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Amide-linked local anesthetics preferentially target leukemia stem cell through inhibition of Wnt/β-catenin

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ABSTRACT

The anti-cancer activities of amide-linked local anesthetics have been demonstrated in various types of bulky/differentiated cancer cells. However, whether these anesthetics also affect biological functions of cancer stem cells is largely unknown. In this study, we systematically investigated the effects of three commonly used amide-linked local anesthetics (ropivacaine, lidocaine and bupivacaine) on leukemia stem cell (LSC) derived from two different leukemia diseases (acute myeloid leukemia, n = 8 and chronic myeloid leukemia, n = 8) as well as normal hematopoietic stem cell (HSC) derived from cord blood donors (n = 8) as comparison. We show that all three local anesthetics at clinically achievable concentrations significantly inhibit colony formation and serial replating of LSC in a dose-dependent manner, suggesting their inhibitory effects on LSC differentiation, proliferation and self-renewal. In addition, lidocaine and bupivacaine are more potent than ropivacaine. However, local anesthetics at the same concentrations do not affect LSC and HSC survival, demonstrating the differentiation and self-renewal as the primary effects of local anesthetics on LSC and HSC. Interestingly, local anesthetics display certain selectivity between LSC and HSC by having higher efficacy on LSC than HSC. Mechanism studies using both pharmacological and genetic approaches demonstrate that these local anesthetics target LSC via inhibiting Wnt/β-catenin but not Hedgehog or NF-κB signaling. Our work is the first to demonstrate the possible influence of amide-linked local anesthetics on cancer as well as normal stem cells via inhibiting Wnt/β-catenin signaling. Our findings contribute to the comprehensive understanding of potential implication of amide-linked local aesthesis in tumor biology.

1. Introduction

Although the primary indications of anesthetics are for preoperative, intraoperative and postoperative management, there is emerging evidence indicating the potential implication of anesthetics in cancer biology [1]. Retrospective studies on the cancer patients received anesthetics during surgical tumor removal and management of postoperative chronic pain demonstrate that the type of anesthesia chosen for surgery could be crucial and may potentially influence long-term outcome of the disease [2–4]. Anesthetics such as opioids can adversely interact with the cellular immune system and affect long-term outcome via decreasing patient's defence against tumor progression [5]. However, there is a particular interest at present in the effect of regional anesthesia using local anaesthetics, which appears to be beneficial to cancer patients as shown by the reduced cancer recurrence and improved overall survival [6–8].

Ropivacaine, lidocaine and bupivacaine have a similar chemical structure and belong to a class of amide-linked local anesthetics. They are commonly used in regional anesthesia and inhibit depolarization of the nerve membrane by blocking voltage-gated sodium-channel and therefore interfering with both Na^+ and K^+ currents [9]. In contrast, pre-clinical studies using cell culture and xenograft mouse models demonstrate that amide-linked local anesthetics also have pro-apoptotic, anti-proliferative and anti-migratory effects in various cancer cells [10–13]. The molecular mechanisms of amide-linked local anesthetics on cancer cells are

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not well elucidated but include inhibition of PI3K/Akt/mTOR and Src signaling [12,13].

In this study, we investigated the biological effects of three amide-linked local anaesthetics in cancer stem cell using two leukemia disease models: chronic myeloid leukemia (CML) and acute myeloid leukaemias (AML). We examined the proliferation, differentiation and self-renewal properties of two types of leukemia stem cells (LSC) exposed to local anaesthetics and used hematopoietic stem cell (HSC) from cord blood (CB) as normal cell control. Finally, we investigated the mechanism of the action of three local anaesthetics on LSC.

2. Materials and methods

2.1. Leukemia cell lines and drugs

CML cell line K562 and AML cell line HL60 (ATCC, US) were cultured in RPMI 1640 Medium supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. (Invitrogen, US) in a 5% CO_2 environment at 37 °C. K562 and HL62 cells in the exponential growth phase were used for cellular assays. Ropivacaine, lidocaine, bupivacaine, lithium chloride (LiCl) were obtained from Sigma.

2.2. Primary leukemia stem cell isolation

CD34+or CD34+CD38-cells were purified from bone marrow mononuclear cell of CML or AML patients seen at the Jingzhou Central Hospital. Cord blood (CB) samples were obtained from Jingzhou Central Hospital. Written informed consent was obtained from all patients and normal donors before specimen collection, under a research protocol approved by the Jingzhou Central Hospital Institutional Review Board. Mononuclear cells were isolated using Ficoll-Paque protocol (GE Healthcare Life Sciences, US). CD34+cells were then selected using CD34 MicroBead kit (Miltenyi Biotec, Germany). CD34+cells were labeled with monoclonal PE-conjugated anti-CD34 and allophycocyanin-conjugated anti-CD38 (BD Biosciences), CD34+CD38-cells were then analyzed and sorted using a MoFlo cell sorter (Beckman Coulter). CML and AML stem cells were cultured using in StemPro complete medium (Life Technologies, US) supplemented with cytokines [14] or StemSPAN complete medium (STEMCELL Technologies, US) supplemented with 150 ng/ml FLT3-ligand, 150 ng/ml Stem cell factor and 50 ng/ml Interleukin-3, respectively.

2.3. Measurement apoptosis

Cells were incubated with drugs for 72 h and then labeled using Annexin V/7-AAD kit (Beckman Coulter, US). The percentage of Annexin V-positive population was quantified by using flow cytometry (Beckman Coulter).

2.4. Colony-forming and serial replating assays

CD34+CD38-cells together with drugs were plated in HSC-CFU complete w/o Epo methylcellulose medium (Miltenyi Biotec, Germany). The number of colonies was scored after 10–14 days and clusters with more than 100 cells were counted as a colony. For serial replating assays, individual colonies formed in colony-forming assay were picked and replated in HSC-CFU complete methylcellulose in a 96-well format. After 2-week incubation, wells were scored as positive for the presence of colonies. 1st serial replating capacity is determined by the percentage of number of positive wells among number of colonies plated. Further rounds of serial replating were performed until no more colonies formed. Each serial replating takes 2 weeks.

2.5. Western blot (WB) analysis

Cells were treated with drugs for 24 h and then lysed by RIPA buffer (Life Technologies, US). Protein concentration were determined using the icinchoninic acid assay (Pierce, US). Equal amount of proteins were resolved using denaturing SDS–PAGE and analyzed by WB using antibodies against Axin, β-catenin and β-actin (Santa Cruz Technology, US).

2.6. Plasmid transfection

TOPflash assay was conducted using the same protocol as our previous study described [15]. Cells were treated with drugs for 24 h. NFkB activation was determined using NFkB p65 Transcription Factor Assay Kit (Abcam, US) by ELISA according to manufacturer's instructions. Hedgehog activity was determined by measuring GLI transcription activity using The Cignal GLI Reporter Assay Kit (Qiagen, US) according to manufacturer's instructions. Briefly, cells were transfected with Cignal GLI Reporter or negative control along with Gli1 expression vector or empty vector. After 24 h post-transfection, cells were treated with drugs for 24 h prior to performing a dual-luciferase assay. Promoter activity values are expressed as arbitrary units using a Renilla reporter for internal normalization. For β-catenin overexpression, cells were transfected with 2 μg pcDNA or pcDNA-β-cat (human β-catenin pcDNA3 plasmid, a kind gift from Dr. Hui Li [15]) using the Amaxa NucleofectorTM kit (Lonza, Germany) for 48 h prior to the indicated drug treatment.

2.7. Statistical analyses

All experiments in this study were repeated at least three times with similar results. The data are expressed as mean and standard deviation (SD). Statistical analyses were performed by unpaired Student's *t*-test, with p-value < 0.05 considered statistically significant.

3. Results

3.1. Amide-linked local anesthetics preferentially inhibit colony formation of leukemia stem cells (LSC)

The three unique characterization of hematopoietic stem cells are long term self-renewal, proliferation and multi-lineage differentiation [16]. We firstly investigated the effects of amide-linked local anesthetics on the proliferation and differentiation of leukemia stem cells (LSC) using colony formation assay. CD34+CD38-LSC cells are purified from AML or CML bone marrow mononuclear cells using CD34 microbeads selection followed by sorting using anti-CD38 antibody. CD34+CD38-cells were also purified from cord blood (CB) as a normal hematopoietic stem cell (HSC) and used as a comparison with LSC.

As expected, CD34+CD38-from AML or CML patients as well as CB samples proliferated and differentiated into colonies in a semi-solid methycellulose media containing various growth factors and cytokines (Fig. 1A). However, in the presence of ropivacaine, lidocaine or bupivacaine, a remarkable reduction on the number of colonies formed were observed (Fig. 1A). In addition, lidocaine and bupivacaine at 100 μM completely abolished the abilities of LSC to form colonies. Interestingly, we noted that the size of the colonies formed by CB CD34+CD38-cells was smaller than the colonies formed by AML or CML CD34+CD38-cells (Fig. 1A), suggesting the difference between HSC and LSC. Quantification of colony forming assay showed that local anesthetics at 10–100 μM significantly inhibited colony formation of LSC in a dose-dependent manner

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