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Morphine decreases the function of primary human natural killer cells by both TLR4 and opioid receptor signaling

Dermot P. Maher*, Deepa Walia, Nicola M. Heller

Department of Anesthesiology and Critical Care Medicine, Johns Hopkins University, Baltimore, MD, United States

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ABSTRACT

Background: Opioids are commonly used to provide analgesia for cancer pain, and functional opioid receptors have been identified on natural killer (NK) cells, the lymphocytes responsible for surveillance and elimination of cancer cells. Opioids also bind to other lymphocyte receptors, such as Toll-like receptor (TLR)-4. Here, we characterized the effects of morphine on primary human NK cell cytotoxicity and mediator release, which occur through classical opioid receptor or TLR4 signaling.

Methods: Purified primary human NK cells were pretreated with inhibitors of opioid receptors or TLR4 before being cultured with target tumor cell line K562 in the presence or absence of morphine. Apoptosis of K562 cells in each treatment condition was measured with an Annexin V flow cytometry-based assay and compared to that of K562 cells cultured with NK cells alone. Supernatant concentrations of 13 cytokines and cytotoxic mediators were measured with a multiplex bead-based flow cytometry assay.

Results: Exposure of NK cells to morphine decreased their ability to induce apoptosis in K562 cells. Pretreating the NK cells with either naloxone, a mu- and kappa-opioid receptor antagonist, or TAK-242, a selective inhibitor of TLR4 signaling, prevented this effect. Paradoxically, morphine increased the concentration of interleukin-6, granzyme A, and granzyme B in cell supernatants. Pretreatment of NK cells with TAK-242 prevented the morphine-induced increase in interleukin-6, whereas pretreatment with naloxone inhibited the morphine-induced increase in granzymes A and B.

Conclusions: Both classical opioid receptors and TLR4 participate in morphine-induced suppression of NK cell cytotoxic activity. These studies have important implications for maintenance of immune function during management of cancer pain.

1. Introduction

The clinical course of many cancers unfortunately results in the development of pain and the need for pain treatment, often with medications such as opioids (Portenoy, 2011). Morphine and other structurally related and clinically used opioids modulate cellular function through interaction with both opioid receptors and other receptors such as Toll-like receptor 4 (TLR4) (Hutchinson et al., 2010). The classic function of TLR4 is to recognize lipopolysaccharide, a component of Gram-negative and some Gram-positive bacteria, and trigger a potent immune signaling response (Lu et al., 2008). The “non-classical” function has the potential to modulate neuroinflammatory responses and reduce analgesia (Hutchinson et al., 2012; Wang et al., 2012). Many opioids have been found to inhibit the cytolytic function of human natural killer (NK) cells against the target tumor K562 cell line in vitro through agonism of the mu- and kappa-opioid receptors (Maher et al., 2019). However, it is unclear whether this decreased function is due to downstream signaling through the classical opioid receptors or TLR4. Purified primary human NK cells express mRNA for all opioid receptors and TLR4 (Maher et al., 2019). However, functional modulation was observed only with mu- and kappa-opioid receptor-specific agonists and not agonists of delta-opioid receptors (Maher et al., 2019). Previously, we showed that many nonspecific and clinically used agonists, such as morphine, decrease human NK cell function in vitro but that pretreatment of NK cells with mu- and kappa-specific-antagonists prevented this opioid-induced suppression of NK cell activity (Maher et al., 2019).

We hypothesized that the morphine-associated changes in the ability of human NK cells to induce apoptosis in target tumor cells differs depending on whether opioids engage the classical opioid

* Corresponding author at: Johns Hopkins Blaustein Pain Treatment Center, Johns Hopkins University School of Medicine, 601 North Caroline Street, Baltimore, MD, United States.
E-mail address: dmaher3@jhmi.edu (D.P. Maher).

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receptors or TLR4. The primary objective of this study was to examine the contribution of TLR4 and classical opioid receptors to modulation of NK cell cytokine function and secreted cytokine profiles.

2. Materials and methods

The protocol for blood donation was approved by the Johns Hopkins Institutional Review Board. A certified phlebotomist collected 150 mL of whole blood from healthy male volunteers between the ages of 25 and 39 into heparinized syringes. No subjects had a history of cancer; immunosuppression; HIV; chronic pain; or use of opioids, steroids, or other immunosuppressant medication in the past 12 months. A relatively homogenous cohort was chosen because NK cell function, even among healthy individuals, can change as a function of age and sex (Phan et al., 2017). A similar protocol has already been optimized and previously reported by our group (Maher et al., 2019).

2.1. Isolation and culture of NK cells

Whole blood was washed with phosphate-buffered saline (PBS; Gibco, Gaithersburg, MD) and then layered over Lympholyte-H human cell separation medium (Cedarlane, Burlington, ON) and centrifuged. Buffy coats were collected, and peripheral blood mononuclear cells were counted. NK cells were isolated by a negative selection magnetic-activated cell sorting (MACS) kit according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The isolated NK cells were then counted, and viability was confirmed with 0.4% trypan blue exclusion dye (Gibco). Cells were stained with conjugated surface antibodies, including CD3-pacific blue (0.4 ug/100 ul), CD45-Alexa 700 (0.4 ug/100 ul), CD56-PE (0.4 ug/100 ul) (BioLegend, San Diego, CA), and live/dead cell viability stain (Thermo Fisher, Waltham, MA). Flow cytometry revealed a purity of 95% for NK cells. Viability dyes were used according to the manufacturer's instructions.

NK cells were maintained in 96-well plates at a concentration of 2×10^4 per 50 µL. Growth medium consisted of RPMI-1640 (Gibco) containing 10% fetal bovine serum (FBS; Gemini Bio Products, West Sacramento, CA) and 100 U/mL of penicillin and streptomycin (Gibco).

2.2. Drug treatment

Our previous work examined concentration escalation of morphine concentrations from 25 to 250 ng/ml (Maher et al., 2019). This work also demonstrated that 250 ng/ml of morphine reduced NK cell cytotoxicity and that pretreatment with 100 ng/ml of naloxone effectively prevented this observation (Maher et al., 2019). Further while the concentration is the same, the affinity of naloxone for the opioid receptors is greater than that of morphine (k_i 0.8 +/- 0.1 vs. 3.6 +/−0.3) (Mestek et al., 1995). Unlike opioids, which are often used at different doses, naloxone is most frequently used at a single clinical dose. For the present work, we chose to focus on one concentration of morphine and naloxone that can be reasonably achieved for pain treatment in the clinical setting (Boland and Pockley, 2016). Stock solutions of morphine and naloxone were obtained from the hospital pharmacy and diluted in PBS. All clinically used medications in this study, including naloxone, were racemic mixtures. A cell-permeable inhibitor of TLR-4 known as TAK-242 was used (Cayman Chemicals, Ann Arbor, MI). TAK-242 inhibits the association of TLR4 with Toll/interleukin-1 receptor domain-containing adaptor protein (TIRAP) or Toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon-β-related adaptor molecule (TRAM) (Matsunaga et al., 2011).

NK cells were incubated first with TAK-242 100 ng/mL or an equivalent volume of PBS for 2 h, then with naloxone 100 ng/mL or an equivalent volume of PBS for 2 h, and finally with morphine 250 ng/mL for an additional 2 h. We chose a 2 h incubation because it represents a biologically plausible NK cell exposure duration after parenteral administration.

2.3. K562 cell culture and staining

We chose K562, a chronic myelogenous leukemia-derived cell line devoid of MHC I complexes, as the standardized target tumor cell for the NK cell apoptosis assay because it is non-adherent and can be analyzed by flow cytometry without the need for physical manipulation. Further, use of the K562 cell line has been extensively described in NK cell apoptosis assays (Fischer et al., 2002; Aubry et al., 1999). K562 cells (ATCC, Manassas, VA) were maintained at a concentration of 1×10^6/mL in Iscove's Modified Dulbecco's Medium (ATCC) with 10% FBS and penicillin and streptomycin and used between passages 0 and 2. They were tested with a universal mycoplasma detection kit (ATCC) and confirmed to be negative. Before using the cells in an apoptosis assay, we counted them and checked their viability with 0.4% trypan blue exclusion dye (Gibco). Then we labelled 2 x10^6 of the K562 cells with 5 µM carboxyfluorescein succinimidyl ester (CFSE; Thermo Fisher) according to the manufacturer's instructions.

2.4. Apoptosis assay, annexin V staining, and flow cytometry

We added 2×10^4 CFSE-labelled K562 target cells in 50 µL of media to each well of a 96-well plate containing 2×10^4 NK cells. The cells were mixed with gentle pipetting, and the plate was placed in an incubator for 30 min. Three wells containing only CFSE-labelled K562 cells were used as negative controls. Three wells that contained untreated NK cells and K562 cells were included as positive controls. Finally, as a positive control for apoptosis, we included three wells of K562 cells exposed to 100 mM staurosporine, which has been shown to induce apoptosis in K562 cells (Oliver et al., 2011).

After the 30-min incubation, the cells were stained with annexin V-APC (BioLegend) according to the manufacturer's instructions and 50 µM propidium iodide (PI; Sigma-Aldrich). Cells were analyzed immediately on a CytoFLEX flow cytometer with a 96-well plate reader and CytEXPERT software (version 2.1; Beckman-Coulter, Indianapolis, IN). A sample of 1×10^4 CFSE-positive cells was collected from each well. The percent of CFSE-gated cells that stained positive for annexin V was determined (i.e., the percent of K562 cells undergoing apoptosis). The percent of CFSE-gated cells that stained positive for PI was also determined (i.e., the percent of K562 cells undergoing necrosis).

2.5. Cytokine analysis

The flow cytometry-based CD8/NK cell LEGENDplex panel (BioLegend) was used for analysis of cytokine profiles in acellular sample supernatants. Cytokine analysis was repeated in duplicate according to the manufacturer's instructions, and results were compared to a standardized concentration curve, allowing for absolute cytokine quantification. We analyzed the supernatants of unstimulated K562 cells, unstimulated NK cells, and NK cells that were exposed to morphine, naloxone, and/or TAK-242 as described above but before exposure to K562 cells.

Supernatants collected from the previously described apoptosis assays of NK cells co-incubated with K562 cells were also analyzed. Of the triplicate samples, supernatant from the one that gave the result closest to the median value for the three repeated samples was subsequently analyzed in the cytokine assay. All LEGENDplex analyses were conducted in duplicate, and the average was used for final statistical analysis. The cytokine data were then analyzed with LEGENDplex software version 7 (BioLegend).

2.6. Analysis and statistics

The primary objective was to characterize changes in the ability of NK cells to induce apoptosis after binding of morphine to either the