Overexpression of the μ-Opioid Receptor in Human Non-Small Cell Lung Cancer Promotes Akt and mTOR Activation, Tumor Growth, and Metastasis

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ABSTRACT

Background: Recent epidemiologic studies suggesting that there were differences in cancer recurrence contingent on anesthetic regimens have raised the possibility that μ-opioid agonists can influence cancer progression. Based on our previous studies indicating the μ-opioid receptor (MOR) is up-regulated in several types of non-small cell lung cancer, this study examined the functional significance of MOR overexpression to elucidate a possible mechanism for the epidemiologic findings.

Methods: Stable vector control and MOR1 overexpressing human bronchioloalveolar carcinoma cells were evaluated using immunoblot analysis, proliferation and transendothelial extravasation assays with or without Akt inhibitor, mTOR inhibitor (temsirolimus), or the peripheral MOR antagonist, methylaltrexone. In human lung cancer xenograft models, primary tumor growth rates and lung metastasis were analyzed using consecutive tumor volume measurements and nestin immunoreactivity in lungs of the nude mouse model.

Results: The authors provide evidence that MOR is an important regulator of lung cancer progression. MOR overexpression increased Akt and mTOR activation, proliferation, and extravasation in human bronchioloalveolar carcinoma cells. In vivo, overexpression of MOR in human bronchoalveolar carcinoma cells increased primary tumor growth rates in nude mice by approximately 2.5-fold and lung metastasis by approximately 20-fold compared with vector control cells (n = 4 per condition).

Conclusions: The overexpression data suggest a possible direct effect of MOR on Akt and mTOR activation and lung cancer progression. Such an effect provides a plausible explanation for the epidemiologic findings. The authors’ observations further suggest that exploration of MOR in non-small cell lung carcinoma merits further study both as a diagnostic and therapeutic option.

What This Article Tells Us That Is New

Recent epidemiologic studies indicate a positive association between perioperative opioid administration and tumor progression, but the mechanisms are unclear

Previous studies show up-regulation of μ-opioid receptors in specific cancer cells including non-small cell lung cancer

What We Already Know about This Topic

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There is currently much debate in the literature regarding the role of anesthesia and analgesia in the recurrence and metastasis rate of numerous malignancies.1–3 Several retrospective studies have shown a reduced incidence of cancer recurrence after regional anesthesia with reduced doses of opioids after surgery for breast, prostate, and colon cancer and melanoma, although other studies have failed to detect any significant differences.4–7 Several hypotheses have emerged to explain the differences in recurrence rates, including immunosuppressive effects and direct effects on tumor cell growth.8–10 Our research has focused on the μ-opioid receptor (MOR) and its role in directly regulating tumor growth and metastasis.11 The opioid receptors are divided into three major subgroups, μ, κ, and δ and are members of the G-protein coupled receptor superfamily. MOR is the main target for opioids such as morphine, fentanyl, and heroin. MOR is

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responsible for mediating the clinically beneficial effects of opioids, including analgesia, but also mediates the secondary side effects such as addiction, respiratory depression, and constipation. 12,13 Endogenous MOR ligands include endomorphin-1 and -2, β-endorphin, enkephalins, and dynorphin A.14 MOR is expressed in both the central nervous system and peripheral tissues. The exact mechanisms governing MOR expression are unclear but a number of OPRM transcription factors have been identified, including AP-1, NF-κB and STAT-6.15 OPRM is also subject to epigenetic regulation including promoter methylation and histone modification.15,16 The human MOR gene OPRM1 is 236 kb and consists of 11 exons, which give rise to at least 17 splice variants. MOR1 is the most abundant transcript and consists of exons 1, 2, 3, and 4 and gives rise to a protein with a molecular weight of approximately 44 kDa.15 Acute opioid activation can lead to phosphorylation of the receptor (although this may be ligand dependent), inhibition of adenylate cyclase activity, and activation of mitogen-activated protein kinase, phosphotidylinositol 3-kinase, and phospholipase C.17 After activation, MOR may undergo endocytosis and be recycled. The rate and mechanisms of receptor densensitization and endocytosis are believed to be important in the development of opioid tolerance and addiction and are an area of ongoing investigation.18 It should be noted that there are a large number of agonist ligands for MOR, each of which may induce different cellular and physiologic outcomes.13,18

Previous studies have also reported on the role of MOR in angiogenesis, tumor cell growth, and metastatic spread.9,10 A number of reports have indicated that both endogenous opioids (endomorphin-1,-2) and those used clinically (morphine) can stimulate angiogenesis both in vitro and in vivo.19–21 Morphine has also been reported to stimulate tumor growth and metastasis, although the results vary and may be dependent on the cell type and the model used.12 However, an early report by Zagon and McLaughlin reported that the μ-opioid antagonist, naltrexone, inhibited the growth of metastatic murine neuroblastoma, resulting in increased survival times.22 In our own work, we have previously shown that methylnaltrexone, a peripheral MOR antagonist, can inhibit angiogenesis and potentiate the effects of mTOR inhibitors in vivo.23–25

Effective therapeutic strategies for lung cancer, the leading cause of cancer-associated mortality worldwide, are extremely limited, exemplifying the need for early diagnosis and novel therapeutic interventions.26,27 We have previously reported that the MOR is up-regulated in several types of human non-small cell lung cancer (NSCLC).11 However, the functional consequences of MOR overexpression in NSCLC are poorly defined. In this study, we investigated the effects of stable overexpression of MOR1 on Akt and mTOR signaling pathways and the downstream functional consequences in human NSCLC cells. Here we present evidence of a role for MOR in the regulation of NSCLC tumor cell proliferation, migration, and invasion in the absence of exogenous opioid stimulation. Further, through the use of tumor xenograft models and the MOR peripheral antagonist methylnaltrexone, our results suggest a possible therapeutic role for MOR antagonism on cancer growth and metastasis that merits further research.

Materials and Methods

Cell Culture and Reagents

Human NSCLC cell lines A549, SKLU-1, H1703, H358, H1993, H661, SW1573, H522, H226, H1437, H1838, H1975, H2170, and noncancerous BEAS-2B were obtained from ATCC (Walkersville, MD) and cultured in Roswell Park Memorial Institute complete medium (Cambrex, East Rutherford, NJ) at 37°C in a humidified atmosphere of 5% CO2, 95% air, with passages 6–10 used for experimentation. Unless otherwise specified, reagents were obtained from Sigma Chemical Company (St. Louis, MO). Methylnaltrexone bromide or methylnaltrexone was purchased from Mallinckrodt Specialty Chemicals (Phillipsburg, NJ). Temsirolimus was acquired through Wyeth Pharmaceuticals (Madison, NJ). Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad (Richmond, CA) and Immobilon-P transfer membrane was purchased from Millipore Corporation (Bedford, MA). Rabbit anti-pSer73 Akt, rabbit anti-pThr389Akt, rabbit anti-Akt, rabbit anti-pThr389mTOR, rabbit anti-epidermal growth factor receptor, and rabbit anti-mTOR antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Rabbit anti-pTyr1173 epidermal growth factor receptor antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Akt Inhibitor XIII was purchased from EMD Biosciences (Gibbstown, NJ). Mouse anti-human nestin antibody that does not react with mouse or rat nestin (clone 10C2) was purchased from Millipore Corporation. Mouse anti-β-actin antibody was purchased from Sigma Chemical Company. Secondary horseradish peroxidase-labeled antibodies were purchased from Amersham Biosciences (Piscataway, NJ).

Immunoblotting

Immunoblotting was performed as we have previously described.11,23 Cellular materials from treated or untreated human NSCLC cells were incubated with lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl2, 1% Triton X-100, 0.1% SDS, 0.4 mM Na3VO4, 40 mM NaF, 50 μM okadaic acid, 0.2 mM phenylmethylsulfonyl fluoride, 1:250 dilution of Calbiochem protease inhibitor mixture 3). The samples were then run on SDS-PAGE in 4–15% polyacrylamide gels, transferred onto Immobilon® membranes, and developed with specific primary and secondary antibodies. Visualization of immunoreactive bands was achieved using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). In some instances, immunoreactive bands were quantitated using computer-assisted densitometry.

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Stable Vector Control and MOR1 Overexpression in NSCLC Cells

The pCMV6-XL5 human MOR1 overexpression vector and vector control (pCMV6-AC-GFP) were transfected from Origene (Rockville, MD). H358 cells were transfected with small hairpin RNA (shRNA) using FuGENE HD® as the transfection reagent (Roche Applied Sciences, Indianapolis, IN) according to the protocol provided by Roche as we have previously described. Stable vector control or MOR1 overexpressing H358 cells (approximately 1 × 10^5 cells/well) were incubated with 0.2 ml serum-free media containing either vehicle (control), 1 μM Akt Inhibitor XIII, 10 nM temsirolimus or 100 nM methylaltrexone for 72 h at 37°C in 5%CO₂/95% air in 96-well culture plates. The in vitro cell proliferation assay was analyzed by measuring increases in cell number using the CellTiter96® MTS assay (Promega, San Luis Obispo, CA) and read at 492 nm. Percent invasion was defined as the number of cells in both the upper and lower chamber. Each assay was set up in triplicate and repeated at least five times.

NSCLC Cell Migration Assay

Measurement of in vitro NSCLC cell migration was performed as we have previously described. Stable vector control or MOR1 overexpressing H358 cells (5 × 10^5 cells/well) were incubated with 0.2 ml serum-free media containing either vehicle (control), 1 μM Akt Inhibitor XIII, 10 nM temsirolimus or 100 nM methylaltrexone for 18 h. Cells from the lower chamber were quantitated using the CellTiter96® MTS assay (Promega, San Luis Obispo, CA) and read at 492 nm. Percent migration was defined as the number of cells in both chambers divided by the number of cells in both the upper and lower chamber. Each assay was set up in triplicate and repeated at least five times.

NSCLC Cell Invasion Assay

Measurement of in vitro NSCLC cell invasion was performed as we have previously described. Stable vector control or MOR1 overexpressing H358 cells (5 × 10^5 cells/well) were incubated with 0.2 ml serum-free media containing either vehicle (control), 1 μM Akt Inhibitor XIII, 10 nM temsirolimus, or 100 nM methylaltrexone were plated with various treatments (methylaltrexone, control shRNA, MOR shRNA) to the upper chamber and media with serum was added to the lower chamber. Cells were allowed to invade through the Matrigel and pores for 18 h. Cells from the upper and lower chamber were quantitated using the CellTiter96® MTS assay (Promega, San Luis Obispo, CA) and read at 492 nm. Percent invasion was defined as the number of cells in the lower chamber divided by the number of cells in both the upper and lower chamber. Each assay was set up in triplicate and repeated at least five times.

Transendothelial Extravasation Assay

The ability of NSCLC cells to invade through a layer of endothelial cells was quantified using transendothelial monolayer resistance measurements using an electrical substrate-impedence sensing system (Applied Biophysics, Troy, NY) as previously described. Briefly, human pulmonary microvascular endothelial cells were grown to confluence on gold-plated microelectrodes connected to a phase-sensitive, lock-in amplifier. Stable vector control or MOR1 overexpressing H358 cells (5 × 10^5 cells/well) untreated or treated with 1 μM Akt Inhibitor XIII, 10 nM temsirolimus, or 100 nM methylaltrexone or 5% serum media-only control were added to the confluent endothelial monolayers on the electrodes. The electrical substrate-impedence sensing system allows for continuous measurement of the endothelial monolayer resistance as the H358 cells attach and begin to invade into the monolayer. A decrease in transendothelial monolayer resistance indicates a disrupted endothelial monolayer barrier via transendothelial extravasation of NSCLC cells. Resistance readings were normalized relative to an undisturbed confluent endothelial monolayer.

Human NSCLC Xenograft Studies in Nude Mice

All animal procedures were carried out in accordance with the guidelines provided by the Institutional Animal Care and Use Committee of the University of Chicago (Chicago, Illinois). All mice were 8- to 12-week-old males obtained from Harlan Laboratories (Indianapolis, IN). At the end of each experiment, lungs and primary tumors were collected, fixed in formalin, and embedded in paraffin or solubilized in extraction buffer for immunoblot analysis. 1.0 × 10^6 stable vector control or MOR1 overexpressing H358 cells were mixed with Matrigel supplemented with 100 ng/ml epidermal growth factor (conditions we previously established to produce optimal H358 tumor growth) and injected subcutaneously into the flank of Ncr-nude mice. Tumor nodules were measured regularly for 31 days using calipers, and tumor volume Vₜ (mm³) was calculated using the ellipsoid formula.
formula $A^2 \times B \times \pi/6$, where $A$ represents the smaller diameter.$^{31}$

Hematoxylin and Eosin Staining of Mouse Lungs

To characterize the morphologic changes due to metastasis from primary tumors growing in mouse hind flank, lungs from vector control or MOR1 overexpressing H358 hind flank-injected mice were formalin fixed and 5 μm paraffin section were subjected to hematoxylin and eosin histostaining. The sections of entire lungs were photographed using a Leica Axioscope (Leica, Bannockburn, IL) and analyzed. Slide images were processed using Imagej (National Institutes of Health, Bethesda, MD) software.

Quantification of Lung Metastasis

To characterize metastasis from primary tumors growing in mouse hind flank, lungs from vector control or MOR1 overexpressing H358 hind flank-injected mice were solubilized in extraction buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl$_2$, 1% Triton X-100, 0.2% SDS, 0.4 mM Na$_3$VO$_4$, 40 mM NaF, 50 μM okadaic acid, 0.2 mM phenylmethylsulfonyl fluoride, 1:250 dilution of Calbiochem protease inhibitor mixture 3) with sonication. The resulting material was run on SDS-PAGE in 4–15% polyacrylamide gels, transferred onto Immobilon® membranes, and developed with specific primary and secondary antibodies. An anti-human nestin antibody (clone 10C2, Millipore) that does not react with mouse or rat nestin was used for quantitation of H358 cell metastasis to the lung.

Statistical Analysis

Data are expressed as means ± SD. Two-way analysis of variance and Student-Newman-Keuls tests were used for data analysis with a $P$ value less than 0.05 considered statistically significant. All statistical analyses were performed using the program SPSS 17.0 for Windows (SPSS, Chicago, IL).

Results

NSCLC, which accounts for approximately 80% of all lung cancers, is heterogeneous disease composed of several types including adenocarcinoma, bronchioloalveolar carcinoma, squamous cell carcinoma, adenosquamous carcinoma, and large cell carcinoma.$^{32,33}$ We determined the relative levels of MOR expression in human NSCLC cell lines representing these various types and observed a fivefold to tenfold increase in MOR expression is most cell lines relative to control non-cancerous BEAS-2B cells (fig. 1). These results are consistent with our previously published data indicating the MOR is up-regulated in lung tissue from patients with NSCLC.$^{11}$ To test the functional significance of this MOR up-regulation, we generated stable vector control and MOR1 (the most abundant MOR transcript that consists of exons 1, 2, 3, and 4)$^{34}$ overexpressing human H358 BAC cells. We chose the human H358 bronchioloalveolar carcinoma cell line to study MOR overexpression based on its low basal metastatic potential.$^{35}$ Our results indicate that stable transfection of vector control plasmid in H358 cells does not change the levels of MOR expression (fig. 2). However, we observe robust MOR immunoreactivity in stable overexpressing MOR.
H358 cells. Because there is an intimate relationship between the MOR and the epidermal growth factor receptor often resulting in transactivation of these receptors in HEK293 cells, astrocytes, and H2009 human NSCLC cells, we analyzed the effect of MOR overexpression on epidermal growth factor receptor expression and activation. Our results indicate MOR overexpression in H358 NSCLC cells did not change basal epidermal growth factor receptor expression or activation (pTyr1173 epidermal growth factor receptor phosphorylation)38(fig. 2A). Phase-contrast images of stable MOR overexpressing, but not control or stable vector control H358 cells, had elongated cellular projections suggestive of a migratory phenotype (fig. 2B). We therefore focused our consequent studies on comparing stable vector control and MOR overexpressing H358 functional analysis.

Interestingly, we observed that overexpression of MOR induced activation (phosphorylation) of the serine/threonine kinases Akt and mTOR, which are implicated in cancer progression39 (fig. 3). Specifically, we observed increased basal serine and to a lesser extent threonine phosphorylation of Akt and serine phosphorylation of mTOR in MOR overexpressing H358 cells. Akt and mTOR are implicated in numerous cancer cell functions, including proliferation and invasion.40,41 We next tested the contributions of Akt and mTOR to in vitro NSCLC proliferation, migration, and invasion, which are reflective of in vivo tumor growth. Our results indicate that MOR overexpressing NSCLC cells have an approximately 45% increase in basal proliferation in serum-free media (fig. 4). Addition of 1 μM Akt Inhibitor XIII, a cell-permeable allosteric inhibitor of Akt 1/2, inhibited vector control cell proliferation by approximately 20% and MOR overexpressing cell proliferation by approximately 50%. In addition, the mTOR complex 1 inhibitor, temsirolimus,42 decreased vector control cell proliferation by approximately 30% and MOR overexpressing NSCLC proliferation by approximately 60%. These data indicate the importance of Akt and mTOR in these processes and suggest a potential increased sensitivity to Akt and mTOR inhibition in MOR overexpressing NSCLC cells.43 Further, addition of 100 nM of the peripheral MOR antagonist, methylnaltrexone, exhibited a potent inhibition of basal proliferation in vector control (approximately 40%) and MOR overexpressing (approximately 60%) cells. We observe a similar trend in our migration and invasion assays in which MOR overexpression increases activity that is inhibited by Akt inhibitor, mTOR inhibitor, and the peripheral MOR antagonist methylnaltrexone (fig. 4-B, C).

In order for many tumor cells to metastasize, they need to breach the endothelium to enter the bloodstream. We tested the in vitro ability of vector control and MOR overexpressing H358 cells to disrupt a confluent human pulmonary microvascular endothelial cell monolayer using an electrical substrate-impedance sensing system.28–30 This system continu-

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**Fig. 2.** Characterization of MOR1 overexpressing human H358 non-small cell lung cancer cells. (A) Control (nontransfected, C), stable vector control (VC) and μ-opioid receptor 1 (MOR1, the most abundant MOR transcript that consists of exons 1, 2, 3, and 4) overexpressing (O/E) H358 cell lines were generated, cell lysates obtained and immunoblotted with anti-μ-opioid receptor (a), anti-epidermal growth factor receptor (b), anti-pTyr1173 epidermal growth factor receptor (c), and anti-actin (2-day) antibodies. (B) Phase-contrast images of H358 control (nontransfected), stable VC, or stable MOR1 O/E cells. The arrows indicate elongated cellular projections suggestive of a migratory phenotype.
ously measures endothelial monolayer resistance as the H358 cells attach and begin to invade into the monolayer. A decrease in resistance indicates a disrupted endothelial monolayer barrier via transendothelial extravasation of the NSCLC cells. Figure 5 indicates MOR overexpressing H358 cells have increased extravasation properties compared with vector control cells, which becomes apparent approximately 7 h after NSCLC cell addition to the confluent endothelial monolayer. These effects are inhibited by Akt Inhibitor XIII, temsirolimus, and methylnaltrexone with MOR cells exhibiting increased inhibitor sensitivity (fig. 5B), results similar to in vitro proliferation, migration, and invasion.

We next translated our in vitro results to an in vivo human NSCLC xenograft model. Vector control and MOR overexpressing H358 cells were injected into the hind flank of nude

Fig. 3. Overexpression of MOR1 in human H358 non-small cell lung cancer cells increases Akt and mTOR Activation. (A) Stable vector control (VC) and μ-opioid receptor 1 (MOR1) (the most abundant MOR transcript that consists of exons 1, 2, 3 and 4) overexpressing (O/E) H358 cell lines were generated, cell lysates obtained and immunoblotted with anti-pSer473 Akt (a), anti-pThr308 Akt (b), anti-Akt (c), anti-pSer2448 mTOR (d), anti-mTOR (e), and anti-actin (f) antibodies. (B) Graphic representation of immunoblot quantitation of the ratio of phosphoprotein to total protein (pSer473 Akt/total Akt, pThr308 Akt/total Akt and pSer2448 mTOR/total mTOR), n = 3 per group. The asterisks indicate a statistically significant (P < 0.05) difference between vector control and MOR1 overexpression.

Fig. 4. MOR1 Overexpression increases proliferation, migration, and invasion and enhances sensitivity to Akt and mTOR inhibitors in human H358 non-small cell lung cancer cells. (A–C) Graphic representations of the percent proliferation (A), migration (B), or invasion (C) of stable vector control (VC) and μ-opioid receptor 1 (MOR1, the most abundant MOR transcript that consists of exons 1, 2, 3, and 4) overexpressing (O/E) H358 cells either untreated (control) or treated with 1 μM Akt Inhibitor XIII, 10 nM temsirolimus (mTOR complex 1 inhibitor), or 100 nM methylnaltrexone (MNTX, peripheral MOR antagonist) as described in the Methods section. Proliferation, migration, or invasion of untreated vector control H358 cells was set at 100%. There is a statistically significant difference (P < 0.05) in proliferation, migration, and invasion between basal VC versus MOR1 O/E H358 cells. The asterisks indicate a statistically significant difference (P < 0.05) between treatment and corresponding control groups.
mice and tumor volumes were measured using calipers for 31 days (fig. 6) as described in the Methods section. MOR overexpressing H358 cells had an approximately 2.5 fold increase in primary tumor growth rate compared with vector control cells (fig. 6B), suggesting a role of MOR overexpression in the proliferative properties of NSCLC.

Considering the differential growth rates of the primary tumors in nude mice, we next examined the metastatic potential of vector control and MOR overexpressing H358 primary flank tumors. Human bronchioloalveolar carcinoma tends to have low metastatic potential. However, as time progresses and/or mutations arise, the invasive properties of bronchioloalveolar carcinoma can increase. The histologic analysis of lungs from nude mice with hind flank tumors from vector control H358 cells revealed minimal cellular infiltration with normal alveolar morphology (fig. 7). In contrast, lungs from nude mice with MOR overexpressing H358 hind flank tumors exhibited areas of atypical cellular density, dysplastic morphology, and disrupted alveolar epithelium. Because these areas did not exhibit well-demarcated tumors, we quantitated lung metastasis using an anti-human nestin antibody that does not react with mouse. Figure 8 indicates robust nestin immunoreactivity in the lung homogenates of MOR overexpressing, but not vector control, mice. Further, there is increased MOR expression in lung homogenates from mice with MOR overexpressing H358 hind flank tumors. Quantitation of nestin immunoreactivity revealed an approximately 20-fold increase in lung metastasis from MOR overexpressing primary tumors (fig. 8B).
7,12-dimethylbenzanthracene-induced rat mammary tumors. Further, naltrexone inhibited the incidence and multiplicity of primary tumor growth rates and lung metastasis in human NSCLC cells increased primary tumor growth rates and lung metastasis in human NSCLC cells increased proliferation, migration, invasion, and transendothelial migration and activation of two serine/threonine kinases implicated in cancer progression, Akt and mTOR. Consistent with this kinase activation, MOR overexpressing H358 cells exhibited increased sensitivity to Akt and mTOR inhibitors in our in vitro cellular assays. In addition, overexpression of MOR in H358 cells increased primary tumor growth rates and lung metastasis in human NSCLC xenografts. Taken as a whole, our data suggest that MOR overexpression promotes lung cancer progression potentially through Akt and mTOR-regulated pathways.

Our results are consistent with other studies indicating a MOR antagonist can inhibit various parameters of cancer progression. Specifically, Zagon et al. reported that daily subcutaneous injection of 0.1 mg/kg naltrexone inhibited tumor appearance and increased survival time in A/Jax mice inoculated with S20Y neuroblastoma cells. Further, naltrexone inhibited the incidence and multiplicity of 7,12-dimethylbenz[a]anthracene-induced rat mammary tumors. In addition, incubation of 100 nM naloxone inhibited MCF-7 breast cancer cell proliferation. Exogenous opioids have been reported to either inhibit or enhance cancer growth and angiogenesis depending on the concentration and model used. A study by Gupta et al. demonstrated stimulatory effect of morphine on tumor growth and angiogenesis. This group found that clinically relevant doses of morphine led to increased tumor volumes and increased tumor vascularization. Although we did not add exogenous opioids in our study, a role for endogenous opioids cannot be ruled out. The endogenous MOR ligands, endomorphin-1 and endomorphin-2, increased angiogenesis and endothelial cell proliferation, migration, and adhesion in vitro, effects that were reversed by the MOR antagonist naltrexone. Further, β-endorphin has been implicated in malignant melanoma progression.

Our observations that MOR overexpression increases Akt and mTOR activation can have clinical significance because these molecules are targets for anticancer drugs. We have previously demonstrated that methylnaltrexone inhibits vascular endothelial growth factor-induced Akt activation and that methylnaltrexone acts synergistically with temsirolimus on inhibition of angiogenesis both in vitro and in vivo. In this study, we demonstrate that temsirolimus inhibits NSCLC proliferation and transendothelial extravasation in NSCLC cells with MOR overexpression conferring increased sensitivity to these drugs. Temsirolimus, currently used to treat renal cell carcinoma, exerts its action by binding to the intracellular protein, FKBP12, and inhibiting mTOR complex 1 formation. However, mTOR can still form a complex with other proteins including SIN1 and Rictor (mTOR complex 2), leading to investigation of drugs that can directly inhibit the mTOR kinase. The mTOR complex 2 serine phosphorylates Akt and is involved in actin cytoskeletal regulation, proliferation, and cell survival. In contrast, PI3 kinase activation of PDK1 induces threonine phosphorylation of Akt. Interestingly, our results indicate MOR overexpression activates serine phosphorylation of Akt to a greater extent than threonine phosphorylation, suggesting a potentially greater role for mTOR in this process. However, we cannot rule out the contributions of other kinases in Akt activation.

Fig. 7. Histochemical analysis of lungs from vector control and MOR1 overexpressing nude mouse hind flank tumors. Representative hematoxylin and eosin stained lung sections from vector control and µ-opioid receptor 1 (MOR1, the most abundant MOR transcript that consists of exons 1, 2, 3, and 4) overexpressing H358 hind flank tumors in nude mice illustrated at 1×, 2.5×, and 20×. The vector control lung sections exhibit normal morphology with little cellular infiltration. In contrast, the MOR1 overexpressing lung sections lungs exhibit areas of atypical cellular density, dysplastic morphology, and disrupted alveolar epithelium (arrows).

Discussion

Based on the recent interest of potential effects of anesthesia and analgesia regimens on the recurrence and metastatic potential of various cancers and our previously published data indicating MOR is increased in lung cancer, this study investigated the functional effects of MOR overexpression in human NSCLC cells both in vitro and in vivo. We observed that MOR1 (the most abundant MOR transcript that consists of exons 1, 2, 3, and 4) overexpression in H358 human NSCLC cells increased proliferation, migration, invasion, and transendothelial migration and activation of two serine/threonine kinases implicated in cancer progression, Akt and mTOR. Consistent with this kinase activation, MOR overexpressing H358 cells exhibited increased sensitivity to Akt and mTOR inhibitors in our in vitro cellular assays. In addition, overexpression of MOR in H358 cells increased primary tumor growth rates and lung metastasis in human NSCLC xenografts. Taken as a whole, our data suggest that MOR upregulation promotes lung cancer progression potentially through Akt and mTOR-regulated pathways.

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We have recently reported that silencing of MOR in lung cancer cells (Lewis lung carcinoma) reduces tumor growth and metastasis in mouse models. These findings are in agreement with the current study indicating overexpression of MOR in human NSCLC increases tumor growth and metastasis. In addition, our previous studies indicate the peripheral MOR antagonist, methylnaltrexone, inhibits Lewis lung carcinoma growth both in vitro and in vivo. We have extended these findings by demonstrating that methylnaltrexone inhibits human NSCLC cell proliferation and trans-endothelial extravasation. Although our studies used methylnaltrexone because it can be coadministered with opiates without reversing analgesia and is often used clinically in patients with advanced cancer, the effects appear to extend more generally to the class of MOR antagonists. Naloxone and naltrexone can inhibit opioid-induced angiogenesis. In addition, naltrexone is being studied as a possible adjuvant therapy for pancreatic cancer and as a treatment for solid metastatic tumors.

Given our previously published data indicating MOR is increased in lung cancer, we undertook a series of in vitro and in vivo experiments to examine the direct effect of MOR overexpression in lung cancer progression using human NSCLC cells. We hypothesized that overexpression and activation of MOR might be a plausible explanation for the differences in recurrence rates observed in the epidemiologic studies. This is supported by our results indicating that MOR overexpression increases the proliferative and metastatic properties of human NSCLC cells. Importantly, our observations that MOR overexpression activates Akt and mTOR suggest a potential therapeutic advantage using combination MOR, Akt and/or mTOR inhibitors in the treatment of NSCLC. Taken together, our data suggest a possible direct effect of MOR overexpression on lung cancer progression, and provides a plausible explanation for the epidemiologic findings. Our observations further suggest a possible therapeutic role for opioid antagonists that merits further evaluation.

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Fig. 8. Overexpression of MOR1 increases lung metastasis in human non-small cell lung cancer (NSCLC) xenograft models. (A) Representative immunoblot analysis of human H358 NSCLC cells alone, control nude mouse lung homogenate, lung homogenate from nude mouse with vector control H358 hind flank tumor or lung homogenates from nude mice with μ-opioid receptor 1 (MOR1, the most abundant MOR transcript that consists of exons 1, 2, 3, and 4) overexpressing H358 hind flank tumors using anti-nestin (a), anti-MOR (b), or anti-actin (c) antibodies. (B) Graphic representation of lung metastasis from vector control or MOR1 overexpressing H358 hind flank tumors in nude mice using quantitation of nestin immunoreactivity as depicted in A. There is a statistically significant difference (P < 0.05) between groups with n = 4 per group.
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