Local Anesthetics with High Lipophilicity are Toxic, While Local Anesthetics with Low pKa Induce More Apoptosis in Human Leukemia Cells

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Abstract

Many studies have indicated that local anesthetics are cytotoxic and can induce apoptosis; however, the types of local anesthetics and the induction rates of apoptosis remain unclear. The aim of this study was to clarify the local anesthetics that induce apoptosis or necrosis and their induction-related factors.

Methods: Lidocaine, mepivacaine, bupivacaine, ropivacaine, tetracaine, dibucaine, procaine, and QX-314 were evaluated for apoptosis and necrosis in HL-60 human leukemia cell lines. Apoptosis and necrosis were analyzed by double-staining assay with propidium iodide (PI) and annexin-V and were measured by flow cytometry (FACS). DNA fragmentation was used for the analysis of apoptosis.

Results: In the double-staining assay by flow cytometry, drugs with high lipophilicity were most cytotoxic. The comparative LD50 values were dibucaine > tetracaine > bupivacaine > ropivacaine > mepivacaine > lidocaine > procaine > QX-314. The LD50 were correlated with lipophilicity (\(\log{P}\)). The comparative maximum rates of annexin-positive and PI-negative apoptotic cells were lidocaine > mepivacaine > ropivacaine > bupivacaine > procaine > tetracaine > dibucaine > QX-314 and were correlated with pKa. Lidocaine and mepivacaine significantly induced DNA fragmentation. DNA fragmentation was also correlated with pKa.

Conclusion: The results indicate that local anesthetics with high lipophilicity are highly toxic and induce mainly necrosis, while local anesthetics with low pKa induce more apoptosis.

Keywords: Local anesthetics; Apoptosis; Lipophilicity; pKa

Introduction

Many local anesthetics are commonly used for regional anesthesia, postoperative pain relief, and anti-arithmetic and are often used in examinations such as biopsies. It is well known that these local anesthetics are cytotoxic [1-4]. Clinical profiles of the toxicities of local anesthetics, especially lidocaine-induced neurotoxicity, have been based on the reported incidence of cauda equina syndrome or transient neurologic syndrome (TNS) after spinal anesthesia [5-8]. Clinically, lidocaine is most often linked to local anesthetic-induced neurotoxicity, although experimental evidence has demonstrated that local anesthetics with high lipophilicity are more toxic than lidocaine [9,10]. Werdehausen et al. [12] compared the toxicity of local anesthetics by double-staining assay with 7-amino-actinomycin D (7-AAD) and annexin-V and were measured by flow cytometry and reported that comparison of LD50 values of the different local anesthetics and resulted in that the toxicity correlated with octanol/buffer coefficients represent as lipophilicity [9].

On the other hand, there some results has been reported that not only the lipophilicity but also another factors correlated with the toxicity of local anesthetics. Kasaba et al. [11] compared the neurotoxicity of procaine, mepivacaine, ropivacaine, bupivacaine, lidocaine, tetracaine, and dibucaine by using cultured neurons from the freshwater snail and reported that dibucaine tetracaine, and lidocaine were more toxic than other local anesthetics [11].

However, the differences in and mechanisms of the toxicities of lidocaine and other local anesthetics remain uncertain. It has recently been demonstrated that one of the mechanisms of the cytotoxicity induced by local anesthetics is apoptosis induced by mitochondrial damage [12], caspase activation [13], inhibition of thyrosine kinase [14], activation of p38 mitogen-activated protein kinase (MAPK) [15], calcium metabolism [16] and blockage of sodium channels [17-19]. On the other hand, local anesthetic-induced necrosis has been reported in clinical practice [20-22]. According to one hypothesis, the mechanism of local anesthetic-induced necrosis is calcium metabolism by free radicals [23-25]. It is uncertain which local anesthetic is more toxic and which induces apoptosis or necrosis. Therefore, in the present study, fatality rates, apoptosis and necrosis induction rates, DNA fragmentation, and caspase activity were investigated. We compared the concentration-dependent apoptotic and necrotic potencies of lidocaine, mepivacaine, bupivacaine, ropivacaine, procaine, dibucaine, tetracaine, and QX-314 in HL-60 human T-cell leukemia cell lines not expressing sodium channels [26].

Materials and Methods

Cell culture

Acute myeloblastic leukemia of human (HL-60) cell lines routinely

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maintained in our laboratory were used. Cells were maintained in RPMI 1640 supplemented with 15% heat-inactivated FBS, penicillin (100 units/ml), and streptomycin (10 µg/ml). Cells were grown at 37°C in a humidified atmosphere of 5% CO2/95% air.

**Double-labeling assay with annexin-V and PI**

To differentiate apoptotic cells from necrotic cells, the surface exposure of phosphatidylserine in apoptotic cells was measured with the ApoAlert® Annexin V-FITC apoptosis detection kit (Beckton Dickinson, CA, USA) [27]. Additional exposure to PI facilitated the differentiation of apoptotic (annexin-positive and PI-negative) cells from necrotic (annexin- and PI-positive) cells [28, 29].

**Flow cytometric analysis**

Treated cells were analyzed using a flow cytometer [30]. After treatment with different concentrations of local anesthetics for 24 h, cells were harvested by centrifugation and suspended with PBS. The cell suspension was then double stained with annexin-V (50µg/ml) and PI (50µg/ml) and kept in the dark for 15 min. The fluorescence of each cell was analyzed by a flow cytometer (FACScan, Beckton Dickinson, CA, USA) using a nitrogen argon laser operating at 488/535 nm excitation/emission for annexin-V, with the fluorescence captured on the FL-1H channel, and at 488/620 nm excitation/emission for PI, with the fluorescence captured on FL-2H channel with logarithmic amplification. For each determination, 10,000 cells were counted. Apoptotic cells were defined as annexin positive and PI negative, while necrotic cells were defined as either annexin negative and PI positive or annexin positive and PI positive.

**Assessment of apoptosis by DNA fragmentation**

DNA fragmentation was assessed by agarose gel electrophoresis [31]. After treatment with different concentrations of local anesthetics for 24 h, the sample cells were incubated at 37°C for 1 h in 10 mM Tris-HCL buffer (pH 7.4) containing 10 mM EDTA and 0.5% Triton-X 100 with 400µg/mL of RNase-A and then were incubated again with 400µg/mL of proteinase K. The DNA was extracted with an equal volume of phenol/chloroform and precipitated with an equal volume of 2-propanol. The DNA samples were electrophoresed on 2% agarose gel containing 10 mM EDTA and 0.5% Triton-X 100 with 400µg/mL of proteinase K. The DNA was extracted with an equal volume of phenol/chloroform and precipitated with an equal volume of 2-propanol. The DNA samples were electrophoresed on 2% agarose gel containing 10 mM EDTA and 0.5% Triton-X 100. The DNA samples were electrophoresed on 2% agarose gel containing 10 mM EDTA and 0.5% Triton-X 100. The DNA samples were electrophoresed on 2% agarose gel containing 10 mM EDTA and 0.5% Triton-X 100.

**Statistical analysis**

The results are expressed as the mean ± standard deviation (SD). The results of repeated measurements of each dose for each group of trials were analyzed by repeated measurement of one-way analysis of variance (ANOVA), followed by Scheffe’s test. The LD50 was obtained from probit analysis and compared by means of analysis of variance (ANOVA) with Tukey’s post hoc test. Correlations between LD50 values, percentage of necrotic cells, and apoptotic cells with clinical
Results

Local anesthetics induce apoptosis and necrosis

To examine the effect of local anesthetics on HL-60 cells, cells were exposed to each local anesthetic. Thereafter, cells were double labeled with annexin-V and PI, and each fluorescent value was analyzed by flow cytometry (Figure 1). The fraction of cells positive for annexin-V only indicated apoptotic cells, and cells positive for both annexin-V and PI indicated necrotic cells. All of the local anesthetics that were investigated induced cell death in a concentration-dependent manner (Figure 1A, B). Higher concentrations of many local anesthetics led to increased fractions of cells that stained positive for both annexin-V and PI, indicating that necrosis was induced (Figure 1A, D). The concentrations of local anesthetics that yielded 50% cell death (LD₅₀) were significantly different for each local anesthetic, as shown in Figure 1B, C and Table 1. The comparative rates of LD₅₀ were dibucaine > tetracaine > bupivacaine > ropivacaine > mepivacaine > lidocaine > procaine > QX-314 (p < 0.05). Drugs with high lipophilicity, such as dibucaine, tetracaine, and bupivacaine, were more toxic than drugs with low lipophilicity, such as lidocaine, mepivacaine, procaine, and QX-314 as shown in Table 1 (p < 0.05). The comparative maximum rates of annexin-positive and PI-positive (necrotic) cells were dibucaine > tetracline > bupivacaine > ropivacaine > mepivacaine > lidocaine > procaine > QX-314. Dibucaine, tetracaine, and bupivacaine were significantly decreased in live cells and were increased mainly in annexin-positive and PI-positive cells (Figure 1D). More than 50% of cell deaths were induced by necrosis; and less than 50% were induced by apoptosis. The comparative maximum rates of annexin-positive and PI-negative (apoptotic) cells were lidocaine > mepivacaine > ropivacaine > bupivacaine > procaine > tetracaine > dibucaine > QX314 (Figure 1E, F). Lidocaine and mepivacaine significantly increased the number of apoptotic cells. In order to identify factors affecting necrosis and apoptosis, the LD₅₀, maximum rates of annexin-positive and PI-positive cells, and maximum rates of annexin-positive and PI-positive cells were correlated to a number of known physicochemical properties by Spearman's rank test, as shown in Table 1. The octanol/buffer coefficient (logP value) means that the lipophilicity of the drug and the clinical potency correlated well with fatality rates, the LD₅₀ value, and the rates of annexin-positive and PI-positive (necrotic) cells. On the other hand, the rates of annexin-positive and PI-negative (apoptotic) cells were correlated with pKa. A fraction of a non-ionized local anesthetic at pH 7.4 means that a local anesthetic with a low pKa close to pH 7.4 will more induce apoptosis (Table 1). In contrast, LD₅₀ values did not correlate with pKa values, fractions of non-ionized local anesthetics, or protein binding.

Blocking potency, lipid solubility (logP), pKa, protein binding, non-ionized fraction, and molecular weight were made by means of Spearman's rank correlation test. P < 0.05 was considered significant. JMP (version 8, SAS, NC, USA) was used for these analyses. Values of P < 0.05 were considered statistically significant.
Local anesthetics induce DNA fragmentation and laddering

Local anesthetics, especially lidocaine and mepivacaine at 5 mM, induced DNA fragmentation in HL-60 cells (Figure 2). Lidocaine and mepivacaine significantly increased the fluorescence intensity level of ethidium bromide, which indicated the degree of DNA fragmentation in a dose-dependent manner (Figure 2B). The fluorescence intensity level of ethidium bromide (degree of DNA fragmentation) correlated with the pKa of local anesthetics (P = 0.02).

Discussion

The results of the present study demonstrate that local anesthetics with high lipophilicity are more toxic and induce mainly necrosis. On the other hand, local anesthetics in which pKa has a pH value close to pH 7.4 induce more apoptosis in HL-60 human leukemia cells. Over half of the cell deaths were induced by necrosis, and less than half were induced by apoptosis within the clinical dose of each local anesthetic. These results are very similar to those reported previously. Werdehausen et al. compared the toxicity of local anesthetics by double-staining assay with 7-amino-actinomycin D (7-AAD) and annexin-V and were measured by flow cytometry in human neuroblastoma cell line SK-N-SH and reported that comparison of LD50 values of the different local anesthetics and resulted in that tetracaine-bupivacaine > prilocaine=mepivacaine=ropivacaine=lidocaine > procaine=articaine and the toxicity correlated with octanol/buffer coefficients represent as lipophilicity and their relative clinical potency [9]. Kamiya et al. [35] reported that below 12 mM, lidocaine exposure for 24 h induced about 50% apoptosis in U937 human leukemia cells [32]. Friederich et al. [33] reported that 24-h exposure to 3 mM lidocaine induced about 40% apoptosis in human neuronal SH-SY5Y cells [33]. Lidocaine and mepivacaine exposure increased the number of apoptotic cells significantly more than other anesthetics. At a high concentration (over 5 mM), the number of necrotic cells increased. The toxicity of each local anesthetic was correlated with its lipophilicity, which was presented as the octanol/buffer partition coefficient (Log P) value, and thus its clinical potency. On the other hand, the induction rate of apoptosis was correlated with pKa and the % of free base at pH 7.4. Many studies have indicated that local anesthetics, especially lidocaine, induce apoptosis in neuronal cells; however, only a few drugs were compared. It is therefore the toxic potency of various local anesthetics is difficult to compare. In some of the studies, the clinical potency was adjusted, making it difficult to compare the toxicities of the local anesthetics. In the present study, the toxicities of local anesthetics correlated with their relative clinical potencies. When clinical potencies are adjusted, no differences in the toxicities of local anesthetics can be observed. Lirk et al. [34] compared the neurotoxic potentials of equipotent concentrations of lidocaine, bupivacaine, and ropivacaine set in cell cultures of rat dorsal root ganglion neurons for 24 h [34]. The percentages of cell death did not differ among the local anesthetics. Those results are reconfirmed and generalized to other local anesthetics in the present study. Myers et al. [35] compared the neurotoxicity of equipotent concentrations of lidocaine, etidocaine, 2-chloroprocaine, and procaine on the sciatic nerve of rats 2 days after a single injection, and they found a significant correlation between the nerve-blocking concentration and toxicity [35]. HL-60 cells containing few sodium channels were used in this examination [26]. It is therefore suggested that the effect of sodium-channel blocking by local anesthetics does not correlate with cell toxicity. Instead, the lipophilicity and permeability of local anesthetics, which also affect blocking potentials, are more important factors in the toxicities of local anesthetics. Although many theories have been reported, the mechanisms of local anesthetics that induce apoptosis and necrosis are still unclear. Lipophilicity was correlated with necrosis, supporting Tsuchiya et al. [36] theory that lidocaine induces inflammation through changes in membrane fluidity, thereby causing necrosis [36]. The present results also support Kitagawa et al. [37] theory that cytotoxic local anesthetics are amphiphilic molecules that will melt lipid bilayers as detergents [37]. On the other hand, in the present study, apoptosis was correlated with pKa in local anesthetics. The intracellular pH changes affect homeostasis. Mitochondria are especially affected and depolarized by intracellular pH changes [38]. In addition, many researchers have reported that local anesthetics, especially lidocaine, induce mitochondrial depolarization and apoptosis through mitochondrial pathways [12,39].

In the present investigation, HL-60 human T-cell leukemia cell lines were used in order to rule out the effects of sodium channels and also to obtain more accurate results in FACS analysis because these floating cell lines are uniform and do not require tearing by enzymes such as trypsin. However, these cancer cell lines may be more sensitive to apoptosis or necrosis induced by local anesthetics than are primary cultures of rat dorsal root ganglion neurons. Obviously, the cell culture model used for the present study presents several limitations to translating the data to in vivo situations. Nevertheless, the HL-60 cell lines seem accurate and sensitive in detecting minor differences among local anesthetics.

In conclusion, local anesthetics with high lipophilicity are highly toxic and induce mainly necrosis, while those with low pKa more induce apoptosis.

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