Ropivacaine Inhibits Serum-Induced Proliferation of Colon Adenocarcinoma Cells In Vitro

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ABSTRACT

Ropivacaine, a new long-acting local anesthetic, is currently being investigated for the treatment of ulcerative colitis. In view of the increased incidence of dysplasia and neoplasia associated with ulcerative colitis, it is important that the medical treatment of these patients does not stimulate cell proliferation further. This study was performed to evaluate the effect of ropivacaine on the proliferation of human colon adenocarcinoma cells (HT-29 and Caco-2) in vitro. A serum-induced proliferation assay of human colon adenocarcinoma cells was used. Ropivacaine inhibited the growth of HT-29 and Caco-2 cells in a dose-dependent manner. Fifty percent inhibition of growth was found at a ropivacaine concentration of 250 μM when the HT-29 cells were cultured in 1% fetal calf serum and of 550 μM when the HT-29 cells were cultured in 10% serum. The effective concentrations are within the range of the therapeutic concentrations obtained in the colon of patients treated rectally with ropivacaine. Lidocaine, hydrocortisone, and 5-aminosalicylic acid were found to be less potent than ropivacaine in inhibiting proliferation. Ropivacaine caused a dose-dependent membrane depolarization that appeared to correlate with the inhibited cell proliferation, whereas the effect was not related to inhibition of leukotriene B₄ or prostaglandin E₂.

In conclusion, the antiproliferative activity of ropivacaine, combined with previously reported anti-inflammatory activities, makes this drug an interesting new alternative for the local treatment of ulcerative colitis.

Materials and Methods

Cell Culture. HT-29 and Caco-2, two human colon adenocarcinoma cell lines, were obtained from American Type Culture Collection (Rockville, MD). HT-29 cells were cultured in RPMI 1640...
medium containing 10% fetal calf serum (FCS), glutamine (2 mM), and antibiotics (50 U/ml penicillin/50 µg/ml streptomycin). Caco-2 cells were cultured in Eagle’s minimal essential medium (EMEM) containing 20% FCS, glutamine and antibiotics as described previously and supplemented with nonessential amino acids and 1 mM sodium pyruvate.

The cells were grown in 75-cm² culture flasks, and the medium was changed every other day. HT-29 cells of passages 132 to 134 and Caco-2 cells of passages 20 to 22 were used for the experiments.

**Cell Proliferation Assay.** The cells were detached with a 0.1% trypsin/0.02% EDTA (1:1) solution for 5 min. Subsequently, the cells were seeded onto 24-well plates (3 x 10⁴ cells/well) and allowed to attach for 24 h before the addition of test compounds and serum. Test compounds were diluted in serum-free culture medium. RPMI 1640 medium containing either 1% or 10% FCS (HT-29 cells) or EMEM containing 2% or 20% FCS (Caco-2 cells) was used as control. Six wells were used for controls and each drug concentration. The cells were fed every second day with fresh medium containing either a low or high concentration of FCS and various concentrations of the drugs.

MK-886 (leukotriene synthesis inhibitor), LTB₄, and extracellular elevated K⁺ were also tested in the cell proliferation assay in an attempt to identify the mechanism of the ropivacaine-mediated effect. In the experiments with the addition of LTB₄, fresh LTB₄ was added daily.

After 1 week, the cells grown in 10 and 20% FCS, respectively, had reached confluence and the experiment was terminated. The cells were detached with the trypsin/EDTA solution for 15 min and counted in an automatic cell counter (model 134; Analyse instrument, Stockholm, Sweden). The viability of cells incubated in control and all experimental media at 37°C was measured by the trypan blue exclusion test.

**Measuring Membrane Potential.** HT-29 cells were seeded onto opaque 96-well plates with clear bottoms and grown to confluence. Cultures of passages 146 to 148 were used. On the day of experiment, the cells were washed three times using Hanks’ balanced salt solution (HBSS) supplemented with 10 mM glucose and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Bisoxonol dye (5 µM; bis-(1,3-dibutylbarbituric acid)trimethine oxonol; DiBAC₄(3)) was added to the cells and allowed to equilibrate at 37°C for 20 min. K⁺ (5–50 mM) and ropivacaine (10–1000 µM) were added to the HBSS/bisoxonol dye solution on the cells, and each concentration was tested in 8 wells. Measurements of changes in fluorescence as an indicator of a membrane depolarization were made instantaneously in a Fluorometric Imaging Plate Reader (Molecular Devices Co., Sunnyvale, CA) and is a well established method for measuring membrane potentials in nonexcitable cells (Enkvist et al., 1989; Rink et al., 1990). The membrane potential response was expressed as percentage of the maximal response to ropivacaine (1 mM).

**LTB₄ and Prostaglandin E₂ Assays.** The HT-29 cells were seeded onto 24-well plates (30,000 cells/well) and grown to confluence. The cells were incubated with either ropivacaine or MK-886. After a 15-min preincubation at 37°C, A23187 (5 µM) was added, and the cells were incubated for an additional 30 min at 37°C. The cells were rapidly cooled on ice, and the supernatants were analyzed for either LTB₄ or prostaglandin E₂ (PGE₂) content using enzyme immunoassay kits. The detection limit of the kits were 7 pg/ml (LTB₄ kit) and 15 pg/ml (PGE₂ kit; as stated by the manufacturer).

**Materials.** RPMI 1640 medium, EMEM, HBSS, FCS, glutamine, and antibiotics (10,000 IU/ml penicillin and 10,000 µg/ml streptomycin) were obtained from Gibco Ltd. (Paisley, UK). Culture flasks and culture plates were purchased from Costar (Cambridge, MA). Ropivacaine (Naropin) and lidocaine (Xylocaine) were obtained from ASTRA (Sweden) (Fig. 1). Hydrocortisone and MK-886 were obtained from Calbiochem Corp. (La Jolla, CA), and 5-ASA was purchased from Sigma Chemical Co. (St. Louis, MO). The LTB₄ and PGE₂ enzyme immunoassay kits were obtained from Cayman Chemical (Ann Arbor, MI).

**Statistics.** Student’s *t* test (unpaired, two-tailed) was used for statistical evaluation of data, and the results are expressed as mean ± S.E.M. A value of *p* < .05 was recognized as a significant difference from the control. The experiments were repeated at least three times. For comparison purposes, IC₅₀ values were calculated.

**Results.**

**Effects of Local Anesthetics on HT-29 Cell Proliferation.** The control value in 1% FCS was 2.88 ± 0.69 x 10⁶ cells. Ropivacaine dose-dependently reduced the cell proliferation in 1% FCS (Fig. 2A), with an IC₅₀ of 250 µM (Table 1). Lidocaine also had significant inhibitory effects on the cells grown in 1% FCS (IC₅₀ = 740 µM; Fig. 2A and Table 1). The viability of cells after culture with or without local anesthetics was >96%.

The number of HT-29 cells was approximately doubled (4.02 ± 0.96 x 10⁶ cells) after growth in 10% FCS compared with 1%. Proliferation of the HT-29 cells cultured in 10% FCS was also affected by ropivacaine (Fig. 2B), although at a somewhat higher concentration (IC₅₀ = 550 µM; Table 1). Lidocaine, on the other hand, did not significantly inhibit the proliferation of HT-29 cells grown in 10% FCS (Fig. 2B). To determine whether the ropivacaine mediated effect was reversible, the cells were cultured in the presence of three concentrations of ropivacaine (0.01, 0.1, and 1 µM) and exposed to fresh media. The viability of the HT-29 cells grown in 1% FCS was 66% at 0.01 µM ropivacaine, 80% at 0.1 µM ropivacaine, and 95% at 1 µM ropivacaine. The control value in 1% FCS was 96%.

**Fig. 1. Structure formulas of ropivacaine and lidocaine.**

**Fig. 2. Effects of ropivacaine (●), lidocaine (□), hydrocortisone (▲), and 5-ASA (●) on the proliferation of HT-29 cells cultured in 1% (A) or 10% (B) FCS for 1 week. The results are mean ± S.E.M. (n = 3) expressed as percent of control. *p < .05, **p < .01, ***p < .001.
concentrations of ropivacaine (10, 100, and 1000 μM) for 7 days and thereafter without ropivacaine for an additional 3 days. Removal of ropivacaine lead to a rapid increase in cell numbers (30–60%) for cells previously cultured in ropivacaine compared with control cells cultured in ropivacaine for 10 days (data not shown).

**Effects of Hydrocortisone and 5-ASA on HT-29 Cell Proliferation.** Compared with controls, HT-29 cells treated with hydrocortisone exhibited a concentration-dependent reduction in their proliferation rate, whereas the proliferation of cells grown in 1% FCS was inhibited by 50% at 650 μM hydrocortisone (Fig. 2A and Table 1). For cells cultured in 10% FCS, hydrocortisone showed an IC50 of 540 μM (Fig. 2B and Table 1). In contrast, 5-ASA had no effect on the proliferation (Fig. 2 and Table 1).

**Effects of Local Anesthetics on Caco-2 Cell Proliferation.** The control value in 2% FCS was 2.73 × 10^5 cells/60 mm dish compared with 2% FCS (Fig. 3A), with an IC50 for ropivacaine of 430 μM (Table 1). Lidocaine at 1000 μM had a small significant inhibitory effect on the cells grown in 2% FCS (Fig. 3A and Table 1). The viability of cells after culture in the tested concentrations of local anesthetics was >95%.

The number of Caco-2 cells was increased by 30% (3.54 ± 0.82 × 10^5 cells) after growth in 20% FCS compared with 2% FCS. Ropivacaine inhibited proliferation of the Caco-2 cells cultured in 20% FCS (Fig. 3B), although at somewhat higher concentrations (IC50 = 640 μM; Table 1) compared with the effect on cells cultured in 2% FCS. Lidocaine had significant inhibiting effects only at the highest concentration tested (1 mM), which resulted in a 30% inhibition compared with the control (Fig. 3B).

**Effects of Ropivacaine on Membrane Potential.** The addition of ropivacaine to the HT-29 cells caused a marked, concentration-dependent increase in the bisoxonol fluorescence, denoting a strong depolarization of the cells (Fig. 4). Elevated extracellular K+ also caused a dose-dependent membrane depolarization. Elevated extracellular K+ inhibited HT-29 cell proliferation significantly in a concentration-dependent manner (Fig. 4). The IC50 value was 40 mM in 1% FCS. The effect of K+ on cell proliferation in the presence of 10% FCS was similar to that in 1% FCS (IC50 = 50 mM; data not shown). The K+-induced depolarization seemed to correlate with the inhibition of HT-29 cell proliferation because ropivacaine and elevated extracellular K+ dose-dependently induced membrane depolarization with the same potency sequence as for the inhibition of HT-29 cell proliferation (Fig. 4).

**Effects of MK-886 and LTB4 on HT-29 Cell Proliferation.** MK-886 dose-dependently inhibited the proliferation of HT-29 cells grown in either 1% FCS (IC50 = 6 μM) or 10% FCS (IC50 = 20 μM; Fig. 5). However, LTB4 added extracellularly had no effect on basal cell proliferation (data not shown).
shown) and was not able to reverse the inhibitory effects exerted by ropivacaine (data not shown). Furthermore, the HT-29 cells could not be stimulated to release increased levels of LTB4 using A23187, and ropivacaine had no effects on basal LTB4 release from HT-29 cells (data not shown).

**Effects of Ropivacaine on PGE2 Release from HT-29 Cells.** We examined the effect of ropivacaine on PGE2 release by HT-29 cells. The basal level of PGE2 produced by the cells was 214 ± 186 pg/10^6 cells. A23187 stimulation resulted in double the amount of PGE2. All the concentrations of ropivacaine tested (10–1000 μM) showed a trend toward reduced PGE2 release for both basal and A23187-stimulated release (data not shown); however, the reduction was the same at every concentration of ropivacaine studied, whereas the proliferation decreased proportionately with increasing concentrations.

**Discussion**

In the present study, ropivacaine and lidocaine were tested for their effects on serum-induced proliferation of two human colon adenocarcinoma cell lines in vitro: HT-29 and Caco-2. It was found that both local anesthetics inhibited the growth of these cell types in a dose-dependent manner. The inhibitory effect of ropivacaine on growth was reversible because removal of the drug by media change after 7 days of treatment lead to rapid regrowth. This suggests that the effects of ropivacaine are not simply via induction of cell death. For comparison purposes, the commonly used therapeutic agents for UC, hydrocortisone and 5-ASA, were included in the study of HT-29 cell proliferation. The relative order of potency of the tested compounds in limiting cell proliferation was ropivacaine > hydrocortisone > lidocaine > 5-ASA. The ropivacaine concentration in the gel used clinically is approximately 20 mM. Due to the damaged mucosa in UC, the absorbance is greatly facilitated and increased compared with the normal colon mucosa, and it is possible that the concentration of ropivacaine reaching the epithelial cells is within the range of the concentrations tested.

The mechanism by which the local anesthetics inhibited proliferation is unknown. These drugs are known to act on ion channels to decrease membrane permeability to Na^+ and K^+ in nerves and may act in a similar way on other cell types. HT-29 cells have previously been shown to express ion channels (Morris and Frizzell, 1993a,b; Sand et al., 1997). Thus, one possibility is that the local anesthetics interacted with ion channels on the tumor cells. In fact, there is increasing evidence that membrane ion channels are involved in cell differentiation and cell-cycle control (see below). Our results showed that ropivacaine and elevated extracellular K^+ caused dose-dependent membrane depolarization of the HT-29 cells. The induced membrane depolarization seemed to be of the same relative potency and magnitude as for the inhibition of HT-29 cell proliferation. The discrepancy between the antiproliferative and depolarizing effect of the high concentrations of K^+ may be due to the hyperosmolarity of the cell medium after K^+ addition. In lymphocytes, the activation of K^+ currents belongs to the early events after mitotic stimulation (Brent et al., 1990; Chandy et al., 1984; DeCoursey et al., 1984), and it has been shown that the membrane potential of resting T cells is set by voltage-activated channels and that blockage of these channels is sufficient to depolarize resting human T cells and prevent their activation (Leonard et al., 1992). Moreover, K^+ channels and membrane voltage have been shown to interfere with proliferation in a variety of different cell lines derived from breast carcinoma (Wegman et al., 1991), small-cell lung cancer (Pancrazio et al., 1993), neuroblastoma (Rouzaire-Dubois and Dubois, 1991), renal epithelium (Teulon et al., 1992), and melanoma (Lepple-Wienhues et al., 1996). Furthermore, the HT-29 cells have previously been shown to be regulated by the membrane potential (Fischer et al., 1992). Thus, if the membrane potential is part of the HT-29 and Caco-2 cell growth regulatory system, the underlying mechanism for the observed inhibition of colon adenocarcinoma cell proliferation by local anesthetics could be explained in these terms.

The effect on membrane potential may in turn affect messengers in the mitogenic signal cascade, such as eicosanoids. Colonic epithelial cells are capable of synthesizing LTB4 (Dias et al., 1992), which has previously been shown to stimulate the proliferation of HT-29 cells (Bortuzzo et al., 1996). Moreover, 5-lipoxygenase inhibitors have been shown to be potent inhibitors of the growth of murine adenocarcinomas (Hussey and Tisdale, 1996). Ropivacaine has also been shown to inhibit the release of LTB4 from human leukocytes (Martinsson et al., 1997b). In the present study, we found that MK-886, which blocks the activation of 5-lipoxygenase, dose-dependently inhibited cell proliferation of the HT-29 cells. However, our findings demonstrate that the antiproliferative effect by local anesthetics is mediated through messengers other than LTB4. Two lines of evidence supported this conclusion: 1) exogenously added LTB4 did not increase HT-29 cell proliferation, and 2) exogenously added LTB4 did not reverse the effect of ropivacaine. The lack of effect of ropivacaine regarding inhibition of LTB4 release further supported this conclusion.

Recent observations indicate that many colonic adenomatous polyps and cancers overexpress cyclooxygenase 2 (Kutchera et al., 1996; Sano et al., 1995) and that inhibition of this enzyme by nonsteroidal anti-inflammatory drugs decreases the risk of colon neoplasia (Giardello et al., 1993; Giovannucci et al., 1995; Shiff et al., 1996). This suggests the presence of a cyclooxygenase pathway for regulation of gastrointestinal epithelial cell growth. We therefore examined PGE2 release from the HT-29 cells and whether ropivacaine could affect this prostaglandin production. Our data suggest that the antiproliferative effect by ropivacaine or lidocaine is not mediated via PGE2 production because PGE2 release was not concentration-dependently inhibited.
In conclusion, ropivacaine, previously shown to be anti-inflammatory, was found to inhibit the proliferation of colon cancer cells in vitro in a dose-dependent manner. It is suggested that this effect is caused by depolarization of the cell membrane. Moreover, our findings showed that ropivacaine inhibited the proliferation with a potency exceeding that of lidocaine, hydrocortisone, and 5-ASA. Because disturbed control of cell proliferation and increased frequency of colon cancer have been demonstrated in UC, the combined anti-inflammatory and antiproliferative activity of ropivacaine makes this drug a promising new alternative for local UC treatment.

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References

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