Schwann Cell-conditioned Medium Promotes Neuroblastoma Survival and Differentiation

Janet L. Kwiatkowski, J. Lynn Rutkowski, Darrell J. Yamashiro, Gihan I. Tennekoon, and Garrett M. Brodeur


Abstract

Neuroblastomas are histopathologically heterogeneous, ranging from immature malignant tumors to benign ganglionneuromas. The amount of Schwann cell stroma greatly increases with neuroblastoma differentiation, and these Schwann cells appear to be normal cells that infiltrate the tumor. To determine whether Schwann cells influence neuroblast differentiation, four human neuroblastoma cell lines were cultured in the presence or absence of human Schwann cell-conditioned medium for 7 days. Neuroblastoma cell survival, as determined by a colorimetric assay, more than doubled in three of the four neuroblastoma cell lines in the Schwann cell-conditioned medium. There was a corresponding reduction in neurite outgrowth in all of the neuroblastoma cell lines, and these processes contained mature neurofilament in three of the cell lines. These results indicate that Schwann cells produce soluble substances capable of supporting survival and differentiation in neuroblastoma cell lines. This may represent a biological mechanism responsible for neuronal differentiation in stroma-rich neuroblastomas.

Introduction

Neuroblastoma, a pediatric malignancy of neural crest origin, is unique in its wide spectrum of clinical behavior. Tumors can regress spontaneously, particularly in infants, or differentiate into ganglionneuromas. Unfortunately, in approximately one-half of all of the patients, the tumor is relentlessly progressive despite intensive therapy (1). Although certain genetic features such as MYCN amplification (2), deletion of the distal short arm of chromosome 1 (3), and neurotrophin receptor expression (4) have been shown to correlate with outcome, the biological mechanisms that underlie these disparate clinical behaviors remain enigmatic.

Neuroblastomas represent a spectrum of histologies from immature neuroblastoma to fully mature benign ganglionneuroma. Immature neuroblastomas are composed of small, undifferentiated neuronal cells with minimal neurite outgrowth and a paucity of stroma. With maturation, the neuronal cells differentiate into larger ganglion-like cells with increased neurite outgrowth, concomitant with a dramatic increase in the amount of Schwann cell stroma (5). Because neuroblasts and Schwann cells are both of neural crest origin, the two cell types were both initially thought to be derived from the malignant clone. However, recent work by Ambros et al. (6) revealed diploid DNA content in the Schwann cells in contrast to near-triploidy in the neuroblastic component of differentiating neuroblastomas. This implies that Schwann cells are not of malignant origin but are normal cells that infiltrate the tumor.

Schwann cells and neuronal cells exhibit reciprocal supportive interactions integral to the development of the peripheral nervous system. Neurons influence Schwann cell migration and proliferation, whereas Schwann cells promote neuronal survival and differentiation (7). We hypothesize that the Schwann cells in stroma-rich neuroblastomas may similarly influence neuroblast survival and differentiation and that this effect is mediated, at least partly, through soluble factors. Therefore, we examined the ability of human Schwann cell-conditioned medium to affect the survival and differentiation of human neuroblastoma cell lines. Primary human Schwann cells were propagated in short-term culture, and the ability of the medium that was collected from these Schwann cells to promote neuronal survival and differentiation was assessed. Our results suggest that Schwann cells produce soluble substances(s) that are capable of fulfilling these functions.

Materials and Methods

Cell Lines and Culture. Four human neuroblastoma cell lines—SH-SY5Y (a neuronal clone from the SK-N-SH neuroblastoma cell line), LA-N-6, NLF, and SK-N-BE (2)—were studied. NLF and SK-N-BE (2) have MYCN amplification, but SH-SY5Y and LA-N-6 have a normal MYCN copy number. Each of these cell lines and their culture conditions have been described previously (8). Primary human Schwann cells were purified from adult nerves and expanded as described previously (9, 10). Schwann cells were maintained at 10% CO2 in DMEM supplemented with 10% FBS.4 50 ng/ml glial growth factor 2 (GGF-2; Cambridge Neuroscience), 50 ng/ml growth arrest specific protein 6 (GAS-6; Amgen), 1% penicillin/streptomycin, and 2 µM forskolin. Forearm skin fibroblast culture (provided by Dr. E. Hiraya, Thomas Jefferson University, Philadelphia, PA) was maintained at 10% CO2 in DMEM supplemented with 10% FBS, 1% l-glutamine, and 1% penicillin/streptomycin.

Collection of Conditioned Media. Conditioned media were collected from the human Schwann cell and skin fibroblast cell lines. Cells were refed without growth factors in the medium 48 h before collection. Confluent 10-cm plates were washed with Dulbecco's PBS, and 4 ml of DMEM supplemented with 2% FBS were added. This medium was then harvested after 24 h and centrifuged at 4500 RPM, and the supernatant was collected and used as conditioned medium. Cell counts were performed to ensure equal numbers of cells contributing to the production of the conditioned media from each cell type.

Neuroblastoma Cultures. Neuroblastoma cells suspended in DMEM with 2% FBS were plated on glass coverslips coated with 100 µg/ml poly-L-lysine and placed in a 24-well plate. The cell lines were seeded at different densities based on their growth rates. SH-SY5Y and LA-N-6 were seeded at a density of 1 X 104 cells, NLF at a density of 5 X 103, and SK-N-BE (2) at a density of 2.5 X 103 cells per coverslip. After 1 h to allow for cell attachment, 370 µl of medium were added per well. Six wells for each of the following conditions were carried out: (a) Schwann cell-conditioned medium; (b) skin fibroblast-conditioned medium; (c) DMEM with 2% FBS; and (d) DMEM with 2% FBS.

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2 Current address: Division of Pediatric Oncology, College of Physicians & Surgeons of Columbia University, New York, NY 10032.

4 The abbreviations used are: FBS, fetal bovine serum; BrdUrd, bromodeoxyuridine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, TdT-mediated dUTP nick-end labeling; NF-H, high molecular weight neurofilament; NGF, nerve growth factor.
supplemented with NGF (100 ng/ml). The cells were refed on the 4th day and cultured for a total of 7 days. Cells were treated with 30 μM BrdUrd for 5 h to assess mitotic activity. Cultures were examined daily under phase microscopy for evaluation of morphology and neurite outgrowth.

Cell Survival Assay. SH-SY5Y, LA-N-6, and NLF were seeded into 96 well plates at a density of 1 × 10⁴ cells/well, and SK-N-BE (2) cells were seeded at 0.5 × 10⁴ cells/well, and cultured in each of the above four conditions. The process for each condition was performed in triplicate. Medium was replaced at 4 days, and cultures were maintained for 7 days. A colorimetric MTT assay was then performed according to previously published methods (11). A multiwell scanner was used to measure the absorbance at 570-nm wavelength.

Apoptosis Assay. Cells on coverslips fixed in 4% paraformaldehyde were assayed by TUNEL assay using a fluorescein apoptosis detection system (Promega) according to the protocol provided, except that the volume was scaled down to 40 μl for coverslips. Cells were counterstained with diamidino-phenylindole, and the percentage undergoing apoptosis in a total of 400 cells was determined. All of the studies were replicated with similar results.

Immunofluorescent Staining. Cells on glass coverslips were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 2 min at room temperature, and further treated with 2N HCl at 37°C for 20 min for BrdUrd localization. The primary antibodies and dilutions used were as follows: (a) TA51 (provided by J. Trojanowski, University of Pennsylvania, Philadelphia, PA), which recognizes NF-H, undiluted; and (b) anti-BrdUrd (Boehringer Mannheim) at a concentration of 6 μg/ml. Coverslips were incubated with primary antibodies for 1 h at 37°C, washed in three changes of medium, and then incubated with a fluorescein-conjugated secondary antibody using anti-rat IgG (Cappel) for TA51 and antimouse IgG (Cappel) for BrdUrd, both at a dilution of 1:200 for 30 min at 37°C. The cells were then washed and postfixed in 95% ethanol and 5% acetic acid. BrdUrd-stained coverslips were counterstained with 15 μM propidium iodine. Cells were then mounted in glycerol with antifadant (Citifluor) and examined with a Leitz fluorescent microscope. The proportion of neuroblastoma cells in S-phase was determined by counting the number of cells double-labeled for BrdUrd and propidium iodine in a total of 400 cells.

Statistical Analysis. The results of the MTT assay were analyzed by unpaired t test and the mitotic activity and TUNEL assay were analyzed by the exact conditional χ² test.

Results

Schwann Cell-conditioned Medium Enhances Survival of Neuroblastoma Cell Lines. There was a significant increase in cell number in three of the four neuroblastoma cell lines when grown in Schwann cell-conditioned medium compared with the controls, as measured by MTT assay (Fig. 1A). For SH-SY5Y, cell survival doubled in the Schwann cell-conditioned medium compared with the 2% FBS control (P = 0.006). For the MYCN amplified cell lines, cell number was increased by a factor of 3.3 (P = 0.006) and 3.5 (P = 0.001) for SK-N-BE (2) and NLF, respectively. The addition of NGF did not significantly improve survival above the 2% FBS control. Similarly, conditioned medium from skin fibroblasts conferred no survival benefit, and cell number was actually reduced in the fibroblast-conditioned medium compared with the basal medium in the LA-N-6 (P = 0.012) and NLF (P = 0.031) lines. There was no
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Fig. 2. Phase microscopy of SH-SY5Y (A–D) and NLF (E–H) at 7 days. ×20. Cells appeared flat, and neurite outgrowth was minimal in basal medium (A, E) and in basal medium with NGF (100 ng/ml; B, F). Both lines survived poorly, and there was minimal differentiation in fibroblast-conditioned medium (C, G). When cultured in Schwann cell-conditioned medium (D, H), the cells polarized and extended long neurites.

significant change in cell survival in the Schwann cell-conditioned medium for the LA-N-6 cell line, but survival advantage under minimal serum conditions may be more difficult to demonstrate in this slow-growing cell line.

Schwann Cell-conditioned Medium Does Not Affect Proliferation of Neuroblastoma Cell Lines. The percentage of mitotically active neuroblastoma cells, as determined by BrdUrd labeling, was not significantly different in Schwann cell-conditioned medium compared with the basal medium with or without NGF for the SH-SY5Y, LA-N-6, and NLF cell lines (Fig. 1B). However, in SK-N-BE (2), cellular proliferation was slightly enhanced by Schwann-conditioned medium compared with the controls. In all of the four cell lines, the mitotic activity was significantly decreased in the fibroblast-conditioned medium. This was attributed to the depletion of essential nutrients from the medium or to the possible production of a toxic substance by the metabolically active skin fibroblasts.

Apoptosis in Neuroblastoma Cell Lines Is Suppressed by Schwann Cell-conditioned Medium. The percentage of TUNEL positive cells was consistently less in the Schwann cell-conditioned medium compared with the controls (Fig. 1C). In the SH-SY5Y LA-N-6, and SK-N-BE(2) cell lines, there was a 10-fold reduction ($P<0.001$) and in the NLF cell line, there was a 7-fold reduction ($P<0.001$) in apoptosis compared with the basal medium control. In the SH-SY5Y, there was also a significant reduction in apoptosis in the medium supplemented with NGF ($P = 0.002$) and in the skin fibroblast-conditioned medium ($P = 0.02$) compared with the 2% FBS control. The reduction in apoptosis in Schwann cell-conditioned medium remained significant compared with the basal medium sup-
Schwann Cell-conditioned Medium Induces Morphological Changes Consistent with Differentiation in Neuroblastoma Cell Lines. Phase microscopy showed a change in cellular morphology to a more bipolar cell with extensive neurite outgrowth in all of the four neuroblastoma cell lines cultured in Schwann cell-conditioned medium, compared with minimal-to-no neurite outgrowth in the control media (Fig. 2). Neurite outgrowth was first evident at 3–4 days in culture. Neurite number and length were greatest in SH-SY5Y (Fig. 2, A–D), although LA-N-6 and both MYCN-amplified lines demonstrated substantial differentiation. Immunofluorescent staining revealed the presence of NF-H in the neurites of the SH-SY5Y, LA-N-6, and SK-N-BE (2) cell lines treated with Schwann cell-conditioned medium (Fig. 3). In contrast, in the NLF cell line, although there was substantial neurite outgrowth (Fig. 2, E–H), there was no apparent NF-H staining in the processes in the cultures treated with Schwann cell-conditioned medium (data not shown).

Discussion

Our data show a dramatic effect of Schwann cell-conditioned medium on neuroblastoma cells in vitro. Overall, cell viability was greatly augmented in Schwann cell-conditioned medium. Schwann cell-conditioned medium promoted survival primarily by a reduction in apoptosis with little if any effect on proliferation. Concomitantly, Schwann cell-conditioned medium induced dramatic neurite outgrowth in all of the neuroblastoma cell lines tested, with accumulation of NF-Hs typically expressed by mature neurons (12) within the processes in three of the four cell lines studied. Developmentally, both neuronal differentiation and cell survival are supported by neurotrophic factors (13). It follows that in promoting differentiation of neuroblasts, Schwann cell factors also facilitate neuroblast survival. Despite some expected individual variability between cell lines with respect to basal growth rate, morphological appearance, and expression of neurofilament, all of the neuroblastoma cell lines showed a similar response to Schwann cell-conditioned medium.

Schwann cells are the source of a number of neurotrophic factors including NGF (14), leukemia inhibitory factor (LIF; Ref. 7), brain-derived neurotrophic factor (BDNF), and ciliary neurotrophic factor (CNTF; Ref. 15). It is likely that a combination of these and perhaps other unknown factors are responsible for the effects we have shown. NGF was an attractive candidate because the expression of TrkA in neuroblastoma has been shown to correlate positively with survival (4), and NGF can induce differentiation in some primary neuroblastoma cultures (4, 16, 17). However, NGF alone did not induce significant differentiation in the neuroblastoma cell lines in our experiments. These results are not surprising in view of previous work which demonstrated that NLF and other cell lines do not respond to NGF despite the expression of the TrkA receptor, suggesting a possible defect in the receptor signaling pathway (18). Therefore, NGF cannot be the sole cause of neuroblastoma differentiation in Schwann cell-conditioned medium. However, it is still possible that NGF/TrkA interactions could contribute to differentiation of neuroblastoma in vivo.

Ganglioneuromas and ganglioneuroblastomas are composed of two populations of cells: neuroblasts and Schwann cells. Data from Ambros et al. (6) suggest that Schwann cells in these tumors are normal cells that infiltrate differentiating neuroblastomas rather than derivatives of the malignant clone. Our results support the concept that Schwann cells play a significant role in the induction of neuroblastoma differentiation. There are several reasons to expect this maturational influence would be further increased in primary neuroblastomas in vivo. First, the ability of neuroblasts to become established as a cell line in long-term culture is associated with a poor prognosis (8). Cell lines frequently acquire additional genetic alterations that may make them less susceptible to differentiation, yet marked differentiation was induced even in this setting. Second, we...
were able to demonstrate substantial differentiation in the MYCN-amplified cell lines despite the association of MYCN amplification with a particularly aggressive phenotype. Finally, the interaction between Schwann cells and neurons in the nervous system is complex and is mediated by cell-cell contact, extracellular matrix interactions, and up-regulation of growth factor receptors, in addition to the production of soluble factors (8). These other mechanisms would be expected to augment the differentiation effects of the Schwann cell secretory products.

We postulate that stroma-rich neuroblastomas differ from unfavorable neuroblastomas by the production of one or more Schwann cell chemoattractants and that these Schwann cells, in turn, promote neuroblast differentiation. Neurons of the peripheral nervous system can promote Schwann cell migration and proliferation in part through the production of soluble factors (19). Similar substances may be involved in the neuroblast-Schwann cell interactions in stroma-rich tumors. This suggests future treatment approaches aimed at using Schwann cells as differentiating agents as an alternative to cytotoxic therapy.

References

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