Cellular and Molecular Neurobiology, Vol. 23, No. 2, April 2003 (© 2003)

Rapid Communication

A Novel Method of Eliminating Non-Neuronal Proliferating Cells From Cultures of Mouse Dorsal Root Ganglia

Parker L. Andersen,[1] J. Ronald Doucette,[2] and Adil J. Nazarali[1,3]

Received November 5, 2002; accepted December 18, 2002

SUMMARY

1. We hypothesized that non-neuronal cells could be eliminated from primary dorsal root ganglion (DRG) cultures by including a DNA topoisomerase inhibitor (camptothecin) during culture.
2. Exposure to 20 μM camptothecin for 48 h, beginning at 3 days in vitro, reliably eliminates proliferating non-neuronal cells.
3. Following camptothecin treatment, neurons survived and continued to extend neurites for several weeks without obvious defects in morphology or viability.
4. Transient camptothecin exposure is therefore an efficient and fast-acting method to purify DRG neurons in culture.

KEY WORDS: DRG neuron; Schwann cell; fibroblast; camptothecin culture.

INTRODUCTION

Unlike neurons of the central nervous system, neurons derived from the dorsal root ganglia (DRG) can be cultured from embryonic (Wood and Bunge, 1986; Wood and Williams, 1984), postnatal (Horie and Kim, 1984), and adult (Scott, 1977) rodents. Because these neurons exhibit great regeneration activity both in vivo and in vitro, and are capable of being myelinated in vitro (Bunge and Wood, 1987;

[1] Laboratory of Molecular Biology, College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.
[2] Department of Anatomy and Cell Biology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.
[3] To whom correspondence should be addressed at Laboratory of Molecular Biology, College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5C9; e-mail: nazarali@duke.usask.ca.

0272-4340/03/0400-0205/0

Devon and Doucette, 1992; Wood and Bunge, 1986; Wood and Williams, 1984), they serve as an excellent experimental model system. One disadvantage when using these cells is that the cell cultures are routinely contaminated with Schwann cells and fibroblasts, which makes experimental interpretations difficult. Existing methods to purify DRG neurons usually involve introducing antimitotic agents such as cytosine arabinoside or fluorodeoxyuridine into the culture medium (Masuko *et al*., 1979; Wood and Bunge, 1986). However these agents are often required for prolonged exposure and thus have limited success. Alternately, DRG neurons can be partially purified because of their unique large size using Percoll density centrifugation (Goldenberg and De Boni, 1983). This method has the disadvantage of a low cell yield, likely due to selection of the larger neurons and partial loss of the smaller neurons normally residing in DRG. Furthermore, the contaminating satellite cells tightly associated with the neuronal somas (from which some Schwann cells are derived) have a tendency for uncontrolled proliferation. A method to quickly and reliably produce pure cultures of DRG neurons would significantly aid studies in determining the molecular mechanisms of regulating myelinating phenotype by Schwann cells, olfactory ensheathing cells, and cells of the oligodendrocyte lineage.

One possible method to eliminate contaminating cells would be to specifically inhibit some activity essential for proliferating cells but which is not essential for the postmitotic neurons. DNA topoisomerase 1 (TOP1) is an important DNA metabolic enzyme and a molecular target of antitumor drugs (Desai *et al*., 1997). TOP1 enzyme regulates DNA supercoiling by controlling strand cleavage. The enzyme cleaves a single strand of the duplex DNA, relaxing the DNA supercoil, while the complementary DNA strand is rotated before religation (Wang, 1985). This "breakage–reunion" reaction by TOP1 relaxes torsional stress generated by the progression of the transcriptional/replication machinery along the DNA double-helix. Camptothecin (CPT) is a plant alkaloid that inhibits TOP1 activity by blocking the religation step of the cleavage/religation reaction of TOP1 (Liu *et al*., 2000) progressing to DNA double strand breaks during DNA synthesis (Ryan *et al*., 1991). The alkaloid leaves the cell deficient in TOP1 (Beidler and Cheng, 1995) inducing TOP1 downregulation via the ubiquitin/26 proteasome pathway (Desai *et al*., 1997, 2001). The primary mechanism of cell death induced by CPT is S-phase-specific (Liu *et al*., 2000). Small doses of CPT stall proliferating cells in S-phase, whereas higher doses induce apoptosis (Kaufmann, 1998; Morris and Geller, 1996). However, cortical neurons are sensitive to higher camptothecin doses resulting in apoptosis, which may correlate with their transcriptional activity and level of TOP1 protein expression (Morris and Geller, 1996).

We hypothesized that transiently exposing primary cultures of DRG neurons to CPT would have damaging effects on DNA replication of the contaminating Schwann cells and fibroblasts, causing their death while simultaneously having little or no effect on the postmitotic DRG neurons. Our experiments demonstrate that CPT treatment can serve as a rapid and reliable method to eliminate proliferating cells from DRG cell cultures without affecting neuron viability.

MATERIALS AND METHODS

All chemicals were obtained from Sigma, Oakville, ON, unless otherwise specified. Routine cultures of mouse DRG were obtained from newborn CD1 mice bred on the University campus. Briefly, newborn CD1 mice were killed by decapitation and submerged in 95% ethanol for approximately 2 min. DRG were aseptically removed and meticulously cleaned of rootlets and connective tissue. The tissue was disaggregated by incubating in L15 nutrient mixture (pH corrected to 7.4) containing 0.1% collagenase for 90 min at 37°C with light trituration every 30 min. Following digestion, horse serum was added to 10% and the cell suspension centrifuged at low speed for 8 min. The cell pellet was resuspended in complete growth medium consisting of 10% horse serum and 50 ng/mL NGF (Cedar Lane Laboratories, Canada) in DMEM (4500 mg/mL glucose and $NaHCO_3$ reduced to 2 g/L). Cells were seeded onto surfaces precoated with poly-L-lysine (50 μg/mL 70,000–150,000 Da). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air; the medium was replaced routinely every 2 days. CPT dissolved in DMSO (10 mM stock) was included on day 3 of the cultures to a final concentration of 20 μM for a total period of 48 h. Sister cultures used as controls received an equivalent volume of DMSO or media alone.

Phase contrast images of live cultures were captured using an Olympus IX 70 inverted microscope (Carsen Group, Inc., ON) to investigate the presence of non-neuronal cells and to observe any morphological change in the neurons due to either CPT or DMSO treatment. To quantitate changes in neuron viability following treatment, cultures were established in 96-well plates at 400 nigrosine excluding neurons per well and neuron density determined by manually counting formaldehyde fixed sister cultures before and after treatments. The results were expressed as mean $\pm$ standard deviations (SD), and statistical analysis was performed using Student's *t* test.

RESULTS AND DISCUSSIONS

By 6 days in vitro (6 DIV), control DRG cultures become confluent with Schwann cells and fibroblasts (Fig. 1(A)). If the Schwann cells and fibroblasts are allowed to proliferate for extended periods, all cells lose adherence to the culture surface. Cultures containing DMSO alone are indistinguishable from untreated cultures (data not shown). Following 20 μM CPT treatment initiated at day 3 of culture for 48 h, the majority of the non-neuronal cells were eliminated by 6 DIV (Fig. 1(B)) with occasional non-neuronal cells (approximately 1 in 200 neurons at best) present. Purity is dependent on removal of all connective tissue during dissection. The neurons retained a large nucleous with smooth soma and an extensive neurite network. DRG neurons are easily identifiable by their large spherical soma, large nucleus, distinct nucleolus, long neurites, and expression of the neuronal growth associated protein GAP-43 immunostaining (Fig. 1(C)). These are characteristics of viable neurons. In lower-density cultures individual neurons possess extensive neurites confirming that the neurons remain healthy following both removal of the non-neuronal cells and

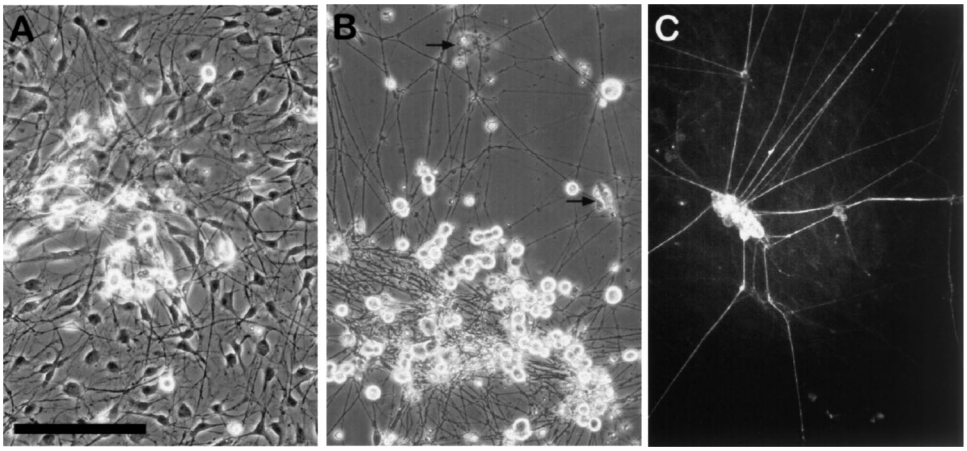

Fig. 1. Phase contrast micrographs of newborn mouse dorsal root ganglia culture (A) at 6 days in vitro (DIV) and (B) a comparable culture at 6 DIV following 20 μM camptothecin (CPT) treatment initiated on day 3 for 48 h. Figure 1(C) illustrates 10 DIV culture stained with GAP-43 (mouse monoclonal 9-1E12 (a kind gift from Dr D. Schreyer) followed with an Alexa conjugated anti-mouse antibody (A-11001, Molecular Probes, Inc., U.S.A.)). Bar = 100 μm for Fig. (A)–(C).

following transient CPT exposure. The few remaining non-neuronal cells likely have not passed through S-phase within the 48 h treatment period and therefore are likely quiescent fibroblast cells, although their exact phenotype has not been investigated (see arrows in Fig. 1(B)).

To investigate the effects of CPT treatment on neuron viability, neuron counts were made at several time points during the culture period, before (3 DIV) and after CPT treatment or DMSO (vehicle) treatment or medium alone (6 and 10 DIV; Table I). No significant difference in numbers of neurons was observed following either CPT or DMSO treatment. The major effect on the DRG cultures from CPT exposure was induced killing of the mitotic non-neuronal cells, with no observable effect on the neurons. Initially dose and time responses were performed to maximize the efficiency of CPT treatment. Either increasing the CPT concentration to 1 mM or exposure to 20 μM CPT initiated at the time of plating eliminated not only the non-neuronal cells but also a significant number of the neurons. Additional prolonged exposure to CPT (7 days) also diminished the neuronal count (data not shown). Hence a dose of 20 μm CPT and exposure of 48 h initiated on day 3 of culture

Table I. Quantitation of Neurite-Expressing Cells From Newborn Mouse Dorsal Root Ganglia (DRG)

DRG culture days in vitro (DIV)	Control (untreated) media	Culture treated with vehicle (DMSO)	Culture treated with 20 μM CPT
3	411 (14)	424 (8)	417 (6)
6	339 (45)	372 (20)	376 (22)
10	422 (14)	411 (21)	420 (19)

Note. DRG cultures untreated (control) or treated with 20 μM camptothecin (CPT) or vehicle (DMSO). For details see Methods. Culture was continued for 3, 6, and 10 days in vitro (DIV). Values represent the average of three wells. Standard deviation is indicated in parentheses. No significant differences were observed between treatments. Additional cell counts were conducted in four separate independent experiments in triplicate, and similar findings were observed.

were found to be optimal (Fig. 1(B)), whereas higher doses or longer exposures were detrimental to the culture. Additional transient treatment with CPT can be applied at later stages if needed to remove remaining proliferating cells without detrimental effects to the neurons (data not shown). The possibility of unobserved actions by CPT, such as an effect on transcription, cannot be ruled out. However, DRG neurons remained healthy (as determined by the appearance of large distinct nucleoli, smooth soma, and neurites) for at least 21 DIV (data not shown) following the 48 h transient exposure to CPT.

The DNA double-strand breaks and apoptosis induced by ara C (1-β-arabinofuranosylcytosine) in sympathetic neurons is reported to be similar to that induced by the topoisomerase 2 inhibitors (Tomkins *et al*., 1994). Fetal CNS neurons have been described to be much more susceptible to either CPT (Morris and Geller, 1996) or etoposide, an inhibitor of topoisomerase 2 (Nakajima *et al*., 1994), than cocultured astrocytes. The susceptibility in both cases has been ascribed to a higher transcription activity in the neurons. A direct link between CPT-induced DNA double-strand breaks in the G_2 phase of the cell cycle of CHO cells and transcription has also been described (Mosesso *et al*., 2000). The DNA topoisomerase 2-β has recently been reported to be involved in early stage cerebellar neuron differentiation by potentiating transcription of neuronal genes (Tsutsui *et al*., 2001).

DRG neurons from the newborn mice are postmitotic; an effect on embryonic DRG neurons was not investigated. CPT induces reversible protein-linked DNA breaks (PLDB) which is an important step in the toxicity of CPT (Beidler and Cheng, 1995). Since cells in the S-phase are significantly more sensitive to the killing effects of CPT, it has been proposed that PLDB collide with the replication fork during DNA synthesis to produce a double-stranded DNA (dsDNA) break, transforming the reversible PLDB to a more permanent dsDNA break (Beidler and Cheng, 1995; Ryan *et al.*, 1991). CPT also induces a reduction in TOP1 protein and this downregulation in TOP1 may be a resistance mechanism to avoid the toxic levels of PLDB (Beidler and Cheng, 1995). In fact there is a general negative correlation between CPT-induced TOP1 downregulation and CPT resistance in certain cancer cell lines. The breast cancer line ZR-75-1, most susceptible to CPT, was also completely defective in CPT-induced TOP1 downregulation (Desai *et al*., 2001). To our knowledge, expression of TOP1 in DRG neurons has not been documented, but it is possible that the DRG exhibit effective CPT-induced TOP1 downregulation and may explain the relative insensitivity of postmitotic DRG to CPT even at the higher concentration used (20 μM).

In conclusion, our results demonstrate that DRG neurons are less susceptible to transient (48 h) CPT treatment than are Schwann cells or fibroblasts. This represents an important new method that can be quickly and easily used by researchers to purify DRG neurons in vitro. The method is being used routinely in our laboratory to purify DRG neurons in vitro in significant numbers.

ACKNOWLEDGMENTS

Supported by a research grant from the Canadian Institutes of Health Research to A.J.N and J.R.D. We also acknowledge support from the Multiple Sclerosis Society of Canada for technical personnel.

REFERENCES

Beidler, D. R., and Cheng, Y.-C. (1995). Camptothecin induction of a time- and concentration-dependent decrease of topoisomerase I and its implication in camptothecin activity. *Mol. Pharmacol.* **47:**907–914.

Bunge, R. P., and Wood, P. M. (1987). Tissue culture studies of interactions between axons and myelinating cells of the central and peripheral nervous system. In Seil, F. J., Herbert, E., and Carlson, B. M. (eds.), *Progress in Brain Research*, *Vol. 71*, Elsevier, New York, pp. 143–152.

Desai, S. D., Li, T.-K., Rodriguez-Bauman, A., Rubin, E. H., and Liu, L. F. (2001). Ubiquitin/26S proteasome-mediated degradation of topoisomerase I as a resistance mechanism to camptothecin in tumor cells. *Cancer Res*. **61:**5926–5932.

Desai, S. D., Liu, L. F., Vazquez-Abad, D., and D'Arpa, P. (1997). Ubiquitin-dependent destruction of topoisomerase I is stimulated by the antitumor drug camptothecin. *J. Biochem. Chem.* **272:**24159–24164.

Devon, R., and Doucette, R. (1992). Olfactory ensheathing cells myelinate dorsal root ganglion neurites. *Brain Res*. **589:**175–179.

Goldenberg, S. S., and De Boni, U. (1983). Pure population of viable neurons from rabbit dorsal root ganglia, using gradients of Percoll. *J. Neurobiol.* **14:**195–206.

Horie, H., and Kim, S. U. (1984). Improved survival and differentiation of newborn and adult mouse neurons in F12 defined medium by fibronectin. *Brain Res*.**294:**178–181.

Kaufmann, S. H. (1998). Cell death induced by topoisomerase-targeted drugs: More questions than answers. *Biochim. Biophys. Acta* **1400:**195–211.

Liu, L. F., Desai, S. D., Li, T.-K., Mao, Y., Sun, M., and Sim, S.-P. (2000). Mechanism of action of camptothecin. *Ann. N.Y. Acad. Sci.* **922:**1–10.

Masuko, S., Kuromi, H., and Shimada, Y. (1979). Isolation and culture of motor neurons from embryonic chicken spinal cords. *Proc. Natl. Acad. Sci. U.S.A.* **76:**3537–3541.

Morris, E. J., and Geller, H. M. (1996). Induction of neuronal apoptosis by camptothecin, an inhibitor of DNA topoisomerase-1: Evidence for cell-cycle-independent toxicity. *J. Cell Biol*. **134:**757–770.

Mosesso, P., Pichierri, P., Franchitto, A., and Palitti, F. (2000). Evidence that camptothecin-induced aberrations in the G_2 phase of the cell cycle of Chinese hamster ovary (CHO) cell lines is associated with transcription. *Mutat. Res.* **452:**189–195.

Nakajima, M., Kashiwagi, K., Ohta J., Furukawa, S., Hayashi, K., Kawashima, T., and Hayashi, Y. (1994). Etoposide induces programmed cell death in neurons cultured from the fetal rat central nervous system. *Brain Res*. **641:**350–352.

Ryan, A. J., Squires, S., Strutt, H. L., and Johnson, R. T. (1991). Camptothecin cytotoxicity in mammalian cells is associated with the induction of persistent double strand breaks in replicating DNA. *Nucleic Acids Res.* **19:**3295–3300.

Scott, B. S. (1977). Adult mouse dorsal root ganglia neurons in cell culture. *J. Neurobiol.* **8:**417–427.

Tomkins, C. E., Edwards, S. N., and Tolkovsky, A. M. (1994). Apoptosis is induced in postmitotic rat sympathetic neurons by arabinosides and topoisomerase II inhibitors in the presence of NGF. *J. Cell Sci.* **107:**1499–1507.

Tsutsui, K., Tsutsui, K., Sano, K., Kikuchi, A., and Tokunaga, A. (2001). Involvement of DNA topoisomerase IIβ in neuronal differentiation. *J. Biol. Chem.* **276:**5769–5778.

Wang, J. C. (1985). DNA topoisomerases. *Annu. Rev. Biochem.* **54:**665–697.

Wood, P. M., and Bunge, R. P. (1986). Myelination of cultured dorsal root ganglion neurons by oligodendrocytes obtained from adult rats. *J. Neurol. Sci.* **74:**153–169.

Wood, P. M., and Williams, A. K. (1984). Oligodendrocyte proliferation and CNS myelination in cultures containing dissociated embryonic neuroglia and dorsal root ganglion neurons. *Brain Res.* **314:**225–241.