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ORIGINAL ARTICLE

Clinically relevant concentrations of lidocaine inhibit tumor angiogenesis through suppressing VEGF/VEGFR2 signaling

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Abstract

Background Angiogenesis, the formation of blood vessel, is required for invasive tumor growth and metastasis. In this study, the effects of lidocaine, an amide-link local anesthetic, on angiogenesis and tumor growth were investigated.

Methods In vitro angiogenesis assays were conducted using human umbilical vascular endothelial cell (HUVEC). Essential molecules involved in vascular endothelial growth factor (VEGF) signaling were analyzed. Tumor angiogenesis was analyzed using in vivo mouse tumor model.

Results Lidocaine at clinically relevant concentrations inhibited angiogenesis. Lidocaine inhibited endothelial cell in vitro capillary network formation on Matrigel through interfering early stage of angiogenesis. In addition, lidocaine inhibited vascular endothelial growth factor (VEGF)-stimulated endothelial cell migration and proliferation without affecting cell adhesion. Lidocaine also induced endothelial cell apoptosis in the presence of VEGF. Lidocaine suppressed VEGF-activated phosphorylation of VEGF receptor 2 (VEGFR2), PLCγ-PKC-MAPK and FAK-paxillin in endothelial cells, demonstrating that VEGF, PLC, MAPK and FAK-paxillin suppression is associated with the antiangiogenic effect of lidocaine. Importantly, the in vitro observations were translatable to in vivo B16 melanoma mouse model. Lidocaine significantly inhibited tumor angiogenesis, leading to delay of tumor growth.

Conclusions This study is the first to report that lidocaine acts as an angiogenesis inhibitor. The findings provide preclinical evidence into the potential mechanisms by which lidocaine may negatively affect cancer growth and metastasis.

Keywords Lidocaine · Local anesthetics · Tumor angiogenesis · VEGF · VEGFR2

Introduction

Angiogenesis, the new blood formation from the pre-existing vessels, is closely associated with pathogenesis of several pathological conditions. Particularly, tumorigenesis and metastasis are largely dependent on angiogenesis which guarantees adequate supply of tumor cells with nutrients and oxygen and provides efficient drainage of metabolites [1]. In addition, the degree of tumor vascularization is correlated with tumor grade as well as aggressiveness [2, 3]. Among many angiogenesis activators, vascular endothelial growth factor (VEGF) which is highly expressed in tumor microenvironment are the most potent and specific ones to induce tumor angiogenesis [4]. VEGF regulates angiogenesis mainly through VEGF receptor 2 (VEGFR2) and VEGF/VEGFR2 preferentially utilizes the PLCγ-PKC-MAPK pathway for signaling [5]. The VEGF/VEGFR system is an important target for anti-angiogenic therapy in cancer. FDA-approved drug bevacizumab, a VEGF-A antibody, has been used to treat a number of types of cancers including hepatocellular carcinoma, lung cancer and renal cell carcinoma [6]. Other angiogenesis inhibitors, such as sunitinib, are currently used for the treatment of renal cell carcinoma and gastrointestinal tumor [7, 8].

Lidocaine is an amide-linked local anesthetic commonly used for regional anesthesia and pain relief. Intraarterial administration of lidocaine has been reported to improve postoperative rehabilitation in cancer patients [9]. Notably, retrospective studies have suggested that the administration

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of local anesthesia during cancer surgery could reduce cancer recurrence [10–12]. In line with the clinical observations, increasing preclinical evidence have also shown that lidocaine and other local anesthetics have direct inhibitory effects on tumor cells [13–18]. Additionally, local anesthesia (e.g., lidocaine and ropivacaine) have been reported to augment chemotherapeutic agents' efficacy in cell culture system and tumor xenograft models in various cancers [14, 17]. However, the effects of local anesthetic on tumor angiogenesis have not been revealed. In this study, the effects of lidocaine at clinically relevant concentrations on tumor angiogenesis using in vitro and in vivo angiogenesis assays were investigated. The associated underlying mechanisms of lidocaine's action on angiogenesis were also systematically analyzed.

Materials and methods

Cell culture and compounds

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and cultured in CSC complete growth medium (Cell System Corporation, US) that contained 10% fetal bovine serum (FBS). Only cells of passages 2–6 were used in experiments. HUVECs used for all the assays were starved in CSC basal medium containing 2% FBS for 3 h before drug treatment. The experimental condition for endothelial cell starvation is the same as previously reported [19, 20]. B16 melanoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Human recombinant VEGF (R&D Systems, US) and lidocaine (Sigma, US) were dissolved in water.

Cell proliferation and apoptosis

HUVEC or B16 (10^4 cells per well in 96-well plate for proliferation and 5×10^4 cells per well in 24-well plate for apoptosis) were treated with lidocaine in the absence/presence of 10 ng/ml human VEGF for 3 days. Cell proliferation was determined using BrdU Cell Proliferation Assay kit (Abcam, US). Cell apoptosis was determined using TUNEL Assay kit (R&D Systems, US). The absorbance value was measured using Spectramax M5 microplate reader (Molecular Devices).

Boyden chamber migration assay

To perform migration assay, HUVEC (10^4 cells per well) together with lidocaine were seeded into the upper well of a Boyden chamber system (Cell Biolabs, US) on polyethylene terephthalate membrane with 8-μm pores. 10 ng/ml human VEGF was added as a chemo-attractant into the lower well. After 8 h incubation, cells migrating through the membrane were fixed and stained with 0.4% Giemsa. The migrated cells from five random fields were chosen in each insert, and the cell number was counted under microscope.

Cell adhesion assay

Quantitative HUVEC adhesion was measured using Vybrant™ Cell Adhesion Assay kit according to manufacturer's instructions. Briefly, calcein-labeled HUVEC (10^3 cells per well) was prepared and then added together with lidocaine onto a diluted Matrigel-coated 96-well plate. After 1 h drug treatment, non-adherent calcein-labeled cells were removed by gentle washing. The calcein absorbance was measured on fluorescence microplate reader.

In vitro capillary network formation assay

HUVEC (2×10^4 cells per well) together with lidocaine were plated onto the polymerized Matrigel (Chemicon International Inc. US) in 96-well plate. After 8 h incubation in cell culture incubator, tube-like structure formation was documented using an inverted microscope (Zeiss, Germany). To investigate at which stage lidocaine interfered with in vitro angiogenesis, time-course analysis was performed where lidocaine was added into culture medium at time-points of 0, 0.5, 1, 2, and 4 h after seeding cells onto Matrigel. Capillary length was quantified by measuring the length of branches from representative fields using NIH Image J software.

Denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot (WB) analyses

Cells were treated with lidocaine for 24 h. Cells were lysed with lysis buffer (Invitrogen, US) supplemented with protease and phosphatase inhibitors (Roche, US). Proteins (50 μg) were resolved using denaturing SDS–PAGE and analyzed by WB. Antibodies used in WB analyses are against phosphor and total VEGFR2, PLCλ1, PKCζ, MAPK, FAK, paxillin and β-actin (Cell Signaling Technologies, US). Immunoblots shown were representative of three independent experiments and quantified using Image J software.

Xenograft experiments in nude mice

Animal experiments were performed in accordance with protocols approved by the Animal Research Committee of Hubei College of Arts and Science. B16 (5×10^6) cells were injected in the right flank of 6-week-old male SCID BALB/c nude mice. After development of palpable tumor, mice were randomly divided into two groups: saline as control and 30 mg/kg lidocaine twice a week through intraperitoneal

injection. After 3 weeks, tumors were isolated to prepare frozen section. Tumor-frozen section slides were fixed with 4% paraformaldehyde. The slides were incubated with primary antibodies against CD31 (Cell Signaling, US), and then secondary antibody conjugated with horseradish peroxidase-DAB. The nuclei were counterstained with hematoxylin (Sigma, US).

Statistical analyses

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. All in vitro experiments were performed at least three times. The in vivo mouse experiment was performed twice independently. The results presented in graphs were relative to control. We setup control as 1 for each independent experiment and, therefore, SEM for control was zero. P value < 0.05 was defined as statistically significant.

Results

Clinically relevant concentrations of lidocaine inhibit angiogenesis in vitro

To investigate the effect of lidocaine on angiogenesis, a well-established in vitro angiogenesis assay was performed. Coined capillary network formation, the assay was conducted using Matrigel matrix which are enriched in extracellular matrix (e.g., 60% laminin, 30% collagen IV and 8% entactin) and growth factors (e.g., VEGF, epidermal growth factor and fibroblast growth factor). Endothelial cells can rapidly align and form tubular structures within 8 h when cultured on complete Matrigel [21]. As shown in Fig. 1a, b, lidocaine at 0.05, 0.1 and 0.2 mM, significantly inhibited HUVEC capillary network formation in a dose-dependent manner. Kaba et al. demonstrated that up to 20 μM (4.6 ug/ml) of lidocaine was detected in patients given continuous lidocaine infusion at a rate of 1.33 mg/kg/h [22]. A starting concentration of 50 μM of lidocaine was an effective dose to inhibit angiogenesis in this study, suggesting that the effective does of lidocaine is clinically relevant.

The capillary network formation is a multi-step and dynamic process including cell attachment to matrix, migration, spreading, cell–cell adhesion and morphogenesis [23]. While cell attachment, migration and spreading occur in the early stage of in vitro tube formation, cell–cell interaction and morphogenesis occur in the late stage. Time course analysis was performed to determine which stage lidocaine interfered with capillary network formation. 0.2 mM lidocaine was added at 0, 0.5, 1, 2, 4 and 8 h, respectively, after HUVEC were plated onto Matrigel matrix. As shown in Fig. 1c, d, hardly any tubular network was formed when lidocaine was added together with HUVEC to Matrigel (0 h) or after 0.5 h after plating HUVEC to Matrigel. There was a gradual loss of angiogenesis inhibition when lidocaine was added at 1 or 2 h. Additionally, lidocaine became ineffective in inhibiting tube formation when it was added at 4 h after plating HUVEC to Matrigel. These demonstrate that lidocaine inhibits angiogenesis mainly by disrupting the early stages of angiogenesis, possibly through interfering endothelial cell adhesion and migration.

Lidocaine inhibits endothelial cell migration, growth and survival without affecting cell attachment and spreading

To further understand how lidocaine inhibits angiogenesis, the effects of lidocaine on the multiple biological activities of endothelial cell, including adhesion, migration, growth and survival were investigated. Calcein has been proven to be an excellent reagent for measuring integrin or other extracellular matrix-mediated endothelial cell adhesion [24]. Interestingly, lidocaine did not affect HUVEC adhesion to diluted Matrigel as assessed by calcein (Fig. 2a). VEGF is the most essential and specific angiogenic growth factor, promoting endothelial cell migration, growth and survival [25]. To mimic the physiological conditions of angiogenesis, the effects of lidocaine on endothelial cells in the presence of VEGF were examined. As expected, VEGF significantly stimulated HUVEC migration and proliferation, and protects HUVEC apoptosis from starvation (2% FBS) (Fig. 2b–d). Furthermore, it was found that lidocaine significantly inhibited VEGF-stimulated endothelial cell migration and proliferation (Fig. 2b, c). In addition, lidocaine induced endothelial cell apoptosis in the presence of VEGF (Fig. 2d). Time course analysis of apoptosis demonstrated that lidocaine induced endothelial cell apoptosis starting from 24-h treatment (Fig. 2e).

Lidocaine inhibits VEGF/VEGFR2 signaling in endothelial cells

VEGF and its receptor VEGFR-regulated signaling have been shown to play critical role in angiogenesis and VEGF-VEGFR system is an important target for anti-angiogenic therapy in cancer [4]. VEGFR-2, as the major signal transducer for VEGF-regulated angiogenesis, preferentially utilizes the PLCγ-PKC-MAPK pathway for signaling. VEGFR2 phosphorylation at Tyrosine 951(T951) and 1175 (T1175) mainly activates downstream signaling and leads to endothelial cell migration and proliferation to form vasculature [26]. Therefore, the phosphorylation levels of the molecules involved in VEGF-VEGFR2 signaling in endothelial cell exposed to lidocaine were examined.

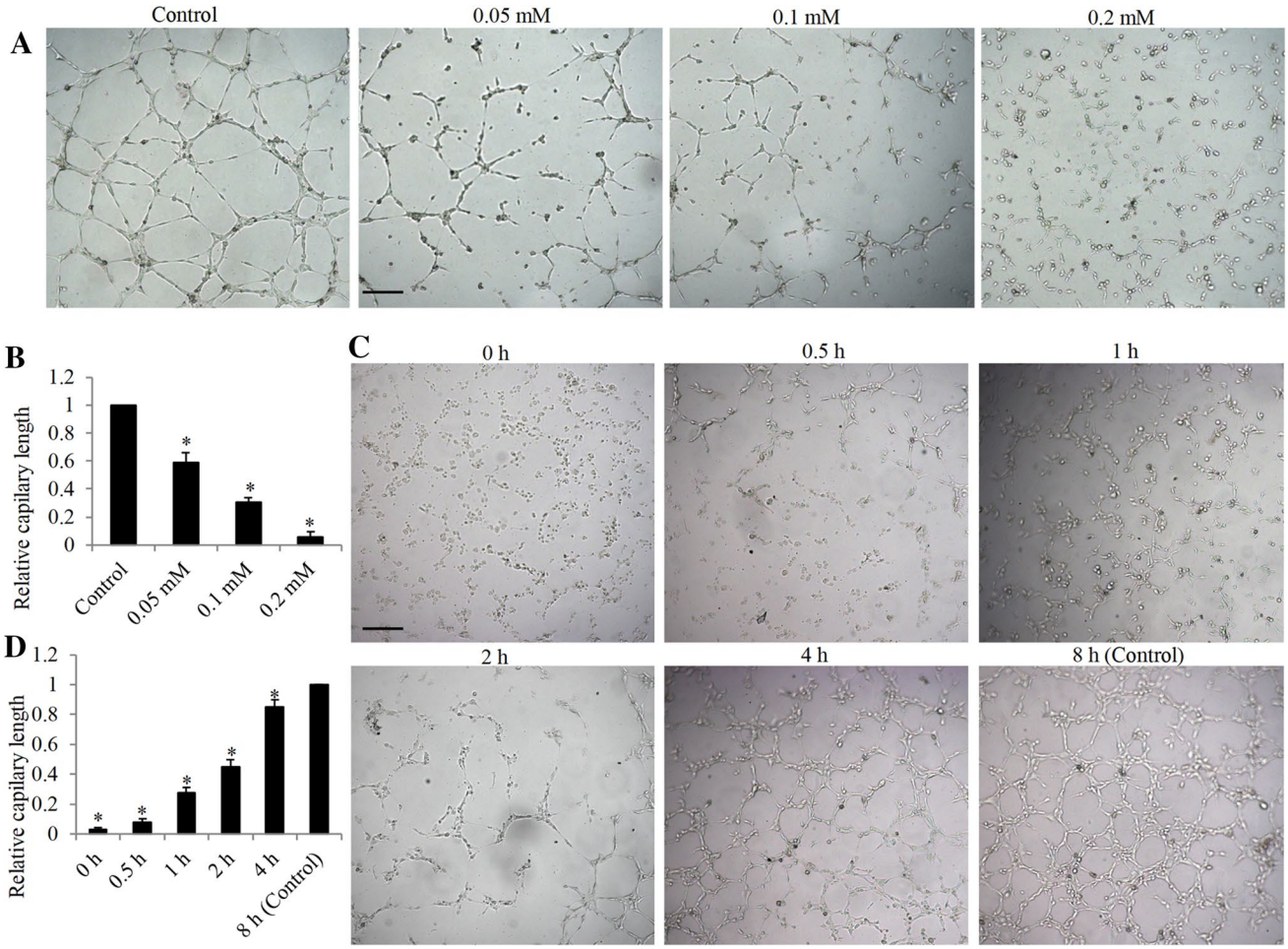

Fig. 1 Clinically relevant concentrations of lidocaine inhibits in vitro angiogenesis. Representative images (**a**) and quantification of capillary length (**b**) using Image J software showing that lidocaine at 0.05, 0.1 and 0.2 mM inhibits endothelial cell tube formation. Representative images (**c**) and quantification of capillary length (**d**) showing that lidocaine (0.2 mM) inhibits endothelial cell capillary network formation in a time-dependent-manner. Lidocaine is required to be present from the early stages of in vitro angiogenesis assay to prevent capillary network formation. Lidocaine was added at 0, 0.5, 1, 2, 4 and 8 h, respectively, after HUVEC were plated onto diluted Matrigel matrix. All capillary network formation was documented at 8 h after endothelial cell plating onto diluted Matrigel matrix. Scale bar represents 200 μm. The mean and SEM of capillary length were based on five independent experiments

In line with the previous publication [26], VEGF increased p-VEGFR2 (T951) and (T1175) levels in HUVEC (Fig. 3a, b), demonstrating the activation of VEGF-VEGFR2 signaling. Notably, lidocaine suppressed VEGF-increased phosphorylation of VEGFR2 (Fig. 3a, b). Consistently, lidocaine suppressed VEGF-stimulated phosphorylation of PLCγ, PKC and MAPK in endothelial cells (Fig. 3a, c). In addition, lidocaine suppressed VEGF-stimulated phosphorylation of focal adhesion kinase (FAK) and paxillin in endothelial cells (Fig. 3a, d). Taken together, these results clearly indicate that lidocaine inhibits angiogenesis through suppressing VEGF/VEGFR2 signaling.

Lidocaine inhibits tumor growth via suppressing tumor angiogenesis in vivo

Tumor growth is largely dependent on tumor angiogenesis [27]. Since lidocaine acts as an angiogenesis inhibitor, it was hypothesized that lidocaine could inhibit tumor angiogenesis, leading to inhibition of tumor growth. Using a mouse B16 graft model, the development of tumor growth was investigated by monitoring its volume and tumor angiogenesis by labeling the blood vessel using endothelial cell marker CD31 [28]. The mice tolerated 30 mg/kg lidocaine well and no significant body weight loss was observed (data not shown). In addition, it was found that lidocaine significantly

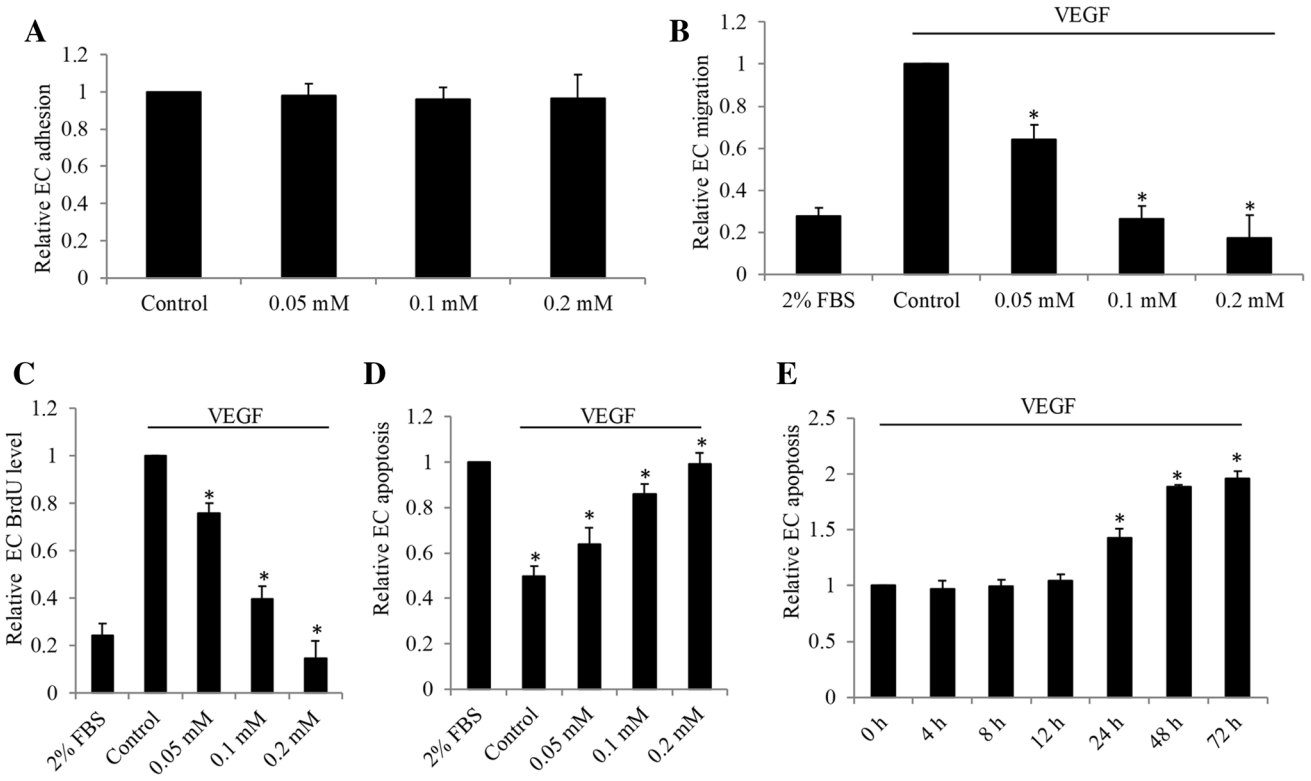

Fig. 2 Lidocaine inhibits endothelial cell migration, growth and survival without affecting endothelial cell attachment and spreading. **a** Lidocaine does not affect EC adhesion onto 10× diluted Matrigel matrix. Lidocaine dose-dependently inhibits endothelial cell migration (**b**) and proliferation (**c**), and induces endothelial cell apoptosis (**d**). **e** Lidocaine (0.2 mM) induces endothelial cell apoptosis after 24 h treatment. *$P < 0.05$, compared to control or 0 h

suppressed B16 tumor growth throughout the duration of treatment (Fig. 4a, b). Moreover, lidocaine significantly disrupted blood vessel formation in tumors as shown by the significantly lesser lumen structures in lidocaine-treated group than control (Fig. 4c, d). Interestingly, compared to endothelial cells, lidocaine at the same concentration did not affect B16 melanoma cell growth and survival (Fig. 5), suggesting that endothelial cells were more susceptible than B16 cells to lidocaine treatment.

Discussion

Since retrospective studies have suggested that perioperative anesthetic management of cancer patients could potentially affect the risk of recurrence and metastases [11], the effects of anesthetics on tumor have gained increasing attention. There are increasing studies demonstrating the biological effects of various types of anesthetics (e.g., local and intravenous anesthetics and opioids) on tumor growth, metastasis, angiogenesis and chemoresistance [13, 17, 29–31]. Using cell culture system and xenograft mouse models, the anti-proliferative, pro-apoptotic and anti-metastatic activities of amide-linked local anesthetics have been consistently shown in a panel of cancers [17, 32, 33]. However, little is known on whether local anesthetics affect tumor angiogenesis which plays a critical role in both tumor development and metastasis. To fully explore the role of amide-linked local anesthetics in tumor biology, this study systematically investigated the effect of lidocaine on angiogenesis and is the first to demonstrate that lidocaine acts as an angiogenesis inhibitor.

At clinically relevant concentrations [22], lidocaine inhibited endothelial cell capillary network formation in a dose- and time-dependent manner (Fig. 1). Due to the potential limitations of in vitro angiogenesis [34], it was demonstrated that these effects of lidocaine translated into decreased tumor neovascularization in vivo in a B16 melanoma model (Fig. 4c, d). Expectedly, the significant reduction of B16 melanoma growth was observed (Fig. 4a, b) which supports the well-known notion on the important role of tumor angiogenesis in tumor development. Overall, the effects of anesthetics on tumor angiogenesis are not fully explored. The findings on the effects of opioid anesthetic morphine seem to be contradictory. While Lisa et al.'s work demonstrated that morphine suppressed tumor angiogenesis associated with tumor growth [35], Gupta et al.'s work showed that morphine stimulated angiogenesis by activating

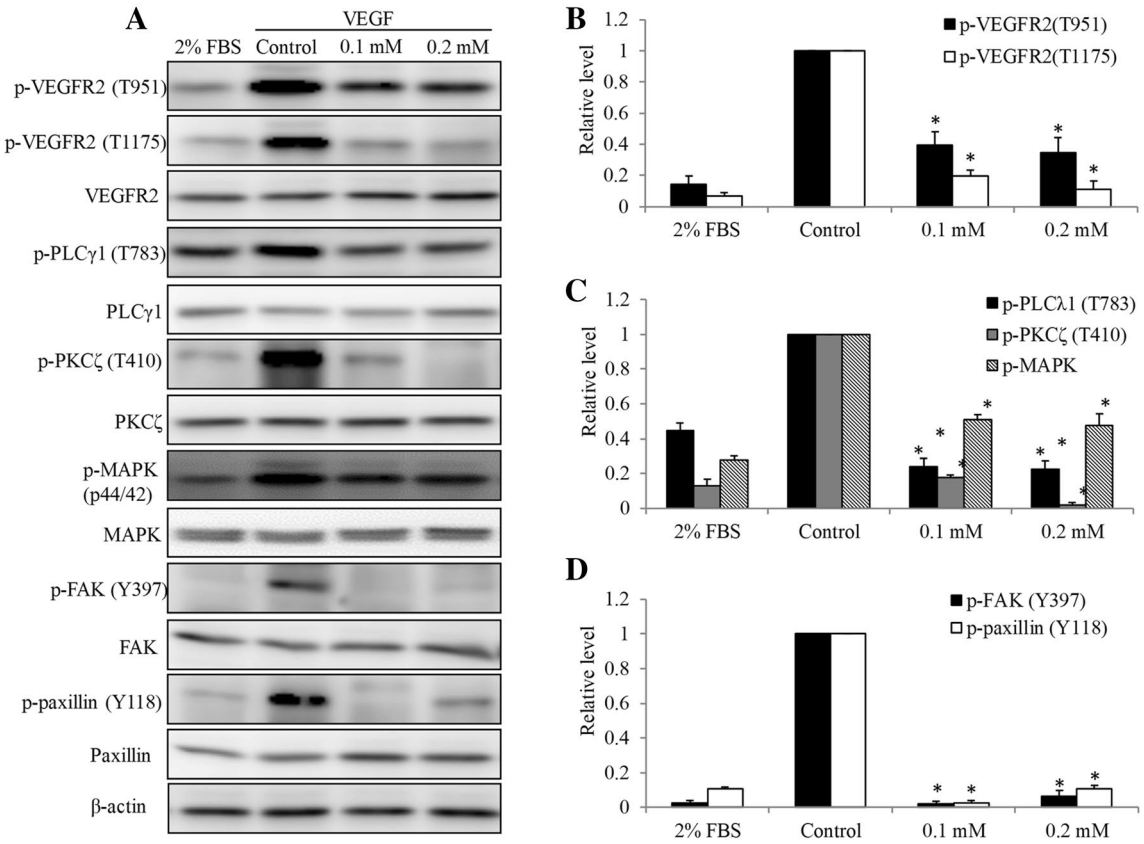

Fig. 3 Lidocaine inhibits VEGF/VEGFR2 signaling in endothelial cells. **a** Western blots of HUVEC treated with lidocaine in the presence of VEGF for 24 h. Antibodies used in western blot analyses include anti-p-VEGFR2 (T951), anti-p-VEGFR2 (T1175), anti-VEGFR2, anti-p-PLCλ1 (T783), anti-PLCλ1, anti-p-PKCζ (T410), anti-PKCζ, anti-MAPK (p44/42), anti-MAPK, anti-p-FAK (Y397), anti-FAK, anti-p-paxillin (Y118), anti-paxillin and anti-β-actin. Representative western blot photos were shown. **b–d** Quantitative evaluation of the effect of lidocaine by western blotting. Normalization of expression of p-VEGFR2, p-PLCλ1, p-PKCζ, p-MAPK, p-FAK and p-paxillin is addressed by their corresponding total protein level *$P < 0.05$, compared to control

proangiogenic and survival-promoting signaling and promotes breast tumor growth [31]. Only two studies reported that intravenous anesthetics propofol inhibits angiogenesis [36, 37]. This study adds local anesthetic lidocaine to the list of anesthetics that display anti-angiogenic potential.

Angiogenesis is a multi-step and dynamic process, including endothelial cell attachment, spreading, migration and morphogenesis. In particular, lidocaine inhibited VEGF-stimulated migration in endothelial cell (Fig. 2b). Since cell attachment to matrix, migration and spreading are the early steps of endothelial capillary network formation, the anti-migration by lidocaine in endothelial cells is supported by the time course analysis that lidocaine inhibited angiogenesis mainly by disrupting the early stage of angiogenesis (Fig. 1b). This finding supports the previous work on the anti-migratory effect by lidocaine at micromolar concentrations in lung cancer cells [15]. In addition, although lidocaine disrupted early steps of angiogenesis, it is noted that lidocaine did not affect endothelial cell adhesion to the diluted Matrigel matrix (Fig. 2a). Lidocaine also significantly inhibited VEGF-stimulated proliferation with IC50 ~ 0.1 mM and induced apoptosis in endothelial cells in the presence of VEGF (Fig. 2c, d). The anti-proliferative and pro-apoptotic effects of lidocaine in cancer cells have been shown by various studies [13, 14]. However, lidocaine only inhibited proliferation and survival in cancer cells at millimolar concentrations (e.g., 5 mM) [14], suggesting that endothelial cells are more sensitive than cancer cells to lidocaine treatment. This is also supported by the results that lidocaine at micromolar concentrations does not affect B16 melanoma cell growth and survival (Fig. 5). These suggest that it is highly likely that lidocaine inhibits B16 tumor growth via suppressing angiogenesis.

The potent anti-angiogenic activity of lidocaine is additionally supported by the observations that lidocaine almost completely suppressed the VEGF-induced activation of VEGFR2, PLCγ-PKC-MAPK and FAK-paxillin in endothelial cells (Fig. 3). VEGF, the most specific and potent angiogenesis growth factor, induces angiogenesis through activating VEGF-VEGFR2 at VEGFR2 (T951) and (T1175) and

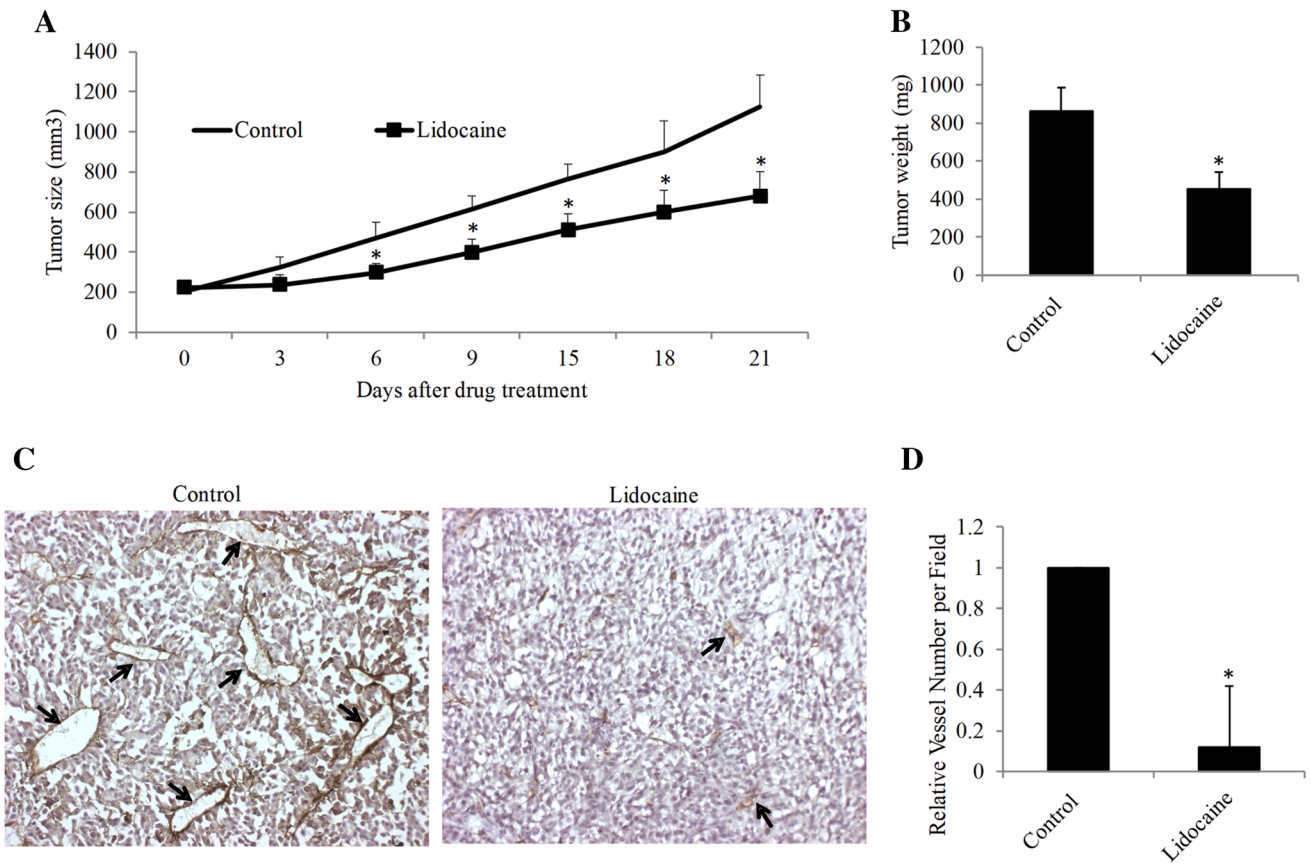

Fig. 4 Lidocaine inhibits melanoma growth and suppress tumor angiogenesis in vivo. Lidocaine significantly decreases B16 melanoma size (**a**) and weight (**b**) throughout the duration of treatment. Representative immunohistochemistry photos of tumor tissues (**c**) and quantification (**d**) show that lidocaine significantly inhibits tumor angiogenesis. Tumor blood vessels (indicator by arrows) were visualized by CD31 staining. For vessel quantification, the average number of vessels per microscopic field, from three microscopic fields per tumor section was analyzed. $^{*}P < 0.05$, compared to control

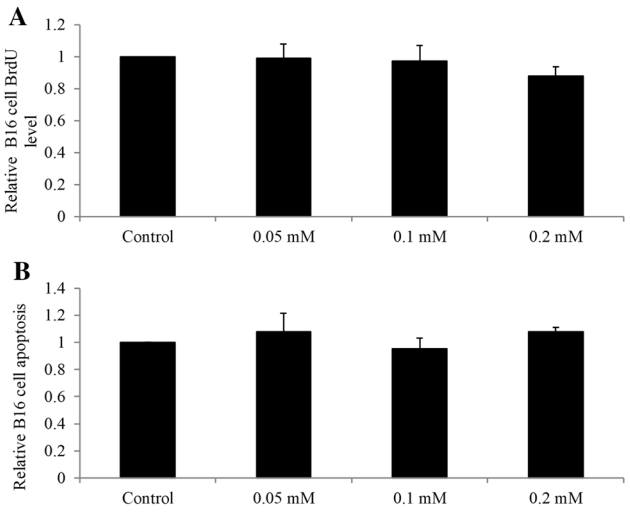

Fig. 5 Lidocaine does not affect B16 melanoma cell growth and survival. Lidocaine at 0.05, 0.1 and 0.2 mM does not affect B16 cell growth (**a**) and apoptosis (**b**). B16 cells were treated with lidocaine for 3 days prior to proliferation and apoptosis analysis

triggering the downstream signaling pathway PLCγ-PKC-MAPK [4]. The findings in this study on the inhibition of VEGF-VEGFR2 activation as well as PLCγ-PKC-MAPK activation by lidocaine in the presence of VEGF in endothelial cells clearly demonstrate the underlying mechanisms on how lidocaine suppresses VEGF-stimulated angiogenesis. In addition, lidocaine also inhibits VEGF-stimulated FAK-paxillin focal adhesion (Fig. 3a, d), which is supported by the previous work that lidocaine inhibits TNFα-induced activation of FAK in lung cancer cells [15]. Lidocaine has been reported to inhibit tyrosine kinase activity of the epidermal growth factor receptor (EGFR) in epithelial cells [38]. The inhibitory effects of lidocaine on in vitro capillary network formation may be partially attributed to its ability in inhibiting EGF/EGFR signaling since Matrigel matrix contains EGF. Giving the essential role of VEGF in angiogenesis [4] and the findings on the inhibitory effects of lidocaine in VEGF-stimulated cellular and molecular changes in endothelial cells, the suppression of VEGF-stimulated PLC, MAPK and FAK-paxillin is highly likely to be the

predominant underlying mechanism of lidocaine's action in endothelial cells.

In conclusion, this work provides preclinical evidence to demonstrate the inhibitory effects of lidocaine on tumor angiogenesis. The findings guide the better understanding on the potential mechanisms by which lidocaine or other amide-linked local anesthetics may negatively affect cancer growth and metastasis.

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Compliance with ethical standards

Conflict of interest All the authors declare no conflict of interest.

Research involving human participants and/or animals All the procedures involving human participants were performed in accordance with the ethical standards of the national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All the procedures involving animals were conducted according to the guidelines approved by the Institutional Animal Care and Use Committee of Hubei University of Arts and Science

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