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Compartmentalized microfluidic culture platform to study mechanism of paclitaxel-induced axonal degeneration

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Abstract

Chemotherapy induced peripheral neuropathy is a common and dose-limiting side effect of anticancer drugs. Studies aimed at understanding the underlying mechanism of neurotoxicity of chemotherapeutic drugs have been hampered by lack of suitable culture systems that can differentiate between neuronal cell body, axon or associated glial cells. Here, we have developed an *in vitro* compartmentalized microfluidic culture system to examine the site of toxicity of chemotherapeutic drugs. To test the culture platform, we used paclitaxel, a widely used anticancer drug for breast cancer, because it causes sensory polyneuropathy in a large proportion of patients and there is no effective treatment. In previous *in vitro* studies, paclitaxel induced distal axonal degeneration but it was unclear if this was due to direct toxicity on the axon or a consequence of toxicity on the neuronal cell body. Using microfluidic channels that allow compartmentalized culturing of neurons and axons, we demonstrate that the axons are much more susceptible to toxic effects of paclitaxel. When paclitaxel was applied to the axonal side, there was clear degeneration of axons; but when paclitaxel was applied to the soma side, there was no change in axon length. Furthermore, we show that recombinant human erythropoietin, which had been shown to be neuroprotective against paclitaxel neurotoxicity, provides neuroprotection whether it is applied to the cell body or the axons directly. This observation has implications for development of neuroprotective drugs for chemotherapy induced peripheral neuropathies as dorsal root ganglia do not possess blood–nerve-barrier, eliminating one of the cardinal requirements of drug development for the nervous system. This compartmentalized microfluidic culture system can be used for studies aimed at understanding axon degeneration, neuroprotection and development of the nervous system.

Keywords

Peripheral neuropathy; Axonal degeneration; Paclitaxel; Erythropoietin; Microfluidic; Compartmentalized culture; Microfabrication; Neuroprotection

Introduction

Peripheral neuropathy is a relatively common disease that affects at least 2.4% of the population in the US (Hughes, 2002). Prevalence increases with aging population and reaches as high as 26% of people older than 65 years of age (Mold, Vesely et al., 2004). Typical symptoms of peripheral neuropathy reflect sensory, motor, or autonomic nerve fiber dysfunction. In particular, sensory symptoms include paresthesias, some of which are painful, sensory loss and numbness. In most polyneuropathies, these symptoms begin distally in extremities and progress proximally (Lacomis, 2002; Periquet et al., 1999). The pathologic changes in most of these polyneuropathies are those of a distal to proximal axonal degeneration, which have been referred to as “dying-back neuropathies” (Dyck et al., 1986). Currently there are no effective therapies aimed at the underlying mechanism of axonal degeneration, except for inflammatory neuropathies characterized by infiltration of peripheral nerves with lymphocytes and macrophages (Merkies et al., 2003).

Paclitaxel, a diterpene alkaloid drug, is a commonly used chemotherapeutic agent against breast, lung and ovarian cancer. One of the major dose-limiting side effects is distal axonal, mainly sensory, polyneuropathy (Lipton et al., 1989; Sahenk et al., 1994). The symptoms of paclitaxel-induced neuropathy are tingling, numbness, loss of balance and burning pain. The mechanisms of neurotoxicity of paclitaxel are largely unknown and effective treatments for axonal neuropathy caused by paclitaxel are currently not available. Paclitaxel binds to beta-tubulin and stabilizes its polymerization. This leads to disruption of the mitotic spindle and arrest of the cell division (Schiff and Horwitz, 1980, 1981). It has been suggested that paclitaxel may lead to an increase and altered distribution of detyrosinated tubulin, a marker for stable microtubules (Laferriere et al., 1997). In addition to these *in vitro* studies, animal models of paclitaxel neuropathy have been developed in rodents (Cavaletti et al., 1995; Melli et al., 2006a,b; Polomano et al., 2001; Sahenk et al., 1994; Wang et al., 2002), but the underlying mechanism of distal axonal degeneration induced by paclitaxel remains to be determined.

Although multiple *in vitro* models of peripheral neuropathies exist only two groups have attempted to use compartmentalized culture systems to ask whether axonal degeneration is due to local axonal disturbances or a consequence of derangement in the neuronal cell body (Melli et al., 2006a,b; Silva et al., 2006). These groups used Campenot chambers that consist of a Teflon divider attached to collagen-coated Petri dish with silicone grease (Campenot, 1977). Campenot chambers require great skill, as leakage between chambers is a common problem, limiting efficiency and reproducibility. Chamber systems, other than Campenot chambers, have been developed to isolate hippocampal (Ivins et al., 1998) and motor axons (Harper et al., 2004) from soma using thin coverslips. However, these had similar problems as the Campenot chambers, leakage between chambers being the most common one. In contrast, advances in microtechnology and biomaterials have led to numerous approaches that precisely control the positioning of cells on substrates (Chen et al., 1997; McDonald and Whitesides, 2002; Taylor et al., 2006; Weibel et al., 2005). Presenting cells with controlled topographical and chemical cues have allowed us to understand a great deal about how cells respond to their local microenvironments (Rhee et al., 2005; Sorribas et al., 2002; Yang et al., 2005a,b). Microfabrication technology, utilizing photolithography, micro-contact

printing, and microfluidics, is used to construct chambers in which precise *in vitro* cellular patterning is achieved. Such microfabrication techniques have been used to create chemical and biochemical analysis platforms (Chen et al., 2002; McDonald and Whitesides, 2002; Mrksich et al., 1997).

In this study, we aim to help elucidate the underlying mechanism of paclitaxel-induced axonal degeneration through the use of microfluidic platforms that allow us to physically and fluidically isolate cellular compartments, as well as to gauge the protective role of recombinant human erythropoietin. Probing different cellular compartments allows us to determine whether the site of action is on the cell body or axonal side. Since most polyneuropathies are “dying-back” neuropathies, we sought to determine if paclitaxel caused the most degeneration when applied to the distal axon as compared to the cell body. Erythropoietin is a glycoprotein hormone that has effects on multiple organs and tissues. We have previously shown that this hormone is involved in an endogenous neuroprotective pathway through Schwann cell-derived erythropoietin (Keswani et al., 2004), and demonstrated this effect both *in vitro* and *in vivo* (Melli et al., 2006a,b). The mechanism of action is not precisely known, and thus we wish to determine if there is a differential effect based on application of the hormone to cell body or axonal side for its implications in the treatment for polyneuropathies. We used microfabrication techniques to develop a novel microfluidic platform to efficiently load and isolate neuronal cell bodies from axons with independent manipulation of the neuronal cell body and axons. Using this platform we demonstrate that chemotherapeutic drug paclitaxel is toxic at clinically relevant pharmacological doses when applied to the axons but not to neuronal cell bodies. Furthermore, we show that recombinant human erythropoietin can protect against this axonal toxicity even when it is applied to the neuronal cell body compartment.

Materials and methods

Microfabricated chamber preparation

A two-step photolithographic process was utilized to create the master mold as depicted in the schematic in Fig. 1. Silicon wafers (University Wafer, MA) were coated with SU-8 2002 (Microchem; MA), spun, and soft baked using parameters specified by the manufacturer to yield a resist thickness of 2.5 μm . An array of microchannels (Fig. 1A), each with dimensions: width = 10 μm , length = 500 μm , were defined by UV light exposure through a high resolution DPI transparency (Cad/Art, OR). The exposed substrate was once again baked, to enhance polymer cross-linking post exposure, and developed as stated in the resist technical sheet to fully define the microchannels. The process was immediately repeated with SU-8 3050 (Microchem; MA) to define the fluidic reservoirs with dimensions: width = 3 mm, length = 13 mm (Fig. 1B). The master mold was then treated with trichlorosilane (United Chemical Technologies; PA) for 30 min to create a nonstick surface for subsequent processing. Standard soft lithography was performed using Sylgard 184 polydimethylsiloxane (PDMS) (Dow Corning, MI) as described previously (Ng et al., 2003). After curing, the PDMS was carefully removed from the master and access ports were created using a suite of dermal biopsy punch tools (3–6 mm) (Huot Instruments, WI).

Cell preparation

All experiments involving animals were conducted according to protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. Unless otherwise noted tissue culture supplies were obtained from Invitrogen (Carlsbad, CA). Dorsal root ganglia (DRG) neuronal cultures were prepared as previously described (Hoke et al., 2003). Briefly, DRGs were dissected from decapitated embryonic age day 15 rats. Once obtained, cells were enzymatically dissociated with 0.25% Trypsin in L15 medium and then suspended in media. The DRGs were maintained in Neurobasal medium containing 10% fetal bovine serum, 20% glucose, 1% penicillin/streptomycin, B-27 supplement, 2 M L-glutamine, and 10 ng/ml glial derived nerve growth factor (GDNF). Two days after seeding cells, neurobasal media containing 10 μ M of Cytosine arabinoside was added to the cultures in order to decrease the amount of glial cells. Paclitaxel (Sigma-Aldrich) was dissolved in cremophor EL/ethanol (50/50 v/v) for a stock concentration of 5.0 mg/ml and stored at -20°C . Recombinant human erythropoietin (EPO) was obtained from R & D Systems (Minneapolis, MN) and dissolved in phosphate buffered saline and stored at -20°C .

DRG neurons were loaded into the soma side of devices and grown for 5–7 days to allow axons sufficient time to grow through channels and into the axonal side at a sufficient length. Paclitaxel was diluted in neurobasal media to achieve a concentration of 25 ng/mL and applied to either the neuronal cell body or axonal side. EPO was diluted in culture medium and applied to neuronal cell body or axonal side. Cells were subsequently stained with calcein AM (green) at a concentration of 2.5 μ M for 1 h and imaged using a fluorescent microscope.

Results

In order to identify the susceptibility of axons and cell bodies to paclitaxel, we added 25 ng/mL of paclitaxel to either axon or cell body chambers and continued to culture the DRGs for another 24 h. Once images of the fluorescently labeled cells and axons were captured, we used ImageJ to calculate axon lengths and calculated percent change in axon length compared to 24 h before taxol exposure. In Fig. 2, we see images of the DRGs before and after paclitaxel exposure, all taken at the same magnification. In these images, we see the axons exiting channels on the left side and going into the axonal compartment on the right. Figs. 2A and B show axons before and after paclitaxel was administered to the axonal compartment, respectively. We can see a noticeable difference in the axon length, as well as morphology. Figs. 2C and D show axons before and after paclitaxel was applied to the neuronal cell body compartment. There is not as noticeable a difference in the axon morphology or length. From these images we see that paclitaxel caused axonal degeneration when applied to the axonal compartment, but not when applied only to the cell body compartment.

Studies on the neuroprotective effect of EPO were performed with concurrent administration of paclitaxel, which was applied to either the neuronal cell body or axonal side. In order to study the effect of EPO on different cellular compartments, we also applied the hormone to either the neuronal cell body or axonal side of the chambers. Figs. 3A and B show axons

before and after administration of EPO and paclitaxel to the axonal side of the chamber, respectively. From these images we see that there is not a large difference in axon morphology or length, demonstrating the neuroprotective effect of EPO as it seems to prevent axon degeneration. This effect was also seen when EPO was applied to the neuronal cell body compartment when paclitaxel was applied to the axonal compartment. Quantification of the results can be seen in Fig. 4. Axon degeneration was defined as the change in axon length over the total axon length expressed as a percentage. When only paclitaxel was applied to the axonal side, we saw a $28.6 \pm 11.2\%$ decrease in the length of axons, compared to a control in which no paclitaxel was added that showed a $0.42 \pm 0.25\%$ increase in axon length. Paclitaxel applied in combination with EPO on the axon side showed a $1.02 \pm 0.42\%$ decrease in axon length, which was more comparable to the control than to the paclitaxel-induced degeneration condition. When paclitaxel was applied on the axon side while EPO was applied to the soma side, we saw a $1.34 \pm 0.59\%$ decrease in axon length, again demonstrating a neuroprotective effect.

Discussion

Paclitaxel-induced sensory neuropathy is a frequent and disabling side effect, and can potentially lead to the discontinuation of chemotherapy. The microfluidic platform used in this study allowed us to better clarify the mechanism for paclitaxel-induced degeneration. The device used in this study does not allow mixture of culture fluids between chambers and provide glass substrate for better optical microscopy compared to Campenot chambers and its derivatives. Furthermore, the microfluidic platform is based on the PDMS, which has excellent gas exchange properties. Recently, Taylor and colleagues developed a microfluidic chamber for the compartmentalized culture of neurons (Taylor et al., 2005). However, these chambers had difficulties in cell loading close to microchannels and low number of axons per microchannel. In contrast, the microfluidic platforms used in this study are simple to load with sufficiently high-density inside cells and corresponding higher amounts of axons microchannels. There was ample segregation of neuronal cell bodies and axons.

In a previous study, we had shown that paclitaxel can cause axonal degeneration but not neuronal death at pharmacological doses used in chemotherapy (Melli et al., 2006a,b). What we did not know at that time was whether this axon degeneration was due to local toxicity of paclitaxel in the axon or a consequence of disruption of cellular events within the neuronal cell body. This study clearly shows that paclitaxel causes axon degeneration through local mechanisms, but that this local toxicity can be controlled by intracellular events induced at the cell body as shown by the EPO data. We know that paclitaxel induces an increase in detyrosinated tubulin, thereby leading to cold-stable microtubule assembly within the axons (Melli et al., 2006a,b; Schiff and Horwitz, 1980; Shea, 1999). How this leads to axonal degeneration is still unknown, although prevailing hypothesis is that it interferes with axonal transport depriving distal axons of their vital nutrients and cellular substrates. Rapid degeneration seen in previous studies and in our culture system suggests perhaps a different mechanism. A curious observation we had in our axonal chambers was presence of axonal blebbing even in the most proximal segments of axons in paclitaxel-treated axonal chambers (data not shown). Axonal blebbing is often regarded as a prelude to axonal degeneration but it can be a reversible process (Johnson and Uhl, 2004). Local axonal activation of protein

degradation pathways, such as caspases or calpains could lead to axonal blebbing and eventual degeneration.

We used the microfluidic chamber platform to examine the site of axon protective action of EPO, expecting that local axonal application of EPO would prevent axonal degeneration induced by paclitaxel. We, however, found that EPO was able to prevent paclitaxel-axonal degeneration even when it was applied to the cell body, away from the axon and paclitaxel. Although we do not know the mechanism of this axon protection, it is possible that EPO-induced changes in intracellular signaling events are transported down the axon using fast axonal transport and block the toxicity of paclitaxel. We do not know if this type of potential mechanism of neuroprotection may apply to other axon protective therapies, but if it does, then the implications for drug development for peripheral neuropathies are immense. One of the limitations of developing therapies for nervous system indications is that axons and neurons are behind a blood–brain/nerve-barrier. This requires that the drugs be able to cross the blood–brain-barrier. However, there are exceptions to this rule and the blood–brain-barrier within the dorsal root ganglia is very leaky. If axon protection can be achieved by action of a drug on the neuronal cell body, even for toxins that cause local axonal degeneration, there would be a less stringent requirement for the drug to cross the blood–brain-barrier. Future studies will help us define if this is a general principle.

In summary, we have developed a novel microfabricated platform, composed of a microfluidic culture system, that is robust, easy to manufacture and reliable. It allows separation of axons from neuronal cell bodies and independent manipulation of each compartment. It can be used to study mechanisms of axonal degeneration, protection against axonal degeneration and developmental events such as myelination. Furthermore, the manufacturing process is scalable to generate templates with more than hundred chambers that can be independently manipulated, thus allowing high-content studies including drug screening. Through the use of this device, we have demonstrated that paclitaxel causes degeneration of axons through local mechanisms. We have also shown that this effect can be counteracted through the administration of EPO both at the cell body and at the axon, indicating exciting implications for drug development for polyneuropathies.

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Fig. 1. Microfluidic culture platform. The device mold was constructed using standard SU-8 photolithography. (A) The first resist layer ($h = 2.5 \mu\text{m}$) defined an array of microchannels while the (B) subsequent step ($h = 150 \mu\text{m}$) defined larger fluidic ports and reservoirs (not drawn to scale). (C) Silicone rubber was then poured and cured over the mold to yield the final device structure. (not drawn to scale). A picture of the final device can be seen in (D).

Fig. 2.

Paclitaxel induced local axonal degeneration. Axonal degeneration upon axonal administration of paclitaxel for 24 h is seen only with local axonal application (A = before paclitaxel; B = after paclitaxel) but not with paclitaxel application to the neuronal cell body chamber (C = before paclitaxel; D = after paclitaxel).

Fig. 3. Neuroprotective effect of EPO. A protective effect of EPO is observed 24 h after concurrent administration with paclitaxel to the axon compartment (A = before paclitaxel + EPO; B = after paclitaxel + EPO).

Fig. 4. Quantification of axon degeneration with paclitaxel administration and axon protection by EPO. Axon lengths were measured on the axonal chamber before and 24 h after application of paclitaxel with or without EPO and expressed as a percent change from baseline. Average $N = 10$ for conditions. (* denotes $p < 0.05$).