

Inhibition of DNA synthesis in mouse epidermis by topical imiquimod is dependent on opioid receptors

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Abstract

The imidazoquinolines are immune response modifiers that have potent antiviral and antitumor properties. The mechanism by which they exert their effects on cell replication has been investigated *in vitro* and is related to the upregulation of the opioid growth factor receptor (OGFr) and modulation of opioid growth factor (OGF; [Met⁵]-enkephalin). The OGF–OGFr axis regulates cell proliferative events through a cyclin-dependent kinase inhibitory pathway. The present study examined the mechanism whereby imiquimod repressed cell proliferation *in vivo*. Using a nude mouse model that has a compromised T-cell immune system, as well as C57BL/6 mice with an intact immune system, the effects of topical imiquimod (Aldara[®]) on DNA synthesis of basal epithelial cells in skin were examined. Imiquimod's effects on DNA synthesis were detected 24 h after application, and could be observed for one week after a single treatment. The magnitude of change in DNA synthesis following imiquimod was similar for one, three or six applications. Naloxone, an opioid antagonist, blocked the inhibitory effect of imiquimod. Imiquimod in combination with OGF or a low dose of naltrexone (LDN; known to upregulate the OGF–OGFr axis) had no greater inhibitory response on DNA synthesis than either OGF or LDN alone. Both OGF and OGFr were upregulated in basal epithelium after imiquimod treatment. Both nude and C57BL/6 mice exhibited the same repressive action of imiquimod on epithelial DNA synthesis. Imiquimod was neither an opioid agonist nor antagonist using nociceptive testing, and did not induce apoptosis or necrosis. Exposure to imiquimod was found to depress DNA synthesis in cells located in distant epithelium from day 3 and lasted until day 5. These results suggest that the target of imiquimod on DNA synthesis is dependent on an opioid receptor-mediated pathway, and infers that imiquimod is reliant on the OGF–OGFr axis for modulating cell proliferation.

Keywords: opioid growth factor, imiquimod, cell proliferation, epithelium, immunity, bystander effect, opioid

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Introduction

Imiquimod (Aldara[®], R-837, S-26308), 1-(2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine, belongs to the imidazoquinoline family, and is a synthetic local immune response modifier that has potent antiviral and antitumor properties.^{1–4} Imiquimod is highly efficacious in the treatment of external and anal warts, basal cell carcinoma, actinic keratoses, Kaposi's sarcoma, chronic hepatitis C infection and intraepithelial carcinoma. This drug is a ligand for toll-like receptor (TLR)-7 and TLR-8, and increases proinflammatory cytokines and chemokines, including interferon (IFN)- α , IFN- γ , tumor necrosis factor- α , interleukin (IL)-1 α and IL-12.^{3,4} In addition, imiquimod, but not another imidazoquinoline, resiquimod, has the potential to induce apoptosis.^{5,6}

In an effort to understand the direct molecular mechanism of imiquimod's action, Urosevic *et al.*⁷ found an upregulation of gene and protein expression for the opioid growth factor receptor (OGFr).⁸ OGFr is a 62-kDa protein, with several nuclear localization signals,^{8,9} and serves as the receptor for the opioid growth factor (OGF).⁸ OGF, chemically termed [Met⁵]-enkephalin, is a pentapeptide that is constitutively expressed, autocrine produced and secreted.^{8,10} The action of this inhibitory peptide is tonic, stereospecific, reversible, and not associated with differentiation, apoptosis, necrosis, migration, invasion or adhesion.^{8,10–13} The peptide and receptor associate to form the OGF–OGFr axis that is involved in cell proliferation, targeting the cyclin-dependent inhibitory kinases and upregulating p16 and/or p21,^{14–16} which leads to a

delay in the cell cycle. Zagon *et al.*,¹⁷ using tumor cells in tissue culture, which eliminated the immune system and TLRs, showed that imiquimod, as well as resiquimod, not only upregulates OGF α , but also stimulates the interaction of the OGF–OGF α axis. Cell proliferation following imiquimod exposure was repressed in a dose-dependent manner. Neutralization of OGF by antibody to this peptide, or knockdown of OGF α using siRNA, diminished the effect of imiquimod.

Given that the mechanism of imiquimod operates through an upregulation of an endogenous opioid system as recorded in our *in vitro* studies,¹⁷ the present study addresses the question of imiquimod action on cell proliferation *in vivo*. Using a nude mouse model with compromised T-cell immunity, as well as C57BL/6 mice with a complete immune system, the effects of imiquimod on DNA synthesis in the epithelium were monitored. The temporal course of activity of imiquimod on DNA synthesis, opioid receptor involvement, as well as evidence that imiquimod is neither an opioid agonist nor antagonist were investigated. The results provide compelling evidence that topical application of imiquimod to the dorsal skin of mice decreases DNA synthesis in the epithelium within circumscribed temporal boundaries and in an opioid receptor-mediated manner, upregulates OGF and OGF α , and does not act as an opioid agonist or antagonist.

Materials and methods

Animals

Athymic nu/nu nude mice, 4–5 weeks old, were purchased from Harlan Laboratories (Indianapolis, IN, USA). C57BL/6 mice, six weeks old, were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Nude mice were housed in a sterile environment in flexible film isolators, whereas C57BL/6 mice were housed under standard laboratory conditions; all animals had water and Purina 5010 rodent chow available *ad libitum*. C57BL/6 mice, but not nude mice, were shaved one day prior to experimentation. Three independent studies were performed, and all conformed to the regulations of the National Institutes of Health and the guidelines of the Institutional Animal Care and Use Committee of the Pennsylvania State University College of Medicine.

Imiquimod treatment

Topical applications of 5% imiquimod cream (Aldara; 3M Pharmaceuticals, St Paul, MN, USA) were placed on the epithelium of the dorsum of the animal using a cotton swab; approximately 50–100 μ L volumes were applied over a 1 cm diameter area on the dorsal surface. Unless otherwise noted, animals received only one application of imiquimod. Some mice were treated with a commercial moisturizing cream (Neutrogena, Johnson & Johnson, Brunswick, NJ, USA), while other animals received no treatment.

Opioid receptor mediation

To examine opioid receptor mediation of imiquimod action on DNA synthesis, nude mice received a single

intraperitoneal injection of the opioid antagonist naloxone hydrochloride (15 mg/kg, Sigma-Aldrich, Indianapolis, IN, USA) simultaneously with the application of imiquimod or moisturizing cream. Some mice were given an intraperitoneal injection of a low dosage (0.1 mg/kg) of naltrexone (LDN, Sigma-Aldrich) to investigate the repercussions of intermittent opioid receptor blockade. To study the effects of OGF, animals received intraperitoneal injections of either 10 or 20 mg/kg [Met⁵]-enkephalin (Sigma-Aldrich) with or without treatment with imiquimod.

DNA synthesis

BrdU incorporation was used to assess DNA synthesis in the basal layer of the epithelium in both nu/nu and C57BL/6 mice, as well as the basal epithelial layer of the cornea of the nu/nu mice. Animals were injected with 100 mg/kg BrdU intraperitoneally at three and six hours preceding euthanasia by intraperitoneal injection of pentobarbital. Skin that was topically treated with imiquimod, as well as skin 3 cm from the treated region and considered 'untreated' was removed, fixed in 10% neutral buffered formalin for 24 h and processed for paraffin embedding. Sections were stained with anti-BrdU-BOD antibodies (Invitrogen, Carlsbad, CA, USA) followed by visualization and counterstaining with hematoxylin and eosin. The number of DNA-positive cells in the basal layer of the epidermis was counted using an ocular grid and a Nikon BH-2 microscope. Labeling indexes were computed as the ratio of labeled to total cells. At least three sections/mouse were evaluated.

Immunohistochemistry

The presence of OGF and its receptor (OGF α) in the epidermis of nude and C57BL/6 mice was determined using well-characterized polyclonal antibodies and published procedures.¹⁷ Levels of peptide and receptor were assessed with semi-quantitative methodology and Optimas 6.0 software (Optimas Corporation, Bothell, WA, USA). At least three sections/mouse from three mice/group were evaluated, with 10 or more readings recorded per section.

Nociception

To evaluate whether exposure to imiquimod was associated with a change in nociception, C57BL/6 mice were monitored for their response (e.g. licking of paws) on an analgesia meter (55°C, Analgesia Meter, Columbia Instruments, Columbus, OH, USA). Mice were measured at zero hours, and one, two and four hours after treatment with 12 mg/kg (intraperitoneal) imiquimod (Sequoia Research Products Ltd, Panbourn, UK) or an equivalent volume of saline. In addition, some animals treated with imiquimod or saline were injected with the opioid agonist morphine (10 mg/kg; Sigma-Aldrich) and tested one hour after treatment.

Statistical analyses

Labeling indexes, latency times on the hot-plate and mean gray values for immunohistochemical optical density

measurements were compared using Student's unpaired *t*-test or analysis of variance with subsequent comparisons made using Newman-Keuls tests.

Results

DNA synthesis in epithelium after acute imiquimod application in nude mice

The temporal course of imiquimod's effect on DNA synthesis was determined in the skin of nude mice. No differences in labeling index were recorded in the skin of animals receiving vehicle only (18.4 ± 0.9) and mice that were not subjected to any treatment (16.7 ± 0.9). For further comparisons, mice not subject to any treatment (i.e. Neutrogena) were considered controls.

In contrast to time zero hour (baseline), wherein an 18% labeling index was recorded, the levels of DNA synthesis following an acute application of 5% imiquimod were not altered at 3, 5, 7, 18 or 20 h (Figure 1a). However, at 24 h, the labeling index of the skin from imiquimod-treated mice was decreased 32% from baseline levels. DNA synthesis remained subnormal (decreases from 25% to 43%) in imiquimod-treated skin for up to one week, but was comparable to control values on days 10, 14 and 21 (Figure 1b).

Untreated skin 3 cm from the imiquimod exposed area had labeling indexes on days 1 and 2 that were comparable

to control levels (Figure 1b). However, on days 3, 4 and 5, DNA synthesis in untreated skin was decreased 27–44% from control values. On days 7, 10, 14 and 21, the labeling index of untreated skin was similar to control levels.

The effects of imiquimod on DNA synthesis in the basal epithelial layer in the cornea of nu/nu mice were assessed at 72 h after treatment with a single application of imiquimod or control cream (i.e. Neutrogena) on the skin. The labeling index of peripheral corneal epithelium for mice exposed to control cream was $12.6 \pm 1.1\%$ compared with $6.7 \pm 0.7\%$ for mice treated with imiquimod; this difference was significant at $P < 0.001$.

The effect of acute imiquimod treatment on reducing DNA synthesis is opioid receptor-mediated

To evaluate whether the decrease in DNA synthesis in the basal epithelium of imiquimod-treated skin was related to the opioid receptor pathway, mice simultaneously received a single topical application of imiquimod as well as a systemic injection of naloxone, a general, short-acting opioid receptor antagonist. At 24 h, imiquimod-exposed skin had a decrease of 37% in labeling index from control levels (Figure 2), whereas imiquimod-treated skin examined from animals receiving naloxone had levels of DNA synthesis comparable to control levels. The basal layer of epithelium exposed to imiquimod had reductions in DNA synthesis of 28% and 33%, relative to naloxone-injected ($P < 0.001$) or imiquimod + naloxone-treated ($P < 0.001$) mice, respectively.

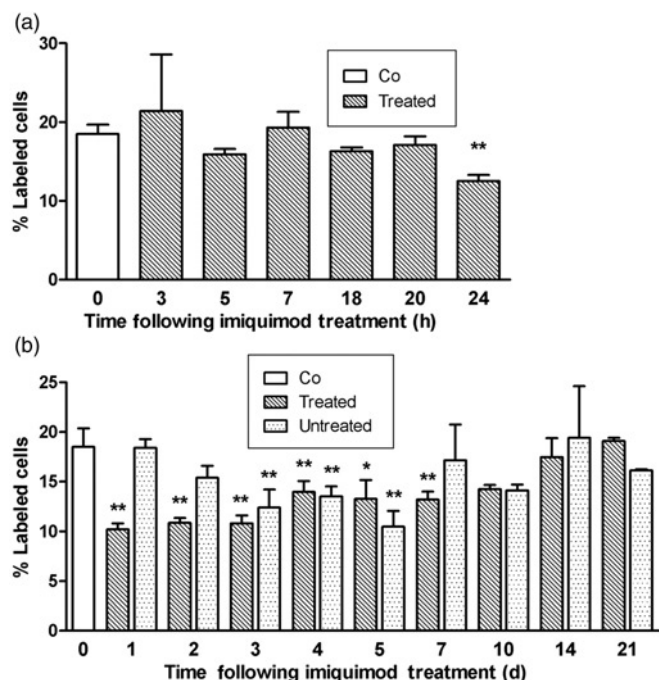


Figure 1 Inhibition of DNA synthesis in the basal epithelium following a single topical application of imiquimod to skin of nude mice. Histograms of BrdU incorporation into basal epithelial cells (a) 3–24 h and (b) 1–21 days following a single topical application of imiquimod (Treated). The untreated skin of mice receiving topical imiquimod was examined beginning on day 1 (Untreated). BrdU measurements from untreated mice served as control (Co). Experiments were conducted three times, with at least 2–3 mice/time point/experiment. Values represent means $\pm$ SE. Significantly different from Co at $P < 0.05$ (*) or $P < 0.01$ (**)

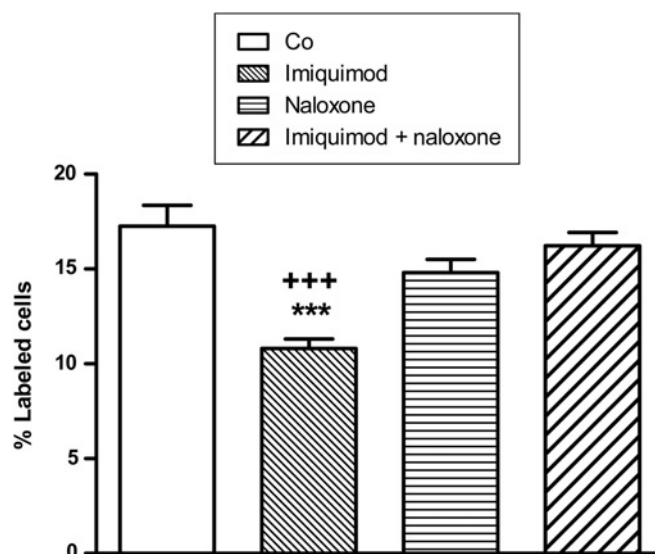


Figure 2 Inhibition of DNA synthesis in basal epithelium following a single topical application of imiquimod is mediated by opioid receptors. BrdU incorporation in the basal epithelium following topical application of imiquimod to mice also receiving injection (intraperitoneal) of 15 mg/kg naloxone (Imiquimod + naloxone) or saline (Imiquimod). Some mice received intraperitoneal injections of only 10 mg/kg naloxone or saline (Co). All tissues were collected 24 h after injections. Three experiments were conducted, with two mice/treatment/experiment. Values represent means $\pm$ SE. Significantly different from Co at $P < 0.001$ (***), and from the Imiquimod + naloxone or Naloxone only groups at $P < 0.001$ (+++)

The combination therapy of topical imiquimod and OGF displayed inhibitory, but not additive, effects on DNA synthesis

To study the repercussion of imiquimod upregulation of OGF α and the addition of exogenous OGF on DNA synthesis, an acute topical application of imiquimod was combined with intraperitoneal injections of 10 or 20 mg/kg OGF. In contrast to a 23% decrease in labeled cells recorded in the epithelium of imiquimod-treated mice compared with controls, a single exposure to 10 or 20 mg/kg OGF depressed cells by 70% and 81%, respectively (Figure 3a). Mice injected with a combination of imiquimod and 10 or 20 mg/kg of OGF were reduced by 49% and 72%, respectively, from animals treated with imiquimod alone. However, mice given a combination of imiquimod and OGF did not differ from animals subjected to OGF alone at either 10 or 20 mg/kg.

Short-term exposure to LDN did not enhance the inhibitory effects of imiquimod on DNA synthesis

In order to examine whether short-term exposure to LDN, another pathway that upregulates the OGF–OGF α axis, has greater efficacy if combined with imiquimod, a study was performed whereby mice received either one

treatment of LDN (systemically), imiquimod (topically) or both LDN and imiquimod. Although treatment with imiquimod alone or LDN alone reduced the labeling index by 39% and 46%, respectively, from control levels, the magnitude of decrease in DNA synthesis for mice exposed to both imiquimod and LDN (44% below control values) was comparable to that for either agent alone (Figure 3b).

Multiple applications of imiquimod did not produce greater inhibitory effects on DNA synthesis

To address the question of whether multiple applications of imiquimod had a greater effect in reducing DNA synthesis of basal epithelial cells than only one application, animals were subjected to three or six treatments with imiquimod every other day over a one- or two-week period of time. The results revealed that one, three or six applications of imiquimod reduced the labeling indexes by 26%, 32% and 19%, respectively, from control values (17.4 ± 0.8) (Figure 4), but did not differ from an acute application of imiquimod.

OGF and OGF α levels are upregulated in epithelium of nude mice by imiquimod treatment

The effects of topical application of imiquimod on the levels of OGF and OGF α in the epidermis were studied by semi-quantitative immunohistochemistry. Skin sections from mice receiving a single application of imiquimod, as well as epithelium from mice that were untreated with imiquimod, were stained with antibodies to either OGF or OGF α and compared with the distribution and quantity of peptide and receptor in control animals 24 and 72 h after treatment. The results show no differences between groups in the cellular distribution of staining for OGF or OGF α in epithelial cells (Figure 5). However, both the treated and untreated regions of the skin from the imiquimod group had marked increases in OGF (5- and 2.3-fold, respectively) and OGF α (2.8- and 2.0-fold, respectively) compared with control values at 24 h. At 72 h after exposure to imiquimod, OGF levels were increased in treated and untreated regions relative to controls, with a ~ 1.5 -fold elevation in OGF and a ~ 2.0 -fold elevation in OGF α .

The effects of imiquimod on OGF α and DNA synthesis are not restricted to nude mice

To determine whether the inhibitory effects of imiquimod on DNA synthesis recorded in nude mice also occur in mice with a complete immunological system, C57BL/6 mice were studied. At 24 h following a single application of imiquimod, the treated skin had a decrease of 43% in cells undergoing DNA synthesis compared with control values, with no change recorded in the untreated epithelium (Figure 6a). Semi-quantitative immunohistochemistry of OGF and OGF α revealed that at 24 h, both treated and untreated skin had a 16% and 25%, respectively, increase in OGF α but OGF levels were comparable to controls (Figure 6b).

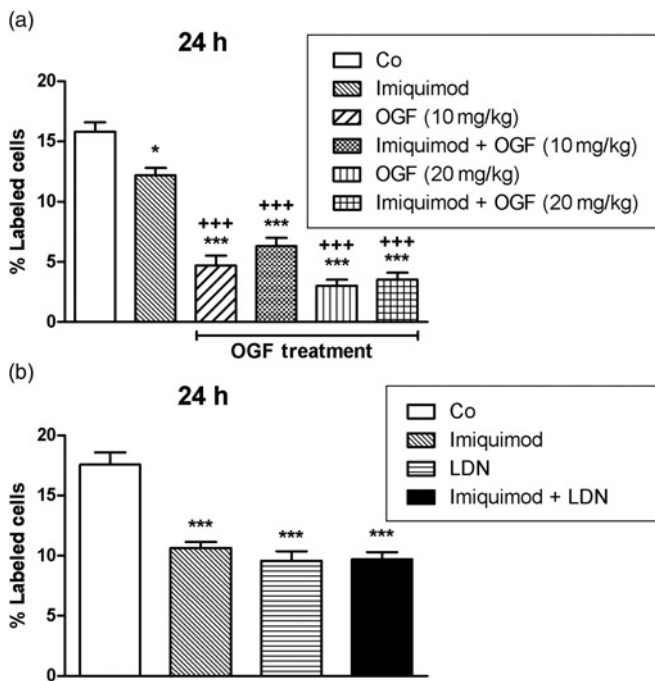


Figure 3 The combination of imiquimod and OGF, or imiquimod and LDN, depresses DNA synthesis of epithelial cells but not to any extent greater than OGF or LDN alone. BrdU incorporation into basal epithelial cells in mice receiving one topical application of imiquimod in combination with (a) 10 or 20 mg/kg OGF and examined 24 h later, or (b) one topical application of imiquimod in combination with 0.1 mg/kg LDN. Animals were examined 24 h later. Some mice received saline (Co). Experiments were conducted three times, with three mice/treatment/experiment. Values represent means $\pm$ SE. Significantly different from Co at $P < 0.05$ (*) or $P < 0.001$ (***). Significantly different from mice receiving only imiquimod at $P < 0.001$ (+++). OGF, opioid growth factor; LDN, naltrexone; Co, control

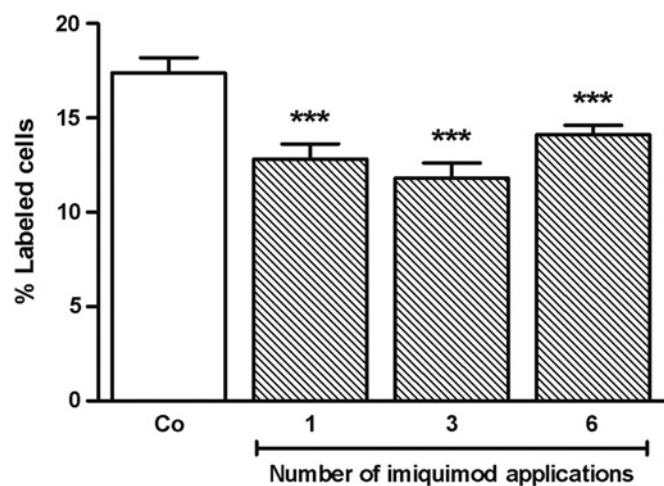


Figure 4 Application of imiquimod one, three or six times depresses DNA synthesis to a similar magnitude. BrdU incorporation in the basal epithelium 24 h following topical application of imiquimod once, three or six times. Experiments were conducted twice with two mice/treatment/experiment. Values represent means $\pm$ SE. Significantly different from Co at $P < 0.001$ (***). OGF, opioid growth factor; OGFr, opioid growth factor receptor; Co, control

Imiquimod does not act as either an opioid receptor antagonist or opioid agonist with respect to nociception

To investigate whether imiquimod acts as an opioid agonist, nociception was monitored after a topical application of imiquimod. At one, two and four hours after treatment with imiquimod, baseline and treated values of nociception were similar (Figure 7). In order to address whether imiquimod was an opioid antagonist, imiquimod-treated mice received an injection of morphine. The mice exposed to imiquimod and challenged with morphine had a marked elevation in nociception time compared with baseline values, but exhibited no difference from control animals subjected to morphine.

Discussion

This study shows for the first time that imiquimod, a clinically important drug, represses cell proliferation in the epithelium of the skin by an opioid receptor-mediated mechanism. Imiquimod's effects on DNA synthesis occurred 24 h after application, and could be observed for as long as one week after a single treatment. The decrease and magnitude of change in DNA synthesis from exposure to imiquimod was not dependent on the number of applications of this drug. The effect of imiquimod on cell replicative events, however, was receptor-mediated, with the opioid antagonist naloxone blocking imiquimod activity at a dosage of naloxone that had no effect by itself. Despite the fact that imiquimod is known to upregulate OGFr, the combination of imiquimod and OGF were not additive in depressing cell proliferation beyond OGF alone, but did decrease DNA synthesis relative to imiquimod treatment alone. These data could suggest that the amount of exogenous OGF optimizes the OGF-OGFr pathway, and any

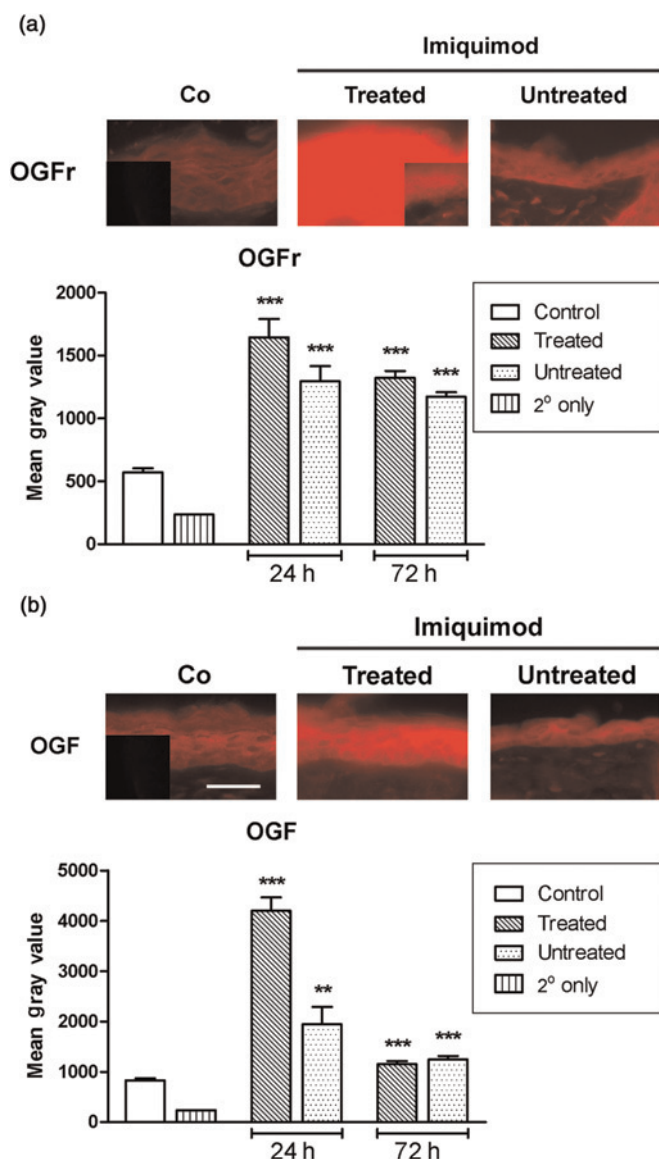


Figure 5 Imiquimod upregulates OGFr and OGF in the epithelium as detected by immunohistochemistry and semi-quantitative analysis. Mice received a single topical application of imiquimod and skin sampled at 24 and 72 h. The untreated skin of mice receiving topical imiquimod was included for examination (Untreated). Sections were stained with antibodies to OGF or OGFr, or secondary antibodies only (inset in Co). Because of the intense staining with OGFr antibodies from sections of imiquimod-treated mice, exposure time was restricted to show cellular details (inset of Treated). Sections from three mice/group were stained. Values (10 or more readings/section) represent mean gray values $\pm$ SE. Significantly different from Co at $P < 0.01$ (**) or $P < 0.001$ (***). Bar = 30 μ m. OGF, opioid growth factor; OGFr, opioid growth factor receptor; Co, control (A color version of this figure is available in the online journal)

additional alterations (e.g. imiquimod stimulation of OGFr) are not capable of producing an even greater inhibitory effect. Indeed, modulation of the opioid system by imiquimod and LDN (known to upregulate OGFr and OGF) also was no more effective than either LDN or imiquimod alone. Both OGF and OGFr were detected in the epithelium, and were markedly increased from baseline in animals exposed to imiquimod, indicating that the receptor as well as the peptide is altered by this agent. The effect of imiquimod on cell proliferation was ubiquitous and not just

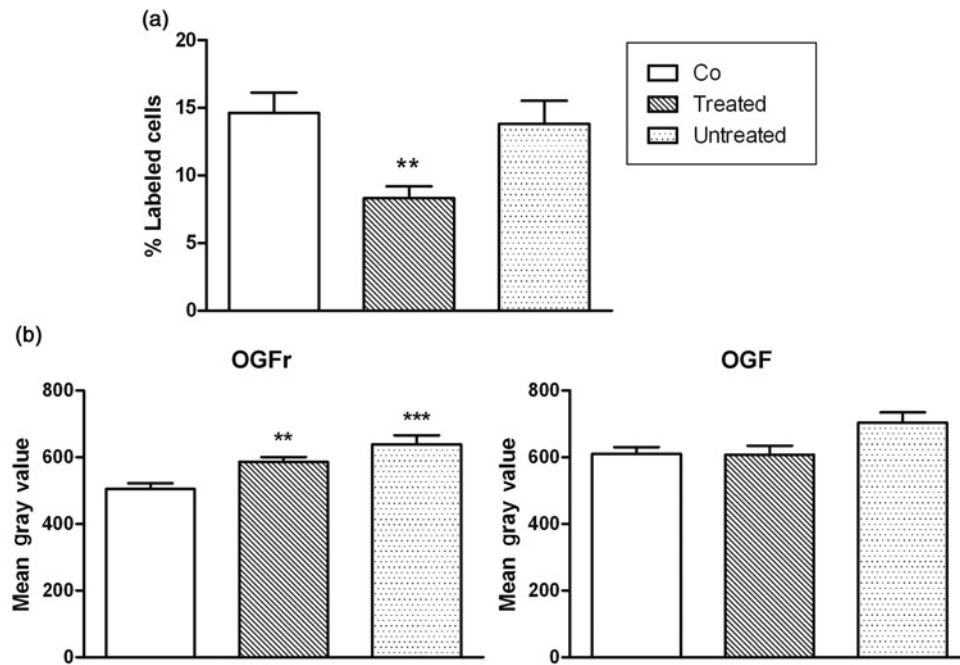


Figure 6 Topical application of imiquimod depresses DNA synthesis, and elevates the levels of OGFr, in the epithelium of C57BL/6 mice at 24 h. The labeling index of BrdU (a) and mean gray values for semi-quantitative densitometry for OGF and OGFr (b). The untreated skin of mice receiving topical imiquimod was included (Untreated). Three mice/treatment were examined. Values represent means $\pm$ SE. Significantly different from Co at $P < 0.01$ (**) or $P < 0.001$ (***). OGF, opioid growth factor; OGFr, opioid growth factor receptor; Co, control

related to nude mice, which are hairless and have a compromised immune system. Furthermore, C57BL/6 mice, with an intact immune system, responded to imiquimod in a similar manner to that of nude animals. Finally, testing of nociception – with and without a challenge by the opioid agonist morphine – demonstrated that imiquimod had no effect on sensitivity to perception of heat. These results

imply that imiquimod did not act as either an opioid agonist or an antagonist. Thus, our investigation supports the novel finding that imiquimod depresses cell proliferation in an opioid-dependent manner *in vivo*, and does so in animals with complete or compromised (T-cell deficient) immune systems.

The present study shows that imiquimod inhibits processes of cell proliferation (i.e. DNA synthesis) in the epithelium. A number of previous reports also have reported a similar decrease in cell replication, such as in mouse hemangioendothelioma,¹⁸ human melanocytes¹⁹ and human squamous cell carcinoma.⁵ However, these earlier studies found an increase in apoptosis in cells exposed to imiquimod, presumably accounting for at least part of the depression in cell number. Previous *in vitro* studies demonstrated that imiquimod depressed DNA synthesis in the absence of altering apoptosis or necrosis.¹⁷ In the present study, the effect of imiquimod on reducing DNA synthesis was neutralized by concomitant treatment with the opioid antagonist naloxone.

An unexpected effect of topical imiquimod was that epithelium in locations distant from the drug application exhibited a subnormal number of cells undergoing DNA synthesis. This decrease in DNA synthesis was initially noted three days after a single imiquimod treatment, and extended until at least day 5. By day 7, the reduction in cells exhibiting DNA synthesis in epithelium located distant from the site of imiquimod treatment was extinguished, and the labeling index of these cells was comparable to that of cells from animals that were not treated with imiquimod. Although this is the first time that such a 'bystander' effect has been reported with imiquimod, it is of interest to note that imiquimod is known to exacerbate

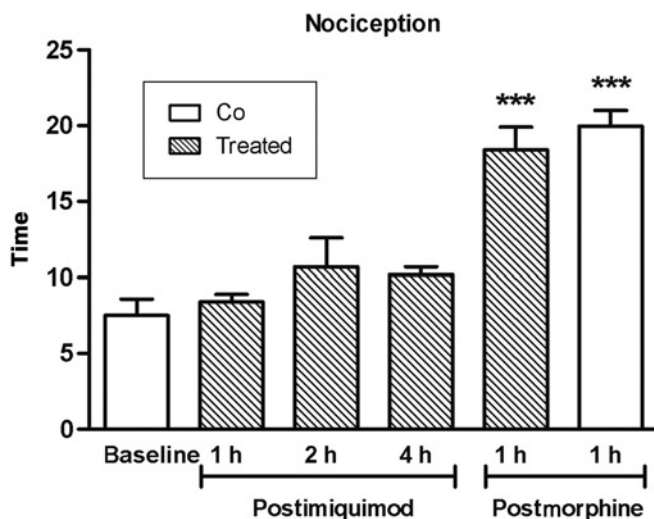


Figure 7 Imiquimod is neither an opioid agonist nor antagonist as determined by nociceptive response. Nociception in C57BL/6 mice was monitored by the hot-plate test at zero hours (baseline), and one, two or four hours after injection with 12 mg/kg imiquimod (postimiquimod). Some mice were injected with 10 mg/kg morphine or an equivalent volume of saline (Co) and examined one hour later (postmorphine). $n = 5$ mice/group. Values represent means $\pm$ SE. Significantly different from baseline at $P < 0.001$ (***). Co, control

psoriasis at distant skin sites.^{20–23} The mechanism underlying the bystander effect of imiquimod as to altering cell proliferation is unclear but our experiments showed that the corneal epithelium also was subnormal in DNA synthesis. Thus, the effect of imiquimod beyond the site of application may be due to systemic absorption. However, further studies are needed to clarify the mechanism of the bystander effect with imiquimod.

Our findings in the present study showing that imiquimod depresses cell proliferation of epithelial cells in an opioid-receptor-dependent manner under *in vivo* conditions extends previous observations¹⁷ on imiquimod, as well as this drug's relationship to endogenous opioid systems, in the epithelium. OGF and OGF α have been documented in the epithelium, and this peptide-receptor complex maintains homeostasis of cellular renewal through an inhibitory pathway to DNA synthesis.^{24,25} The interaction of imiquimod with OGF α was discovered by Urošević *et al.*⁷ initially by microarray analysis and subsequently by protein assessment that documented a notable elevation in OGF α with imiquimod exposure. Zagon *et al.*¹⁷ demonstrated that imiquimod's elevation in OGF α enhanced the interaction of OGF with OGF α and produced a robust inhibitory response that repressed cell proliferation using a tissue culture model. Moreover, the decrease in cell replication found to be associated with an increase in apoptosis related to imiquimod treatment, was not detected by Zagon *et al.*¹⁷ Additionally, resiquimod, which also depresses cell proliferation but does not cause apoptosis,¹⁷ functioned through the OGF–OGF α axis. A defining experiment in these previous studies was documenting that the knockdown of OGF α using siRNA technology diminished the inhibitory effect of imiquimod on cell proliferation. The present investigation has taken this earlier information a step further by showing that imiquimod action is dependent on opioid receptor mediation *in vivo*. Thus, placing all of these previous studies into perspective, the present results can be explained by an upregulation of OGF α by imiquimod that leads to an intensification of OGF–OGF α interactions, which in turn produces an exaggeration in function: depression of cell proliferation.

Imiquimod is an important drug in the clinical setting,^{1–5} and is ranked as the 101st pharmaceutical drug in 2009 with sales at over \$366 million dollars (Drugs.com). The information from the present study offers a number of insights into the mechanism – and utilization – of this agent. First, we now understand that though imiquimod is an immune response modifier, this drug works through an endogenous opioid system. As such, problems in the pathways of the OGF–OGF α axis (e.g. p16, karyopherin β) will reflect upon the efficacy of imiquimod in patients. Second, though imiquimod is usually administered a few times/week, in fact we now see that drug action can persist up to seven days after a single administration. This would suggest that to minimize problems (e.g. ulcers) that may occur with imiquimod, a longer interval (once or twice a week) between applications may avoid these complications. Third, individuals on naltrexone therapy for substance abuse may not benefit from imiquimod treatment since the upregulation of opioid receptors would be negated because of opioid receptor blockade by this drug. Fourth,

OGF is an immunosuppressant so that exacerbation of the OGF–OGF α axis by imiquimod may neutralize agents that are immunostimulants. Moreover, to avoid problems from chronic immunosuppression, less frequent application of imiquimod would be preferable. Fifth, the concentrations of imiquimod used for clinical practice may not be necessary, and investigation of a reduction in dosages should be explored. Sixth, there is no benefit from combining imiquimod with OGF or LDN to enhance imiquimod action. Seventh, at least for the two and one-half weeks tested, there is no tolerance of imiquimod's effects on cell replication. Eighth, imiquimod is neither an opioid agonist nor an opioid antagonist so there is no concern for accompanying problems such as physical dependence (in the case of an opioid agonist) or withdrawal (in the case of an opioid antagonist used in individuals with chronic consumption of opioids). Ninth, the bystander effect of imiquimod may suggest that an application of drug at one site holds significance at distant locations. Thus, application of imiquimod at one site may suffice for treatment of skin abnormalities located at some distance from this location.

Author contributions: All authors participated in the conception and overall design of the experiments. PJM conducted the experiments and supervised other laboratory personnel. PJM analyzed data presented in this manuscript. ISZ and PJM interpreted the results and wrote the manuscript.

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