Selective blockade of the OGF–OGFr pathway by naltrexone accelerates fibroblast proliferation and wound healing

Jessica A Immonen, Ian S Zagon and Patricia J McLaughlin

Department of Neural & Behavioral Sciences, The Pennsylvania State University College of Medicine, Hershey, PY 17033, USA
Corresponding author: Patricia J McLaughlin. Email: pxm9@psu.edu

Abstract

Naltrexone (NTX) is an opioid receptor antagonist that acts at classical and non-classical opioid receptors including the opioid growth factor receptor (OGFr). Animal models of type 1 and type 2 diabetes, as well as normal rodents, have shown that topical NTX enhances the healing rates of corneal epithelium and full-thickness cutaneous wounds. The mechanism of this general opioid antagonist on growth, and in particular the specific receptor pathway involved, is not understood. Tissue culture studies using NIH 3T3 fibroblasts and primary rat auricular fibroblasts were established to evaluate growth following opioid receptor antagonist treatment. Treatment of cells with CTOP, naltrindole, or nalmefene, selective antagonists for mu, delta, and kappa opioid receptors, respectively, did not accelerate cell replication. Addition of the classical opioid receptor peptides DAMGO, DPDPE, or EKC did not alter cell growth, suggesting that the classical opioid receptors were not involved in cutaneous wound healing. However, NTX (10^{-6} M) increased the growth of NIH 3T3 fibroblasts in culture over a 96-h period, and the specific ligand OGF decreased cell growth, supporting that the OGF-OGFr axis is tonically active and constitutively expressed in fibroblasts, the primary cell type in granulation tissue of the skin. Transfection of NIH 3T3 cells with OGFr siRNA reduced receptor protein; subsequent treatment with NTX did not accelerate cell proliferation. These data indicate that blockade of the OGFr pathway enhances proliferation of fibroblasts *in vitro*, and in a primary culture of auricular fibroblasts, suggesting that the effect of NTX on growth is mediated through the OGF-OGFr axis. Finally, antagonists for classical opioid receptors as well as NTX were topically applied to cutaneous wounds in type 1 diabetic rats; only NTX accelerated wound closure. These studies indicate that the mechanistic pathway underlying the effects of NTX to enhance cutaneous wound closure in diabetic and nondiabetic subjects is specific blockade of the OGF–OGFr regulatory axis.

Keywords: Diabetes, cutaneous wound closure, naltrexone

***Experimental Biology and Medicine* 2014; 239: 1300–1309. DOI: 10.1177/1535370214543061**

Introduction

Diabetes mellitus affects more than 26 million individuals in the USA[1] and 382 million persons worldwide.[2] Type 1 and type 2 diabetes are accompanied by complications related to delayed epithelialization during wound healing, leading to infection, ulceration, and even amputation.[1,2] Delayed repair of foot ulcers is a leading cause of hospital admissions for patients with diabetes in the developing world, with up to 15% of the diabetic population experiencing delayed wound closure.[3]

Topical administration of naltrexone (NTX) has been studied in animal models with type 1 and type 2 diabetes for treatment of full-thickness cutaneous wound closure, as well as re-epithelialization of the corneal surface.[4–7] NTX in Vigamox accelerates corneal epithelialization.[8–11] NTX dissolved in moisturizing cream enhances cutaneous wound closure[4] and accelerates proliferation of endothelial and smooth muscle cells related to angiogenesis.[5] Collagen deposition and organization of wounds are promoted in rats with type 1 diabetes treated with NTX,[6] and the primary cell type involved in collagen formation is the fibroblast. Fibroblasts are an integral cell type involved in cutaneous wound healing and migrate into the wound bed to deposit new extracellular matrix.[12] In the diabetic animal, complications associated with fibroblast proliferation and collagen deposition are observed.[4–7] Alterations include decreases or impairments in growth factor production, collagen accumulation, and fibroblast migration and proliferation along with modifications in the balance between extracellular matrix accumulation and matrix remodeling by matrix metalloproteinases (MMPs).[13–17]

NTX is a non-specific opioid receptor antagonist capable of binding to classical and non-classical opioid

receptors.[18,19] NTX lacks intrinsic opiate activity but is capable of blocking the interactions of opioid peptides and receptors.[20] and was originally discovered as a treatment of opiate addiction.[19] Systemic and topical exposure to NTX, administered in a high dose, produces a continuous blockade of opioid receptors that accelerate DNA synthesis, cell proliferation, and in the case of diabetic animal models, wound healing.[21–24] One of the peptides is opioid growth factor (OGF), which interacts with the nuclear-associated receptor, OGFr. OGF is a pentapeptide (MW∼574; Tyr-Gly-Gly-Phe-Met), chemically termed [Met^5]-enkephalin that has growth inhibitory action mediated by cyclin-dependent inhibitory kinase pathways. OGF is not tissue specific and has been identified in normal and neoplastic tissues, and depresses cell replication in homeostasis, wound closure, cancer, and normal development.[4,8]

Classical opioid receptors are present in skin[25–27] and function in peripheral nerve fibers innervating skin, melanocytes, and hair follicles. Activation of pain receptors is relevant in the treatment of psoriasis[28] and pruritus,[29] and interactions between cutaneous stimulation of immune cells and pain receptors are important for management (i.e. differentiation and cell death) of inflamed and/or damaged skin.[30] Oral epithelial cells are stimulated to migrate following morphine binding to delta opioid receptors.[31] The specific receptor pathway targeted by NTX for advancing wound healing requires characterization. Utilizing tissue culture models of fibroblasts, as well as a cutaneous wound model in type 1 diabetic rats, the present study determined that the opioid receptor blocked by NTX to enhance proliferation rat model of type 1 diabetic rat is OGFr.

Materials and methods

Cell culture

NIH 3T3 fibroblast cells, originally derived from the Swiss mouse and spontaneously immortalized, were utilized. NIH 3T3 fibroblasts were grown in a humidified atmosphere of 5% CO_2/95% air at 37°C in Dulbecco's modified Eagle's medium (DMEM). Media was supplemented with 10% fetal calf serum, 1.2% sodium bicarbonate, and antibiotics (5000 units/mL penicillin, 5 μg/mL streptomycin, and 10 mg/mL neomycin).

Primary rat fibroblasts were isolated from auricular cartilage of 8- to 12-week-old male Sprague-Dawley rats. Animals were euthanized using intraperitoneal (i.p.) injections of Euthasol®. The ears were swabbed with ethanol and antiseptic prior to dissection, placed in 70% ethanol for 3 min, and rinsed in sterile DMEM. Using sterile single-edged razor blades, the ears were minced and tissue trypsinized (2 mL of 0.25% trypsin) at 37°C for 1 h, with vortexing every 10 min. Tissue was centrifuged, resuspended in fresh DMEM, and plated; media was replaced after 48 h. Primary fibroblasts were grown in a humidified atmosphere of 5% CO_2/95% air at 37°C, and passaged eight times before new cultures were established.

Immunocytochemistry

To confirm the purity of fibroblast cultures, cells grown on 22 × 22 mm cover slips were stained with vimentin, an intermediate filament specific to fibroblasts, and counterstained with DAPI.

The presence and distribution of OGF and OGFr in NIH 3T3 fibroblast cells and primary auricular fibroblast cells were assessed by semiquantitative immunocytochemistry.[32] Log-phase cells were fixed in 95% ethanol, and stained with anti-OGF (CO-172) and anti-OGFr (Santa Cruz, Dallas, TX, USA) polyclonal antibodies followed by staining with rhodamine-conjugated secondary antibodies. The OGF antibody was generated and characterized by our laboratory.[33] Cultures incubated with secondary antibody only served as controls. All cells were examined for semiquantitative imaging using an Olympus IX-81 epifluorescence microscope.

Cell growth

To establish the growth characteristics of fibroblast cells, NIH 3T3 cells were utilized. Cells were seeded in 6- or 24-well plates and counted after 24 h (time 0); triplicate wells were established for each treatment. All compounds were prepared in sterile water and dilutions represent final concentrations; control wells received sterile water. Unless otherwise stated, media and compounds were replaced daily. At designated times, cells were harvested by 0.25% trypsin-EDTA and counted with a hemacytometer using the trypan blue exclusion methodology. Two aliquots per well and two or three wells per treatment were counted at each time point. At least two independent experiments were conducted for each assay.

Naltrexone selectivity of opioid receptors

To determine the receptor pathway utilized by NTX to enhance cell replication, NIH 3T3 cells were subjected to a variety of concentrations of CTOP, naltrindole, or nalmefene, specific for μ, δ, or κ classic opioid receptors, respectively. NTX was added as a general antagonist. Cell number was monitored after 72 h or 96 h of growth in the presence of antagonists at concentrations of 10^{-4} M to 10^{-10} M, or an equivalent volume of sterile water. Binding affinities (K_1) for all of the antagonists to their selected receptor were comparable.[34,35] For growth curves, cultures were treated with NTX (10^{-6} M), OGF (10^{-6} M), or an equivalent volume of sterile water at 24, 48, 72, and 96 h.

Specificity of OGFr

To confirm that the specific receptor associated with increased cell growth was OGFr, siRNA technology was utilized to knockdown OGFr and measure cell number in the presence of agonists and antagonists. NIH 3T3 fibroblast cells were transfected for 48 h with 20 nmol/L concentrations of OGFr siRNA or scrambled siRNA (Ambion, Austin, TX, USA) using Oligofectamine reagent (Invitrogen, Carlsbad, CA, USA).[32,36] In addition, untransfected and mock treatment groups were prepared. Mock transfection wells received only Oligofectamine, whereas

untransfected wells were treated with equivalent volumes of sterile water. siRNAs were administered to wells at 24 h and 48 h after seeding. Following transfection, cells were treated for 72 h with 10^{-6} M concentration of the general opioid antagonist NTX, or [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO), [D-Pen^{2,5}]-enkephalin (DPDPE), or ethylketocyclazocine, agonists specific for μ, δ, and κ opioid receptors, respectively. In addition, OGF was tested as the specific agonist for OGFr.[36] Cells were harvested for Western blot analysis of protein expression or growth assays. The level of protein knockdown resulting from OGFr siRNA transfection following treatment with NTX and classical opioid receptor agonists DAMGO, DPDPE, or EKC was determined by Western blotting following published procedure.[32,36]

In vitro function

Scratch wound assays

To assess function *in vitro*, scratch wound assays were established to determine whether any specific opioid receptor antagonist or the general antagonist NTX altered migration of NIH 3T3 fibroblasts. Cells were seeded (70,000/well) in 24-well plates and maintained for four days until confluent. Uniform scratches to remove cells were made using a sterile plastic 10 μL pipette tip.[37] Cultures (in triplicate) were exposed to 10^{-6} M concentrations of NTX, CTOP, nalmefene, or naltrindole, or an equal volume of sterile water. Phase contrast microscopy (10×) was used to visualize the scratched pathway at 3, 6, 16, and 20 h. For each treatment group, three images were taken of each scratch from three wells per treatment. The width of the scratch was measured using Image-Pro Plus 6.2 software. All analysis was completed prior to 24 h to ensure cell proliferation was not contributing to scratch closure.

Cell death analysis

To evaluate whether selective antagonists altered cell number by inducing or inhibiting cell death, a trypan blue exclusion test was conducted at 24, 48, and 72 h. Cell death was assessed on attached cells, as well as the media. Supernatants from individual wells were collected, centrifuged, and pelleted cells resuspended in trypan blue stain and counted. Two aliquots per well (four samples/aliquot) were counted; viable cells exclude the stain and necrotic cells are stained blue.

Selective receptor studies: *in vivo* wound closure in diabetes

Information obtained from the *in vitro* assays was confirmed by animal studies utilizing a full-thickness cutaneous wound model of type 1 diabetes.[4–6]

Animals and induction of diabetes

To induce type 1 diabetes, six-week old Sprague-Dawley rats (~150 g; Charles River Laboratories, Wilmington, MA, USA) received intraperitoneal injections of 40 mg/kg streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO, USA) or citrate buffer for two consecutive days.[9,10] Rats receiving citrate buffer were considered normal (Normal). Blood glucose levels were monitored from the dorsal tail vein using a True Track Smart System glucometer (Home Diagnostics, Ft. Lauderdale, FL, USA) and measured prior to induction of hyperglycemia by STZ and at one, four, and eight weeks after STZ injection. STZ-injected rats had blood glucose levels of >350 mg/dL within four days of STZ injection. If an animal had sustained blood glucose measurements of 600 mg/dL or greater, and appeared lethargic and unable to eat, it was not included in the study. All rats were housed at the Central Animal Quarters of the Penn State Hershey College of Medicine in an environmentally controlled animal room, and provided with water and food (2018 Global Rodent Diet, Teklad®, Indianapolis, IN, USA) *ad-libitum*. All surgical procedures followed an approved protocol and the guidelines of The Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

Cutaneous wound surgery

Full thickness cutaneous wounds were created as described previously.[5,6] In brief, rats were anesthetized using a mixture of ketamine (60 mg/kg), xylazine (10 mg/kg), and acepromazine (1 mg/kg). Under sterile conditions, four excisional circular (6 mm diameter) skin wounds were created on the dorsum (two on each side of the midline) to the level of the panniculus muscle. Wounds and surrounding tissue were treated with antiseptic surgical scrub and left uncovered.

Photographs of wound closure were taken immediately after surgery and on days 2, 4, 6, 8, 10, 12, 14, and 16.[5] A metric ruler was photographed with the wound to determine magnification. Areas of residual wound were monitored by capturing images with a digital camera mounted on a tripod located ~15 cm from the surface. For photography, rats were lightly sedated by isoflurane regulated with a mixture of oxygen. Areal analyses of the images were performed using Image ProPlus 6.2 (Media Cybernetics, Inc., Bethesda, MD, USA), and the percent residual wound was calculated at each time point for every rat.

Treatment with opioid receptor antagonists

Opioid antagonists dissolved in water were mixed in moisturizing cream to a final concentration of 10^{-5} M; sterile saline dissolved in moisturizing cream served as control. NTX, CTOP, nalmefene, and naltrindole, specific antagonists for OGFr, μ, κ, and δ opioid receptors, respectively, were topically applied three times daily (0800, 1300, and 1800) for 20 days to the wounded area. Placement of treatments was randomized across all four wounds. Three wounds per rat were treated with the same antagonist and one wound received control cream; six to eight wounds within each cohort of type 1 diabetic ($N = 9$) and normal rats ($N = 6$) were established for each treatment.

Statistical analysis

All data were analyzed using GraphPad Prism software (GraphPad Software, Inc.). Data were subjected to one-way analysis of variance (ANOVA), with Newman–Keuls *post hoc* tests for subsequent comparisons.

Results

Antagonists for classical opioid receptors do not stimulate fibroblast proliferation

Immunocytochemistry staining for vimentin showed that >99% of cultured NIH 3T3 cells were fibroblasts (Figure 1a). A seven-fold dosage range of selective antagonists for classical opioid receptors revealed that CTOP (Figure 1b), nalmefene (Figure 1c), and naltrindole (Figure 1d) did not accelerate cell number. In fact, the highest dosages (10^{-4}–10^{5} M) of the kappa opioid receptor antagonist nalmefene resulted in depressed cell number. Naltrindole, the selective antagonist for delta opioid receptors, inhibited cell number at 10^{-4} M to 10^{-6} M range. The reduction in cell number in cultures treated with nalmefene or naltrindole was related to cell death. Addition of 10^{-7} M to 10^{-4} M NTX to cell cultures resulted in 18% to 35% more cells after 72 h of treatment relative to sterile water-treated controls.

Agonists selective for classical opioid receptors do not alter growth

Fibroblast cultures treated with DAMGO, EKC, or DPDPE, selective agonists for the μ, κ, and δ classical opioid receptors, respectively, were not altered in growth (Figure 2). Exposure to OGF, a specific agonist for the OGFr inhibited growth inhibited growth by nearly 24%.

Silencing of OGFr blocks the stimulatory action of NTX

The requirement of OGFr for NTX's stimulatory action was evaluated using a siRNA knockdown technique (Figure 3). The addition of NTX to NIH 3T3 cultures treated with mock siRNA, scrambled siRNA (Figure 3a), or an equivalent volume of sterile water resulted in 20–34% increase in cell number at 72 h. Western blot analyses of cultures transfected with OGFr siRNA indicated that OGFr protein was reduced approximately 38%. Addition of NTX to fibroblast cells with decreased expression of OGFr did not alter growth.

Exposure of mock-transfected cultures to selective agonists DAMGO, DPDPE, EKC, or OGF revealed that in the presence of intact receptors, OGF inhibited growth (Figure 3b), but knockdown of OGFr nullified the inhibitory effect of OGF (Figure 3c).

The OGF-OGFr axis is present and functional in NIH 3T3 fibroblasts

OGF and OGFr are present in NIH 3T3 fibroblasts (Figure 4a). OGFr immunostaining was located in the perinuclear area, whereas OGF immunolabeling was diffuse throughout the cytoplasm.

To determine the effects of OGF on growth of fibroblast cells, cell number was calculated after 96 h of exposure to 10^{-4} M to 10^{-10} M OGF. Concentrations of 10^{-4} M to 10^{-6} M OGF reduced cell number by 32–34% relative to cultures treated with sterile water (Figure 4b). Cell growth over a period of four days was recorded following treatment with 10^{-6} M OGF or NTX. OGF inhibited cell number at 72 and 96 h by 14 and 21%, respectively. Exposure to NTX significantly increased cell proliferation by approximately 26–34% compared to cultures treated with sterile water over the 96-h period.

NIH 3T3 fibroblast doubling times were calculated from growth curves. Cells treated with NTX had a doubling time of approximately 22 h in comparison to 27 h for control cultures; OGF exposure extend the doubling time to approximately 30 h.

Cell migration and cell death

Closure rates of scratch wounds created in confluent fibroblast cultures revealed no significant differences between residual widths of scratches at 20 h in cultures treated with specific antagonists for classical opioid receptors, the general opioid antagonist NTX, or the selective agonist for OGFr, OGF.

At 24, 48, and 72 h, there were no differences in cell death of attached fibroblasts by the trypan blue exclusion test with treatment of any compound. However, analysis of cells in the supernatant 48 and 72 h showed substantial cell death following 10^{-4} M, 10^{-5} M and 10^{-6} M concentrations of naltrindole. At 72 h of treatment, the range of dead cells floating in the supernatant was 2.5×10^3 to 17.5×10^3 for fibroblasts treated with 10^{-6} M to 10^{-4} M naltrindole. There were no notable dead cells in the supernatant of fibroblasts treated with nalmefene.

The OGF-OGFr axis is present and functional in primary rat fibroblasts

Primary rat fibroblast cultures were stained with vimentin to determine that cultures were approximately 99% pure. Immunostaining with antibodies to OGF and OGFr revealed that rat fibroblasts expressed peptide and receptor. OGFr presented diffusely throughout the cytoplasm. OGF was heavily present in the perinuclear area and diffusely present throughout the remainder of the cytoplasm (Figure 5a).

Exposure to OGF at concentrations of 10^{-4} M–10^{-7} M decreased fibroblast cell number by 27–37% relative to controls (Figure 5b). NTX treatment accelerated proliferation of primary fibroblasts in a dose-related manner, with cell number increasing approximately 33–39% relative to controls (Figure 5c). Within 48 h of treatment, primary rat fibroblast growth was increased 17% by 10^{-6} M NTX and decreased 30% by 10^{-6} M OGF in comparison to controls (Figure 5d). At 72 and 96 h, NTX increased cell proliferation by approximately 12% at each time point, and OGF decreased growth of fibroblasts between 24 and 40% relative to sterile water-treated control cultures. Primary fibroblast cells doubled within 32 h, whereas NTX treatment reduced the doubling time to 28 h and OGF required 38 h for doubling the number of cells (Figure 5d).

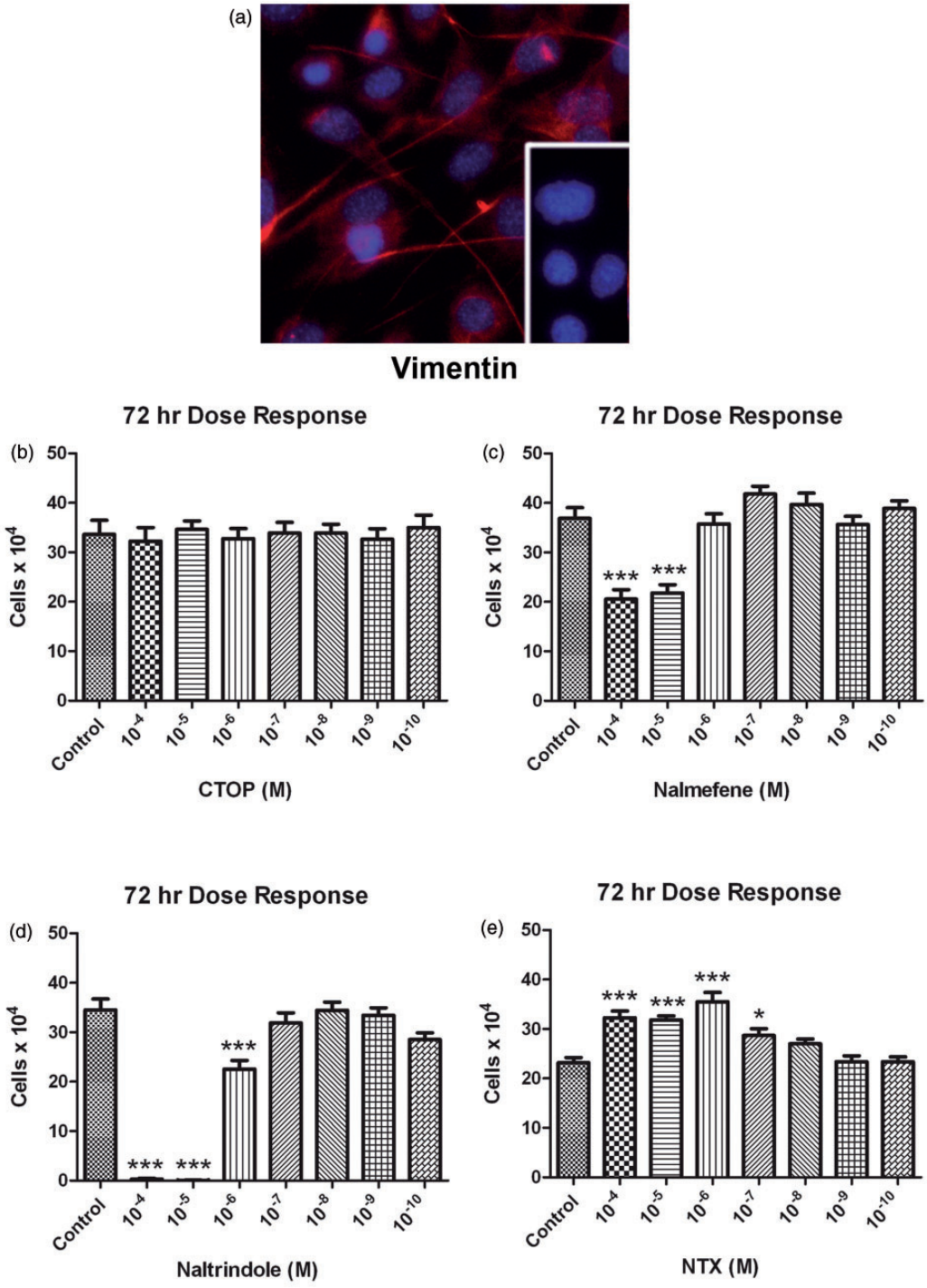

Figure 1 (a) Photomicrograph of NIH 3T3 fibroblast cells stained for vimentin, and counter-stained with DAPI to demonstrate purity of cultures. Inset: NIH 3T3 cells stained with secondary antibody only and DAPI. Bar = 5 μm. (b–e) Cell number of NIH 3T3 fibroblasts grown for 72 h in the presence of various concentrations of CTOP (b), nalmefene (c), or naltrindole (d), specific antagonists for μ, κ, and δ classical opioid receptors, respectively, as well as naltrexone (e), a general opioid antagonist. Control cells were treated with sterile water; compounds and media were replaced daily. Data represent means ± SEM for at least two aliquots/well and three wells/treatment group. *$P < 0.05$ or ***$P < 0.001$ significantly different from sterile water-treated controls. (A color version of this figure is available in the online journal)

Full-thickness wound closure was accelerated only with NTX

Diabetic rats had residual wounds that were larger than those recorded in non-diabetic rats throughout the observation period. Residual wound areas in diabetic rats topically treated with nalmefene, naltrindole, CTOP or sterile water were comparable in size to each other, and markedly larger than wounds treated with NTX at each time photographed between day 2 and 16. Wounds on day 18 and 20 were too small for statistical differences to be recorded (Figure 6).

Type 1 diabetic animals treated with NTX had 10% to 51% smaller wound areas than control-treated diabetic rats. For example, on day 8, residual wound sizes were 65% of the original in μ, κ, δ, or control-treated animals in comparison to a 14% wound area remaining in the NTX-treated group. By day 14, NTX-treated wounds were closed, whereas wounds treated with other antagonists or control cream required at least six additional days for closure (Figure 6).

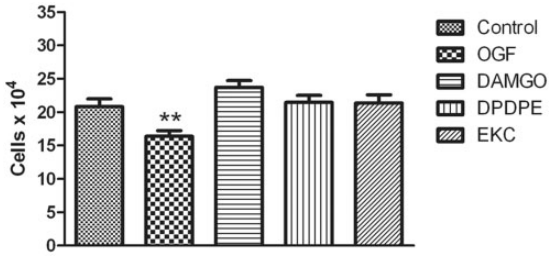

Figure 2 OGFr is required for NTX's stimulatory action on growth of NIH 3T3 fibroblast cells. Log phase cells were transfected for 48 h with OGFr siRNA or scrambled siRNA; some cultures received only Oligofectamine (mock-transfected), or were untreated (Control). Following transfection, cultures were treated with 10^{-6} M NTX for 72 h. Media and compounds were replaced daily. Data represent means ± SEM for at least two aliquots/well and three wells/treatment group. **Significantly different from sterile water-treated controls

Discussion

NTX application to full-thickness wounds accelerates closure by increasing proliferation of cells related to blood vessel formation, connective tissue, and the epithelium.[4,5] Topical NTX dissolved in moisturizing cream advances wound repair in normal mice and rats, as well as animal models of type 1 and type 2 diabetes.[4–7] NTX is a general opioid antagonist, and the specific opioid receptor pathway mediating these effects was unknown. This study demonstrated that the selective opioid receptor blocked by NTX and stimulating growth is OGFr. Using a fibroblast tissue culture model, application of selective agonists (DPDPE, DAMGO, EKC) and antagonists (CTOP, nalmefene, naltrindole) for μ, δ, and κ opioid receptors did not enhance cell proliferation. Knockdown of OGFr and the addition of selected agonists had no effect of cell proliferation. However, the loss of OGFr protein nullified the stimulated growth observed following NTX treatment. Selective antagonists had no effect on fibroblast migration or cell death; the general antagonist NTX also did not induce cell death or fibroblast migration in a scratch wound assay. Selective antagonists for classical opioid receptors did not enhance cutaneous wound closure in type 1 diabetic rats, whereas NTX application expedited wound closure.

This study revealed that independent of systemic biology, the stimulatory effect of NTX on fibroblasts is mediated

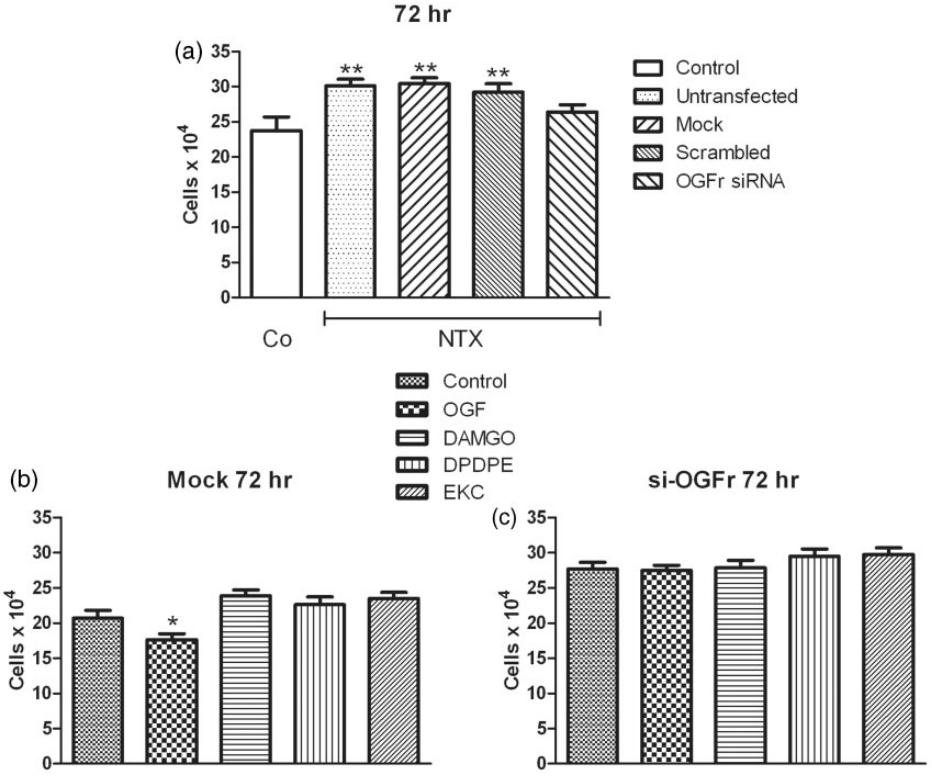

Figure 3 OGF is the specific agonist that mediates growth at the OGFr. (a) Cell number of NIH 3T3 fibroblasts treated for 72 h with specific opioid receptor agonists including OGF, DAMGO, DPDPE, or EKC, specific for OGFr, μ, δ, or κ classical opioid receptors, respectively. (b) siOGFr transfected or (c) mock-transfected NIH 3T3 cells treated for 72 h with 10^{-6} M concentrations of OGF, DAMGO, DPDPE, or EKC which are selective agonists for OGFr, μ, δ, or κ classical opioid receptors, respectively. Media and compounds were replaced daily. Data (cell number) represent means ± SEM for at least two aliquots/well and three wells/treatment group. $^{**}P < 0.01$ or $^{*}P < 0.05$ significantly different from sterile water-treated controls

by the OGF-OGFr axis. Blockade of three other classical opioid peptide-receptor pathways is not involved in wound closure. Using a tissue culture model of fibroblasts, this study shows that NTX is capable of accelerating proliferation of a major cell type involved in the wound-healing process. Fibroblast cells are critical to contraction and remodeling of a full-thickness wounds and their role in wound healing is altered in the diabetic model.[13–17]

The opioid peptide OGF and its receptor OGFr are present and functional in the immortalized mouse fibroblast cell line, as well as in primary cultures of rat fibroblasts. Interaction between this opioid peptide and receptor was tonically active. The addition of OGF inhibited growth in a dose-related manner, and NTX blocked the interaction and accelerated growth. OGF was found to be the only opioid peptide capable of inhibiting cell growth in fibroblasts; this is consistent with studies on neoplasia where OGF is the only synthetic or natural opioid peptide that alters growth.[36] Exposure to siRNAs for μ, κ, and δ opioid receptors indicates that classical opioid receptors did not play a

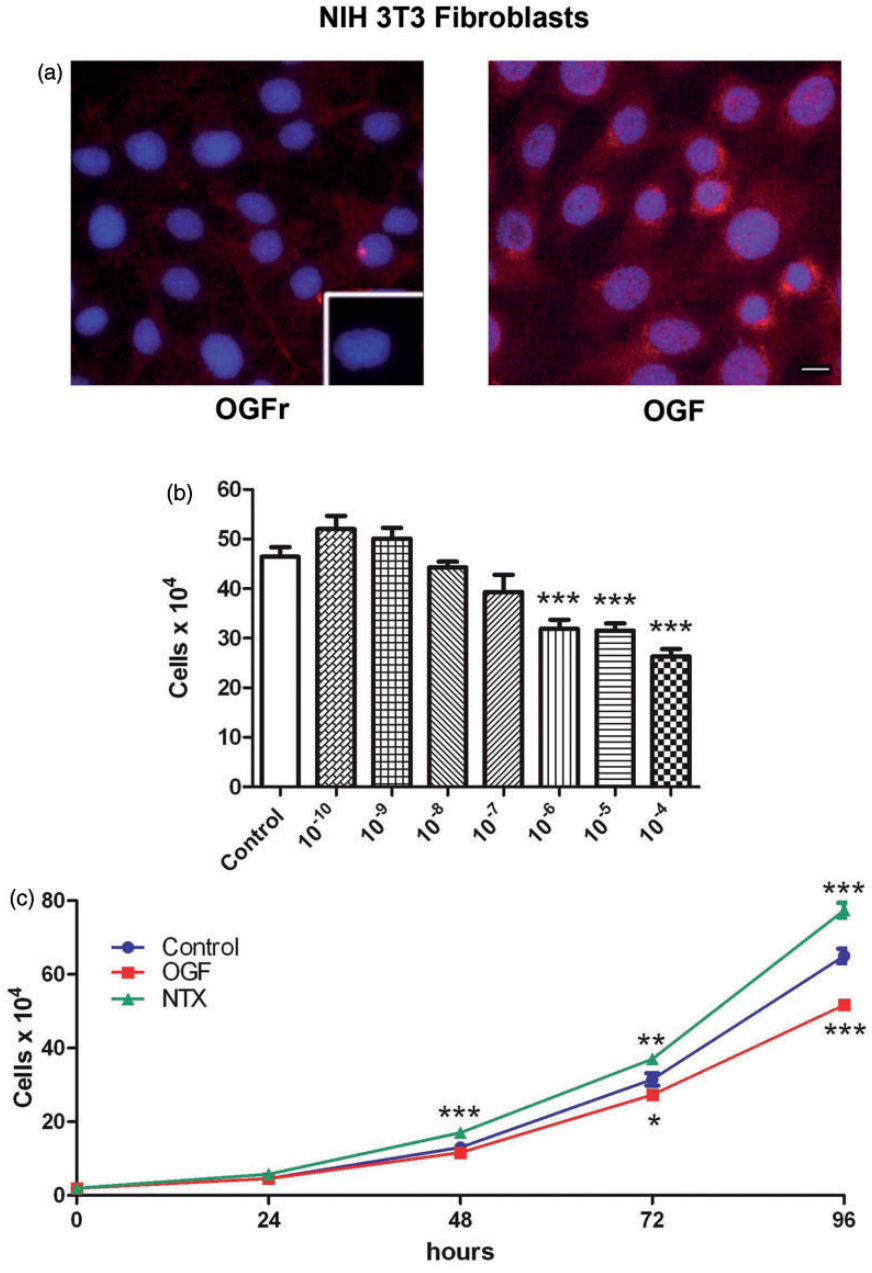

Figure 4 Presence and function of the OGF-OGFr axis in NIH 3T3 fibroblasts. (a) Photomicrographs of anti-OGFr (left panel) and anti-OGF (right panel) antibody staining of log phase fibroblasts. Inset: image of cultures stained with secondary antibody only. Bar = 5 μm. (b) Fibroblast growth was inhibited by OGF in a dose-dependent manner. Concentrations of OGF (10^{-4} M to 10^{-10} M) were added to cells for 72 h. (c) OGF inhibits growth and NTX accelerates growth of NIH 3T3 cells over a 96-h period of time. Data (cell number) represent means ± SEM for at least two aliquots/well and three wells/treatment group. *$P < 0.05$, **$P < 0.01$, or ***$P < 0.001$ significantly different from sterile-water treated controls. (A color version of this figure is available in the online journal)

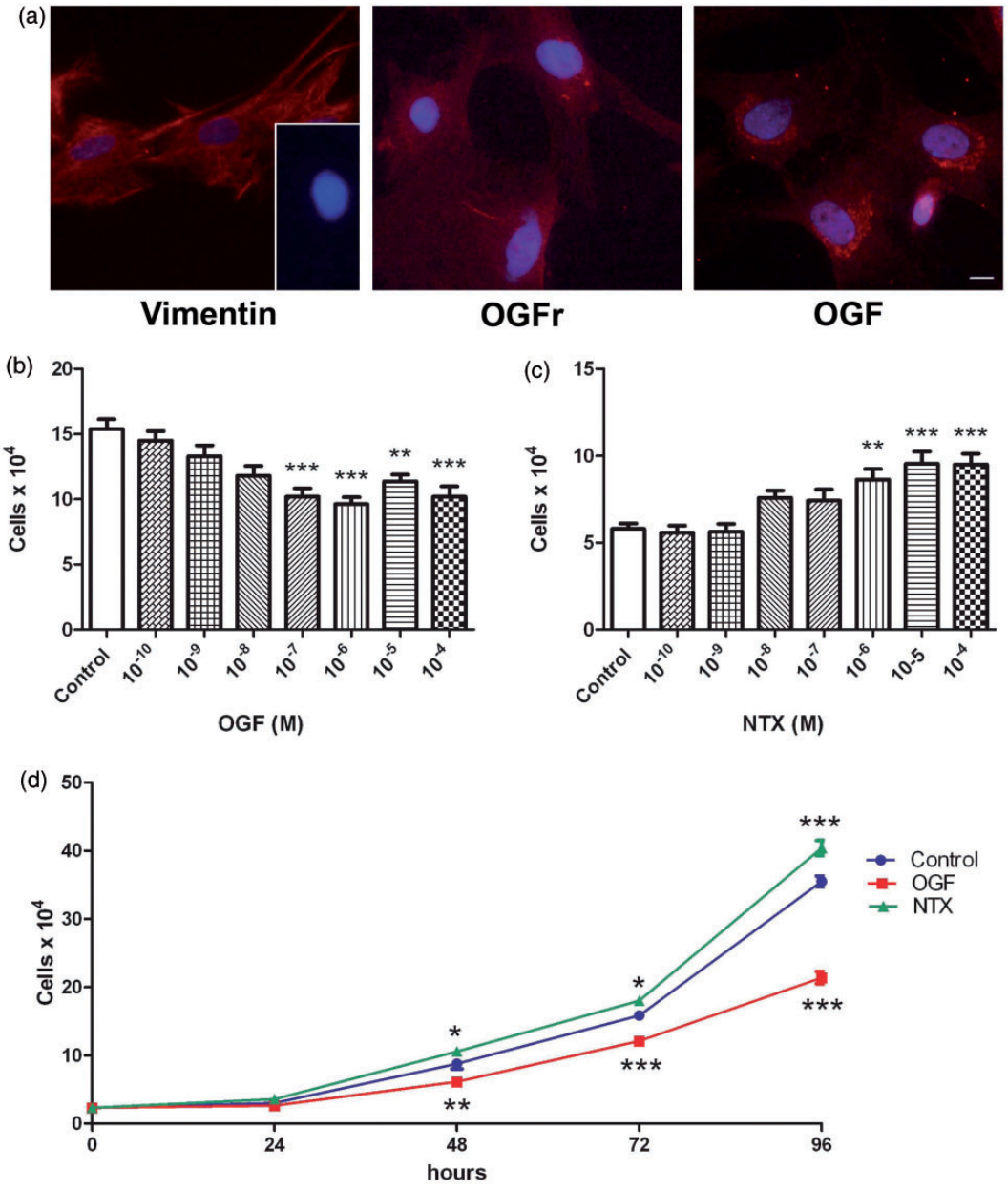

Figure 5 The OGF-OGFr axis is present and functioning in primary rat cartilage fibroblast cultures. (a) Fibroblasts isolated from adult rat auricular cartilage were purified (vimentin) and grown to log phase before staining with anti-OGFr or anti-OGF antibodies. Inset is image of cultures stained with secondary antibody only. Bar = 5 μm. Cell growth of primary fibroblast cultures treated for 72 h with various concentrations of OGF (b) and NTX (c) or for 96 h (d) with 10^{-6} M concentrations of OGF or NTX; sterile water served as control for all growth assays. Drugs and media were replaced daily. Data (cell number) represent means ± SEM for at least two aliquots/well and three wells/treatment group. *$P < 0.05$, **$P < 0.01$, or ***$P < 0.001$ significantly different from sterile water-treated controls. (A color version of this figure is available in the online journal)

role in growth regulation of several neoplastic cell lines.[32,36,38] When administered to NIH 3T3 and primary fibroblast cultures, OGF depressed cell proliferation, while treatment with NTX increased proliferation by interfacing with the OGFr.

OGF and OGFr have been documented in human and mouse skin,[39] with NTX regulating epithelial cell proliferation in a circadian rhythm-dependent manner as measured by tritiated thymidine incorporation.[40] The effects of systemic injections of OGF were constitutively functioning because concomitant administration of naloxone blocked the inhibitory effects of the peptide.[39]

Blockade of opioid receptors by NTX was critical in the discovery of the OGF – opioid growth factor receptor (OGFr) axis.[41–43] Systemic administration of NTX resulted in divergent outcomes dependent on the dosage of NTX utilized. Low dosages of NTX (e.g. 0.1 mg/kg) administered systemically to rats reduced DNA synthesis and produced smaller body and brain weights. Higher dosages of NTX (e.g. 50 mg/kg) or repetitive application of lower dosages, resulted in significantly larger body, brain, and organ weights.[44,45] Further study revealed that the duration of opioid receptor blockade by NTX, and not the dosage of NTX, is important in determining the biological outcome.[22]

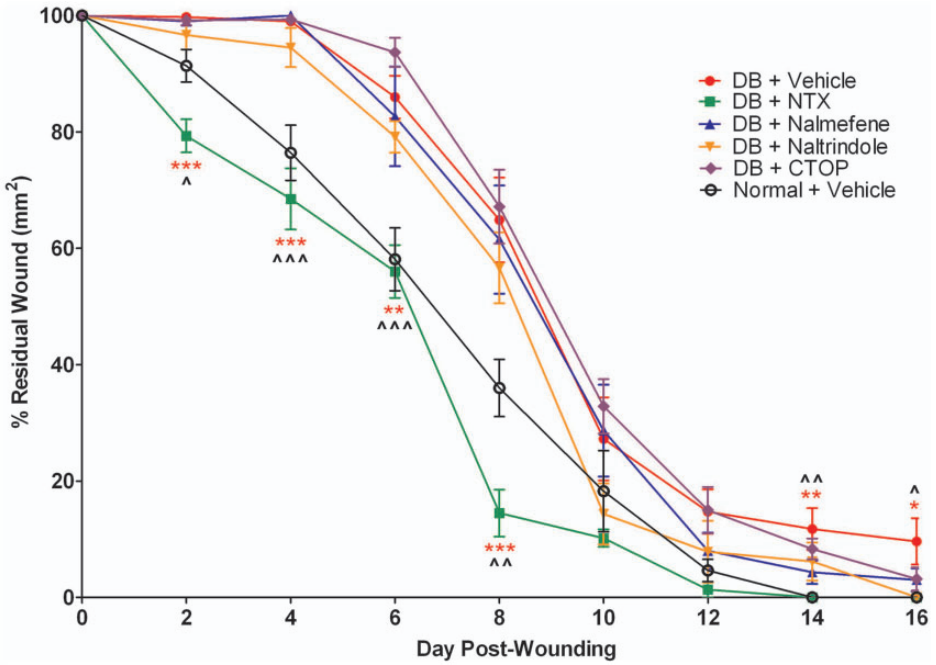

Figure 6 OGFr is the specific opioid receptor pathway blocked by NTX for enhancement of cutaneous wound closure. Adult male rats, rendered hyperglycemic by injections of streptozotocin, were surgically wounded on the dorsum. Selective antagonists for classical opioid receptors (CTOP, nalmefene, naltrindole) or NTX were topically applied three times daily to the wounds. Control diabetic rats received only moisturizing cream (Normal + Vehicle); normal rats receiving vehicle only were included for comparison (normal + vehicle). Wounds were photographed and areal analysis used to determine the percent residual wound. Values represent means ± SEM for 8–12 wounds per treatment. All data from diabetic rats treated with vehicle or classical opioid antagonists differed significantly from the DB + NTX group at $P < 0.01$ (**) and $P < 0.001$ (***). Residual wound areas for DB+ Vehicle rats differed significantly from normal + vehicle animals at $P < 0.05$ (^) and $P < 0.01$ (^^). (A color version of this figure is available in the online journal)

The OGF–OGFr axis is an endogenous opioid receptor system that is tonically active and a determinant of cell proliferation. Topical or systemic administration of NTX has been shown to block this pathway in normal development as well as cancer. Intermittent blockade invoked by low dosages of naltrexone (LDN) results in upregulation of OGF and OGFr, and subsequent reduction in cell number and proliferation of human ovarian cancer cells in vitro and in nude mice. However, continuous blockade induced by higher dosages of NTX or repetitive application of low dosages of NTX enhances cell replication and tissue organization.[46,47]

In summary, the findings from this study provide understanding of the regulation of cell growth by NTX. Modulation of the OGF-OGFr axis was accomplished in primary fibroblast cells. These cells have undergone limited passages and are representative of a functional component of the *in vivo* state compared to an immortalized cell line like NIH 3T3 fibroblast cells. The ability to manipulate the growth of primary mammalian fibroblasts suggests that NTX can alter skin repair in patients with impairments in wound healing. Fibroblasts are a critical cell type in the healing process, and modifications to their physiology have been observed in studies of non-healing wounds, a common complication experienced with diabetes mellitus. The ability to increase the quantity of fibroblast cells present in a diabetic ulcer may reduce the morbidity associated with this complication of diabetes and lessen the number of amputations preformed each year on individuals with the disease.

Author contributions: JAI designed experiments, performed the research, analyzed data and prepared the figures as part of her doctoral thesis research. PJM designed the study, analyzed data, and wrote the paper. ISZ designed the research and critically revised the manuscript.

ACKNOWLEDGEMENTS

The work was supported in part by a grant from the American Diabetes Association (PJM), and a generous gift from The Shockey Family Foundation.

REFERENCES

1. http://www.idf.org/diabetesatlas
2. http://www.diabetes.org/
3. Reiber GE, Vileikyte L, Boyko EJ, del Aguila M, Smith DG, Lavery LA, Boulton AJ. Causal pathways for incident lower-extremity ulcers in patients with diabetes from two settings. *Diabetes Care* 1999;**22**:157–62
4. McLaughlin PJ, Pothering CA, Immonen JA, Zagon IS. Topical treatment with the opioid antagonist naltrexone facilitates closure of full-thickness wounds in diabetic rats. *Exp Biol Med* 2011;**236**:1122–32
5. McLaughlin PJ, Immonen JA, Zagon IS. Topical naltrexone accelerates full-thickness wound closure in type 1 diabetic rats by stimulating angiogenesis. *Exp Biol Med* 2013;**0**:1–11

6. Immonen JA, McLaughlin PJ, Zagon IS. Topical treatment with the opioid antagonist naltrexone accelerates the remodeling phase of full-thickness wound healing in type 1 diabetic rats. *Exp Biol Med* 2013;**238**:1127–35
7. Immonen JA, Zagon IS, McLaughlin PJ. Topical naltrexone as treatment for type 2 diabetic cutaneous wounds. *Adv Wound Care* 2014 (in press)
8. Zagon IS, Sassani JW, McLaughlin PJ. Reepithelialization of the rat cornea is accelerated by blockade of opioid receptors. *Brain Res* 1998;**798**:254–60
9. Zagon IS, Klocek MS, Sassani JW, McLaughlin PJ. Use of topical naltrexone to normalize corneal epithelial healing in diabetes mellitus. *Arch Ophthalmol* 2007;**125**:1082–8
10. Klocek MS, Sassani JW, McLaughlin PJ, Zagon IS. Topically applied naltrexone restores corneal reepithelialization in diabetic rats. *J Ocular Pharmacol Ther* 2007;**23**:89–102
11. Zagon IS, Sassani JW, Carroll MA, McLaughlin PJ. Topical application of naltrexone facilitates reepithelialization of the cornea in diabetic rabbits. *Brain* Res Bull 2010;**81**:248–55
12. Diegelmann RF, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. *Frontiers Biosci* 2004;**9**:283–9
13. Galkowska H, Wojewodzka U, Olszewski WL. Chemokines, cytokines and growth factors in keratinocytes and dermal endothelial cells in the margin of chronic diabetic foot ulcers. *Wound Repair Regen* 2006;**14**:558–65
14. Goren I, Muller E, Pfeilschifter J, Frank S. Severely impaired insulin signaling in chronic wounds of diabetic ob/ob mice: a potential role of tumor necrosis factor-alpha. *Am J Pathol* 2006;**168**:765–77
15. Falanga V. Wound healing and its impairment in the diabetic foot. *Lancet* 2005;**366**:1736–43
16. Gibran NS, Jang YC, Isik FF, *et al*. Diminished neuropeptide levels contribute to the impaired cutaneous healing response associated with diabetes mellitus. *J Surg Res* 2002;**108**:122–8
17. Lobmann R, Ambrosch A, Schultz G, Waldmann K, Schiweck S, Lehnert H. Expression of matrix metalloproteinases and their inhibitors in the wounds of diabetic and non-diabetic patients. *Diabetologia* 2002;**45**:1011–16
18. Blumberg H, Dayton HB. Naloxone, naltrexone, and related noroxymorphones. In: Braude MC, Harris LC, May EL, Smith JP, Villarreal JE (eds). *Advances in biochemical psychopharmacology*. 2nd ed. *Narcotic Antagonists*. New York: Raven Press, 1974, pp. 33–43
19. Gutstein HB, Akil H. Opioid analgesics. In: Hardman JG, Limbard LE (eds). *The pharmacological basis of therapeutics*, 10th ed. New York: McGraw Hill, 2001, pp. 569–619
20. Leslie FM. Methods used for the study of opioid receptors. *Pharmacol Rev* 1987;**39**:197–249
21. Zagon IS, McLaughlin PJ. Duration of opiate receptor blockade determines tumorigenic response in mice with neuroblastoma: a role for endogenous opioid systems in cancer. Life *Sci* 1984;**35**:409–16
22. Zagon IS, McLaughlin PJ. Increased brain size and cellular content in infant rats treated with an opiate antagonist. *Science* 1983;**221**:1179–80
23. Zagon IS, McLaughlin PJ. Naltrexone modulates tumor response in mice with neuroblastoma. *Science* 1983;**221**:671–3
24. Zagon IS, McLaughlin PJ. Gene-peptide relationships in the developing rat brain: the response of preproenkephalin mRNA and [Met^5]-enkephalin to acute opioid antagonist (naltrexone) exposure. *Mol Brain Res* 1995;**33**:111–20
25. Bigliardi PL, Sumanovski LT, Buchner S, Rufli T, Bigliardi-Qi M. Different expression of μ-opiate receptor in chronic and acute wounds and the effect of β-endorphin on transforming growth factor β II receptor and cytokeratin 16 expression. *J Invest Dermatol* 2003;**120**:145–52
26. Bigliardi PL, Bigliardi-Qi M, Buechner S, Rufli T. Expression of μ-opiate receptor in human epidermis and keratinocytes. *J Invest Dermatol* 1998;**111**:297–301
27. Bigliardi PL, Tobin DJ, Caveriaux-Ruff C, Bigliardi-Qi M. Opioids and the skin – where do we stand? *Exp Dermatol* 2009;**18**:424–3
28. Taneda K, Tominaga M, Negi O, Gengara S, Kamo A, Ogawa H, Takamori K. Evaluation of epidermal nerve density and opioid receptor levels in psoriatic itch. *Brit J Dermatol* 2011;**165**:277–284.
29. Tominaga M, Ogawa H, Takamori K. Possible roles of epidermal opioid systems in pruritus at atopic dermatitis. *J Invest Dermatol* 2007;**127**:2228–35
30. Nissen JR, Kragbelle E. Enkephalins modulate differentiation of normal human keratinocytes *in vitro*. *Exp Dermatol* 1997;**6**:222–9
31. Charbaji N, Schafer-Korting M, Kuchler S. Morphine stimulates cell migration of oral epithelial cells by delta-opioid receptor activation. *PLOS One* 7:e42616
32. Donahue RD, McLaughlin PJ, Zagon IS. Cell proliferation of human ovarian cancer is regulated by the opioid growth factor – opioid growth factor receptor axis. *Am J Physiol Regul Integr Comp Physiol* 2009;**296**:R1716–25
33. Zagon IS, McLaughlin PJ. Production and characterization of polyclonal and monoclonal antibodies to zeta (ζ) opioid receptor. *Brain Res* 1993;**630**:295–302
34. Emmerson PJ, Liu MR, Woods JH, Medzihradsky F. Binding affinity and selectivity of opioids at mu, delta and kappa receptors in monkey brain membranes. *J Pharmacol Exp Ther* 1994;**271**:1630–7
35. Raynor K, Kong H, Chen Y, Yasuda K, Yu L, Bell GI, Reisine T. Pharmacological characterization of the cloned kappa-, delta-, and mu-opioid receptors. *Mol Pharmacol* 1994;**45**:330–4
36. Zagon IS, Donahue RN, McLaughlin PJ. Opioid growth factor – opioid growth factor receptor axis is a physiological determinant on cell proliferation in diverse human cancers. *Am J Physiol Regul Integr Comp Physiol* 2009;**297**:R1154–61
37. Campbell AM, Zagon IS, McLaughlin PJ. Astrocyte proliferation is regulated by the OGF-OGFr axis in vitro and in experimental autoimmune encephalomyelitis. *Brain Res Bull* 2013;**90**:43–51
38. Donahue RN, McLaughlin PJ, Zagon IS. Low-dose naltrexone targets the opioid growth factor-opioid growth factor receptor pathway to inhibit cell proliferation: mechanistic evidence from a tissue culture model. *Exp Biol Med* 2011;**236**:1036–50
39. Zagon IS, Wu Y, McLaughlin. The opioid growth factor, [Met^5]-enkephalin, and the ζ (zeta) opioid receptor are present in human and mouse skin and tonically act to inhibit DNA synthesis in the epidermis. *J Invest Dermatol* 1996;**106**:490–7
40. Zagon IS, Wu Y, McLaughlin PJ. Opioid antagonist modulation of DNA synthesis in mouse tongue epithelium is circadian dependent. *Pharmacol Biochem Behav* 1994;**48**:709–14
41. Zagon IS, McLaughlin PJ. Opioid antagonist (naltrexone) stimulation of cell proliferation in human and animal neuroblastoma and human fibrosarcoma cells in culture. *Neuroscience* 1990;**37**:223–6
42. Zagon IS, Goodman SR, McLaughlin PJ. Zeta (ζ), the opioid growth factor receptor: identification and characterization of binding subunits. *Brain Res* 1993;**605**:50–6
43. Zagon IS, Verderame MF, McLaughlin PJ. The biology of the opioid growth factor receptor(OGFr). *Brain Res Rev* 2002;**38**:351–76
44. Zagon IS, McLaughlin PJ. Opioid antagonist (naltrexone) modulation of cerebellar development: histological and morphometric studies. *J Neurosci* 1986;**6**:1424–32
45. Zagon IS, McLaughlin PJ. Opioid antagonist-induced modulation of cerebral and hippocampal development: histological and morphometric studies. *Devel Brain Res* 1986;**28**:233–46
46. Zagon IS, Sassani JW, McLaughlin PJ. Opioid growth factor modulates corneal epithelial outgrowth in tissue culture. *Am J Physiol Integr Comp Physiol* 1995;**268**:R942–50
47. Zagon IS, McLaughlin PJ. Identification of opioid peptides regulating proliferation of neurons and glia in the developing nervous system. *Brain Res* 1991;**542**:318–23

(Received April 19, 2014, Accepted June 4, 2014)