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Ropivacaine inhibits the migration of esophageal cancer cells via sodium-channel-independent but prenylation-dependent inhibition of Rac1/JNK/paxillin/FAK

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ABSTRACT

The direct anti-proliferative and pro-apoptotic effects of local anesthetics have been well documented in various cancers. However, whether local anesthetics affect cancer metastasis and their underlying molecular mechanisms are not well understood. In this work, we show that ropivacaine at the clinically relevant concentration significantly inhibits esophageal cancer cell migration. Interestingly, ropivacaine at the same concentration does not display inhibitory effects on esophageal cancer cell growth and survival. We further demonstrate that ropivacaine significantly decreases activities of GTPases including RhoA, Rac1 and Ras, and inhibits prenylation in esophageal cancer cells. In addition, the inhibitory effects of ropivacaine on GTPases activities and migration are abolished in the presence of geranylgeraniol and farnesol, demonstrating that ropivacaine inhibits GTPases activities via prenylation inhibition. Finally, we demonstrate that ropivacaine-inhibited esophageal cancer cell inhibition occurs independently of sodium channel but via suppressing Rac1/JNK/paxillin/FAK pathway. Our work demonstrates the potent anti-migratory effect of ropivacaine in esophageal cancer by attenuating prenylation-dependent migratory signalling events. These findings provide significant insight into the potential mechanisms by which local anaesthetics may negatively affect metastasis.

1. Introduction

Esophageal cancer is one of the most common cancers worldwide with five-year survival rate less than 25% [1]. Surgery in combination of chemoradiotherapy is the standard treatment for esophageal cancer but is usually not effective to advanced esophageal cancer patients. Recurrence of cancer after surgery remains a significant clinical challenging. Interestingly, the role of anesthesia during cancer surgery has recently gained attention as substantial retrospective studies have suggested that local anaesthetics can be beneficial to cancer patients [2]. Local anaesthetic use has been shown to reduce the chance of cancer recurrence, minimize the need to use opioid analgesics which suppress the immune system and improve overall survival [3,4]. Pre-clinical studies also demonstrate the direct inhibitory effects of local anaesthetics on various types of tumor cells [5–9].

Ropivacaine is an amide-linked local anesthetics used in the relief of pain in cancer patients during and after tumor removal surgery through blocking voltage-gated sodium channel (VGSC) influx and therefore preventing the generation of propagated action potentials in axons [10]. Of note, VGSC is functionally overexpressed in a variety of carcinomas, including colon, breast, lung and prostate cancers [11]. VGSC has been shown to be critically involved in the colon and breast cancer invasion and metastasis [12,13]. However, it seems that the inhibitory effects of local anesthetics on the multiple biological aspects of tumor cells are through diverse molecular mechanisms, both dependent and independent of VGSC, such as blocking PI3K/Akt/mTOR and focal adhesion kinase [14,15].

In this study, we investigated the effect of ropivacaine on esophageal cancer focusing on migration and systematically annotated its detailed molecular mechanisms. We show that the inhibition of migration rather than growth and survival is the key event by ropivacaine at clinically achievable concentrations in esophageal cancer cells. We further show that ropivacaine inhibits

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esophageal cancer cell migration via VGSC-independent but prenylation-dependent suppression of Rac1/JNK/paxillin/FAK pathway.

2. Materials and methods

2.1. Cell culture and drug reconstitution

Three human esophageal carcinoma cell lines OE33, ESO26 and FLO-1 (Sigma, US) were cultured in RPMI 1640 medium (Invitrogen, US) supplemented with 2 mM glutamine and 10% heat-inactivated fetal bovine serum (Gibco, US). Ropivacaine (Sigma, US) was dissolved in water at 100 mM as a stock. Geranylgerniol (GGOH, Sigma, US), farnesol (FOH, Sigma, US), Veratridine (R&D Systems, US) and tetrodotoxin (Sigma, US) were dissolved in DMSO and citrate buffer, respectively.

2.2. Proliferation and apoptosis assays

Cells (5×10^3 cells/well in a 96-well plate for proliferation and 10^5 cells/well in a 12-well plate for apoptosis) were incubated with Ropivacaine for 72 h. Cells proliferation activities were measured using MTS Cell Proliferation Assay Kit (Abcam, US). Apoptosis was quantified using a cell death detection ELISA kit (Roche Applied Science).

2.3. Boyden Chamber migration assay

Migration assay was performed using the Boyden chamber (Cell Biolabs Inc. US) as described previously with modifications [16]. Cells together with ropivacaine were pre-incubated in MEM medium supplemented with 2% FBS for 30 min at 37 °C and were added onto the Matrigel (BD Biosciences, US)-coated cell culture inserts. MEM medium supplemented with 10% FBS was placed onto the lower chamber. After 6–8 h, the non-migratory cells on the upper surface of the insert were removed with a cotton swab. Migratory cells on the lower surface of inserts were fixed with 4% formaldehyde (Sigma, US) and stained with 0.4% Giemsa. The migrated cells from five random fields were counted under microscope (Zeiss, Germany).

2.4. Western blot analyses

Cells were treated with ropivacaine for 24 h prior to total protein extraction using lysis buffer contained 4% SDS, protease inhibitor cocktail and phosphatase inhibitor (Roche, US). Bicinchoninic acid (Thermo Scientific, US) protein assay was performed to determine protein concentrations. Proteins were resolved using denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blot. Antibodies against Rap1, DnaJ, β-actin, total and phosphor-JNK, -paxillin and -FAK (Santa Cruz Inc, US) were used to perform the western blot analyses.

2.5. Measurement of RhoA, Rac1 and Ras activity

Cells were treated with ropivacaine for 24 h. Cellular RhoA, Rac1 and Ras activities were determined using RhoA G-LISA Activation Assay Kit, Rac1 G-LISA Activation Assay Kit and Ras G-LISA Activation Assay Kit (Cytoskeleton Inc. US).

2.6. Plasmid transfection

pMX GFP Rac(G12V) overexpressing constitutively active Rac1 (a kind gift from Dr. Joan Brugge) was transfected into OE33 cells using Lipofectamine 2000 transfection reagent (Invitrogen) as per the manufacture's protocol. After 48 h transfection, cells were harvested for determining Rac1 activity level or treated with ropivacaine for cell migration assay.

2.7. Statistical analyses

All data are expressed as the mean and standard deviation (SD) to indicate data variability. Statistical analyses of the differences between two groups were performed using the one-way analysis of variance (ANOVA) and subsequently by unpaired Student's t-test. P-value < 0.05 was defined as statistically significant.

3. Results

3.1. Ropivacaine potently inhibits esophageal cancer cell migration without affecting growth and survival

Local anesthetics have been shown to negatively affect cancer development via inhibiting growth and inducing apoptosis [5,6]. To understand the potential role of ropivacaine in esophageal cancer metastasis, we performed Boyden Chamber assay using multiple esophageal cancer cell lines: OE33, ESO26 and FLO-1 [17]. Boyden Chamber assay is the most accepted technique used to analyze cell migration and invasion [18]. We found that ropivacaine at 50, 100 and 200 μM significantly inhibited migration in OE33, ESO26 and FLO-1 cells in a dose-dependent manner (Fig. 1A and B). Similar to ropivacaine, we further found that other local anesthetics (eg, bupivacaine and lidocaine) at micromolar concentration significantly inhibited migration of esophageal cancer cells (Supplementary Fig. S1). It is consistent with the recent publication that bupivacaine at micromolar concentration inhibits migration of gastric cancer cells [19]. Interestingly, ropivacaine at the same concentrations has either slight or no inhibition on growth and viability on all tested esophageal cancer cell lines. These results demonstrate that the anti-migratory effect of ropivacaine is specific rather than the consequence of decreased cell viability.

3.2. Ropivacaine decrease RhoA, Rac1 and Ras activities and inhibits prenylation signalling in esophageal cancer cells

Rho GTPases play an essential role in regulating cell spreading, adhesion, and migration downstream of integrin engagement with the extracellular matrix [20]. Among all the Rho family members, RhoA and Rac1 are the dominant and active GTPases capable of signaling to a diverse array of downstream effectors to regulate migration [21]. We therefore investigated whether ropivacaine affected RhoA and Rac1 activities. We found that ropivacaine dose-dependently decreased both RhoA and Rac1 activities in esophageal cancer cells (Fig. 2A and B). Interestingly, we observed that ropivacaine also decreased the activity of other GTPase protein Ras in esophageal cancer cells (Fig. 2C).

It is known that the function of GTPase proteins are largely dependent on prenylation which is post-translational modification that anchors the proteins to the plasma cell membrane, for binding to effector molecules in the various downstream signaling pathways [22]. Our results that ropivacaine effectively decreases activation of RhoA, Rac1 and Ras suggest the potential inhibitory effects of ropivacaine on protein prenylation. We next analysed the prenylation status of DnaJ and Rap1A using immunoblotting because their unprenylated forms display reduced mobility in denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis compared with their prenylated forms. As expected, we observed that DnaJ and Rap1A were in the processed, prenylated forms in control whereas treatment with ropivacaine inhibited the processing, resulting in unprenylated forms with reduced