Published in final edited form as:

Pain. 2011 February ; 152(2): 308–313. doi:10.1016/j.pain.2010.10.030.

Intraepidermal nerve fiber loss corresponds to the development of Taxol-induced hyperalgesia and can be prevented by treatment with minocycline

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Abstract

Loss of intraepidermal nerve fibers (IENFs) has been speculated to play a critical role in the development of various neuropathies. In this study, the density of IENFs were studied over time during the induction of Taxol-induced chemoneuropathy and compared with the changes in IENFs in animals co-treated with Taxol plus the protective agent minocycline. Rats were injected (i.p.) with 2mg/kg of Taxol every other day for 4 injections (Days 1, 3, 5, and 7). Minocycline (25 mg/kg) was given in a separate group of rats 24 hours prior to the first dose of Taxol and every day for the next 9 days (Days 0 through 9). Animals were tested for mechanical paw withdrawal thresholds prior to any drug administrations and again on Days 7, 14, and 30. Immunohistochemistry using the pan-neuronal marker PGP9.5 was performed on glabrous skin of the hind-paw foot pad to stain for IENFs also on Days 7, 14, and 30. The results show that Taxol-treated animals developed mechanical sensitivity and corresponding IENF loss. Animals receiving minocycline plus Taxol showed no hyperalgesia or loss of IENFs. This study confirms, for the first time, that a loss of IENFs occurs as a neuropathy develops, and further shows a protection against both IENF loss and hyperalgesia with minocycline treatment.

Keywords

Intraepidermal nerve fibers; Taxol; Neuropathy; Minocycline; Skin biopsy; Chemotherapy

Paclitaxel (Taxol) is a commonly used chemotherapeutic agent that exerts antitumor effects by causing hyper-stabilization of microtubules [13], and is commonly used as a primary treatment in breast, lung, and ovarian cancers. Unfortunately, there are multiple side effects with Taxol treatment, including alopecia, emesis, diarrhea, and fatigue. While disruptive, these side effects tend to subside following completion of therapy. Conversely, the side effect of peripheral neuropathy is much more disruptive and persistent. Almost all patients

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There are no conflicts of interest to report.

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treated with Taxol will experience symptoms of neuropathy by the third treatment cycle, and up to half of all patients will discontinue therapy as a result [9,11,32].

Currently, the cause of chemoneuropathy is not well understood. However, there are several contributing physiological changes that occur following exposure to Taxol. Administration of Taxol has been found to activate microglia and astrocytes and lead to macrophage infiltration in the spinal cord [28]. Several pro-inflammatory cytokines are increased, including TNF α , IL-1 and IL-6 and IFN α and IFN γ [25,47]. Increased activity and prolonged afterdischarges in dorsal horn neurons have been recorded [4].

Taxol treatment also results in loss of nerve fibers that innervate the epidermis [37]. These intraepidermal nerve fibers (IENFs) are bare nerve endings that enter the epidermis as A δ and C fibers cross the dermal/epidermal junction and shed their myelin ensheathment that was present within the dermis. They are particularly important in transmitting noxious mechanical and thermal information [24], and loss of IENFs is seen in a host of chronic painful neuropathic conditions, including diabetes [15,36], complex regional pain syndrome [27], and post-herpetic neuralgia [30]. While there is much speculation that IENF loss is related to functional impairments, there is a paucity of research investigating this hypothesis. Such studies have found IENF loss is most pronounced in areas specific to pain [26,30,34], and decreased IENF density correlates with alterations to warmth detection [36] and pinprick sensitivity [43]. Theories have been proposed to link decreased IENFs to the generation of chronic pain [26]; however, no studies have looked in parallel at the development of pain and IENF changes.

The present study sought to determine whether loss of intraepidermal nerve fibers coincided with the development of peripheral neuropathy following Taxol treatment, and to further determine if improving neuropathy could result from salvaging IENFs. The development of Taxol-induced mechanical hyperalgesia was assessed, as were changes in IENF density, with the expectation that mechanical hyperalgesia would progress as IENF density decreased. To address whether prevention of neuropathy might be associated with preservation of IENFs, minocycline, a tetracycline derived antibiotic with known immunomodulatory effects and that has been successfully used to prevent Taxol-induced hyperalgesia [5], was tested as a neuroprotective agent.

Methods

Subjects

Thirty-three male Sprague Dawley rats (Harlan) were used in this study. Animals arrived from the supplier at approximately 55 days of age and were allowed to acclimate for approximately 7 days prior to any experimental manipulation. At the beginning of the study, animals were randomly assigned to one of three groups: minocycline/Taxol (n=12), vehicle/Taxol (n=11), and vehicle/vehicle (n=10). Animals were maintained on a 12 hour light/dark cycle with free access to food and water. Animals were weighed daily during treatment just after behavioral testing thereafter. All procedures were approved by the Institutional Animal Care and Use Committee for the University of Texas MD Anderson Cancer Center and adhered to the guidelines set forth by the National Institutes of Health Guidelines for the Use and Care of Laboratory Animals and by the Committee for Research and Ethical Issues of the International Association for the Study of Pain [48].

Drugs

Taxol (Bristol Myers Squibb) was diluted with saline from the supplied 6mg/ml concentration to 1mg/ml and given at a dosage of 2mg/kg (i.p.) every other day for a total of four injections (Days 1, 3, 5, and 7). Control animals received an equivalent volume of

vehicle, which consisted of equal amounts of Cremophor EL and alcohol diluted with saline to reach a concentration of vehicle similar to Taxol treated animals. Minocycline hydrochloride (Sigma) was diluted in saline and buffered using NaOH to a pH of approximately 7.0. The final concentration of minocycline was 15mg/ml, which was given at a dose of 25mg/kg, starting 24 hours prior to the first dose of Taxol. Minocycline was continued every day for the next nine days, for a total of 10 injections. Control animals received saline in similar volumes. On days when both drugs were to be administered (Days 1, 3, 5, and 7) minocycline was given 30 minutes prior to Taxol.

Mechanical paw withdrawal threshold testing

To assess responding to mechanical stimulation, an ascending set of von Frey monofilaments (1, 4, 10, 15, 26, and 60 grams) were applied to the mid-plantar surface of the hind paw. Animals were first placed individually inside Plexiglas containers (10×10×4in) which were set upon an elevated wire mesh stand. After a 15 minute habituation time period, the testing session began by applying the lowest force monofilament (1 gram) to the left and then right paw for approximately 1 second. This was repeated 6 times for each paw. If the animal did not respond at least 3 out of the 6 administrations, the next highest monofilament was administered. The monofilament at which the animal made a response of paw withdrawal, flinching, or licking 3 out of the 6 applications was determined to be the 50% withdrawal threshold. Environmental factors such as noise level, lighting conditions, time of day testing occurred, and experimenter performing the testing were held constant throughout the experiment. Testing was always performed prior to drug administration on days when drugs were injected.

Immunohistochemistry

On Day 7, Day 14, and Day 30 animals were overdosed with pentobarbital and a 3mm biopsy was taken from the foot pad of the right hind paw. These biopsies were immediately placed in Zamboni's fixative, where they were left overnight at 4°C. Tissue was then transferred to 20% sucrose for at least 24 hours and then frozen in Optimal Cutting Temperature compound (OCT) and sliced into 25um sections using a microtome (Leica Microsystems, CM 1850). Following a 1 hour incubation in blocking solution (.1M PBS containing 5% NDS/.3% Triton X-100) at room temperature, liquid was decanted and free floating slices were incubated overnight at 4°C with the primary antibodies: PGP9.5 (AbD Serotec; rabbit; 1:1000) and collagen IV (Southern Biotech; goat; 1:25) in wash buffer (1% NDS/.3% Triton X 100 in 0.1 M PBS). PGP9.5 reliably immunostains intraepidermal nerve fibers, while collagen IV stains the basement membrane between the dermal/epidermal junction. The next day, primary antibody was decanted and slices were washed 3 times for one hour each in wash buffer. Wash buffer was removed and secondary antibodies (Jackson ImmunoResearch) were added for an overnight incubation at 4°C: donkey anti-rabbit Cy3 (1:400) and donkey anti-goat Cy2 (1:200). On the final day, tissue was washed in wash buffer 3 times for one hour each and then mounted onto slides.

Quantification of intraepidermal nerve fibers

Quantification was done using a Nikon Eclipse E600 fluorescence microscope. Five slices from each animal were randomly chosen for quantification. Nerve fibers that crossed the collagen stained dermal/epidermal junction into the epidermis were counted in three fields of view from each slice using a 40x objective. The length of the epidermis within each field of view was measured using Nikon NIS Elements software. IENF density was determined as the total number of fibers per unit length of epidermis (IENF/mm) [21]. Fibers that branched after crossing the basement membrane were counted as a single fiber. The experimenter making these counts was blind to all conditions.

Data analysis

Data for mechanical paw withdrawal threshold values and IENF counts were analyzed using separate repeated measures analysis of variance (ANOVA) tests. Significant effects were further examined using the Tukey HSD test for post hoc comparisons. The significance level was set at $p < .05$ for all tests.

Results

Mechanical Paw Withdrawal Threshold

As can be seen in Figure 1, Taxol treated animals (vehicle/Taxol) developed a significant decrease in mechanical thresholds by Day 14 when compared to vehicle treated rats (vehicle/vehicle). This decrease in threshold was also present at Day 30. On Day 7 there was a trend for Taxol animals (vehicle/Taxol) to have lower thresholds than vehicle animals, but this trend was not significant ($p = .06$). Rats that were treated with both Taxol and minocycline were not significantly different from vehicle animals at any time point, indicating that minocycline treatment prevented the development of Taxol-induced hypersensitivity to mechanical stimulation.

IENF changes

Figure 2 contains representative samples from each of the groups on the three testing days. Vehicle treated animals (vehicle/vehicle) (Figure 2a, d, and g) had brightly stained fibers that were evenly distributed throughout the sample and extended from the dermal/epidermal border and well into the epidermis. This was also found in Taxol treated animals that were co-administered minocycline (Figure 2c, f, and i). Taxol treated animals began to show decreased density on Day 7 (Figure 2b), and by Days 14 (Figure 2e) and 30 (Figure 2h) there were notably fewer fibers crossing the dermal/epidermal junction compared to vehicle/vehicle controls. In addition, fibers that were present tended to be sparsely distributed throughout the sample and showed a spindly low-intensity appearance.

The average total length of epidermis that was measured for each slice of skin was 2.5mm. This did not significantly vary across groups. On Day 7, there was a trend for Taxol treated animals (vehicle/Taxol) to have fewer IENFs when compared to vehicle animals (vehicle/vehicle); however, this trend was not significant (Figure 3). By Day 14, Taxol treated rats had significantly less IENFs ($p = .03$). On the contrary, fiber counts in Taxol treated animals that were co-treated with minocycline were not different from vehicle treated rats ($p = .61$), indicating a protective effect for IENFs with minocycline treatment. These same findings were again detected on Day 30, with Taxol animals having fewer IENFs than vehicle animals ($p = .01$) and minocycline/Taxol rats not being significantly different from vehicle rats ($p = .35$).

Discussion

Painful peripheral neuropathy is a generally chronic condition that negatively impacts both quality of life and treatment outcomes for many patients, yet the cause is still not clearly elucidated. The results of this study contribute to the current understanding of neuropathy, and importantly demonstrate effective prevention of neuropathy symptoms. Taxol produced an exaggerated response to mechanical stimuli in the context of an ongoing loss of nerve fibers innervating the epidermis. Further, both mechanical hyperalgesia and IENF loss were prevented by treatment with minocycline.

Almost all patients will experience some sensory disturbances or pain following Taxol treatment [32], and many researchers have found mechanical hyperalgesia in rats following

Taxol administration [1,3,5,37]. Collectively, the findings from humans and animals given chemotherapy have implicated changes at multiple neuraxial levels. In the spinal cord, chemotherapy treatments evoke the release of several inflammatory chemical mediators, including various pro-inflammatory cytokines and chemokines [16–18,31,45]. This release may underlie the observed activation of central glial cells [28,39] and the expression of glial glutamate transporters GLAST and GLT-1 that are downregulated with chemotherapy treatments [44]. Down-regulation of glutamate transporters would be expected to result in excess synaptic levels of glutamate and consequently result in excess afterdischarges to synaptic inputs [4].

Given that Taxol often poorly penetrates to the CNS [6], many of the central changes may be driven by those observed in the periphery. Within the DRG, substance P is released [22], and there are increased levels of ATF3 and increased macrophage infiltration [28]. There are also changes within peripheral nerve endings that are strongly implicated in chemoneuropathy. Siau et al [37] recently reported a loss of IENFs in animals treated with Taxol on day 30 of the experiment. This effect is confirmed and further detailed here. While there was a trend for IENF loss by Day 7, no significant loss was detected until Day 14. This loss was still present at Day 30. Interestingly, this time course corresponded to the development of hyperalgesia, with a non-significant trend of mechanical hypersensitivity at Day 7 and a robust hyperalgesia at Day 14 and Day 30.

The reason for IENF loss is not understood, but cytokines, acting as pro-inflammatory mediators, may play a key role. *In vitro* exposure to Taxol produces increased levels of multiple cytokines, including TNF α , IL-1 and IL-6, and IFN α [25] [47]. These cytokines are strongly implicated in pain and in damage to neurons and supporting cells. For example, TNF α can inhibit proliferation in Schwann cells [41]. Further, Taxol treatment produces increased ATF3 and hypertrophy in Schwann cells [28]. More recently Taxol was shown to lead to mitochondrial dysfunction [7,8,12], which could also be explained by increases in pro-inflammatory cytokines. For example, IL-1 β produces nitric oxide-induced decreases in mitochondrial function [42] and TNF can cause mitochondria to drastically increase reactive oxygen species (ROS) production, leading to cytotoxicity [10]. The findings reported here with minocycline further support a cytokine hypothesis. Minocycline is a tetracycline derivative that has been shown to inhibit the NF- κ B pathway [23] and subsequent cytokine release [19,20,40], and it can protect against cytokine-related damage to Schwann cells [14]. Minocycline has also been used to ameliorate both surgically-induced [2] and Taxol-induced [5] neuropathy, and minocycline treatment decreased hyperalgesia and pro-inflammatory cytokine release induced by L5 spinal nerve transection [33]. Therefore, the finding that minocycline protects nerve fibers against Taxol-induced loss and prevents Taxol-related mechanical hyperalgesia is not only supported by previous work, but can also be explained by a cytokine hypothesis.

Much work has been done to characterize the mechanism of action of minocycline as a neuroprotective agent. It has both anti-inflammatory and anti-apoptotic actions, and is useful for ameliorating the neurotoxic effects of stroke and spinal cord injury [46]. As mentioned above, many of these effects can be attributed to the moderating effect of minocycline on cytokine release. Further, these effects may be occurring at various levels of the nervous system. Within the brain and spinal cord, minocycline inhibits glial cells [35], known to be major sources of cytokines, and Yong et al [46] proposed that T cell activation, and subsequent cytokine release, is also suppressed in the periphery following minocycline treatment.

Although it cannot be unequivocally determined from these results that IENF loss is the underlying cause of the mechanical hyperalgesia, these data would indicate this is indeed a

possibility. At Day 7, a trend was seen for both IENF counts and mechanical thresholds that indicated worsening of both measures. This trend was significant by Day 14 for both IENF density and mechanical hyperalgesia. Further, treatment with both Taxol and minocycline resulted in protection against IENF loss. Importantly, these animals did not have altered mechanical thresholds. Therefore, it seems that, as IENFs were lost, mechanical sensitivity developed and preventing IENF loss averted the development of hyperalgesia.

The loss of IENFs is not specific to chemoneuropathy, but instead is seen in multiple populations that have neuropathy, and in multiple studies, the loss of IENFs corresponds to pain. In patients with post-herpetic neuralgia, IENF loss is greatest in the area of pain [29] and is most pronounced in patients with more severe pain [26]. Loss of IENFs is also observed in patients with diabetic neuropathy [15,36] and complex regional pain syndrome [27]. While the results of various studies support the findings presented here that IENF loss is frequently associated with neuropathic pain, there are caveats that should be addressed. Some researchers have failed to find a clear association between IENF loss and pain. Sorensen [38] reported IENF loss in patients with diabetes, but surprisingly, this loss was most severe for patients that did *not* have measurable signs of neuropathy. Further, IENF loss was observed in patients with diabetes but without pain. The authors argue that IENF loss does not explicate neuropathic pain; however, it is possible that patients with pronounced IENF loss but little pain or sensory disturbance at the time of the experiment would later develop these symptoms. This explanation would not preclude the hypothesis that IENF loss mediates neuropathic pain. Petersen [30] reported a relationship between IENF loss and changes in thermal perception in patients with post-herpetic neuralgia, but found that pain and aberrant thermal perception resolved before IENFs reinnervated the skin. While the findings of Sorensen can be explained, those of Petersen are more difficult to interpret. Petersen et al point out that DRG cells are frequently abolished by herpes zoster, a finding that is not observed following chemotherapy. It may be then that there are differing mechanisms between IENF loss following chemotherapy and fiber depletion following herpes zoster. This could alter the genesis and progression of pain.

In conclusion, this study is the first to investigate the coinciding development of IENF loss and hyperalgesia. The loss of intraepidermal nerve fibers as a result of Taxol treatment coincided with the development of mechanical hyperalgesia, and treatment with minocycline effectively prevented both mechanical hyperalgesia and IENF loss. These results have interesting applications for better understanding the mechanisms of Taxol-related peripheral neuropathy and the relationship between neuropathy-related pain and loss of epidermal nerve fibers.

Acknowledgments

This work was supported by National Institute of Health grant NS46606 and National Cancer Institute grant CA124787 and by the Astra-Zeneca Corporation.

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Figure 1.

Taxol treated animals had significantly lower mechanical threshold in a test of paw withdrawal, a finding that was prevented by treatment with minocycline. Animals treated with Taxol (vehicle/Taxol; n=11) had a significant decrease in threshold that began on Day 14 when compared to vehicle treated animals (vehicle/vehicle; n=10). This effect was also found on Day 30. Rats receiving both minocycline and Taxol (mino/Taxol; n=12) were not significantly different from vehicle treated animals at any time point, indicating a protective effect of minocycline. (**:p<.001)

Figure 2.

Immunohistochemistry results showing staining of intraepidermal nerve fibers by PGP9.5 (red). Nerve fibers can clearly be seen crossing the collagen-stained basement membrane (green), and extending into the epidermis. On Day 7, vehicle/Taxol treated rats (b) had fewer, although not significantly so, IENFs compared to both vehicle/vehicle (a) and minocycline/Taxol (c) treated animals. By Day 14, there was a significant decrease in the number of fibers in vehicle/Taxol treated animals (e) compared to vehicle/vehicle (d) and minocycline/Taxol (f) treated rats. This was again seen on Day 30 (h-i).

Figure 3.

Taxol treatment (vehicle/Taxol) results in decreased intraepidermal nerve fibers within the footpad of the hind paw on Day 14 and Day 30, but not on Day 7. Rats receiving Taxol with minocycline pretreatment (mino/Taxol) were not significantly different from vehicle treated animals (vehicle/vehicle) in regards to IENF counts, indicating a protective effect of minocycline against Taxol-induced nerve fiber loss. (*:p=.05; **:p=.01)