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Morphine stimulates cancer progression and mast cell activation and impairs survival in transgenic mice with breast cancer

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Editor's key points

- Morphine activates cellular signalling pathways which can promote angiogenesis in mouse models of cancer.
- High levels of MOR expression in metastatic prostate cancer are associated with reduced progression free survival.
- This study uses a transgenic mouse model of cancer and investigates the effect of morphine on cancer onset and progresssion.
- Morphine does not affect tumour onset, but it promotes development of established tumours with increased MOR expression.

Background. Morphine stimulates angiogenesis and cancer progression in mice. We investigated whether morphine influences tumour onset, development, and animal model survival, and whether μ-opioid receptor (MOR), lymphangiogenesis, mast cell activation, and substance P (SP) are associated with the tumour-promoting effects of morphine.

Methods. Transgenic mice with a rat C3(1) simian virus 40 large tumour antigen fusion gene which demonstrate the developmental spectrum of human infiltrating ductal breast carcinoma were used. Mice were treated at different ages with clinically relevant doses of morphine or phosphate-buffered saline to determine the effect on tumour development and progression, and on mouse survival. Tumours were analysed for MOR, angiogenesis, lymphangiogenesis, SP, and mast cell activation by immunofluorescent- or laser scanning confocal-microscopy. Cytokine and SP levels were determined by enzyme-linked immunosorbent assay.

Results. Morphine did not influence tumour development when given before the onset of tumour appearance, but significantly promoted progression of established tumours, and reduced survival. MOR-immunoreactivity (ir) was observed in larger but not in smaller tumours. Morphine treatment resulted in increased tumour angiogenesis, peri-tumoural lymphangiogenesis, mast cell activation, and higher levels of cytokines and SP in tumours. SP-ir co-localized with mast cells and elsewhere in the tumours.

Conclusions. Morphine does not affect the onset of tumour development, but it promotes growth of existing tumours, and reduces overall survival in mice. MOR may be associated with morphine-induced cancer progression, resulting in shorter survival. Mast cell activation by morphine may contribute to increased cytokine and SP levels, leading to cancer progression and refractory pain.

Keywords: analgesics, opioid, morphine; angiogenesis, lymphangiogenesis; cancer; mast cells; μ-opioid receptor

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Morphine and other opioids are used in escalating doses to control severe pain in cancer patients.[1 2] While morphine exerts analgesia via μ-opioid receptor (MOP/MOR, termed MOR here) in the central nervous system (CNS), it also has direct effects on non-neural cells, including endothelial, tumour, and mast cells.[3–6] Previous studies from our laboratory showed that morphine activates mitogen-activated protein kinase/extracellular signal-related kinase and Akt signalling pathways leading to the proliferation and survival of endothelial cells.[3] Activation of these signalling pathways and cycloxygenase-2 signalling led to the promotion of angiogenesis, tumour growth, and metastasis and reduced survival in mice.[7] Methylnaltrexone (MNTX), an opioid receptor antagonist, inhibits morphine-induced endothelial proliferation, angiogenesis, and disruption of endothelial barrier function.[5 8] However, it remains to be determined whether morphine promotes cancer initiation, or whether it stimulates the growth of existing tumours without influencing the development of cancer.

In a retrospective analysis, we found that patients with metastatic prostate cancer requiring higher doses of opioids had shorter progression-free survival (PFS) and overall survival (OS).[9] The presence of high MOR-immunoreactivity (ir) in the malignant areas of the prostate biopsies correlated strongly

with shorter time to progression, PFS, and OS. Both opioid requirement and MOR-ir retained prognostic significance for PFS and OS in multivariable analyses that adjusted for known prognostic factors.

Opioids are known to stimulate mast cell activation resulting in the release of inflammatory cytokines, neuropeptides such as substance P (SP), and tryptase.[6 10–12] High density of tryptase-positive mast cells were found to be associated with advanced stages of colorectal cancer.[13] In murine breast cancer models, mast cells modulate the tumour microenvironment and facilitate metastases via their cell surface receptor c-Kit.[14]

Tryptase and SP released from mast cells activate peripheral nerve terminals leading to the release of more SP.[6] In turn, SP further activates the nerve fibres leading to increased pain. Additionally, SP stimulates angiogenesis and promotes cancer progression.[15] SP and its high affinity receptor neurokinin 1 (NK1) are highly expressed in HER2+ breast cancer and SP stimulates HER2 signalling and may contribute to drug resistance in breast cancer.[16] Thus, mast cell activation by morphine may further exaggerate the pro-inflammatory, pro-nociceptive, and vasoactive tumour microenvironment.

In the present study, we used a transgenic mouse model that mimics the evolutionary spectrum of human breast cancer.[17] In this mouse, distinct phases of tumour development, progression, metastasis, and survival can be evaluated, enabling analysis of the effect of opioids on tumour development and progression and also on survival.

Methods

Mouse studies were performed with approval from the University of Minnesota's Institutional Care and Use Committee (IACUC, Protocol #0703A03589 and 1212-30170A). Detailed procedures are described in the Supplementary Appendix.

Mice

Female, transgenic mice with a rat C3(1) simian virus 40 large tumour antigen fusion gene (called C3TAG mice henceforth), which causes highly invasive breast tumours were used.[17] These mice develop ductal epithelial atypia around 8 weeks of age that progresses to intraepithelial neoplasia around 12 weeks, and to grossly palpable tumours and invasive carcinoma around 16 weeks. Tumours principally metastasize haematogenously to the lungs, but also to the adrenals, liver, and heart. By 6 months of age, mice die due to universal development of multifocal mammary adenocarcinomas. This model was chosen because the effect of morphine could be distinguished on distinct phases of tumour development (between 8 and 12 weeks), tumour growth (12 weeks onwards), metastases (16 weeks onwards), and survival.[18]

Drugs and treatments

Mice were injected subcutaneously with either phosphate-buffered saline (PBS) or morphine sulphate (MS; Baxter Esilerderle Healthcare, Cherry Hill, NJ, USA) at 0.5 mg kg^{-1} day^{-1} for 2 weeks, followed by dose escalation every 2 weeks as follows: 0.75, 1.0, 1.25 mg kg^{-1} day^{-1}, and finally to 1.5 mg kg^{-1} day^{-1} for the duration of each study as indicated. Mice were divided into three groups to examine the effect of morphine on: (i) *tumour development/onset*: 6-week-old mice were injected with morphine for 7 weeks; (ii) *tumour growth/progression*: 3-month-old mice were injected with morphine for 7 weeks; (iii) *survival*: 3-month-old mice were injected with morphine until they became moribund, which was considered the end of survival as per our animal ethics policy.

Tumour burden

At the end of treatment, mice were euthanized and all visible tumours throughout the body were dissected out. Tumour numbers and tumour weight/mouse were recorded as measures of tumour burden.

Immunofluorescent staining for MOR, CD31, and lymphatic vessel endothelium hyaluranon receptor-1

Tumours were cryosectioned into 6 μm thick sections and immunostained with: goat anti-lymphatic vessel endothelium hyaluranon receptor-1 (LYVE-1) (1:500, R&D Systems, Minneapolis, MN, USA), rat anti-CD31 (1:200, Santa Cruz Biotechnology, Dallas, TX, USA), and rabbit anti-MOR (1:100, Millipore, Billerica, MA, USA) as described by us.[6 7 18–20] Anti-MOR antibody was validated for MOR specificity using skin sections from MOR-knockout mice as described earlier (Supplementary Fig. S1).[19]

Mast cell analysis

Approximately 6 μm thick tumour sections were stained with Toluidine blue and enumerated as described previously.[6 21]

Laser scanning confocal microscopy of tumour sections

Approximately 6 μm thick tumour sections were fixed in 4% paraformaldehyde, blocked with 3% donkey serum and permeabilized with 0.03% Triton X-100 (all from Sigma-Aldrich, St Louis, MO, USA). Sections were then incubated with primary antibodies, rabbit anti-SP (1:50, AbD Serotec, Raleigh, NC, USA), rat anti-CD31 (1:200, Santa Cruz, CA, USA), goat anti-c-Kit (1:100, BD Bioscience, San Jose, CA, USA), and rabbit anti-FcεR 1 (eBioscience, San Diego, CA, USA) as described by us.[6]

Enzyme-linked immunosorbent assay for cytokines

Supernatants from tumour lysates were analysed for: tryptase (American Research Products, Inc., Waltham, MA, USA), β-hexosaminidase (Cedarlane Labs, Burlington, NC, USA), granulocyte macrophage colony-stimulating factor (GM-CSF), regulated on activation normal T-cell expressed and secreted (RANTES), SP, and interleukin-6 (IL-6; all from R&D Systems) after the manufacturer's instructions.

Statistical analysis

Data were analysed with Prism software (v 5.0a, GraphPad Prism Inc., San Diego, CA, USA). A P-value of <0.05 was considered significant. Analysis of variance with Bonferroni's