling predominantly via the PI 3-kinase, protects SC from caspase-mediated apoptosis induced by serum withdrawal.

MATERIALS AND METHODS

Reagents

Tissue culture plates were obtained from Corning (Corning, NY). Dulbecco’s modified Eagle’s medium (DMEM), low-glucose DMEM, Ham’s F-12, calf serum (CS), fetal bovine serum (FBS), trypsin–ethylenediaminetetraacetic acid (EDTA), pituitary extract, and Hank’s balanced salt solution (HBSS) were purchased from Gibco-BRL (Gaithersburg, MD). Recombinant human IGF-I was a generous gift of Cephalon (West Chester, PA) and was stored at 100 μg/mL bovine pituitary extract. Cells were maintained in growth media (GM) of DMEM, 10% FBS, 2 μM forskolin, and 10 μg/mL bovine pituitary extract. Cells were passaged upon confluency (1:4) and used for experiments for four passages.

At the beginning of each experiment, cells were washed twice with HBSS and cultured in a serum-free defined media (DM) [low glucose DMEM/Ham’s F-12 1:1, transferrin (10 μg/mL), putrescine (10 μM), progesterone (20 nM), and sodium selenite (30 nM)].

Nuclear Staining

Schwann cells were grown in DM ± 10 nM IGF-I for 24 h and fixed with 10% formalin in phosphate-buffered saline (PBS) (pH 7.4) for 30 min. SC were stained with bisbenzamide (1 μg/mL in PBS) for 15 min, rinsed with PBS, mounted with Gel/Mount (Fort Washington, PA), and viewed under ultraviolet (UV) illumination on a Nikon Microphot fluorescence microscope (Matthews and Feldman, 1996).

Flow Cytometry

DNA fragmentation is a hallmark of apoptosis. The DNA fragments are multiples of 180–200 base pairs and are present in the sub G0 region of DNA content histograms. Flow cytometry is used to determine the cell-cycle phase distribution and quantitate the percentage of cells in sub-G0 as a measure of apoptosis (Kokileva, 1994; Matthews et al., 1997; Sgonc and Wick, 1994). Briefly, after cells were cultured in DM for the appointed times, the culture media and trypsinized cells were combined and washed twice in HBSS. Single-cell suspensions were fixed in ice-cold 70% ethanol and stored up to 1 week at 4°C. Fixed cells were rinsed twice with HBSS and stained with propidium iodide (18 μg/mL) for 1–4 h. Flow cytometry data were collected from an Epics Elite flow cytometry system (Coulter Cytometry, Hialeah, FL).

Caspase Fluorometric Assay

Caspase-3 fluorogenic assay was conducted according to the manufacturer’s instructions. Briefly, media and trypsinized cells were collected and washed twice with PBS. Next, 2 × 10^5 cells in 500 μL PBS were added to 10 μL (10 μg) Ac-DEVD-AMC (fluorogenic substrate) and incubated at 37°C for 1 h. The fluorescence emitted by cleaved substrate was measured on an Epics Elite flow cytometry system (Coulter Cytometry, Hialeah, FL) using UV excitation (440 nm).

RESULTS

Previous reports demonstrate SC undergo apoptosis in vivo both during development and following injury and disease (Berciano et al., 1998; Erdem et al., 1998; Jessen et al., 1994; Nakao et al., 1997; Syroid et al., 1996; Trachtenberg and Thompson et al., 1996). Syroid et al. (1996) examined SC apoptosis in vitro following serum withdrawal. To develop an in vitro system to investigate the intracellular signaling mech-
organisms of SC undergoing apoptosis, we employed a similar serum withdrawal paradigm.

Apoptosis Following Serum Withdrawal

Schwann cells were cultured in serum-free DM for 12, 24, 48, or 72 h, and processed for FACS analysis. Cells in the sub-G₀ region of the DNA content histograms were considered apoptotic. At 12 h, 14 ± 2% of SC were apoptotic, increasing to 57 ± 5% by 72 h [Fig. 1(A)]. Control cultures maintained in standard GM containing serum (see Materials) exhibited a baseline of 5–10% apoptosis at any given time in culture (data not shown). Hence, in SC cultures switched from GM to DM, the proportion of cells that underwent apoptosis increased with time, suggesting that factors in the GM were critical for survival. There was a marked increase in apoptosis at 24 h. Cells were grown for 24 h in DM for further experiments unless otherwise noted.

Serum Blocks Apoptosis

Because GM contains essential components, we cultured SC in DM with increasing concentrations of FBS. Relatively low concentrations of serum, 0.3% and 1.0%, were able to rescue apoptotic cells, and 3.0% and 10% FBS improved on this effect [Fig. 1(B)]. The percentage of apoptotic cells grown in DM plus either 3% or 10% (9 ± 1.7% and 6 ± 0.8% apoptotic, respectively) was similar to those grown in GM. Serum added to DM was sufficient to protect against apoptosis.

Insulin or IGF-I Prevents Apoptosis

There are several reports in the literature in which SC were grown in serum-free DM (Dong et al., 1995; Jessen et al., 1994; Stewart et al., 1996). The main difference between these reports and our DM is the inclusion of insulin, IGF-I, or both (Dong et al., 1995; Jessen et al., 1994; Stewart et al., 1996). To examine whether either of these is sufficient to protect against apoptosis induced by serum withdrawal, SC were cultured for 24 h in DM with the addition of either insulin or IGF-I to the culture media in dose ranges representative of those published concentrations. Figure 2(A) shows that insulin offers some protection from apoptosis in 24 h at higher concentrations (18.8 ± 2.24% at 1000 ng/mL media or 174 μM) compared to DM alone (35.3 ± 2.22%). In contrast, IGF-I was extremely protective in doses as low as 0.3 nM (15.95 ± 3.7%) compared to DM alone (36.8 ± 5.52%). It is likely that these components were responsible for maintaining healthy SC in vitro in those early studies (Dong et al., 1995; Jessen et al., 1994; Stewart et al., 1996). Thus, SC undergo apoptosis following serum withdrawal, and IGF-I is highly protective.

Apoptotic Morphology

To examine nuclear morphology, SC were cultured in DM ± 10 nM IGF-I for 24 h and stained with bisbenzamide. SC grown in DM plus IGF-I had evenly stained, healthy nuclei [Fig. 3(B)]. There were few if any pyknotic nuclei. In contrast, SC grown in DM alone were less dense, probably because a portion of the population had already died and detached from the plate. Some of the cells that remained attached were in a slightly different plane of focus, suggesting they were beginning to detach. These SC displayed apoptotic morphology; the nucleus had condensed and formed small apoptotic clusters [Fig. 3(A)].

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**Figure 1** Serum withdrawal induced apoptosis in SC grown in defined media. SC were grown until confluency in GM and then switched to serum-free DM for 12, 24, 48, or 72 h (A). Cells began to become apoptotic as early as 12 h postserum withdrawal, and this percentage continued to increase until 72 h, the latest time examined. When SC were maintained in DM with the addition of increasing concentrations of serum for 24 h (B), they were rescued from apoptosis.
IGF-I Prevents Apoptosis via PI 3-K Signaling

Previous work done in our laboratory with SH-SY5Y neuroblastoma cells suggested that the protective effect of IGF-I is mediated via the phosphatidylinositol 3-kinase (PI 3-K) as opposed to the MAP kinase pathway (Singleton et al., 1996a). To examine this signaling pathway, SC were cultured for 24 h in DM alone, and with or without IGF-I (10 nM), the PI 3-K inhibitor LY294002 (10 \( \mu M \)), and the MAP kinase inhibitor PD98059 (10 \( \mu M \)) (Fig. 4). Twenty-five percent of the SC were apoptotic when grown in DM alone or either inhibitor. IGF-I decreased the number of apoptotic cells to only 12.9%. LY294002 blocked IGF-I’s protective effect (22.0% apoptosis), but PD98059 did not (only 13.2% apoptotic cells). This demonstrates that IGF-I mediates its protection in SC predominantly via the PI 3-K.

Caspases in SC Apoptosis

Caspases act as downstream effector molecules in many models of cell death (Thornberry and Lazebnik, 1998). To determine whether caspases are active during SC apoptosis, we initiated apoptosis by withdrawing serum and cultured SC in DM alone, with IGF-I, or with the caspase inhibitor BAF (50 \( \mu M \)) (Fig. 5). BAF protected SC from apoptosis. Furthermore, when SC were grown with both IGF-I and BAF, or LY294002 (10 \( \mu M \)) and BAF, the percent apoptotic cells was still about 10%. BAF was not additive to IGF-I’s protection and was not blocked by PI 3-K inhibition, suggesting that caspase activation was downstream from both IGF-I signaling and PI 3-K activity.

Caspases-3-like Activation in SC

Considering the neurologic phenotype of the caspase-3-null mouse, in which the developing brain lacks appropriately timed apoptosis, causing a variety of hyperplasias (Kuida et al., 1996), it seemed possible...
that caspase-3 plays an active role in SC apoptosis. However, we were unable to identify the cleaved product by immunoblotting of whole cell lysate from apoptotic SC (data not shown). As an alternative, a more sensitive fluorometric technique was employed to measure caspase-3 activity. In this assay, activated caspase-3 and caspase-7 cleaved a fluorogenic substrate, and the resulting fluorescence was measured by flow cytometry. SC were cultured in DM with or without IGF-I. In SC cultures grown in DM alone for 1 h, there was a slight increase in fluorescence compared to the control nonapoptotic population (Fig. 6). This difference between the DM alone and control was greatest at 3 h, when 44.2% of cells had activated caspase. At 6 h, this effect decreased to 5.5% of cells, and even further to 2.7% at 12 h. At each time point, the inclusion of IGF-I diminished caspase-3 activation.

Figure 4 IGF-I signals via PI 3-K. SC were grown in DM for 24 h, with or without the PI 3-K inhibitor LY294002 (10 μM) (+LY), the MAP kinase inhibitor PD98059 (10 μM) (+PD), and IGF-I. Neither the LY nor the PD inhibitors had an influence on SC apoptosis compared to DM alone. IGF-I (10 nM) rescued SC from apoptosis, and LY294002 interfered with the ability of IGF-I to prevent PCD. PD98059 slightly diminished the protective effect of IGF-I.

Figure 5 Caspase inhibition prevented apoptosis in SC. SC were grown in DM for 24 h, alone (DM), plus IGF-I (10 nM), plus the caspase inhibitor bok-asp-fmk (50 μM) (+BAF), plus BAF and the PI 3-K inhibitor LY294002 (10 μM) (+BAF +LY), or plus BAF and the MAP kinase inhibitor PD98059 (10 μM) (+BAF +PD). Both IGF-I and BAF were protective. Neither the LY nor the PD interfered with the protective effect of BAF.

Figure 6 Caspase-3 was rapidly activated in SC apoptosis. SC were grown in DM ± IGF-I (10 nM) for 1, 3, 6, or 12 h. Fluorogenic substrate was cleaved as caspase-3 was activated. Percent cells fluorescent is an indication of caspase activation. Caspase-3 was activated as early as 1 h post-serum withdrawal, peaked at 3 h, and decreased at 6 and 12 h. At each time point, the inclusion of IGF-I diminished caspase-3 activation.

DISCUSSION

Our laboratory has established that IGF-I exerts multiple affects on cells of the nervous system, including promoting neurite outgrowth (Kim et al., 1997; 1998), inducing cytoskeletal rearrangement (Kim and Feldman, 1998; Leventhal and Feldman, 1997; Leventhal et al., 1997), and protecting cells from programmed cell death (Cheng and Feldman, 1998; Singleton et al., 1996a,b) (for review, see Feldman et al., 1997). SH-SY5Y cells undergo hyperosmotic-induced PCD, exhibiting nuclear fragmentation and DNA laddering (Matthews and Feldman, 1996; Matthews et al., 1997). IGF-I protects the cells from apoptosis via the type I IGF receptor (Singleton et al., 1996a,b). Current investigations extended this line of inquiry to SC, the myelinating cells of the peripheral nervous system.

Schwann cells grown in serum-containing media underwent apoptosis when switched to serum-free DM. Insulin and particularly IGF-I were effective in preventing this induction of apoptosis. Our results were in agreement with a recent report by Syroid et al. (1999), in which IGF-I promoted survival of postnatal...
SC grown \textit{in vitro} in DM for up to 3 days. In these experiments, cell survival was measured using the MTT assay as an indicator of SC viability. In the current studies, IGF-I rescued mainly via the PI 3-K pathway and prevented caspase activation which rapidly occurred following serum withdrawal. These findings concur with our previous reports for SH-SY5Y cells (Matthews and Feldman, 1996; Matthews et al., 1997; Singleton et al., 1996a). The replication of these findings in SC is of particular interest because of the important role that SC play as the myelinating cells of the peripheral nervous system.

Our findings indicate that serum withdrawal induced PCD in cultured SC beginning as early as 12 h and increasing to 72 h, at which time the majority of cells were dead or detached from the plate. Bisbenzamide staining revealed nuclear condensation characteristic of apoptotic cells. Syroid et al. (1996) made similar observations. Following serum withdrawal, they noted chromatin condensation and TUNEL-positive cells. Interestingly, neither we (data not shown) nor Syroid et al. (1996) were able to identify DNA laddering in SC.

This evidence of SC apoptosis \textit{in vitro} reflects the apoptosis that is naturally occurring \textit{in vivo}. Competition for axonal-derived growth factors such as NRG controls the survival of SC during neonatal and early postnatal development in the rat (Grinspan et al., 1996; Nakao et al., 1997; Syroid et al., 1996). SC death by apoptosis is well documented following nerve injury (Ekstrom, 1995; Grinspan et al., 1996; Kopp et al., 1997; Syroid et al., 1996; Trachtenberg and Thompson, 1996; Zorick and Lemke, 1996) and is apparent in tellurium-induced neuropathy (Berciano et al., 1998) as well as Charcot–Marie–Tooth neuropathy (Erdem et al., 1998), a hereditary disorder in humans. Considering the integral role of SC apoptosis in normal development, experimental neuropathies, and human disease, an \textit{in vitro} model is required so that death mechanisms may be more fully understood at the molecular and biochemical levels.

The data indicate that IGF-I prevents apoptosis predominantly through activation of the PI 3-K. We cannot rule out a role for the MEK pathway, however, since the MEK inhibitor PD98059 causes a small diminution of IGF-I’s ability to rescue SC from PCD. Recent studies suggest a link between PI 3-K and protection from PCD in hematopoietic cells and fibroblasts (Minshall et al., 1996; Yao and Cooper, 1996). Concentrations of insulin which are known to activate IGF-IR (De Meyts et al., 1994) prevent PCD in PC12 cells by activating PI 3-K, independent of the MEK pathway (Yao and Cooper, 1996). In parallel, the ability of insulin to prevent PCD in PC12 cells is blocked by wortmannin, a specific inhibitor of PI 3-K (Yao and Cooper, 1995). IGF-I signaling may follow a linear pathway in apoptotic prevention through the IGF-IR, PI 3-K, Akt, and Bad (Datta et al., 1997; del Peso et al., 1997; Dudek et al., 1997; Kauffmann-Zeh et al., 1997; Kennedy et al., 1997; Kulik et al., 1997; Kulik and Weber, 1998; Zhang et al., 1998). Our own studies in SH-SY5Y cells indicated that IGF-I prevents PCD via the IGF-IR:PI 3-K pathway (Singleton et al., 1996a). Collectively, previous reports (see above references), our own work in SH-SY5Y cells (Singleton et al., 1996a), and the results from SC presented here support the theory that activation of IGF-IR by IGF-I prevents PCD via a PI 3-K signaling pathway.

A number of pro- and anti-apoptotic proteins are involved in the apoptotic pathway (for reviews, see Adams and Cory, 1998; Ashkenazi and Dixit, 1998; Evan and Littlewood, 1998; Green and Reed, 1990). Caspases are almost universally implicated in apoptosis (Thornberry and Lazebnik, 1998). Recent reports in neuronal cells suggest caspases are activated early in the death cycle, followed later by morphological and biochemical evidence of DNA fragmentation (Posmantur et al., 1997). In the current study, we show that caspases are involved in SC apoptosis. Caspase-3-like activity rapidly increases following serum withdrawal, and a caspase inhibitor prevents apoptosis. Increased caspase activity is detected early, followed by later morphological evidence of SC apoptosis (i.e., bisbenzamide staining). That IGF-I decreases caspase-3-like activity following serum withdrawal in SC is in accordance with our own findings in SH-SY5Y cells and DRG neurons (Singleton et al., 1996a; Russell et al., 1998) and reports in cerebellar granule neurons (Tanabe et al., 1998; Tanaka et al., 1998), hypoxic cortical neurons (Tamatani et al., 1998), and cardiomyocytes (Wang et al., 1998a,b).

In addition to IGF-I, several growth factors including NFG, BDNF, NT-3, NT-4/5, LIF, CNTF, TGF-β, GDNF, and the neuregulins (NRG) may play supporting roles in SC development, migration, and survival (for review, see Scherer and Salzer, 1996). The NRG are a group of peptide growth factors that are the product of alternative splicing from a single gene (Marchionni et al., 1993). They exert their biological effects through the EGF-like erbB2, 3, and 4 receptors (reviewed by Marchionni et al., 1997). Significantly, SC express the erbB receptors at critical periods of SC apoptosis during development and following injury (Carroll et al., 1997; Grinspan et al., 1996). NRG prevents apoptosis of SC during development (Dong et al., 1996; Grinspan et al., 1996) and following injury (Carroll et al., 1997; Grinspan et al., 1996; Kopp et al., 1997; Trachtenberg and Thompson, 1996). Indeed, NRG promotes SC motility and neurite formation.
outgrowth (Mahanthappa et al., 1996), findings recapitulated in our laboratory with IGF-I (Cheng et al., 1999). NRG protects against apoptosis following serum-withdrawal in SC (Li et al., 1998). Clearly, IGF-I and NRG have overlapping functions in vivo and in vitro, and how these may relate to one another has not been investigated.

In conclusion, IGF-I, signaling via the PI 3-K, rescues SC from caspase-mediated apoptosis following serum withdrawal. Our results in SC largely duplicate those reported for neurons, emphasizing the need to determine the details of the apoptotic machinery in each cell type of clinical relevance. It is invalid to compare SC to cancer cells, and for the same reason an SC (or other glia) is functionally different from a neuron. SC retain the ability to divide in the adult; neurons are terminally differentiated and do not divide (Korr et al., 1986; McCarthy and Leblond, 1998). In fact, glia are noted for becoming reactive following insults, producing growth factors and responding to cytokines (Ridet et al., 1997). Our long-term goal is to understand the continuum between proliferation and apoptosis in glial cells and the therapeutic value of growth factors such as IGF-I and NRG in the treatment of glial cell disorders.

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