

Imiquimod Upregulates the Opioid Growth Factor Receptor to Inhibit Cell Proliferation Independent of Immune Function

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The imidazoquinoline compounds imiquimod and resiquimod are low-molecular-weight immune response modifiers that have potent anti-viral and anti-tumor properties. The mechanism by which they exert their effects remains unclear. Using pancreatic and colorectal cancer cells, as well as squamous carcinoma cells of the head and neck in tissue culture, which eliminated the immune system and toll-like receptors, we show that the imidazoquinolines upregulate the Opioid Growth Factor receptor (OGFr), which in turn stimulates the interaction of the OGF-OGFr axis. This native, tonically active inhibitory pathway regulates cell proliferation by modulating cyclin dependent kinase inhibitors, resulting in a retardation of cells at the G₁-S interface of the cell cycle. Neutralization of OGF or knockdown of OGFr by siRNA technology eliminates the inhibitory effects of imidazoquinolines on cell replication. This exciting new knowledge of the mechanism of imidazoquinolines has important physiological relevance, and allows strategies to be developed for the use of these agents that will enhance effectiveness as well as attenuate side-effects. *Exp Biol Med* 233:968–979, 2008

Key words: cell proliferation; imiquimod; opioid growth factor; cancer; immunity

Introduction

The imidazoquinoline compounds imiquimod and resiquimod are immune response modifiers with potent anti-viral and anti-tumor properties (1–4). Imiquimod (Aldara, R-837, S-26308), the best characterized and most widely

used, is highly efficacious in the treatment of external genital and anal warts, basal cell carcinoma, actinic keratoses, Kaposi's sarcoma, chronic hepatitis C infection, and intraepithelial carcinoma. Additionally, imiquimod applied systemically in animal experiments has proven efficacy in a variety of transplantable tumors including melanomas, lung sarcomas, and mammary carcinomas (5). The mechanism of imiquimod action remains to be elucidated (1–4). Imiquimod has been reported to be a toll-like receptor-7 agonist (6), and its anti-tumor effect exerted by modification of the immune response and stimulation of apoptosis (7). The effects of imiquimod may be mediated by induction of various cytokines, including interferon (IFN)- α , IFN- γ , tumor necrosis factor- α (TNF α), interleukin (IL)-1 α , and IL-12 (1–8).

Imiquimod treatment has been reported to induce gene and protein expression of the opioid growth factor receptor (OGFr) (8). OGFr is a 62 kDa protein, with several bipartite nuclear localization signals (9). The native ligand for OGFr is the opioid growth factor (OGF). OGF, chemically termed [Met⁵]-enkephalin, is a pentapeptide that is constitutively expressed, autocrine produced, and secreted (10–12). The action of this inhibitory peptide is tonic, stereospecific, reversible, and not associated with differentiation, apoptosis, necrosis, migration, invasion, or adhesion (10–15). In tissue culture, OGF's effects are serum-independent, anchorage-independent, and occur at physiologically relevant concentrations in a wide variety of poorly and well-differentiated human cancer cell lines (10–15). The mechanism of OGF is targeted to DNA synthesis (16, 17), directed toward the G₁-S interface of the cell cycle (18–20), and focused on upregulation of cyclin-dependent kinase inhibitory pathways (19, 20).

The present study investigated whether the OGF-OGFr axis plays a role in the antineoplastic effects of imiquimod and resiquimod. A tissue culture model was chosen for study in order to eliminate the contribution of the immune system, as well as dependence on toll-like receptors (TLR). Three different types of cancers, pancreatic, colorectal, and

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squamous cell carcinoma of the head and neck, were chosen that have both OGF and OGF α , and are documented to have the OGF-OGF α pathway as a determinant of cell proliferation (10–12). The results provide compelling evidence that imiquimod and resiquimod treatment of cancer cells (i) increases OGF α , (ii) the OGF-OGF α axis is the pathway responsible for decreasing cell proliferation, and (iii) neither immunity nor TLR-related events are associated with drug action.

Materials and Methods

Cell Lines. Human pancreatic cancer cell lines MIA PaCa-2, BxPC-3, PANC-1, and Capan-2, human SCCHN cell lines UM-SCC-1 and Cal-27, as well as human colon adenocarcinoma cell lines HT-29 and HCT-116 were utilized. All cell lines except UM-SCC-1 were purchased from the ATCC (Manassas, VA); the UM-SCC-1 cell line (SCC-1) was obtained from The University of Michigan, Cancer Research Laboratory (Thomas E. Carey, Ph.D., Director). Descriptions of the cell lines have been reported in our previous work on OGF inhibition of proliferation (10–12). MIA PaCa-2, PANC-1, Cal-27, and SCC-1 cells were grown in Dulbecco's MEM (modified) media, whereas BxPC-3 and Capan-2 cells were grown in RPMI 1640 media; all media were supplemented with 10% fetal calf serum, 1.2% sodium bicarbonate, and antibiotics (5,000 Units/ml penicillin, 5 mg/ml streptomycin, 10 mg/ml neomycin). HT-29 and HCT-116 were grown in McCoy's 5A media supplemented with 10% fetal calf serum, sodium bicarbonate, and antibiotics. Cell cultures were maintained in a humidified atmosphere of 5% CO $_2$ /95% air at 37°C.

Receptor Binding Assays. Cells were prepared for binding assays as previously described (11). Nuclear-enriched protein samples were incubated for 60 minutes with shaking at 22°C with [3 H]-[Met 5]-enkephalin (custom-synthesized by Perkin-Elmer Life and Analytical Sciences, Boston, MA; specific activity, 41 Ci/mmol). Nonspecific binding was measured in the presence of unlabeled [Met 5]-enkephalin. Saturation isotherms and Scatchard plots were determined using GraphPad Prism software (San Diego, CA); K_d and B_{max} values were calculated by the software.

Cell Growth Assays. Cells were seeded at equivalent amounts into either 6- or 24-well plates (Falcon) and counted 24 hours later to determine plating efficiency. Compounds or sterile water (control) were added beginning 24 hours after seeding (=0 hours). Unless specifically stated, media and compounds were replaced daily. OGF was prepared in sterile water and imiquimod was dissolved in 100% DMSO to 10 $^{-2}$ M, and this solution was further diluted in sterile water to a final concentration of 10 $^{-6}$ M. Experiments treating cells with DMSO diluted in sterile water revealed no effect on growth with these low concentrations of DMSO (data not shown). For assays other than the dose response experiments, imiquimod was

utilized at 2 $\times$ 10 $^{-6}$ M and OGF at 10 $^{-6}$ M, a concentration of OGF that has demonstrated growth inhibition (10–12).

At appropriate times, cells were harvested by trypsinization with 0.25% trypsin/0.53 mM EDTA, centrifuged, and counted with a hemacytometer. Cell viability was determined by trypan blue staining. At least two aliquots per well, and 2–6 wells/treatment, were counted. Experiments were conducted two to five times.

Doubling times were calculated from growth over a 96-hour period of time using linear regression analyses.

DNA Synthesis, Apoptosis and Necrosis. To determine the mechanisms of action of imiquimod, DNA synthesis (BrdU), apoptosis (TUNEL), and necrosis (trypan blue) were evaluated; in some cases, exogenous OGF was added to the cultures to compare the effects of exogenous and endogenous OGF. To examine DNA synthesis, cells were seeded on to 22 mm diameter coverglasses placed in 6-well plates (3 $\times$ 10 3 cells/coverglass) and treated over a 72-hour period. Three hours prior to fixing cells, 30 μ M BrdU was added to the cultures. At appropriate times, cells were rinsed, fixed in 10% neutral buffered formalin, and stained with antibodies to BrdU (Invitrogen, Eugene, OR). The number of positive cells was recorded; at least 1000 cells/treatment at each time were counted.

TUNEL staining was performed to assess apoptosis (15). Cells were seeded on to coverglasses in 6-well plates and treated with imiquimod and/or OGF beginning 24 hours later. Cells were harvested after 72 hours of treatment and stained. Three samples from every treatment were analyzed. The number of positive cells was recorded; at least 1000 cells/treatment at each time were counted.

Necrosis was evaluated at the time of counting cells for growth curves. Trypan blue uptake is indicative of necrosis.

Specificity of Endogenous OGF. To determine the specificity of endogenous OGF, cultures of imiquimod treated cells were exposed to a polyclonal antibody to OGF (1:200; CO172) (10, 21). Controls included cultures exposed to pre-immune rabbit serum (1:200). Compounds and media were changed daily. Cell viability was determined by trypan blue staining. At least two aliquots per well, and 2–6 wells/treatment, were counted.

Immunohistochemistry. To examine for the presence of OGF α , log-phase cells were plated on to 22 mm round coverglasses and collected 72 hours after treatment with 2 $\times$ 10 $^{-6}$ M imiquimod or sterile water. Cells were fixed and permeabilized in 95% ethanol and acetone at –20°C, and stored at –20°C for no more than 7 days before processing. Coverglasses were processed according to previously published procedures (10–12), and stained with ammonium sulfate purified anti-OGF α -IgG (1:100; I0028) (21) diluted in Sorenson's phosphate buffer with 1% normal goat serum in 0.1% Triton X-100 for 18 hours at 4°C. Coverglasses were washed and incubated with goat anti-rabbit IgG (1:1000) conjugated to rhodamine and viewed with fluorescence microscopy. Cells incubated with secondary antibody only served as controls.

Semi-quantitative densitometry (mean gray values) was utilized to determine levels of receptor using Optimas software (Meyer Instruments, Inc., Houston, TX). A random sample of 10 fields/coverglass from at least 2 coverglasses/treatment was captured using a SPOT RT camera (Diagnostic Instruments, Stirling Heights, MI).

siRNA Knockdown of OGFr. The OGFr-targeted siRNAs (antisense: 5'-uagaaacucagguuugcg-3'; sense: 5'-cgccaaaccugaguucua-3') were designed and obtained as ready-annealed, purified duplex probes from Ambion (Austin, TX). For transfection, 5×10^4 cells per well were seeded in 6-well plates containing 1 ml of serum-containing media without antibiotics. In each well, 20 nM OGFr-siRNA or control siRNA solutions in serum-free media were added. Cells were incubated for 4 hours at 37°C prior to the addition of either OGF (10^{-6} M) or an equivalent volume of sterile water. Cultures were incubated for an additional 20 hours, and then 2 ml fresh complete media either lacking or containing OGF was added. At 72 hours, cells were collected for computing growth. Two independent experiments were conducted. The control siRNAs were purchased from Ambion.

Chemicals. Imiquimod was obtained as a crystalline powder (>99.0% purity) from Sequoia Research Products (Oxford, United Kingdom), resiquimod (R-848) was acquired from PharmaTech (Shanghai, China) as powder (98.8% purity), and OGF and naloxone were purchased from Sigma-Aldrich Chemicals (St. Louis, MO).

Statistical Analyses. All data were analyzed using Student's two-tailed *t* test or analysis of variance (ANOVA) with subsequent comparisons made using Newman-Keuls tests.

Results

Imiquimod Treatment Upregulates OGFr Binding in a Dose-Dependent Manner. MIA PaCa-2 cultures treated with 2, 5, or 10×10^{-6} M imiquimod for 3 days had binding capacity values for OGFr that were 2.2- to 2.6-fold more than for control cells (Fig. 1A); no differences were observed between imiquimod treatments. The K_d values for all cultures were comparable to control values (4.9 ± 0.7 nM). Scatchard plots (Fig. 1B) and computer analysis revealed a single binding site. Hence, the lowest dosage of imiquimod (2×10^{-6} M) was utilized for subsequent growth studies to eliminate or reduce any potential side-effects of toxicity and/or apoptosis. The duration of imiquimod effects lasted for 48 hours (Fig. 1C). Chronic (daily) imiquimod treatment revealed significant elevations in receptor number for up to 10 days, with mean B_{max} values elevated up from 2.1- to 5.7-fold from control levels (Fig. 1D). Chronic imiquimod exposure did not alter the binding affinity for OGFr.

Imiquimod Treatment Does Not Disturb Cellular Morphology and Upregulates Expression of Receptor Using Quantitative Immunohistochemis-

try. Log phase MIA PaCa-2 cells treated with imiquimod for 72 hours were comparable in structure to cultures subjected to vehicle (sterile water) as recorded by differential interference microscopy (Fig. 2A). These cells were generally spherical in shape, approximately 6–9 μ m in diameter, and often had one or two small processes. Staining with antibody to OGFr revealed a similar pattern of distribution in control and imiquimod treated cultures (Fig. 2A), with all cells being immunopositive. Analysis of brightness (i.e., fluorescence) utilizing densitometric measurements showed a 2.1-fold greater intensity (mean gray value) in cells subjected to imiquimod relative to control specimens (Fig. 2B).

Overexpression of OGFr Induced by Imiquimod Depresses Cell Growth. Log-phase cultures treated with imiquimod (2, 5, or 10×10^{-6} M) had 40–45% fewer cells than control cultures (Fig. 3A). Examination of growth curves of cells exposed to 2×10^{-6} M imiquimod demonstrated that at 48, 72, and 96 hours, imiquimod-subjected cultures contained 28 to 34% significantly fewer cells than in control cultures; at 24 hours no differences were noted (Fig. 3B). These results show that the autocrine action of endogenous OGF with OGFr has a marked effect on homeostatic cell proliferation.

In order to ascertain whether the inhibitory growth effects were opioid receptor mediated, log-phase cell cultures of MIA PaCa-2 were exposed to imiquimod (2×10^{-6} M) and/or naloxone (5×10^{-5} M); cells treated with sterile water served as control (Fig. 3C). Imiquimod treated cultures were decreased 26% in cell number from control levels. Pancreatic cancer cells subjected to both imiquimod and naloxone had a comparable cell number as controls, indicating that naloxone blocked the growth inhibition in cultures with an overexpression of OGFr. Naloxone alone had no effect on growth, thereby showing that the effect of both compounds was not the sum total of a decrease in growth by imiquimod and an increase of growth by naloxone.

To discern the mechanism(s) underlying the decrease in cell growth by imiquimod exposure, DNA synthesis and cell death were evaluated in cultures treated with imiquimod or sterile water for 72 hours (Fig. 3D). The number of BrdU positive cells in cultures exposed to imiquimod was decreased by 61% of control levels. Evaluation of TUNEL preparations for apoptotic cells, and trypan blue stained material for necrosis, revealed no differences between imiquimod and control cultures (≤ 1 in 1,000 cells were apoptotic or necrotic).

In order to establish whether the endogenous ligand for the upregulated OGFr was OGF, cells were treated with imiquimod and an antibody to OGF. These cultures contained 24% more cells than control preparations, and 45% more cells than those receiving imiquimod only (Fig. 3E). Exposure to pre-immune serum had no effect on growth. These results reveal that neutralization of endogenous OGF by an antibody, attenuates the growth effects of

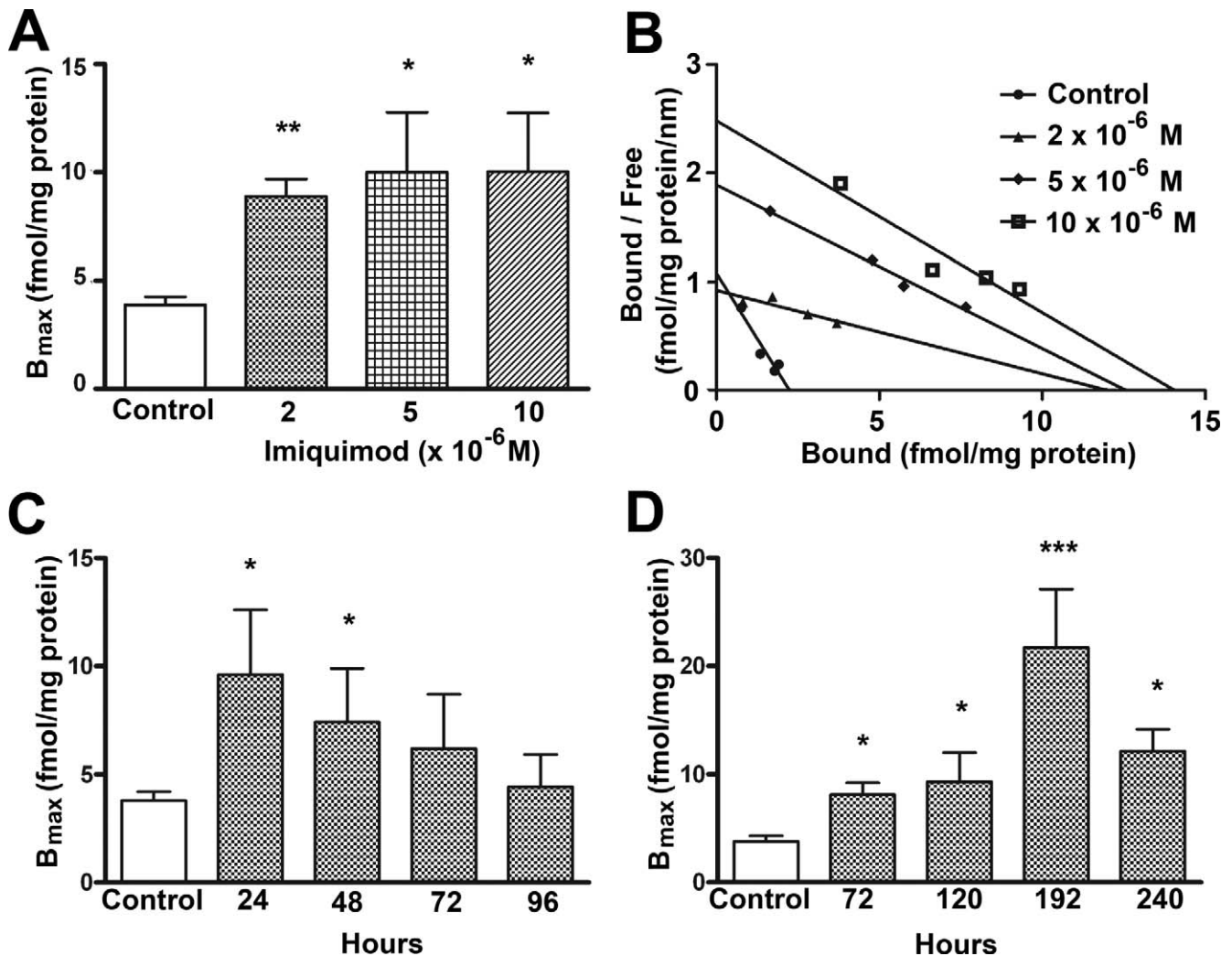


Figure 1. Imiquimod treatment upregulates OGFr in MIA PaCa-2 cells. (A) Dose-response experiments. Histogram of binding capacity values (B_{max}) of log phase cells treated for 3 days with 2, 5, or 10×10^{-6} M imiquimod, or an equivalent volume of sterile water (Control); media and drugs were changed daily. (B) Representative Scatchard plots of specific binding in A. (C) The effects of an acute exposure to imiquimod. Histogram of B_{max} of log phase cells treated for 24 hours with 2×10^{-6} M imiquimod, or an equivalent volume of sterile water (Control). (D) The effects of chronic administration to imiquimod. Histogram of B_{max} of log phase cells treated daily with 2×10^{-6} M imiquimod for up to 10 days, or an equivalent volume of sterile water (Control); media and drugs were changed daily. Values represent means $\pm$ SEM for at least 3 independent binding assays. Significant from Control at $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***).

imiquimod, thereby confirming that this peptide is specifically involved with imiquimod action.

To further define the role of OGF on imiquimod activity, cells were treated with OGF (10^{-6} M), imiquimod (2×10^{-6} M), or both imiquimod and OGF for 72 hours (Fig. 3F). Compared to control values, exogenous OGF depressed cell number by 21%, whereas imiquimod treated cultures contained 26% fewer cells. The latter decrease in cell number is consistent with an earlier experiment (see Fig. 3A), and reflects the activity of endogenous OGF on cells with more OGFr. The combination of imiquimod exposure and addition of exogenous OGF depressed cell number by 46% of control levels. These results of additive growth inhibition by imiquimod and exogenous OGF show that imiquimod generates more OGFr than can be occupied

by endogenous OGF, and thus can interact with additional exogenous OGF and facilitate enhanced growth inhibition.

The Growth Inhibition of Cell Replication by Imiquimod Treatment Is Ubiquitous. To understand whether upregulation of OGFr by imiquimod treatment inhibits the course of cell replication in other human pancreatic cancer cells, Capan-2, PANC-1, and BxPC-3 human pancreatic cell lines were examined (Fig. 4). Cell number was decreased from controls in all 3 cell lines exposed for 72 hours to imiquimod with decreases ranging from 22 to 49%. To further investigate whether the effect of imiquimod was cancer type dependent, several human cell lines of both squamous cell carcinoma of the head and neck (SCCHN) and colorectal adenocarcinoma were studied. In SCCHN, imiquimod treatment depressed growth of CAL-27 and SCC-1 cell lines by 35% and 26%, respectively,

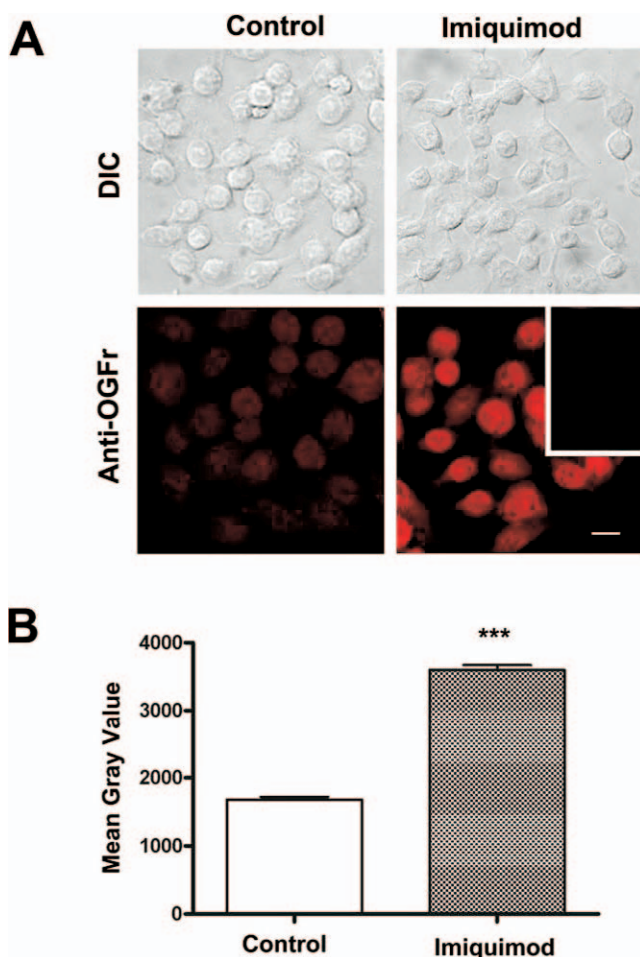


Figure 2. The upregulation of OGFr by imiquimod documented by immunohistochemistry and quantitative analysis. (A) Cells treated with 2×10^{-6} M imiquimod or an equivalent volume of sterile water (Control) for 3 days and photographed with differential interference contrast (DIC) or fluorescent optics following staining with polyclonal antibody to OGFr (Anti-OGFr). Inset = secondary antibody only. Bar = 5 μ m. (B) Quantitation of immunohistochemical preparations of imiquimod-treated and control cells stained with anti-OGFr. Mean gray value $\pm$ SEM for 10 fields/coverslip, and at least 2 coverslips/treatment. Different from control levels at $P < 0.001$ (***). A color version of this figure is available in the online journal.

whereas colorectal cancer lines HCT-116 and HT-29 each exhibited decreases of 46% following 72 hours of treatment with 2×10^{-6} M imiquimod (Fig. 4).

Calculation of doubling times revealed that all cell lines subjected to imiquimod had 12% to 82% longer doubling times than their respective controls (Fig. 5) with 7 of the 8 cell lines having a statistical difference from their control (sterile water) counterparts.

Silencing of OGFr in Imiquimod Treated Cultures Attenuates the Inhibitory Action of Endogenous and Exogenous OGF. The specific molecular nature of the receptor that is upregulated by imiquimod exposure and responsible for alterations in cell proliferation was investigated by siRNA experiments. Pretreating MIA PaCa-2 cells (wild-type and transfected with negative siRNA) with imiquimod for 72 hours upregulated OGFr

mRNA levels by 30% compared to untreated cultures (wild-type or transfected with negative siRNA) (controls) (Fig. 6A, B). Using a 19 nucleotide oligomer, specific for OGFr (9), Northern blot analysis (Fig. 6A, B) showed that in comparison to log phase cells that were not transfected or receiving negative siRNA for 24 hours, cultures subjected to OGFr siRNA had a decrease of 52% in OGFr mRNA. Similarly, in contrast to log phase cultures pretreated with imiquimod for 72 hours and transfected for an additional 24 hours, cells receiving siRNA for OGFr had a 46% decrease in OGFr mRNA in relationship to cultures that were not pretreated with imiquimod, not transfected, or transfected with negative siRNA.

Cultures exposed to OGFr siRNA and exogenous OGF had 65% more cells than those cultures receiving negative siRNA and OGF, and a 35% increase in cell number from untreated cultures that were either not transfected or transfected with negative siRNA (Fig. 6C). Cell number did not differ between untreated cultures that were not transfected or transfected with negative siRNA (Fig. 6C). However, untreated cultures transfected with OGFr siRNA were increased in number by 33%. These results demonstrate that neutralization of OGFr by siRNA removes the inhibitory influence of endogenous or exogenous OGF on cell proliferation.

Cultures pretreated with imiquimod and exposed to OGFr siRNA and exogenous OGF had 54% more cells than those cultures receiving negative siRNA and OGF, and a 17% increase in cell number from imiquimod treated cultures that were either not transfected or transfected with negative siRNA (Fig. 6D). Cell number did not differ between these latter two cultures. However, imiquimod cultures transfected with OGFr siRNA were increased in number by 13%. These data demonstrate that neutralization of OGFr upregulated by imiquimod removes the inhibitory influence of endogenous or exogenous OGF on cell proliferation.

Resiquimod Upregulates OGFr in Two Human Cancer Cell Types. Resiquimod, another member of the imidazoquinoline family, increased OGFr number in log-phase MIA PaCa-2 and SCC-1 cells by 5.8- and 2.1-fold, respectively, relative to control values (Fig. 7A, B); no differences in binding affinity were recorded. Moreover, resiquimod treatment inhibited cell growth by 10–43% in a dose-dependent manner relevant to control cultures (Fig. 7C, D).

To provide further evidence that resiquimod action is also mediated by the OGF-OGFr axis, siRNA technology was utilized to knockdown OGFr and cell growth was monitored. MIA PaCa-2 and SCC-1 cultures exposed to OGFr siRNA and exogenous OGF had 17% and 31%, respectively, more cells than those cultures not transfected (Fig. 7E, F). In comparison to cultures transfected with a negative siRNA but receiving exogenous OGF, there was a 67% and 80% elevation in cell number for MIA PaCa-2 and SCC-1 cultures, respectively; this demonstrates that the inhibitory effect of exogenous OGF was abolished when

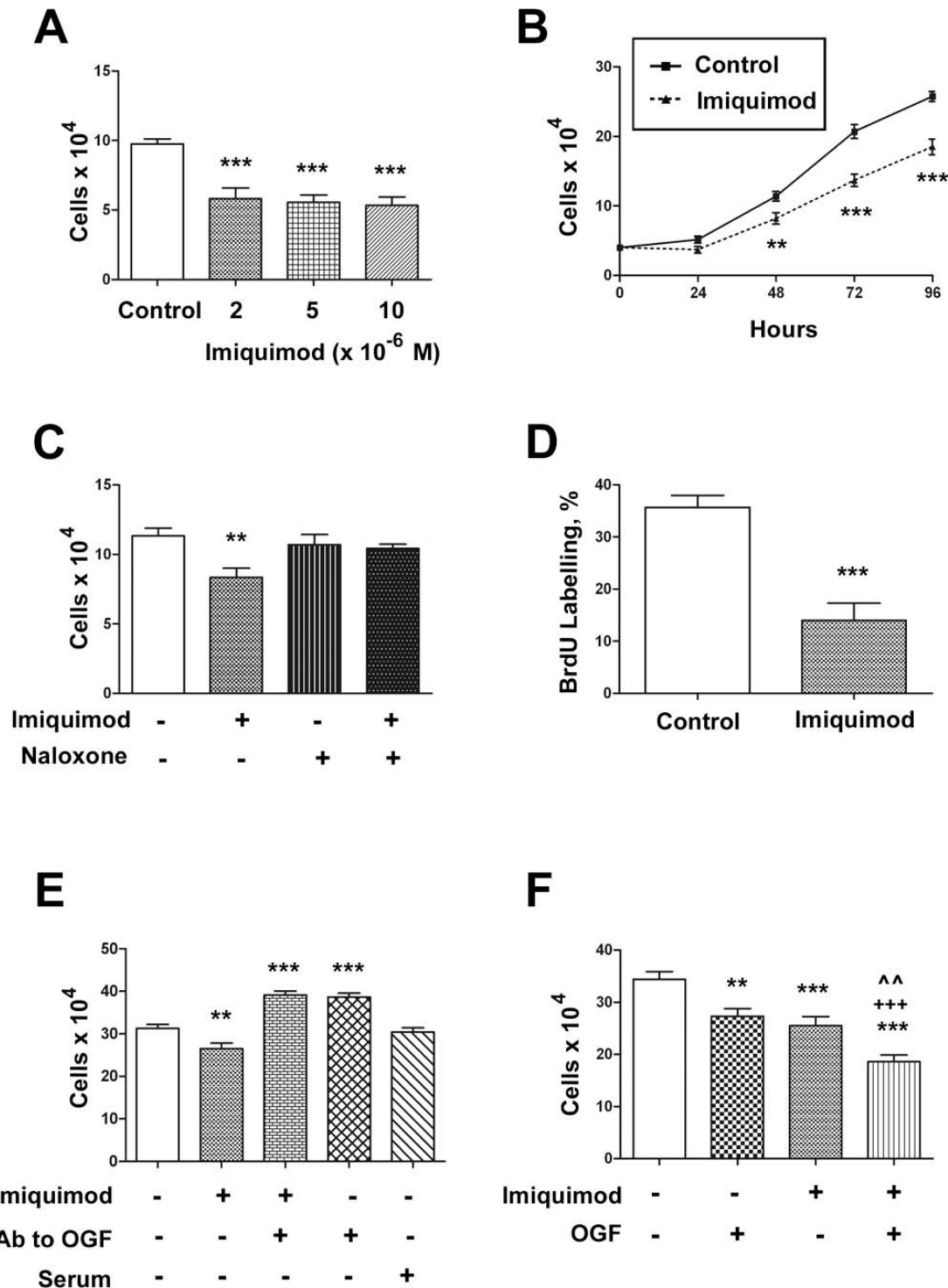


Figure 3. Upregulation of OGFr by imiquimod inhibits cell growth of MIA PaCa-2 cells. (A) Histogram showing 40–45% fewer cells in cultures exposed to 2, 5, or 10×10^{-6} M imiquimod for 72 hours in contrast to control levels. (B) Growth curves of cells treated with 2×10^{-6} M imiquimod or an equivalent volume of sterile water (Control) and examined over a 96-hour period. (C) Histogram of the effects of imiquimod (2×10^{-6} M), the opioid antagonist naloxone (5×10^{-5} M), or imiquimod and naloxone for 72 hours in order to examine opioid receptor mediation. (D) DNA synthesis in cells treated with imiquimod (2×10^{-6} M) or an equivalent volume of sterile water (Control) for 72 hours, and incubated in BrdU for 3 hours prior to fixation. (E) Cells treated with 2×10^{-6} M imiquimod or an equivalent volume of sterile water (Control), and antibody to OGF (1:200) or pre-immune serum (1:200), were examined at 72 hours. (F) Growth effects of exogenous OGF on imiquimod treated cells. Cells with exogenous OGF (2×10^{-6} M), imiquimod (2×10^{-6} M), imiquimod and OGF, or an equivalent volume of sterile water for 72 hours. For all experiments, compounds and media were changed daily. Values in A, B, C, and E represent means $\pm$ SEM for 2–6 wells/treatment group, whereas D represents means $\pm$ SEM for at least 1,000 cells/treatment. Significantly different from controls at $P < 0.01$ (**) or $P < 0.001$ (***), and in Panel F from cultures treated with OGF only at $P < 0.001$ (+++), and from cultures exposed to imiquimod only at $P < 0.01$ (^ ^).

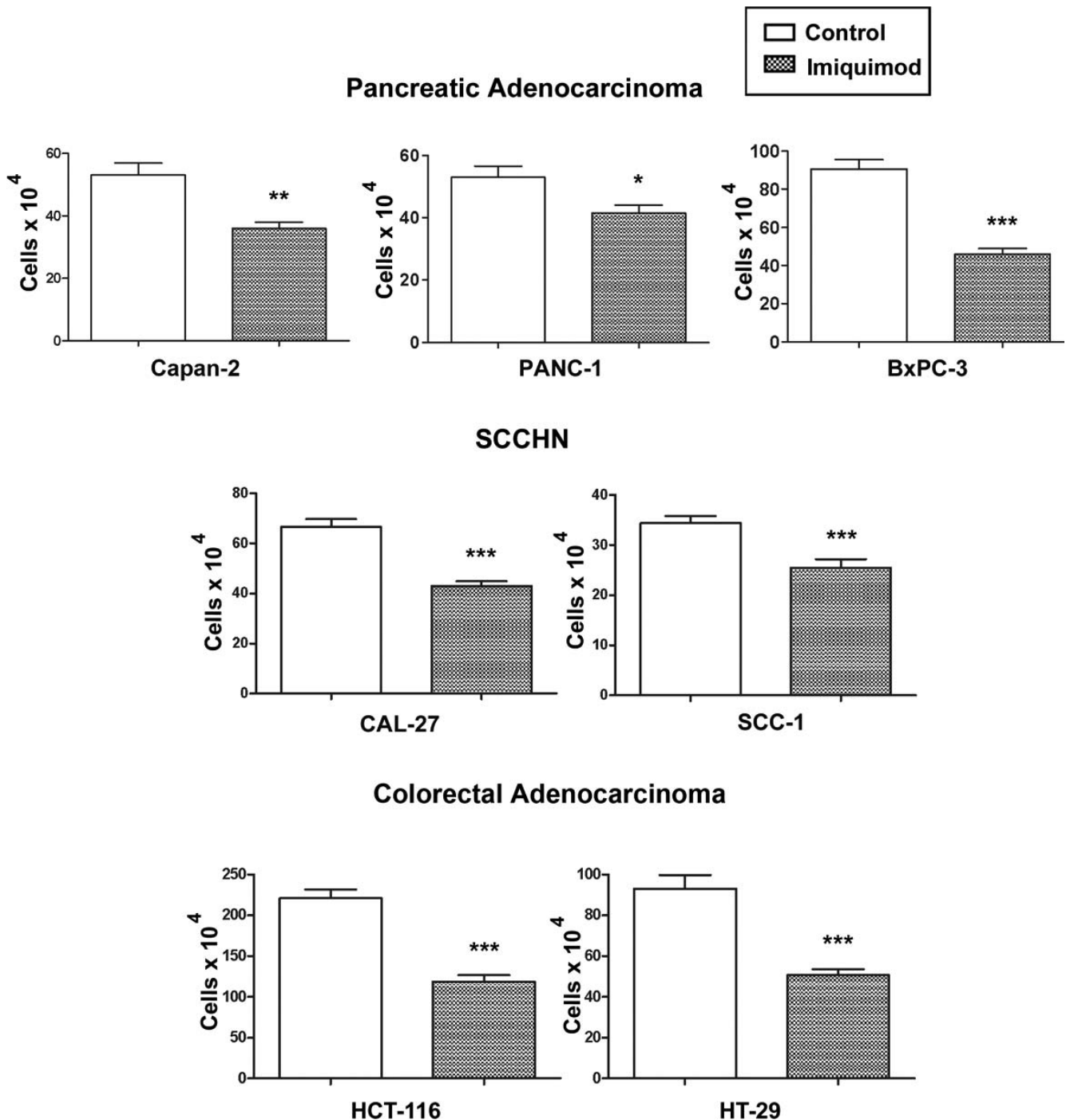


Figure 4. Growth inhibition induced by upregulation of OGF α by imiquimod is ubiquitous across cancer types. Seven cell lines representative of pancreatic adenocarcinoma, SCCHN, and colorectal adenocarcinoma were examined in cultures treated for 72 hours with 2×10^{-6} M or an equivalent volume of sterile water (Control); compounds and media were replaced daily. Values represent mean $\pm$ SEM for cell numbers; 2–6 wells/treatment group were evaluated. Significantly different from Control at $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***).

OGF α is knocked down by siRNA. In both cell types, cell number was not different between groups given sterile water and not transfected or transfected with negative siRNA. MIA PaCa-2 or SCC-1 cells receiving negative siRNA and OGF had 30% and 25%, respectively, fewer cells relative to their respective wild-type cells or those receiving sterile water and either no transfection or negative siRNA.

When resiquimod pretreated cells were transfected with siRNA for OGF α the addition of OGF had no effect, with cell numbers for the MIA PaCa-2 and SCC-1 cultures being 28% and 39%, respectively, greater than non-transfected cultures (Fig. 7G, H). No differences in cell number were observed in resiquimod treated cultures that were not transfected or transfected with negative siRNA. However,

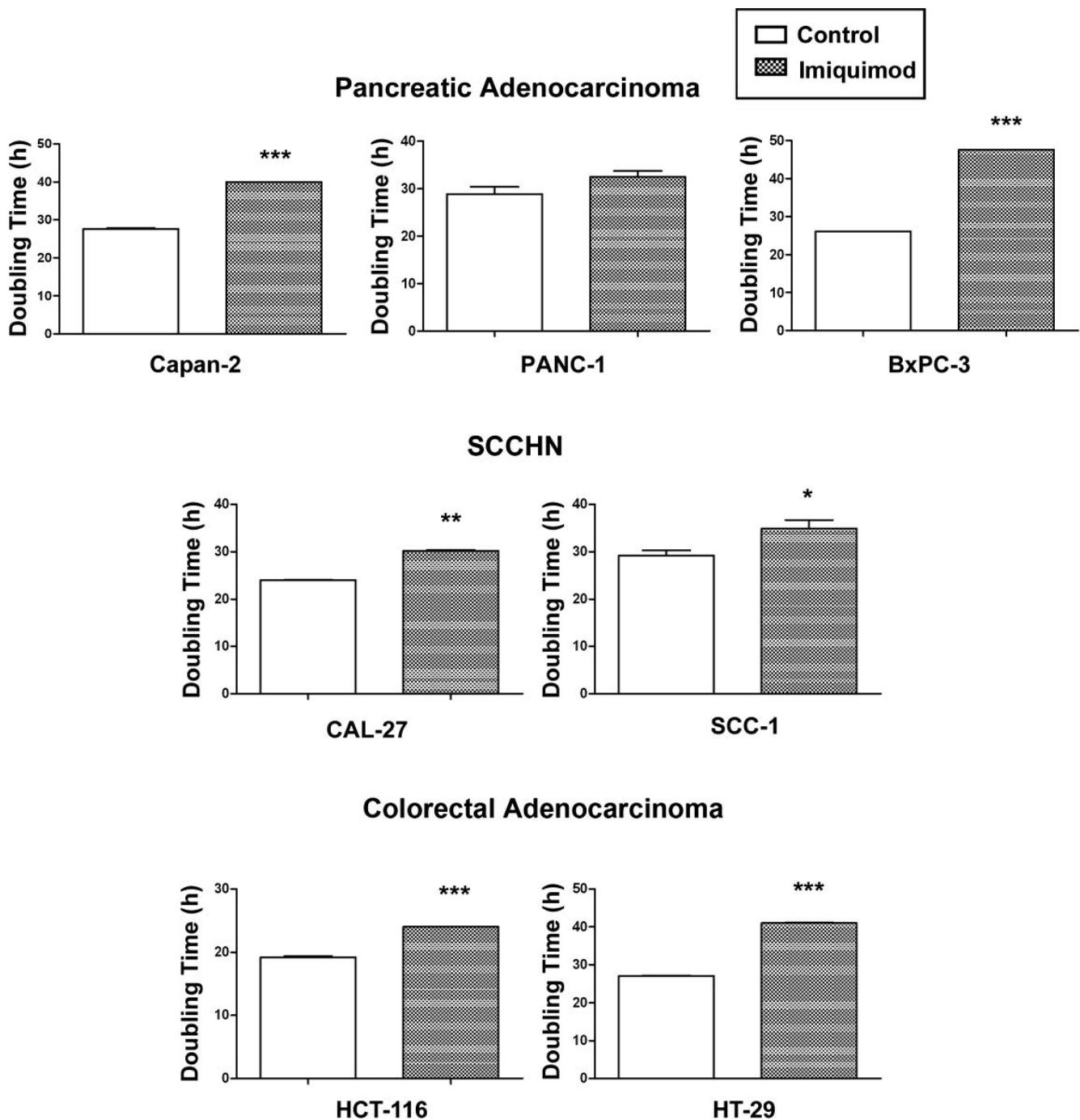


Figure 5. Doubling times of cells treated with imiquimod (2×10^{-6} M) were markedly extended from controls receiving an equivalent volume of sterile water (Control). Doubling times were calculated from 2–8 growth curves/cell line over a 96-hour period of time for human pancreatic adenocarcinoma (A), SCCHN (B), and colorectal (C) cancer cell lines. Values represent mean $\pm$ SEM. Significantly different from respective control doubling times at $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***).

MIA PaCa-2 or SCC-1 cells treated with siRNA for OGF α and receiving sterile water were increased by 31% and 40%, respectively, from cells not transfected or transfected with negative siRNA. In resiquimod pretreated MIA PaCa-2 and SCC-1 cells administration of exogenous OGF to cultures that were transfected with negative siRNA there were

decreases in cell number of 26% and 20%, respectively, compared to non-transfected cultures.

Discussion

This study shows for the first time that imiquimod, a clinically important drug, represses cell proliferation by way

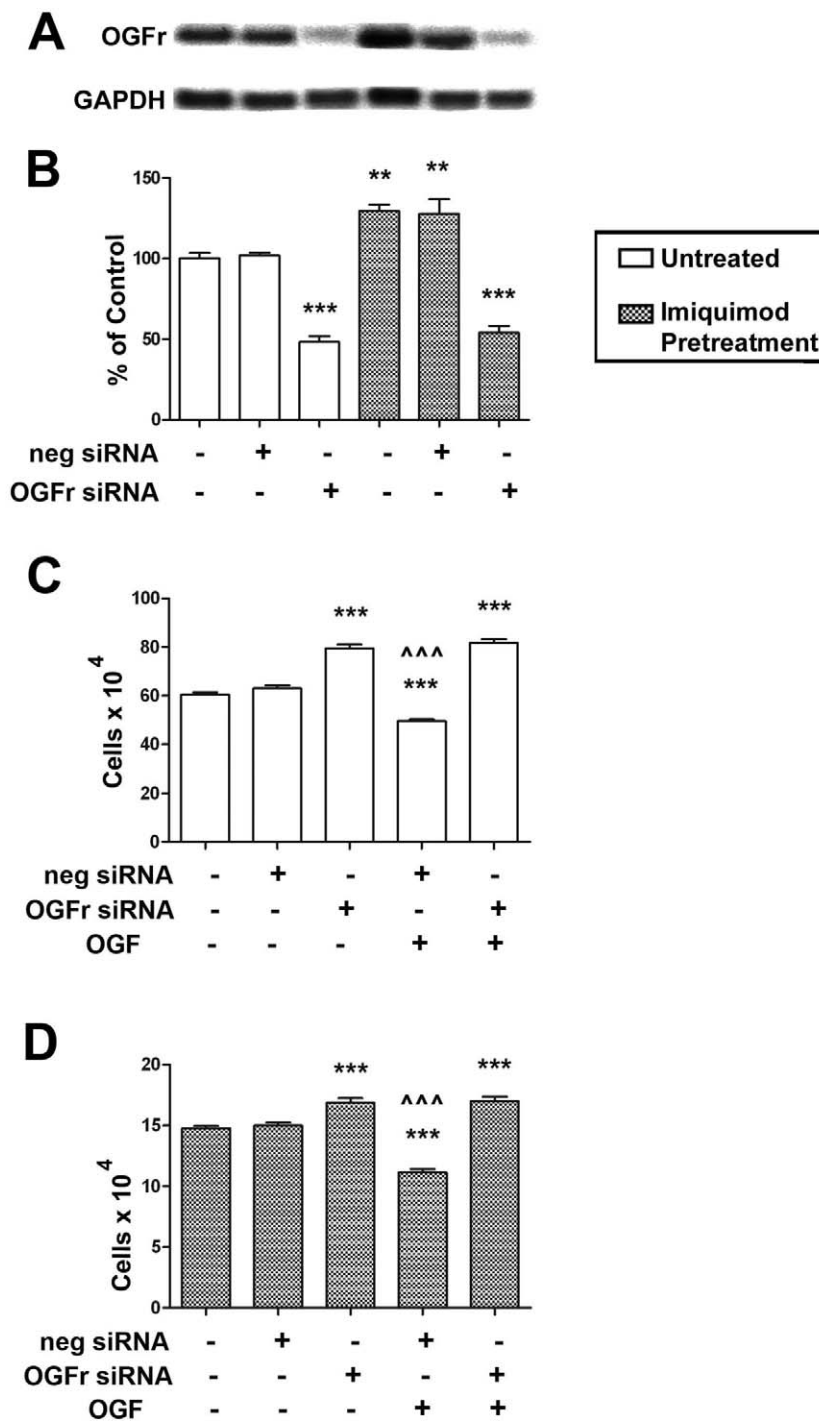


Figure 6. The effects on growth by the upregulation of imiquimod on OGFr are neutralized by silencing the mRNA for OGFr. (A) Northern blot analysis demonstrating the specificity of the OGFr siRNA in untreated and imiquimod-pretreated (Imiquimod Pretreatment) MIA PaCa-2 cells for 72 hours prior to being transfected; imiquimod treatment was discontinued after 72 hours. Cells were reseeded and transfected for 24 hours with either negative siRNA or OGFr siRNA. Three days after transfection, cells were harvested and RNA isolated. (B) Quantitative densitometry of the Northern blots in (A). Data (percent of OGFr/GAPDH ratio for Control (non-transfected cells)) represent mean $\pm$ SEM for 2 blots from independent experiments. Significantly different from non-transfected cultures at $P < 0.01$ (**) or $P < 0.001$ (***). (C, D) Growth of untreated cultures (C) or cells pre-treated for 72 hours with 2×10^{-6} M imiquimod (D). Cultures were transfected for 24 hours and treated with either OGF (10^{-6} M) or an equivalent volume of sterile water for an additional 48 hours; compounds and media were changed daily. Values represent mean $\pm$ SEM cell counts for 2 aliquots/well and 2 wells/treatment. Significantly different from cultures that were not transfected as well as cells that were transfected with negative siRNA at $P < 0.001$ (***). Significantly different from cultures transfected with OGFr siRNA and receiving OGF at $P < 0.001$ (^^^).

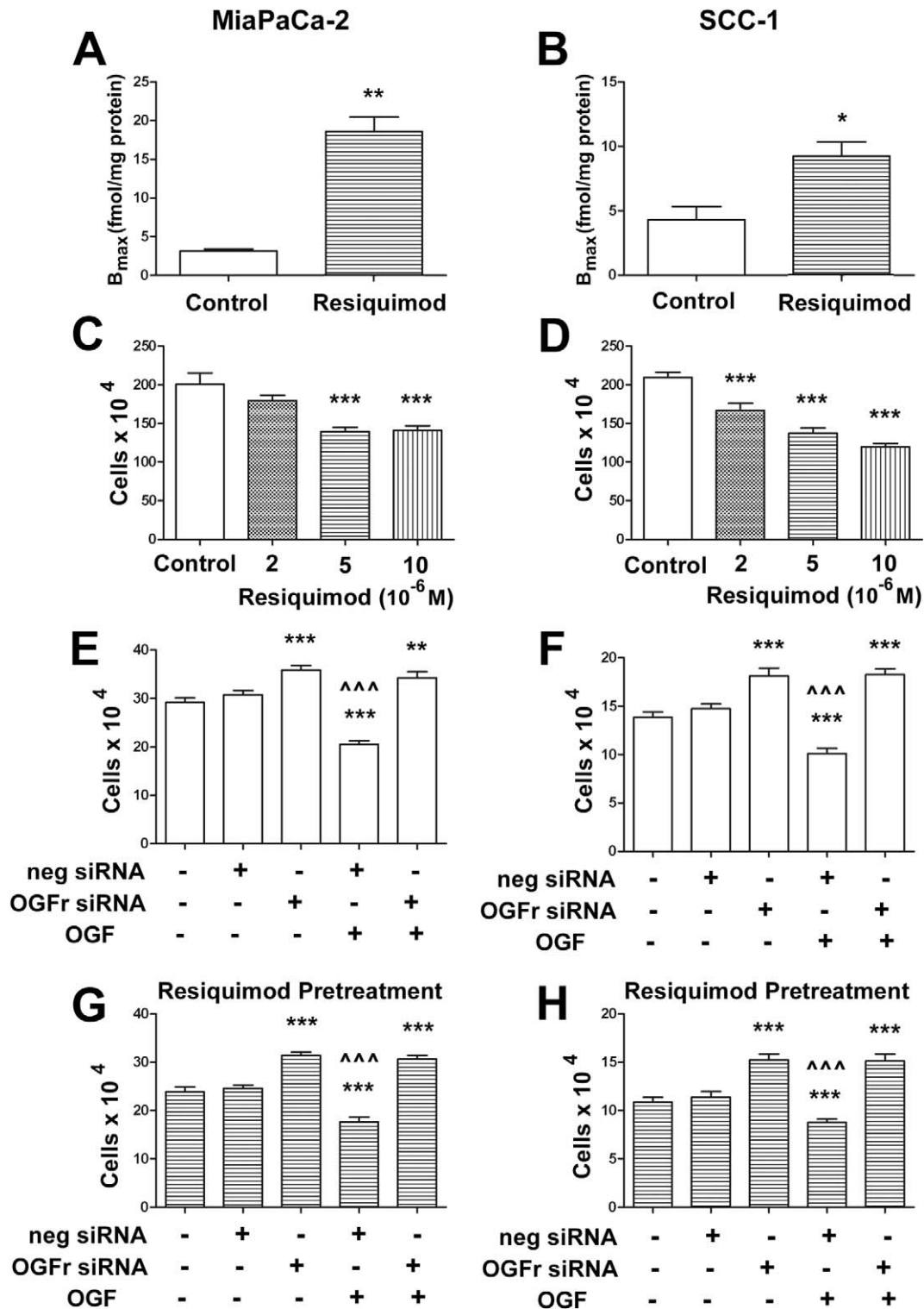


Figure 7. Characterization of MIA PaCa-2 and SCC-1 cells exposed to resiquimod. (A, B) Histograms of binding capacity values (B_{max}) of log-phase cells treated with 5×10^{-6} M resiquimod for 3 days. Values represent means $\pm$ SEM for at least 3 independent experiments. Significantly different from Control levels at $P < 0.05$ (*) or $P < 0.01$ (**). (C, D) Histograms of cell number in cultures exposed to 2, 5, or 10×10^{-6} M resiquimod for 72 hours or sterile water (Control). Values represent means $\pm$ SEM for at least 2 wells/treatment. Significantly different from Controls at $P < 0.001$ (***). (E–H) Growth of cultures either not pretreated (E, F) or pretreated (G, H) for 72 hours with 5×10^{-6} M resiquimod. In some cultures, cells were treated with either OGF (10^{-6} M) or an equivalent volume of sterile water for an additional 48 hours. Compounds and media were changed daily. Data represent mean $\pm$ SEM for 2 aliquots/well, and 2 wells/treatment. Significantly different from cultures with no transfection as well as those transfected with negative siRNA at $P < 0.01$ (**) or $P < 0.001$ (***). Significantly different from cultures transfected with OGFr siRNA and receiving OGF at $P < 0.001$ (^^^).

of the OGF-OGFr axis and does so independent of immune response and TLR agonism. Initial experiments, using rigorous statistical methodology, validated and extended an earlier study (8) based on subjective results that imiquimod upregulates OGFr. Imiquimod exposure increased OGFr gene expression, as well as the number but not the affinity of OGFr at the protein level. The repercussions of this upregulation of OGFr had a marked effect on function. Cell number was subnormal following imiquimod treatment, but this depression was not related to cytotoxicity. Rather, imiquimod exposure inhibits DNA synthesis. The effect of imiquimod on repressing cell replication was dependent on endogenous OGF as shown in antibody neutralization experiments. In fact, exogenous OGF administration even further reduced cell number in imiquimod-treated cultures, suggesting that endogenous OGF was not capable of occupying all of the upregulated OGFr. Knockdown experiments of OGFr in imiquimod treated cells demonstrated that neither endogenous nor exogenous OGF had any effect on growth. Indeed, cell number was markedly elevated in cultures exposed to OGFr siRNA because they were released from regulation by the OGF-OGFr axis. These inhibitory effects of imiquimod were not limited to a single cell line or cancer, but were observed in 8 human cancer cell lines representing 3 different types of neoplasia. Thus, our study makes the novel finding that the upregulation of the OGF receptor by imiquimod stimulates the interfacing of OGF with OGFr, which in turn enhances depression of cell replication. Importantly, all of these functional repercussions are direct and take place in the absence of the immune system.

A decrease in growth and/or an increase in apoptosis has been observed previously in imiquimod treated human cell cultures, including squamous cell carcinomas (7), spontaneously immortalized keratinocytes (7), epithelial cells (22), keratinocytes (22), and bladder cancer cells (23). Our results are consonant with depressed growth of a wide variety of cancer cell lines consequent to imiquimod exposure, even though we used at least 10-fold lower drug concentrations. However, apoptosis (or necrosis) was not detected in our study, perhaps because of these lower imiquimod concentrations. In contrast, Urošević et al. (8) detected no effect of imiquimod on growth or survival of primary human basal cell carcinoma cell lines. A lack of reduced cell number after imiquimod exposure may be due to the human basal cell carcinoma cell lines utilized that had doubling times of 14 to 30 days but the effects of imiquimod were only monitored for 24 hours.

Resiquimod (R-848, S-28463), another imidazoquinoline (1–4, 7), also is an immune response modifier *in vitro* and *in vivo*, and exhibits antiviral and anti-tumor activity as well as increasing cytokine production. In fact, resiquimod has been reported to be more potent at inducing cytokine expression than imiquimod (24). However, unlike imiquimod, resiquimod does not exert pro-apoptotic effects (7), indicating that pro-apoptotic effects by imidazoquinolines

may not be the underlying mechanism of drug action. Yet, we now see herein that this does not hold for the growth inhibitory effects, whereby 2×10^{-6} M imiquimod inhibited MIA PaCa-2 growth by $\sim 50\%$ whereas 5×10^{-6} M resiquimod only repressed growth by $\sim 26\%$. The present study does show that the effects of resiquimod appeared to be mediated at the level of the OGF-OGFr axis. Resiquimod treatment resulted in depressed cell proliferation, and did so in an opioid receptor manner (data not shown). Most importantly, knockdown of OGFr eliminated the inhibitory effects of resiquimod on cell replication. These results demonstrate that the repressive effect on cell replication by imiquimod and resiquimod can be explained by a common mechanism of action that lies at the level of the OGF-OGFr axis.

Upregulation of OGFr, and the functional consequences of increased OGFr, have been demonstrated before using a variety of techniques. OGFr cDNA delivered to the normal and abraded rat cornea by a gene gun has been found to depress DNA synthesis of basal cells in the corneal epithelium, and to delay re-epithelialization (25, 26). Overexpression of OGFr in human cancer cells (27, 28) with cDNA has been shown to diminish cell replication. The pathway of the OGF-OGFr axis has been partially elucidated so that OGFr is known to be synthesized on the outer nuclear envelope where it binds to OGF, and the OGF-OGFr complex is transported to the nucleus by way of nucleocytoplasmic transport (21). The binding of this complex to chromatin appears to elicit an increase in cyclin-dependent kinase inhibitors (CKI), specifically p16 and/or p21, which in turn decreases phosphorylation of retinoblastoma protein and impedes cells from exiting G₁-S. Blockade of CKI activity by siRNA technology resulted in elimination of the OGF's repressive action on cell proliferation (19, 20).

The novel observation showing that the mechanisms of both imiquimod and resiquimod on growth processes in cancer cells *in vitro* by the native OGF-OGFr axis is a paradigm shift in the sense that prior research often has focused on the immunomodulatory capabilities of this class of drugs. Needless to say, the relationship of imidazoquinoline to the OGF-OGFr system *in vivo* merits exploration. The clinical implications of this new knowledge about the mechanism of imidazoquinoline hold exciting possibilities. This class of drugs, particularly imiquimod, has been used in treatment of a wide variety of diseases (1–4, 29–31). The immense impact of these diseases is profound, with over 1 million new nonmelanoma skin cancers alone occurring each year. Knowledge that imiquimod acts directly on an endogenous biological regulator of cell proliferation (i.e., OGFr) may serve to enhance the therapeutic potential of this agent. OGF, the specific ligand for OGFr, has been approved in Phase I trials by the FDA for systemic use (32), and this compound is now in Phase II clinical trials for pancreatic cancer (Smith and Zagon, personal communication). It may be envisioned that a combination of OGF and

imiquimod therapy would enhance the activity of each of these compounds. Moreover, the use of OGF to enrich imiquimod action may facilitate reduction of imiquimod dosage, known to have such side effects as mild to moderate erythematous flaking and edema at the lesion site, burning at the application site and pain in <10% of patients (33). Finally, comprehension of the exact mechanism of imiquimod and resiquimod action may lead to the identification of related compounds that could enhance the capability of OGF-OGFr as a growth inhibitor without proinflammatory potential.

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