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Morphine Stimulates Angiogenesis by Activating Proangiogenic and Survival-promoting Signaling and Promotes Breast Tumor Growth[1]

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ABSTRACT

Morphine is used to treat pain in several medical conditions including cancer. Here we show that morphine, in a concentration typical of that observed in patients' blood, stimulates human microvascular endothelial cell proliferation and angiogenesis *in vitro* and *in vivo*. It does so by activating mitogen-activated protein kinase/extracellular signal-regulated kinase phosphorylation via Gi/Go-coupled G protein receptors and nitric oxide in these microvascular endothelial cells. Other contributing effects of morphine include activation of the survival signal PKB/Akt, inhibition of apoptosis, and promotion of cell cycle progression by increasing cyclin D1. Consistent with these effects, morphine in clinically relevant doses promotes tumor neovascularization in a human breast tumor xenograft model in mice leading to increased tumor progression. These results indicate that clinical use of morphine could potentially be harmful in patients with angiogenesis-dependent cancers.

INTRODUCTION

Angiogenesis is closely associated with the etiology and pathogenesis of several pathological conditions including tumor progression and metastasis (1–4). Despite the widespread use of opioids to treat pain in patients with cancer, little is known about the effect of these drugs on vascular endothelium.

Whereas the pharmacology and functions of opioids have been extensively characterized in the central nervous system, little is known about their effect on non-neuronal systems. In the central nervous system, opioids act via specific, well-defined MOR,[3] DOR, and KOR, and they are associated with several neuro-psychological effects including analgesia, tolerance, and addiction (5–10). Recent studies suggest a possible role for opioids in non-neuronal tissues. For example the presence of specific opioid receptors, including MOR, has been shown on endothelial cells (11, 12), and in the vascular endothelium morphine activates NO via MOR and leads to vasodilatation (12). Opioids also activate MAPK/ERK in nonendothelial cells (13–16) and promote cell proliferation (13–18). These findings are reminiscent of NO-dependent MAPK/ERK phosphorylation and angiogenesis induced by the endothelial cell specific growth and survival factor, vascular permeability factor/VEGF (19–23).

Therefore, we hypothesized that morphine may activate MAPK/ERK phosphorylation via NO in the endothelium and thereby stimulate angiogenesis. Using a variety of angiogenesis assays and a breast tumor model, we show that a medically relevant concentration of morphine sulfate promotes HDMEC proliferation, survival, and angiogenesis, and promotes breast tumor growth by stimulating tumor neovascularization. Morphine does so by stimulating MAPK/ERK phosphorylation via PTX-sensitive GPCRs and NO, and by activating survival signaling via Akt phosphorylation and cyclin D1.

MATERIALS AND METHODS

Cell Culture. We isolated HDMEC from neonatal human foreskins as described (24). Culture medium consisted of medium MCDB 131 (Life Technologies, Inc., Gaithersburg, MD) with 1 μg/ml hydrocortisone acetate, 5×10^{-4} M dibutyryl cyclic AMP, 10 mM L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, 0.25 mg/ml amphotericin B, 0.004% heparin, 10 μg/liter epidermal growth factor, and 20% heat inactivated male human serum. MCF-7 human breast cancer cells were maintained in phenol red-free improved MEM plus insulin and 10% FCS. Wild-type Chinese hamster ovary cells, which do not express any opioid receptors, were used as negative controls.

Proliferation Assays. We seeded HDMEC overnight at 5000/well in a 24-well plate or 50,000/well in a six-well plate. For serum-replete conditions, cells were incubated with inducers/inhibitors for 48 h in complete culture medium without the growth factor. For serum-depleted conditions, cells were serum and growth factor starved overnight and then incubated for an additional 48 h without serum and growth factor but with morphine, opioid agonists, naloxone, or VEGF. Cells were enumerated using a Coulter counter and with WST-8 assay kit (Dojindo Molecular Technologies, Gaithersburg, MD), which forms a colored formazan by the activity of cellular dehydrogenases of viable cells (25). Optical density obtained was extrapolated for the number of cells using calibration curves for known number of cells.

Western Blot Analysis. Cell lysates (30 μg protein) resolved on 10% SDS-PAGE were transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA). For immunoblotting we used antibodies to Phospho-p44/42 MAPK/ERK (Thr202/Tyr204), total MAPK/ERK, phospho-Akt, total Akt (New England Biolabs, Beverly, MA), or to Cyclin D1 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoreactive proteins were visualized with ECF Western blotting system (Amersham Life Sciences, Buckinghamshire, United Kingdom), and chemiluminescent signals were acquired using Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Densitometric analysis was performed using Molecular Analyst Software (Molecular Bioscience Group, Hercules, CA).

Apoptosis and Cell Cycle Analysis. We performed flow cytometric analysis of propidium iodide-labeled cells to quantitate the percentage of cells in A_0, G_0/G_1, S, and G_2/M phases of the cell cycle, as described (26), using a FACScalibur (Becton Dickinson, Mountain View, CA) and FlowJo software (Becton Dickinson). We also confirmed apoptosis by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling to detect DNA strand breaks (Boehringer Mannheim, Indianapolis, IN; Ref. 27).

Tube Formation Assay. We seeded HDMECs (5×10^4) on the surface of growth factor reduced Matrigel (10 mg/ml; Collaborative Research, Bedford, MA) previously polymerized for 30 min at 37°C. Then VEGF, morphine, naloxone, or vehicle were added and cultures incubated for 18 h. Tubes were photographed and enumerated as described previously (28).

Murine Matrigel Angiogenesis Assay. As described, (29) we injected 0.5 ml of Matrigel (Collaborative Research), admixed with 10 μM morphine, 10 μg/ml VEGF, 10 μM naloxone, or vehicle s.c., in the left and right flank of 6–8-week-old female Balb/cJ mice (Jackson Laboratory, Bar Harbor, ME). Ten days later, 25 mg/ml FITC-dextran was injected systemically, and blood samples were collected. After sacrificing the mice, Matrigel implants were resected and photographed under a fluorescent microscope, and then homogenized with 5 units/ml dispase (Life Technologies, Inc., Grand Island, NY). Angiogenic response was expressed as the fluorescence ratio of Matrigel

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[3] The abbreviations used are: MOR, μ opioid receptor; DOR, δ opioid receptor; KOR, κ opioid receptor; NO, nitric oxide; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; VEGF, vascular endothelial growth factor; HDMEC, human dermal microvascular endothelial cell; PTX, pertussis toxin; GPCR, G protein-coupled receptor; DAMGO, [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-Enkephalin; NOS, nitric oxide synthase; PI3k, phosphatidylinositol 3′-kinase; D-NAME, Nω-nitro-D-arginine methyl ester; L-NAME, Nw-nitro-L-arginine methyl ester; PE, phycoerythrin.

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implant:plasma, obtained using a Fluorescence Multi Plate Reader (Applied Biosystems, Foster City, CA).

Breast Tumor Growth. Four- to 6-week-old female nude mice (National Cancer Institute, Frederick, MD) were implanted with a 0.125 mg slow release 17 β-estradiol pellet (Innovative Research of America, Sarasota, FL). MCF-7 cells (5×10^6) were injected into the mammary fat pad. Mice were given s.c. injections of: (*a*) normal saline (control); (*b*) morphine sulfate at 0.714 mg/kg mouse/day for first 15 days and then 1.43 mg/kg mouse/day (equivalent to 50 mg and 100 mg morphine per day, respectively, for a 70 kg human); (*c*) naloxone (equimolar to morphine); or (*d*) both morphine and naloxone.

Tumor growth was measured biweekly and expressed as tumor volume (mm^3) by the formula: volume = (smaller dimension^2 $\times$ large dimension)/2 (30). Animal experimentation was in accordance with institutional guidelines.

Tumor Neovascularization. Serial cryosections of tumors were immunostained with anti-CD31-PE (PharMingen, San Diego, CA). Digital images of at least three different areas of each section (from three different sections per tumor) were binarized and linearized to quantitate total PE-positive pixels and blood vessel length, ends, and nodes using the Image Processing Tool kit, Plug-in Functions for PhotoShop (Reindeer Games, Asheville, NC), as described (31).

Statistical Analysis. All of the data are expressed as mean ± SD. A multivariate ANOVA was conducted for comparisons between different treatment groups. Dunnett's method was used to determine whether any treatment differed from control. Cell cycle data were analyzed by subsequent pair-wise contrasts constructed between treatments for different phases and tested using t statistics. For tumor progression, comparisons between different treatments were made after adjusting for multiple comparisons by Bonferroni's method. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Morphine Stimulates Angiogenesis. We studied the effect of morphine and specific opioid receptor agonists on human microvascular endothelial cells, the type of endothelial cells most relevant to the process of pathophysiological neovascularization. We first confirmed the presence of MOR, DOR, and KOR on HDMEC by reverse transcription-PCR, and sequencing of the amplified products, immunofluorescence microscopy, and Western immunoblotting (data not shown). Morphine as well as MOR, DOR, and KOR agonists (at 1 μM) induced significant HDMEC proliferation under both serum-free and serum-replete conditions, and almost to the same extent as stimulated by 100 ng/ml $VEGF_{165}$ (Fig. 1*a*). The degree of stimulation by most individual agonists was similar in both serum-replete and -depleted conditions, but the MOR agonist DAMGO stimulated HDMEC proliferation by 68% in serum-replete conditions as compared with 37% in serum-free conditions ($P = 0.006$). None of these opioids had any effect on wild-type Chinese hamster ovary cells (data not shown). Therefore, morphine, and MOR, DOR, and KOR agonists induce endothelial proliferation directly, and MOR agonist also potentiates the serum-induced proliferation.

We then examined the effect of morphine concentration (1 nM to 10 mM) on HDMEC proliferation. Morphine is used clinically in doses of 10–2450 mg/day, resulting in serum concentrations that are only 2 nM to 3.5 μM (32, 33). We found that a significant proliferative effect occurred in the range of 10 nM to 100 μM morphine ($P < 0.005$ *versus* control; Fig. 1*b*). At 1 mM and higher concentrations, only ~10% of HDMEC remained viable, as assessed by a cytotoxicity assay and trypan blue dye exclusion. Thus, at medically relevant concentrations morphine stimulates endothelial proliferation, but it is cytotoxic for endothelial cells at higher concentrations that are not clinically relevant.

In addition to endothelial proliferation, endothelial tube formation is necessary for the formation of new vessels. We observed that 1 μM morphine induced endothelial tube formation ($P = 0.0003$ *versus* without morphine) on growth factor-reduced Matrigel, an effect similar to that of $VEGF_{165}$ (Fig. 2*a*). Quantitatively, morphine induced a 2.25-fold higher number of tubes than untreated controls, after 18 h of stimulation, compared with 3-fold for $VEGF_{165}$ (Fig. 2*b*). In keeping with the endothelial cytotoxicity seen with morphine at mM concentrations, Matrigel cultures with a high concentration of morphine (1 and 10 mM), with or without naloxone, showed aggregates of round and dead cells, and no endothelial tubes were formed (data not shown). Naloxone (1 μM) did not antagonize the stimulatory effect of 1 μM morphine. Nonetheless, these results are consistent with the proliferative activity of medically relevant concentrations of morphine described above.

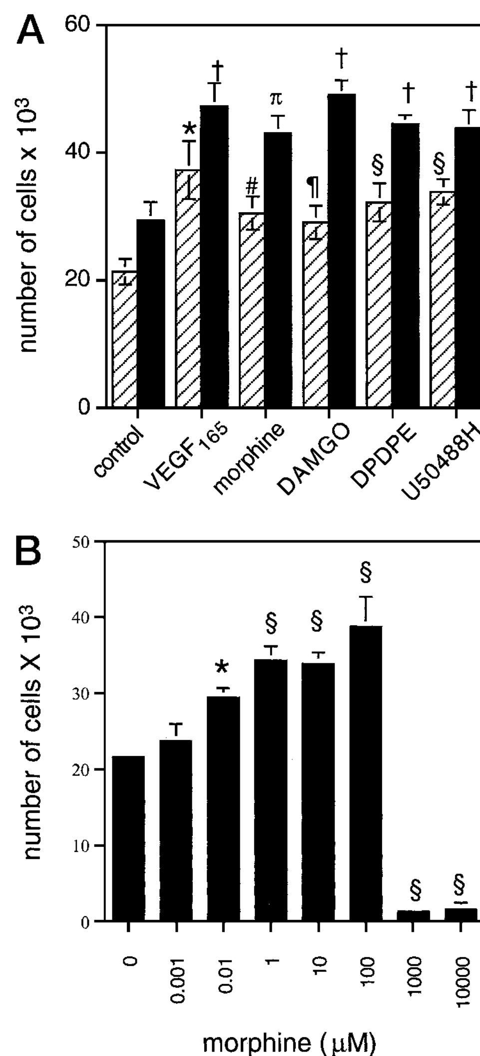

Fig. 1. Morphine and opioid receptor agonists stimulate HDMEC proliferation. *a,* after 48 h of incubation,1 μM each of morphine, MOR, DOR, and KOR agonists (DAMGO, DPDPE {[D-Pen (2, 5)]-Enkephalin} and U-50488H {*trans*-(±)-3,4-Dichloro-*N*-methyl-*N*-(2-[1-Pyrrolidinyl] Cyclohexyl)-Benzeneacetamide}, respectively) stimulated HDMEC proliferation to the same extent as 100 ng/ml $VEGF_{165}$, under serum-free (▨) as well as serum-replete (■) conditions. *, $P < 0.0001$; §, $P < 0.05$; #, $P < 0.01$; ¶, $P < 0.05$, compared with serum-free control; †, $P < 0.0001$; π, $P < 0.0002$, compared with serum-replete control. *b,* morphine concentration-dependent stimulation of HDMEC proliferation after 48 h of incubation. *, $P < 0.005$; §, $P < 0.0001$, compared with without morphine. Each experiment was repeated three times in triplicate, and each value indicates mean; *bars,* ± SD.

The significance of these *in vitro* observations is greatly increased by our observation that morphine stimulates angiogenesis *in vivo* in Matrigel implants in mice. We found that both VEGF ($P < 0.0001$) and morphine ($P = 0.0001$) containing implants showed significantly higher neovascular ingrowth, as compared with vehicle containing controls (Fig. 2, *c* and *d*). Histological analysis of Matrigel plug

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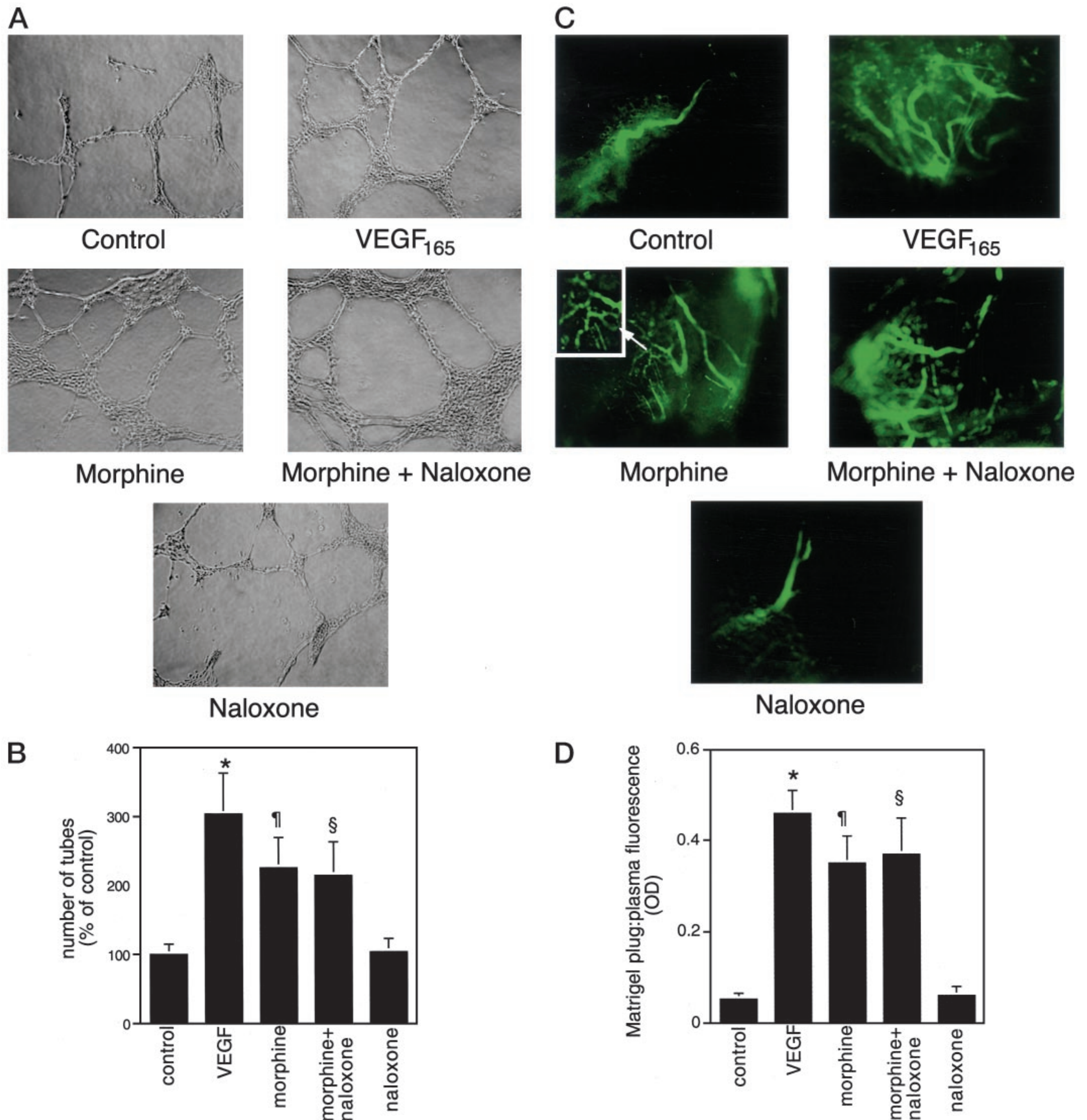

Fig. 2. Morphine stimulates angiogenesis *in vitro* and *in vivo*. *a,* HDMECs were seeded on growth factor reduced Matrigel and incubated for 24 h in serum-free medium containing vehicle (*control*), 100 ng/ml $VEGF_{165}$, 1 μM morphine, 1 μM morphine plus 1 μM naloxone, or 1 μM naloxone alone. Phase contrast micrographs showing stimulation of endothelial tube formation by morphine as well as $VEGF_{165}$. *Top left,* control; *top right,* VEGF165; *middle left,* morphine; *middle right,* morphine plus naloxone; and *bottom,* naloxone alone. Magnification ×100. Representative of three separate experiments, each was performed in triplicate. *b,* mean of the number of tubes counted in 10 fields/well of the above experiments. *, $P < 0.0001$; ¶, $P < 0.0005$; §, $P < 0.005$; compared with control. *c,* Matrigel was admixed with either vehicle (*control*), 10 μg/ml VEGF165, 10 μM morphine, 10 μM morphine plus 10 μM naloxone, or 10 μM naloxone, and then injected into the flanks of mice. Matrigel plugs dissected out 10 days after implantation show FITC-dextran loaded microvessels. Representative figures from three reproducible and independent experiments are shown. Magnification ×100. *d,* FITC-dextran ratio in Plasma *versus* Matrigel plugs were determined to quantify the intact vessels observed in *c* above. Mean of three separate experiments is shown. *, $P < 0.0001$; ¶, $P < 0.0002$; §, $P < 0.0005$, compared with control; *bars,* ± SD.

cryosections from the contralateral flank of the same animal, using anti-CD31 antibody, confirmed the neovascular ingrowth (not shown). Naloxone neither antagonized morphine-induced angiogenesis nor induced angiogenesis by itself (Fig. 2, *c* and *d*). Notably, Matrigel implants with a high (supra-therapeutic) concentration of morphine (10 mM), with or without 10 mM naloxone, did not promote angiogenesis (data not shown). Thus, morphine in medically relevant concentrations also induces angiogenesis *in vivo*.

Morphine Stimulates MAPK/ERK Signaling. We and others have shown earlier that VEGF and other growth factors promote angiogenesis by activating the MAPK/ERK signaling pathway (19, 27). Here, we observed that morphine and opioid receptor agonists

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induced a time-dependent activation of MAPK/ERK in HDMEC (Fig. 3*a*). Morphine-induced MAPK/ERK phosphorylation peaked at 1 min, 10–15 min and later, suggesting that it may be because of the activation of more than one opioid receptor. Indeed, MOR, DOR, and KOR activation by receptor-specific agonists stimulated MAPK/ERK phosphorylation at early, late, and intermediate time points, respectively (Fig. 3*a*). The loading control for total MAPK/ERK on stripped and reprobed membranes did not show any time-induced changes (representative figure for morphine is shown in last row, Fig. 3*a*), indicating that the quantity of MAPK/ERK in HDMEC remained unaltered. Thus, morphine as well as MOR, DOR, and KOR opioid receptor-specific agonists stimulate MAPK/ERK phosphorylation in endothelial cells.

It is known that opioids generally act via PTX-sensitive Gi/Go coupled GPCRs (6) and that in the endothelium morphine stimulates NO production (12). We observed that both PTX and the NOS inhibitor L-NAME blocked morphine-induced MAPK/ERK phosphorylation at early and later time points, as seen by densitometric analysis of the protein bands (Fig. 3, *b and c*). MAPK/ERK phosphorylation was not inhibited by naloxone but was completely blocked by the specific MAPK/ERK inhibitor PD98059 (data not shown). These inhibitors (PTX, L-NAME, and PD98059) also blocked morphine-induced HDMEC proliferation (Fig. 3*d*). Our results indicate that morphine acts through PTX-sensitive GPCRs, and activates NO and MAPK/ERK in a sequential manner to promote angiogenesis.

Morphine Inhibits Endothelial Apoptosis and Promotes Cell Cycle Progression by Activating Akt and Cyclin D1. The coupled relationship between promotion of cell proliferation and inhibition of cell death is a particularly potent driving force for the promotion of angiogenesis (34). We observed that, compared with serum-replete HDMEC, serum-starved HDMEC showed a significant increase ($32 \pm 12\%$) in the A_0 peak that represents apoptotic cells and a significant decrease ($8 \pm 3.5\%$) in the S phase peak that represents DNA synthesis ($P < 0.005$ and <0.05, respectively; Fig. 4*a*). Treatment of serum-starved cells with morphine inhibited apoptosis and promoted cell cycle progression to the same extent as that seen with serum or $VEGF_{165}$ (Fig. 4*a*). The changes in percentage of apoptosis by fluorescence-activated cell sorter analysis of propidium iodide-stained cells were paralleled by changes of similar magnitude in percentage of apoptotic cells by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling assay (data not shown).

One report has shown that morphine stimulates the phosphorylation of survival signal Akt in MOR-expressing cells (35). Akt acts as a regulator of apoptosis, cell growth, and cell cycle progression by inducing the transcription of cyclin D1 (36). We observed that morphine stimulated Akt phosphorylation, which peaked at 1 min and

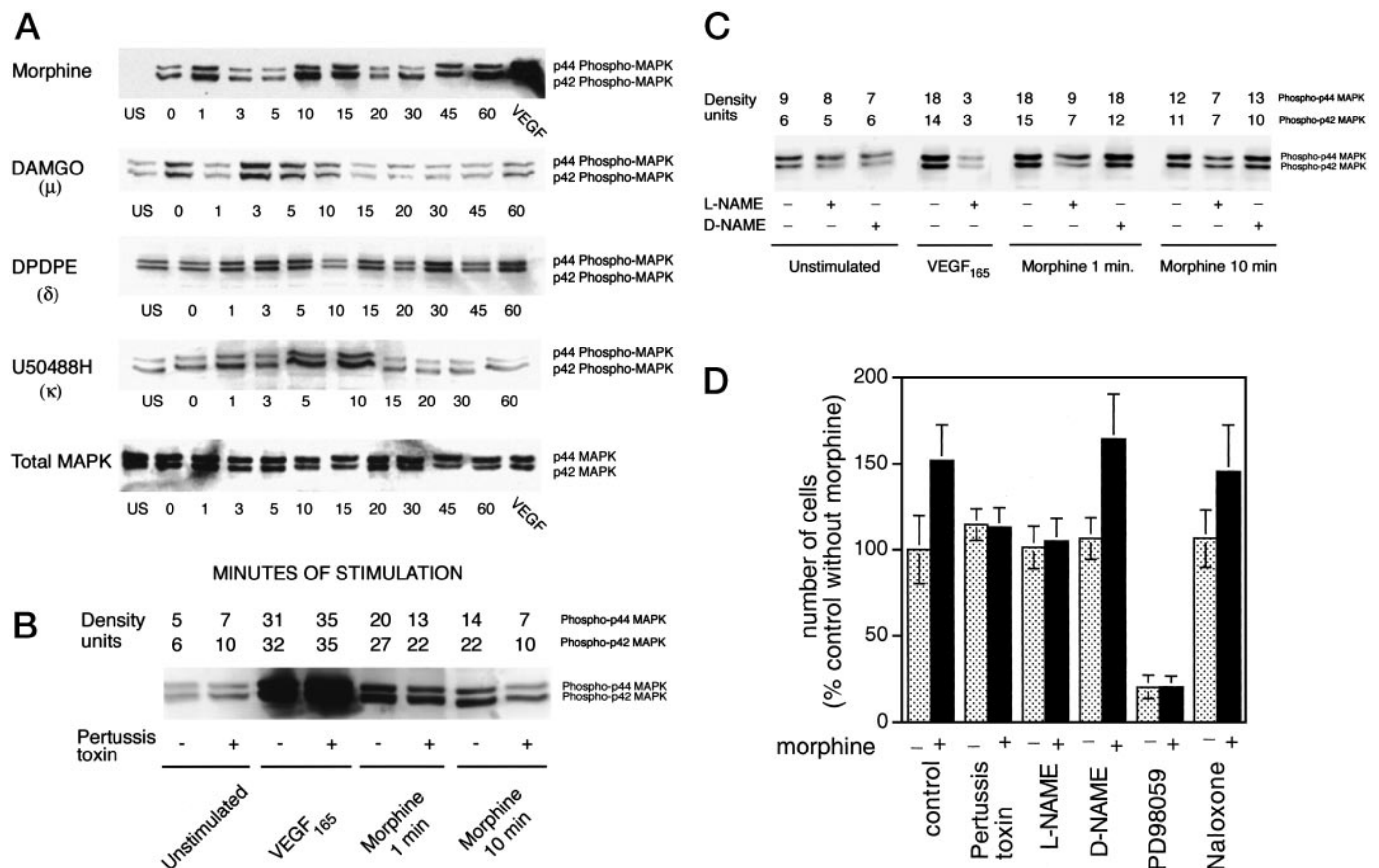

Fig. 3. Morphine stimulates proangiogenic signaling in HDMEC. *a,* time-dependent MAPK/ERK1 and 2 phosphorylation by 1 μM morphine or 1 μM specific opioid receptor agonists. *Left column* shows morphine or specific receptor agonist used (receptor specificity of each agonist is shown in parenthesis). *US* indicates unstimulated cells, and *0* indicates that the agonist was added and removed immediately. *Bottom panel* shows total MAPK/ERK bands obtained after stripping and reprobing the morphine membrane (*top row*). Each represents five to seven experiments performed independently. *b,* pretreatment with 100 ng/ml PTX (blocker of $G_{i/o}$ type GPCR) overnight inhibited morphine-induced MAPK/ERK phosphorylation at both 1 and 10 min, but did not inhibit VEGF165-induced MAPK/ERK phosphorylation. Densitometric analysis of the representative bands is shown as density units $\times 10^3$ in the *top panel*. Each experiment was repeated reproducibly and independently three to five times. *c,* pretreatment with NOS inhibitor L-NAME (100 μM) for 5 min inhibited morphine as well as $VEGF_{165}$-induced MAPK/ERK phosphorylation, but its inactive enantiomer D-NAME did not have any effect. Densitometric analysis is shown as described above, and each experiment is representative of three to five reproducible experiments. *d,* PTX, L-NAME, and MAPK inhibitor PD98059 (1 μM) significantly inhibited morphine-induced HDMEC proliferation ($P < 0.05$), but naloxone or D-NAME did not have any significant effect ($P > 0.05$) as compared with morphine stimulated control. Data from three separate experiments performed in triplicate were normalized to the untreated control, which was set at 100% (shown as mean); *bars,* $\pm$ SD.

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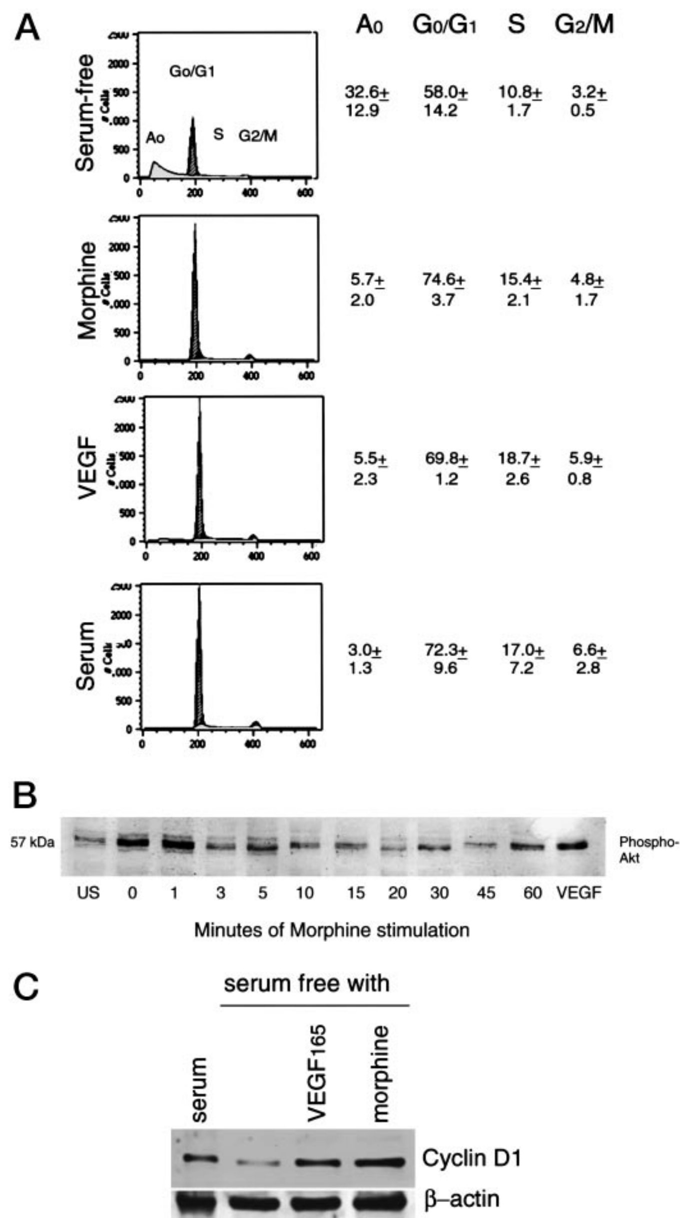

Fig. 4. Morphine promotes HDMEC survival, stimulates Akt phosphorylation, and promotes cyclin D1. *a,* HDMEC were incubated in serum-free medium for 48 h, with 1 μM morphine, 100 ng/ml $VEGF_{165}$, or 10% human serum. Representative histograms (*left panel*; from five separate experiments) show the distribution of cells in different phases of cell cycle. In the *right panel*, percentage of cells in each phase of cell cycle are shown as mean from five separate experiments. Morphine significantly inhibited apoptosis ($P < 0.0001$) and increased the number of cells in both G_1 and S phases of cell cycle ($P < 0.0001$ and 0.05, respectively), as compared with serum-free cells. *b,* time-dependent phosphorylation of survival signal Akt/PKB by 1 μM morphine and also by 10 min stimulation with 100 ng/ml $VEGF_{165}$. In *Lane 1, US* indicates unstimulated cells, and in *Lane 2, 0* indicates that morphine was added and removed immediately. Representative of three separate and reproducible experiments. *c,* HDMEC were treated as described above for 4*a*. Morphine (1 μM) promoted cyclin D1 to the same extent as promoted by $VEGF_{165}$ (100 ng/ml), as compared with serum-free control (*second lane from the left*). Loading control using β-actin antibody (*bottom row*) did not show these changes. Representative of three separate and reproducible experiments is shown.

then declined to the basal levels (Fig. 4*b*), and also induced cyclin D1 protein expression in HDMEC (Fig. 4*c*). This effect of morphine on cyclin D1 expression was comparable with that of $VEGF_{165}$ (Fig. 4*c*). These data indicate that morphine acts as an endothelial cell survival factor in the same fashion as VEGF.

Morphine Stimulates Tumor Angiogenesis and Breast Tumor Growth. Because morphine induced angiogenesis in several *in vitro* and *in vivo* assays, we investigated whether morphine could stimulate angiogenesis in an MCF-7 cell breast tumor xenograft model in mice *in vivo*. Tumors became detectable at day 15 in all of the groups except for the naloxone group, in which measurable tumors first appeared at day 22 (Fig. 5*a*). Repeated measures ANOVA of the log-transformed tumor volumes revealed that tumor volume changed with time at the 5% level of significance (Wilks' λ; $P = 0.0063$). As compared with controls, the morphine group showed a statistically significant increase in tumor volume after 32 days ($P < 0.05$, 0.02, and 0.002 for day 32, 35, and 38, respectively).

Acceleration of tumor growth in morphine-treated mice was associated with increased vascularization. We observed increased microvessel density (as anti-CD31PE-positive pixels) and higher vessel number (ends), increased total length, and more vessel branching in the morphine group as compared with controls ($P = 0.002$ for density and <0.0001 for all of the other parameters; Fig. 5*b*). The observed 1.8–2-fold increase in these angiogenesis parameters closely correlated with the average 1.9-fold increase in tumor volume on day 38 in

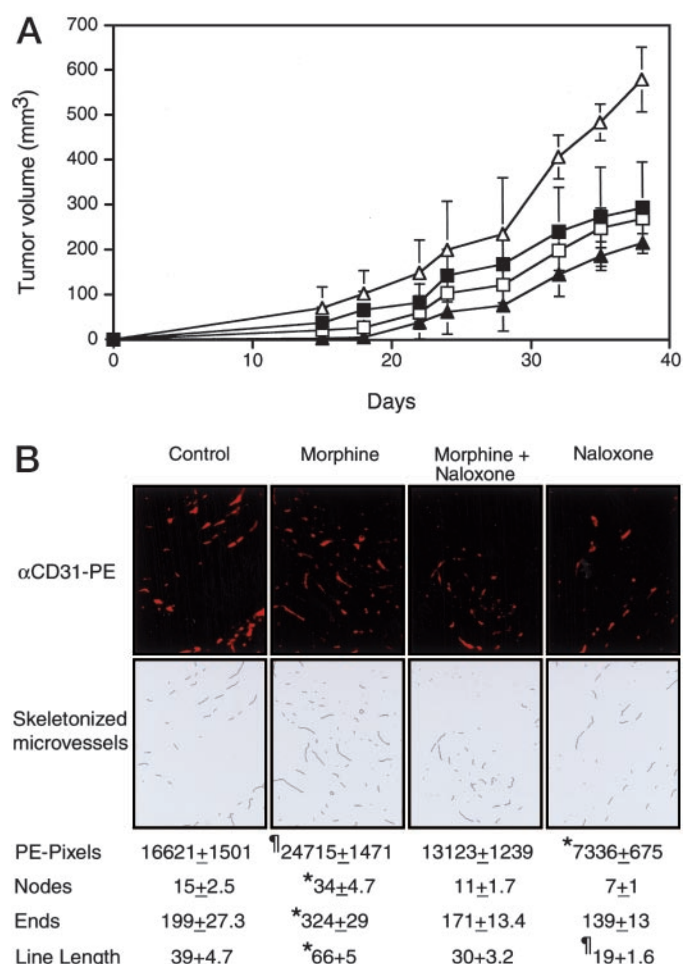

Fig. 5. Morphine stimulates angiogenesis in a breast tumor xenograft model. *a,* breast tumor growth in mice treated with vehicle (■), morphine (△), morphine plus naloxone (□), or naloxone (▲). Repeated measures ANOVA of log-transformed tumor volumes showed significant changes with time at 5% level of confidence (Wilk's λ; $P = 0.0063$). Tumor volumes increased significantly after 28 days of morphine treatment until 38 days ($P < 0.05$) as compared with control (vehicle-treated). Each point represents the mean of five separate experiments. *b,* sections of tumors from treated groups described above were stained with anti-CD31-PE (*top row*). Digital images were binarized (not shown) and then skeletonized (*middle row*) to quantitate PE-positive pixels, number of nodes (equivalent to vessel branching), vessel ends (for the number of vessels), and line length (vessel length), which are shown numerically below the figure. Three different areas from each section and three sections per tumor (from five tumors per group) were analyzed. Each value thus represents the mean of multiple images from five different tumors per treatment group. ¶, $P < 0.005$ and *, $P < 0.0001$, as compared with control. Magnification ×100; *bars,* ± SD.

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the morphine group. Coadministration of naloxone with morphine consistently reduced tumor volumes (morphine *versus* morphine + naloxone, $P = 0.04$, 0.01, and 0.001 for days 32, 35, and 38, respectively) and resulted in slower growth of tumors than controls (Fig. 5*a*), apparently because naloxone by itself inhibited tumor growth as compared with controls. However, naloxone did not have a significant effect on tumor angiogenesis. It did not inhibit vessel branching (nodes) and number of microvessels (ends; $P > 0.05$ *versus* control; Fig. 5*b*), which are specific characteristics of tumor microvessel architecture (31). Together, these results indicate that morphine promotes tumor growth by stimulating tumor angiogenesis. Naloxone directly inhibits tumor growth possibly by an independent mechanism and not by antagonizing the effect of morphine.

DISCUSSION

We show that morphine at medically relevant concentrations stimulates endothelial proliferation, survival, and cell cycle progression, and angiogenesis in both *in vitro* and *in vivo* assays. Because of the potential limitations of *in vitro* angiogenesis assays (37), we also demonstrate that these effects of morphine translate into enhanced tumor neovascularization *in vivo* in a breast tumor model. One previous study showed that high concentrations of morphine [1.65, 3.3, and 16.5 mM morphine (5, 10, or 50 μg/4 μl)] inhibited angiogenesis in the chick chorioallantoic membrane assay (38). We found that morphine is cytotoxic to endothelial cells at high concentrations. Therefore, the inhibition of angiogenesis by mM concentration of morphine, observed by others (38), could be because of its cytotoxic effect at such concentrations. However, as serum/plasma concentrations of morphine in patients reach levels of only between 2 nM and 3.5 μM (32, 33), the proangiogenic activity of 1 μM morphine observed by us in the present study is more likely to be the clinically relevant effect.

The opioid receptor antagonist naloxone did not inhibit the proangiogenic activity of morphine. However, there are many exceptions to the expected antagonist activity of naloxone, both functionally as well as pharmacologically (11, 39). Opioid receptors may be present as several subtypes and spliced variants (7, 40, 41). Opioid receptor homo- and heterodimerization has also been demonstrated (42–45). It is possible that either the opioid receptor subtype or oligomerization or the presence of "nonconventional" receptors on HDMEC allow the morphine activity described here to be nonresponsive to naloxone. In a recent study the immunosuppressive activity of DOR antagonist naltrindole remained unaltered in triple MOR/DOR/KOR receptor-deficient mice, suggesting the presence of yet to be discovered opioid receptors (46). Therefore, it is possible that morphine activity on the endothelium may be mediated by these nonclassical opioid receptors.

The functional proangiogenic activity of morphine and opioid receptors is additionally supported by our observations that morphine and MOR, DOR, and KOR agonists stimulated the MAPK/ERK signaling pathway in parallel with stimulating proliferation of HDMEC. In addition, morphine also stimulated the migration of HDMEC *in vitro* by 23.7 ± 8.4% over control (migration in the absence of morphine; $P < 0.05$). Thus, our results link the morphine-induced signaling to the promotion of angiogenesis. In several other cell types, morphine and these opioid receptors have been shown to stimulate MAPK/ERK phosphorylation (13–16). Similar to classical opioid receptor-mediated mechanisms, we found that morphine-induced MAPK/ERK phosphorylation and proliferation in HDMEC were dependent on PTX-sensitive GPCRs and on NO. Among the several known biological proangiogenic factors, only VEGF is dependent on NO-mediated MAPK/ERK phosphorylation (20, 21). Therefore, it appears that morphine acts in a fashion similar to VEGF in the endothelium. Very recently, MOR activation has been shown to transactivate epidermal growth factor receptor via MAPK/ERK phosphorylation in HEK293 cells (47), leading us to speculate that morphine may be transactivating VEGF receptors in the endothelium. A recent *in vitro* study shows that morphine inhibits hypoxia-induced VEGF expression in human umbilical vein and mouse heart microvascular endothelial cells (48). Because tumors continue to grow in patients receiving morphine for long periods of time, this does not affect either tumor angiogenesis or tumor growth. Indeed, we observe that morphine promotes both tumor angiogenesis as well as tumor growth in a mouse model, which is discussed below.

We have shown previously that MAPK/ERK phosphorylation is also essential for HDMEC survival (27). Indeed, morphine promoted endothelial survival and cell cycle progression by activating the cell survival signal Akt and increasing cell cycle protein cyclin D1 in HDMEC. Akt phosphorylation, which is dependent on PI3k activation, activates NOS and cell cycle progression by modulating cell cycle proteins in the nucleus (36). Morphine has been shown to activate PI3k and Akt phosphorylation in MOR-transfected cells (49). We also observed that Manumycin A and Ly294002, inhibitors of GTP-Ras and PI3k, respectively, completely inhibited morphine-induced HDMEC proliferation (data not shown). The morphine-induced mitogenic and survival signaling that we have observed is comparable with the effect of VEGF on the endothelium (19, 22, 23). Our observations are consistent with reports showing that the KOR agonist U69,593 activates DNA synthesis in glioma cells by activating Ras and MAPK/ERK signaling via PTX-sensitive, Gi/Go-coupled G protein receptors (16). In these glioma cells, KOR agonist-mediated stimulation of DNA synthesis was comparable with that induced by basic fibroblast growth factor (16). Considering these data from the literature together with our present results we propose a signaling mechanism for morphine in the endothelium (Fig. 6). Briefly, morphine, through Gi/Go-coupled G protein receptors, activates Ras, PI3k, NO, and MAPK/ERK, and endothelial proliferation in a sequential manner and promotes endothelial survival by the Akt signaling pathway.

Because angiogenesis actively participates in tumor progression (2), we also examined the effect of morphine in a breast tumor model. We observed that morphine induced tumor neovascularization and increased tumor progression. Other studies have shown that morphine inhibits the proliferation of MCF-7 breast cancer cells (50) used in the

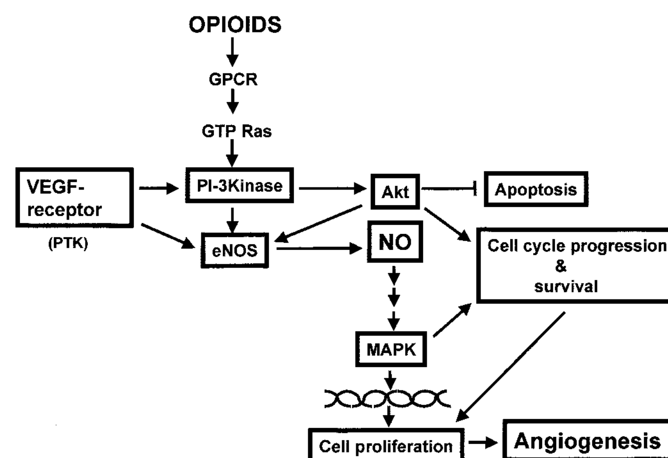

Fig. 6. Proposed model for morphine signaling in endothelium. Morphine can activate MAPK/ERK phosphorylation, a critical signaling pathway in endothelial survival and angiogenesis. Morphine acts via PTX-sensitive GPCRs and NO to activate MAPK/ERK phosphorylation and induce proliferation. It also activates survival signaling by stimulating Akt phosphorylation and increasing cyclin D1. Morphine signaling and angiogenic activity is similar to VEGF signaling and angiogenic activity (*shown in boxes*).

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tumor model in this study. Therefore, the morphine-induced tumor progression we observed appears to be primarily dependent on morphine-induced tumor angiogenesis. This is also supported by our observation that morphine did not influence the initial growth of the tumors, which is less likely to be influenced by angiogenesis. Most of the clinically used opioid analgesics are MOR agonists, and we have shown here that MOR agonist DAMGO had the same effect as morphine on endothelial signaling as well as function. Nevertheless, the effect of other opioid analgesics on angiogenesis needs to be specifically studied. Similar to the observations made by others (51), we also observed that naloxone by itself inhibited breast tumor growth. Because of the therapeutic potential of inhibition of tumor growth by naloxone, we are currently investigating its mechanism of action.

Previously, little was known about the effect of morphine on the vascular endothelium and angiogenesis. The proangiogenic activity of morphine shown here might have implications for its therapeutic application in cardiovascular medicine and wound healing. In contrast, opioid administration to patients with cancer or retinopathy might inadvertently increase angiogenesis, raising concerns about the widespread use of these analgesics in patients with cancer.

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REFERENCES

1. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid, and other diseases. Nat. Med., *1:* 27–31, 1995.
2. Carmeliet, P., and Jain, R. K. Angiogenesis in cancer and other diseases. Nature (Lond.), *407:* 249–257, 2000.
3. Lyden, D., Hattori, K., Dias, S., Costa, C., Blaikie, P., Butros, L., Chadburn, A., Heissig, B., Marks, W., Witte, L., Wu, Y., Hicklin, D., Zhu, Z., Hackett, N. R., Crystal, R. G., Moore, M. A., Hajjar, K. A., Manova, K., Benezra, R., and Rafii, S. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat. Med., *7:* 1194–201, 2001.
4. Risau, W., and Flamme, I. Vasculogenesis. Annu. Rev. Cell Dev. Biol., *11:* 73–91, 1995.
5. Childers, S. R. Opioid receptors: pinning down the opiate targets. Curr. Biol., *7:* R695–R697, 1997.
6. Simon, E. J. Opioid receptors and endogenous opioid peptides. Med. Res. Rev., *11:* 357–374, 1991.
7. Knapp, R. J., Malatynska, E., Collins, N., Fang, L., Wang, J. Y., Hruby, V. J., Roeske, W. R., and Yamamura, H. I. Molecular biology and pharmacology of cloned opioid receptors. FASEB J., *9:* 516–525, 1995.
8. Law, P. Y., and Loh, H. H. Regulation of opioid receptor activities. J. Pharmacol. Exp. Ther., *289:* 607–624, 1999.
9. Harrison, L. M., Kastin, A. J., and Zadina, J. E. Opiate tolerance and dependence: receptors. G-proteins, and antiopiates. Peptides (Elmsford), *19:* 1603–1630, 1998.
10. Massotte, D., and Kieffer, B. L. A molecular basis for opiate action. Essays Biochem., *33:* 65–77, 1998.
11. Arendt, R. M., Schmoeckel, M., Wilbert-Lampen, U., Plasse, A., Heucke, L., and Werdan, K. Bidirectional effects of endogenous opioid peptides on endothelin release rates in porcine aortic endothelial cell culture: mediation by δ opioid receptor and opioid receptor antagonist-insensitive mechanisms. J. Pharmacol. Exp. Ther., *272:* 1–7, 1995.
12. Stefano, G. B., Hartman, A., Bilfinger, T. V., Magazine, H. I., Liu, Y., Casares, F., and Goligorsky, M. S. Presence of the mu3 opiate receptor in endothelial cells. Coupling to nitric oxide production and vasodilation. J. Biol. Chem., *270:* 30290–3, 1995.
13. Chuang, L. F., Killam, K. F., Jr., and Chuang, R. Y. Induction and activation of mitogen-activated protein kinases of human lymphocytes as one of the signaling pathways of the immunomodulatory effects of morphine sulfate. J. Biol. Chem., *272:* 26815–26817, 1997.
14. Li, L. Y., and Chang, K. J. The stimulatory effect of opioids on mitogen-activated protein kinase in Chinese hamster ovary cells transfected to express μ-opioid receptors. Mol. Pharmacol., *50:* 599–602, 1996.
15. Trapaidze, N., Gomes, I., Cvejic, S., Bansinath, M., and Devi, L. A. Opioid receptor endocytosis and activation of MAP kinase pathway. Brain Res. Mol. Brain Res., *76:* 220–228, 2000.
16. Bohn, L. M., Belcheva, M. M., and Coscia, C. J. Mitogenic signaling via endogenous kappa-opioid receptors in C6 glioma cells: evidence for the involvement of protein kinase C and the mitogen- activated protein kinase signaling cascade. J. Neurochem., *74:* 564–573, 2000.
17. Law, P. Y., and Bergsbaken, C. Properties of δ opioid receptor in neuroblastoma NS20Y: receptor activation and neuroblastoma proliferation. J. Pharmacol. Exp. Ther., *272:* 322–332, 1995.
18. Law, P. Y., McGinn, T. M., Campbell, K. M., Erickson, L. E., and Loh, H. H. Agonist activation of δ-opioid receptor but not μ-opioid receptor potentiates fetal calf serum or tyrosine kinase receptor-mediated cell proliferation in a cell-line-specific manner. Mol. Pharmacol., *51:* 152–160, 1997.
19. Kroll, J., and Waltenberger, J. The vascular endothelial growth factor receptor KDR activates multiple signal transduction pathways in porcine aortic endothelial cells. J. Biol Chem., *272:* 32521–32527, 1997.
20. Murohara, T., Asahara, T., Silver, M., Bauters, C., Masuda, H., Kalka, C., Kearney, M., Chen, D., Symes, J. F., Fishman, M. C., Huang, P. L., and Isner, J. M. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. J. Clin. Investig., *101:* 2567–2578, 1998.
21. Ziche, M., Morbidelli, L., Choudhari, R., Zhang, H. T., Donnini, S., Granger, H. J., and Bicknell, R. Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not fibroblast induced angiogenesis. J. Clin. Investig., *99:* 2625–2634, 1997.
22. Gerber, H. P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B. A., Dixit, V., and Ferrara, N. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′–kinase/Akt signal transduction pathway. J. Biol. Chem., *273:* 30336–30343, 1998.
23. Gille, H., Kowalski, J., Li, B., LeCouter, J., Moffat, B., Zioncheck, T. F., Polletier, N., and Ferrara, N. Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). J. Biol. Chem., *276:* 3222–3230, 2001.
24. Gupta, K., Ramakrishnan, S., Browne, P. V., Solovey, A., and Hebbel, R. P. A novel technique for culture of human dermal microvascular endothelial cells under either serum-free or serum-supplemented conditions: isolation by panning and stimulation with vascular endothelial growth factor. Exp. Cell Res., *230:* 244–251, 1997.
25. Matsuoka, M., Wispriyono, B., and Igisu, H. Increased cytotoxicity of cadmium in fibroblasts lacking c-fos. Biochem. Pharmacol., *59:* 1573–1576, 2000.
26. Vindelov, L. L., and Christensen, I. J. Detergent and proteolytic enzyme-based techniques for nuclear isolation and DNA content analysis. Methods Cell Biol., *41:* 219–229, 1994.
27. Gupta, K., Kshirsagar, S., Li, W., Gui, L., Ramakrishnan, S., Gupta, P., Law, P. Y., and Hebbel, R. P. VEGF prevents apoptosis of human microvascular endothelial cells via opposing effects on MAPK/ERK and SAPK/JNK signaling. Exp. Cell Res., *247:* 495–504, 1999.
28. Gupta, K., Gupta, P., Wild, R., Ramakrishnan, S., and Hebbel, R. P. Binding and displacement of vascular endothelial growth factor (VEGF) by thrombospondin: effect on human microvascular endothelial cell proliferation and angiogenesis. Angiogenesis, *3:* 147–158, 1999.
29. Klement, G., Baruchel, S., Rak, J., Man, S., Clark, K., Hicklin, D. J., Bohlen, P., and Kerbel, R. S. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J. Clin. Investig., *105:* R15–R24, 2000.
30. Van den Berg, C. L., Cox, G. N., Stroh, C. A., Hilsenbeck, S. G., Weng, C. N., McDermott, M. J., Pratt, D., Osborne, C. K., Coronado-Heinsohn, E. B., and Yee, D. Polyethylene glycol conjugated insulin-like growth factor binding protein-1 (IGFBP-1) inhibits growth of breast cancer in athymic mice. Eur. J. Cancer, *33:* 1108–1113, 1997.
31. Wild, R., Ramakrishnan, S., Sedgewick, J., and Griffioen, A. W. Quantitative assessment of angiogenesis and tumor vessel architecture by computer-assisted digital image analysis: effects of VEGF-toxin conjugate on tumor microvessel density. Microvasc. Res., *59:* 368–376, 2000.
32. McQuay, H. J., Carroll, D., Faura, C. C., Gavaghan, D. J., Hand, C. W., and Moore, R. A. Oral morphine in cancer pain: influences on morphine and metabolite concentration. Clin. Pharmacol. Ther., *48:* 236–244, 1990.
33. Klepstad, P., Kaasa, S., and Borchgrevink, P. C. Start of oral morphine to cancer patients: effective serum morphine concentrations and contribution from morphine-6-glucuronide to the analgesia produced by morphine. Eur. J. Clin. Pharmacol., *55:* 713–719, 2000.
34. Holmgren, L., O'Reilly, M., and Folkman, J. Dormancy of micrometastases: Balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nat. Med., *1:* 149–153, 1995.
35. Polakiewicz, R. D., Schieferl, S. M., Gingras, A. C., Sonenberg, N., and Comb, M. J. μ-Opioid receptor activates signaling pathways implicated in cell survival and translational control. J. Biol. Chem., *273:* 23534–23541, 1998.
36. Blume-Jensen, P., and Hunter, T. Oncogenic kinase signalling. Nature (Lond.), *411:* 355–365, 2001.
37. Auerbach, R., Akhtar, N., Lewis, R. L., and Shinners, B. L. Angiogenesis assays: problems and pitfalls. Cancer Metastasis Rev., *19:* 167–172, 2000.
38. Pasi, A., Qu, B. X., Steiner, R., Senn, H. J., Bar, W., and Messiha, F. S. Angiogenesis: modulation with opioids. Gen. Pharmacol., *22:* 1077–1079, 1991.
39. Roy, S., Sedqi, M., Ramakrishnan, S., Barke, R. A., and Loh, H. H. Differential effects of opioids on the proliferation of a macrophage cell line. Bac 1.2F5. Cell Immunol., *169:* 271–277, 1996.

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40. Wollemann, M. Recent developments in the research of opioid receptor subtype molecular characterization. J. Neurochem., *54:* 1095–101, 1990.
41. Befort, K., Filliol, D., Decaillot, F. M., Gaveriaux-Ruff, C., Hoehe, M. R., and Kieffer, B. L. A single nucleotide polymorphic mutation in the human [micro]-opioid receptor severely impairs receptor signaling. J. Biol. Chem., *276:* 3130–3137., 2001.
42. Jordan, B. A., and Devi, L. A. G-protein-coupled receptor heterodimerization modulates receptor function. Nature (Lond.), *399:* 697–700, 1999.
43. George, S. R., Fan, T., Xie, Z., Tse, R., Tam, V., Varghese, G., and O'Dowd, B. F. Oligomerization of μ- and δ-opioid receptors. Generation of novel functional properties. J. Biol. Chem., *275:* 26128–26135, 2000.
44. Devi, L. A. Heterodimerization of G-protein-coupled receptors: pharmacology, signaling and trafficking. Trends Pharmacol. Sci., *22:* 532–537, 2001.
45. He, L., Fong, J., Zastrow, M., and Whistler, J. L. Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization. Cell, *108:* 271–282, 2002.
46. Gaveriaux-Ruff, C., Fillol, D., Simonin, F., Matthes, H. W. D., and Kieffer, B. L. Immunosuppression by d-opioid antagonist naltrindole: δ and triple $\mu/\delta/\kappa$-opioid receptor knockout mice reveal a nonopioid activity. J. Pharmacol. Exp. Ther., *298:* 1193–1198, 2001.
47. Belcheva, M. M., Szucs, M., Wang, D., Sadee, W., and Coscia, C. J. μ-Opioid receptor-mediated ERK activation involves calmodulin-dependent epidermal growth factor receptor transactivation. J. Biol. Chem., *276:* 33847–33853, 2001.
48. Balasubramanian, S., Ramakrishnan, S., Charboneau, R., Wang, J., Barke, R. A., and Roy, S. Morphine sulfate inhibits vascular endothelial growth factor expression in endothelial cells and cardiac myocytes. J. Mol. Cell. Cardiol., *33:* 2179–2187, 2001.
49. Polakiewicz, R. D., Schieferl, S. M., Dorner, L. F., Kansra, V., and Comb, M. J. A mitogen-activated protein kinase pathway is required for μ-opioid receptor desensitization. J. Biol. Chem., *273:* 12402–12406, 1998.
50. Maneckjee, R., Biswas, R., and Vonderhaar, B. K. Binding of opioids to human MCF-7 breast cancer cells and their effects on growth. Cancer Res., *50:* 2234–2238, 1990.
51. Aylsworth, C. F., Hodson, C. A., and Meites, J. Opiate antagonists can inhibit mammary tumor growth in rats. Proc. Soc. Exp. Biol. Med., *161:* 18–20, 1979.

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