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局部麻醉藥劑促進腫瘤細胞凋亡之研究

Apoptosis Induced by Local Anesthetics in Tumor Cells

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中文摘要

關鍵詞：乳癌、甲狀腺癌、麻醉、局部麻醉劑、細胞凋亡

乳癌對女性健康和精神方面一直是莫大的威脅。佔台灣青壯年(30~60 歲)女性癌症發生率第一位。值得注意的是，台灣地區婦女乳癌症發生率有逐年升高的趨勢。另外甲狀腺癌的發生率近年來也有顯著增加。外科手術仍是乳癌、甲狀腺癌治療中之極重要一環。吸入性氣體麻醉全身麻醉在接受手術的病人上可能有術後疼痛及噁心、嘔吐的副作用。手術創傷也會引起不當免疫反應，而免疫缺損則可能促成腫瘤生長和擴散。局部麻醉可以阻止外科手術創傷訊息傳入，已經廣泛應用於癌症診斷切片和細胞抽吸。近來更可使用局部麻醉藥品在鎮靜麻醉下應用於乳癌手術。

本研究使用之局部麻醉藥品(Lidocaine, Bupivacaine) 以乳癌腫瘤、甲狀腺癌細胞和利用腫瘤之動物模式，發現其對腫瘤細胞抑制的效果。

本研究發現局部麻醉藥品以低於臨床乳癌手術使用的局部麻醉劑量，影響粒腺體和胞外凋亡路徑而導致的凋亡蛋白酶 7,8,9 活化，就會造成乳癌細胞的死亡，而對正常乳腺細胞株較無影響。

另外也發現局部麻醉藥品能抑制甲狀腺癌腫瘤細胞的生存能力，減少細胞群落形成。藉由瓦解粒線體膜電位，釋放细胞色素 C (Cytochrome C)，活化凋亡蛋白酶 3, 7，升高 Bax/Bcl-2 比例進而引發甲狀腺癌細胞凋亡及壞死。由局部麻醉藥品所誘導之 MAPK 訊息傳遞路徑可能為其促進細胞凋亡之重要機轉。

總結來說，本研究發現局部麻醉劑的抑制癌細胞效果。我們大膽預測，本研究的延伸可能帶來醫界重大的衝擊，提供兼顧腫瘤控制的乳癌和甲狀腺癌手術麻醉模式。

ABSTRACT

Keyword: breast cancer, thyroid cancer, local anesthetics, anesthesia, apoptosis

The incidence of breast and thyroid cancer has remarkably increased in recent years. Local anesthetics are frequently used in fine-needle aspiration of thyroid lesions and locoregional control of persistent or recurrent thyroid cancer. Wound infiltration with local anesthetics reduces postoperative pain after breast surgery. Recent evidence suggests that local anesthetics have a broad spectrum of effects including inhibition of cell proliferation and induction of apoptosis in neuronal and other types of cells.

In this study, we demonstrated that treatment with lidocaine and bupivacaine resulted in decreased cell viability and colony formation of both 8505C and K1 cells in a dose-dependent manner. Lidocaine and bupivacaine induced apoptosis, and necrosis in high concentrations, as determined by flow cytometry. Lidocaine and bupivacaine caused disruption of mitochondrial membrane potential and release of cytochrome c, accompanied by activation of caspase 3 and 7, PARP cleavage, and induction of a higher ratio of Bax/Bcl-2. Based on microarray and pathway analysis, apoptosis is the prominent transcriptional change common to lidocaine and bupivacaine treatment. Furthermore, lidocaine and bupivacaine attenuated extracellular signal-regulated kinase 1/2 (ERK1/2) activity and induced activation of p38 mitogen-activated protein kinase (MAPK) and c-jun N-terminal kinase. Pharmacological inhibitors of MAPK/ERK kinase and p38 MAPK suppressed caspase 3 activation and PARP cleavage.

Taken together, our results for the first time demonstrate the cytotoxic effects of local anesthetics on thyroid cancer cells and implicate the MAPK pathways as an important mechanism.

We also demonstrated that the apoptotic effects of local anesthetics in human breast tumor cells. Treatment of breast tumor cells with lidocaine and bupivacaine at clinically relevant concentrations resulted in inhibition of cell viability via induction of apoptosis. The effects were more prominent in MCF-7 cells than in MCF-10A cells. Treatment with local anesthetics induced caspase 7, 8, 9, and poly ADP-ribose polymerase (PARP) cleavage. The cleavage of caspase 7 and PARP induced by local anesthetics was effectively blocked by caspase inhibitors. Furthermore, treatment of MCF-7 xenografts with local anesthetics resulted in higher expression of cleaved caspase 7 and an increase in terminal deoxynucleotidyl transferase dUTP nick-end labeling staining.

Our findings reveal previously unrecognized beneficial actions of local anesthetics and call for further studies to assess the oncologic advantages of their use during breast cancer surgery and in the management of patients with thyroid cancer.

ACRONYMS AND ABBREVIATIONS

CI	Combination index confidence interval (CI)
CI	Confidence interval
DMEM	Dulbecco's modified Eagle's medium
ED50	Effective dose to induce apoptosis in 50% of cells
ERK	Extracellular signal-regulated kinase
ER	Estrogen receptor
FBS	Fetal bovine serum
FITC	Fetal bovine serum fluorescein isothiocyanate
HER	Human epidermal growth factor receptor
IL	Interleukin
INF	Interferon
IRS	Insulin receptor substrate
JAK	Janus kinase
JNK	Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NK	Natural killer
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PI	Propidium iodide
TNF	Tumor necrosis factor
TdT	Terminal deoxynucleotidyl tranferase
TUNEL	TdT-mediated dUTP nick-end labeling
$\Delta\Psi m$	Mitochondrial membrane potential

INTRODUCTION

Breast cancer

Breast cancer is the most common cancer in women with greater than 1,380,000 cases and estimated to be responsible for almost 460,000 deaths in women each year worldwide., accounting globally for 23% of all cancers in women and 14% of cancer deaths [1]. Worldwide, breast cancer incidence and mortality are expected to increase .by 50% between 2002 and 2020 [2]. Breast cancer incidence rates differ comparing the lowest risk and highest risk countries. These rising cancer rates will be disproportionately high in developing countries and are projected to reach 55% increased incidence and 58% greater mortality in less than 20 years [3]. The increase in breast cancer incidence was striking in women born after the 1960s. In the past two decades, the incidence of breast cancer in young Taiwanese females has been rapidly increasing, approaching the risk level of western countries. Although the incidence of invasive breast cancer in women is lower in Asian countries than in western countries, it has been rapidly increasing in parts of Asia, including Taiwan (Supplemental Fig. S1) [4]. Although some of this variability may be because of differences among countries in screening and reporting, international variability in breast cancer incidence and documented changes in incidence among populations that migrate from low-risk to high-risk countries provide powerful evidence supporting the potential for reducing the burden of breast cancer in our society. Migrant studies of Asian women moving to Hawaii and California, for example, convincingly showed that risk increases among the daughters of the women who migrated [5]. And, in traditionally low-incidence countries in Asia, breast cancer incidence has increased steadily over time as reproductive and lifestyle patterns have changed [6, 7]. For example, in Korea, age at

menarche has decreased from 16.9 years, on average, among women born in between 1920 and 1924 to 13.8 years among women born between 1980 and 1985.10 Fertility has decreased over the past 50 years from an average of 6 births to 1.23 births per woman in 2010. Age-specific breast cancer incidence has increased 3-fold to 140 cases per 100,000 women ages 45 to 49 years [7].

Established modifiable causes of breast cancer include radiation exposure [8] ,alcoholic beverage consumption, postmenopausal obesity, lack of physical activity, and postmenopausal hormone therapy with estrogen plus progestins. For both premenopausal and postmenopausal women who are at high risk of breast cancer as a result of family history or other characteristics (such as a history of atypical hyperplasia of the breast [9], the use of selective estrogen receptor modulators greatly reduces the risk of both invasive breast cancer and noninvasive lesions [10, 11]. Aromatase inhibitors also reduce risk among high-risk women [12]. Randomized trials of tamoxifen and raloxifene show a roughly 50% reduction in the incidence of invasive breast cancer and an even greater reduction in hormone receptor-positive breast cancer [10, 11]. Furthermore, protection persists for several years after treatment cessation [13]. Breast cancer incidence models and age incidence plots show that risk accumulates rapidly from menarche to first birth [14]. The rate of increase then slows after each additional birth, and early menopause reduces subsequent breast cancer risk. Childhood and early adult exposures play an important role in driving breast cancer risk. Risk accumulation through premenopausal years and the burden of disease diagnosed among women aged <50 years both point to the importance of timing for prevention.

Breast cancer typically is detected either during a screening examination or after symptoms have developed. Most breast cancer are invasive, or infiltrating. Ductal carcinoma in situ is a spectrum of abnormal breast changes that start in the cells lining the breast ducts which may or may not progress to invasive cancer. Lobular carcinoma

is much less common than DCIS (accounting for about 12% of in situ cancer) which is not a true cancer, but an indicator of increasing risk for developing invasive cancer. The prognosis of invasive breast cancer is strongly influenced by the stage of the disease. The American Joint Committee on Cancer (AJCC) staging system provides a strategy for grouping patients with respect to prognosis. Therapeutic decisions are formulated in part according to staging categories but primarily according to tumor size, lymph node status, estrogen receptor and progesterone receptor levels in the tumor tissue, human epidermal growth factor receptor 2 (HER2) status, menopausal status, and the general health of the patient.

Breast cancer is increasingly considered to be not one disease but a group of diseases distinguished by different molecular subtypes, risk factors, clinical behaviors, and responses to treatment. Distinct molecular subtypes of breast cancer have been identified using gene expression profiles. In clinical practice, a group of patients may have the same diagnosis and receive the same or different therapeutic recommendations. The recommended course of therapy may be successful and cause toxicity in some patients, but may cause toxicity without demonstrating a therapeutic benefit in others. Meanwhile, others may experience no effect, either detrimental or beneficial. Prognostic tools have been developed to identify patients who require treatment in the first place, as well as to identify patients who will benefit from specific treatments. Dosing is another critical aspect to personalized medicine. Treatment individualization allows clinicians to diagnose subtypes of breast cancer more precisely. A specific treatment can be selected that best targets the disease, avoids adverse drug reactions, and improves efficacy. A biologically-based, individualized approach is critical to select the most potentially effective drug regimens and dosing strategies. Patients with early-stage breast cancer undergo surgery and radiation therapy, but some may be at risk for later disease recurrence and metastasis. Likewise, there are subsets of patients

with node negative or node positive breast cancer who will go on to develop metastatic disease. It is therefore necessary to identify the specific stage of disease, especially for those patients who do not have any readily visible tumor by the currently available pathological or radiologic techniques. In addition, individualized medicine is needed to help better characterize patients who are predisposed to develop breast cancer in the first place. Although additional work is needed, a proof-of-concept has been, in that a better understanding of the molecular basis of cancer has already allowed the community to identify important disease biomarkers and select appropriate therapy.

Treatment options for patients with breast cancer have progressively improved over the past 40 years, from an era of no chemotherapy to the introduction of taxanes, hormonal therapy, and biologic therapy. These advances have resulted in substantial, 15%–20% improvements in clinical outcomes. However, progress has yet to be made to improve the prognosis in many breast cancer patients, and research is currently under way to test new tools, or new applications of older tools, to advance breast cancer management. Chemotherapy clearly remains a cornerstone of adjuvant breast cancer treatment, because breast cancer can be very sensitive to the currently available agents. Chemotherapy clearly remains a critical component of adjuvant breast cancer treatment and will always be in use because breast cancer is very sensitive to the available agents. Meanwhile, the era of a one-size-fits-all approach to breast cancer management is over, and the maximum potential of chemotherapy should now be reached by targeting specific populations, as demonstrated with the trastuzumab story. Effective biomarkers are now needed to optimize chemotherapy, define more selective populations, and clearly tailor treatment. In developed countries, there has been a remarkable improvement in mortality from breast cancer, but almost all of that benefit has occurred in the ER+ and HER-2+ subsets. Triple-negative disease remains the biggest challenge moving forward, but we are close to improved, treatment options and outcomes for

these patients. Predictive markers in estrogen receptor–negative and triple-negative disease will be particularly important because in the absence of therapy, these tumor subtypes tend to have a poor prognosis.

Of more than one million women diagnosed with breast cancer each year, above 60% of patients have hormone receptor disease. Although endocrine therapy has revolutionized breast cancer management and substantially improved outcomes in these patients, the optimal management of these patients remains a significant challenge. For instance, the threshold for adding adjuvant chemotherapy is a topic of continuing debate, and the most effective regimens that include endocrine therapy and chemotherapy are still under debate as well. Tumor markers, such as Ki-67, and host markers, such as cytochrome P450 2D6, are being studied as potential tools to offer more tailored adjuvant endocrine therapy. Current research suggests that luminal A and luminal B cancers are two completely different diseases, and work is being performed to better distinguish between these two disease types and deliver more effective therapy to individual patients. Patients with luminal B cancers are the most interesting subgroup for the exploration of new agents targeting growth factor receptor signaling (such as m-Tor, PI3K and CDK inhibitors), and more research is still needed to fully understand the biology of luminal A cancers.

Approximately 15–20% of breast cancer tumors overexpress human epidermal growth factor receptor 2 [15]. HER2 is a member of the HER superfamily, which is composed of tyrosine kinase receptors involved in the regulation of proliferation and survival of epithelial cells. The family includes four receptors: HER1, HER2, HER3 and HER4. HER2 is considered an orphan receptor as it has no known ligand. However, the other three HER receptors have known ligands and form either homodimers or heterodimers upon ligand binding. HER2 can heterodimerize with any of the other receptors and is considered the preferred dimerization partner. Dimerization results in

the autophosphorylation of tyrosine residues within the cytoplasmic domain of the receptors and initiates signal transduction via the PI3K/AKT and RAS/MAPK pathways. Although HER2-overexpression identifies patients who are likely to respond to therapy with trastuzumab, not all patients benefit from treatment. Approximately 15% of patients relapse after therapy, indicating the presence of de novo or required resistance [16]. Several resistance mechanisms have been proposed including altered receptor–antibody interaction, activating of the downstream pathways by increased signaling from either other members of the HER family or other receptors, or constitutive activation of downstream elements. Multiple putative resistance mechanisms to trastuzumab have been derived from laboratory experiments but very few data support their clinical relevance. It is not clear whether intrinsic and acquired resistance are distinct or overlapping [17].

A major focus in the treatment of HER2-positive BC in recent years has been on developing therapeutic agents to either potentiate the effect of trastuzumab or target cells which have become resistant to trastuzumab. Preclinical evidence suggest that co-inhibition of HER2, other members of the HER family and/or the downstream pathway by giving trastuzumab/lapatinib in combination with other targeted therapies might prevent or at least prolong time to resistance and treatment failure [18, 19]. Dual blockade is likely to represent a substantial advance for patients with HER2-positive breast cancer. The neoadjuvant trials NeoALTTO and NeoSphere investigating the combination of trastuzumab plus lapatinib and trastuzumab plus pertuzumab, respectively, offered the first evidence of dual targeting being superior to single agent therapy with trastuzumab, in terms of pathological complete response, although data of impact on disease-free survival are still needed. The adjuvant trials ALTTO and APHINITY will provide the proof-of-concept for this strategy. However, the relevant subpopulation remains to be defined and side effects including cardiotoxicity might be

a limiting factor to the use. There is an urgent need for prospective biomarker-driven trials to identify patients for whom dual targeting is cost-effective.

Ado-trastuzumab emtansine (antibody-drug conjugate) was approved specifically for treatment of HER2-positive metastatic breast cancer in patients who have been treated previously with trastuzumab and a taxane, and who have already been treated for metastatic breast cancer or developed tumor recurrence within six months of adjuvant therapy. Approval was based on the EMILIA study, a phase III clinical trial that compared trastuzumab emtansine versus capecitabine plus lapatinib in 991 people with unresectable, locally advanced or metastatic HER2-positive breast cancer who had previously been treated with trastuzumab and taxane chemotherapy. This trial showed improved progression-free survival in patients treated with trastuzumab emtansine, along with improved overall survival and safety [20].

Breast cancer patients have benefited from clear advances in treatment options during the past four decades. Expanding novel chemotherapy, endocrine and target therapy options have reflected substantial improvements in clinical outcomes. Although the mortality rates for breast cancer patients have improved over the last decade, the loss of lives each year as a result of metastasis has remained constant. Certain patient do not demonstrate clear, consistent benefits with currently available regimens, and further works are needed to optimize and improve outcome for patients.

Thyroid cancer

Thyroid cancer is the most common malignant endocrine tumor but comprises only about 2% of all malignancies [21]. Most thyroid cancers arise from follicular epithelial cells as well differentiated papillary or follicular types. Other rare forms of thyroid cancer include medullary thyroid cancer arising from parafollicular C-cells.

While the incidence of many head and neck cancers is decreasing, studies have

reported an increasing incidence of thyroid cancer during recent decades [2, 3]. The trend is also observed in East Asia [22]. It is argued that the rising incidence of thyroid cancer is attributable to improved screening by high-resolution ultrasound, fine-needle aspiration biopsy of small nodules, as well as frequent "incidentalomas" found on cross-sectional imaging. However, large cancers and cancers with extrathyroidal extension and with nodal metastases have also more than doubled in incidence [23], suggesting that improved diagnostic identification of small subclinical disease does not explain the observation. Other reasons for this increase, including environmental, hormonal, dietary, and genetic causes, need to be explored.

The overall prognosis for patients with thyroid cancer is one of the best among all cancers. Evaluation of 53,856 thyroid carcinoma cases from the National Cancer Database of the United States revealed that the 10-year overall survival rates with papillary, follicular, Hurthle cell, medullary, and undifferentiated/anaplastic carcinoma was 93%, 85%, 76%, 75%, and 14%, respectively [24]. In a recent study of 589 cases with papillary thyroid cancer (PTC) in Hong Kong, the 10-year cancer specific survival rate was 99%, 87%, 64% and 42% for MACIS stages I, II, III and IV lesions, respectively [22]. Well-differentiated papillary or follicular cancer generally has an excellent prognosis. On the contrary, anaplastic carcinoma is a highly lethal, albeit rare, form of thyroid cancer with a median survival of less than 6 months in most series [25]. Histological type, size of the primary tumor, extrathyroidal tumor extension, and distant metastases are reported to correlate with outcome.

Despite general good prognosis of thyroid cancer, a modest proportion of patients would face the morbidity of recurrences. Lymph node metastasis is well established as a predictor of locoregional recurrence and implicated as a harbinger of distant metastases [26]. The impact of lymph node metastases on survival in thyroid cancer has been the sources of debates and controversies. While some believe that

lymph node metastasis has no major impact on survival of patients with thyroid cancer, in a large population-based, nested case-control study, lymph node metastases were associated with increased mortality (an odds ratio of 1.9 in multivariate analysis controlling for TNM stage) [27]. From a clinical point of view, regional nodal control constitutes an important component in the management of thyroid cancer.

Thyroid cancer is the most common of all endocrine cancers, and the incidence of thyroid cancer is increasing worldwide [28, 29] . The majority of thyroid cancers are well differentiated with papillary and follicular thyroid carcinoma being the most common types. Although well-differentiated thyroid cancer has a generally favorable prognosis, the overall recurrence rates could be as high as 35% [30]. Persistent or recurrent disease occurs largely in the neck. Moreover, development of recurrence is associated with a higher mortality. Thirty-year cancer mortality rates were reported to be about 12% in patients with local recurrence and 43% in those with distant recurrence [30].

Effect of anesthetic technique and surgery on cancer recurrence

The likelihood of tumor metastases depends on the balance between the metastatic potential of the tumor and the anti-metastatic host defenses [31]. Metastatic cells arise from primary tumors and certain tumor types tend to metastasize to specific organs. Stephen Paget's 1889 proposal that metastasis depends on cross-talk between selected cancer cells (the 'seeds') and specific organ microenvironments (the 'soil') still holds forth today [32], describes a progressive growth of the primary tumor, during which time the nutrient supply is initially met by diffusion, but later requires neovascularization. Angiogenic factors are synthesized and secreted, and a capillary network arises from adjacent host tissue. Tumor cells, which are genotypically and phenotypically diverse, then enter the host circulation, most commonly via the

lymphatic system. These tumor cells detach and embolize. Most of these cells will be destroyed by the host immune defenses.

Tumor cells that survive will become trapped in the capillary beds of distant organs, extravasate, proliferate, and ultimately develop their own blood supply. These cells are now micrometastases and in turn have their own metastatic potential. It is now recognized that metastases will only develop in specific organs and this is a result of biologically unique micro-environments [33]. The tumor cells themselves are the product of an evolutionary process during which they randomly mutate and undergo selection. Initially, tumor cells are weakly antigenic and do not elicit an immune response. This is a phase of recognition, elimination, and selection of immune-resistant cells. Ultimately, the tumor cells develop escape mechanisms to evade the host immune response.

Metastases represent the end-products of a multi-step cell-biological process termed the invasion-metastasis cascade, which involves dissemination of cancer cells to anatomically distant organ sites and their subsequent adaptation to foreign tissue microenvironments. Steps of the invasion-metastasis cascade – namely, survival in the circulation, arrest at distant sites, extravasation, initial survival in foreign microenvironments, and/or metastatic colonization – are successfully completed. Each of these events is driven by acquisition of genetic and/or epigenetic alterations within tumor cells and co-option of non-neoplastic stromal cells, which together endow incipient metastatic cells with traits needed to generate macroscopic metastases. Recent advances have provided provocative insights regarding these cell-biological and molecular changes, which carry implications concerning the pathogenesis of metastatic progression and the steps of the invasion-metastasis cascade that appear amenable to therapeutic targeting [34].

Dark side of oncological surgery

While surgical resection and adjuvant therapy can cure well-confined primary tumors, metastatic disease is largely incurable because of its systemic nature and the resistance of disseminated tumor cells to existing therapeutic agents. This explains why >90% of mortality from cancer is attributable to metastases, not the primary tumors from which these malignant lesions arise [35, 36]. The major cause of death from operable cancer is metastatic recurrence, despite successful removal of the primary tumor and the use of adjuvant therapy. Unfortunately, the surgical procedure itself may promote metastasis during the immediate postoperative period. Because of ethical and methodological limitations, this has never been directly tested in patients. The prospect that surgery may promote metastasis has become a greater concern especially given the early detection of cancer and our current understanding of tumor immunology. Encouragingly, advances in surgical procedures and our growing understanding of neuroimmunomodulation may allow the adverse effects of surgery to be reduced, and the hypothesis to be directly tested in cancer patients.

One of the most important aspects of oncological surgery is the removal of the major source of proliferating and mutating malignant cells that give rise to metastases. However, several consequences of surgery may promote metastasis have been proposed. The presence of the primary tumor may itself inhibit angiogenesis, and therefore, tumor removal may eliminate a safeguard against angiogenesis. Tumor cells shed into the circulation due to the physical manipulation of the tumor or its vasculature [37]. Excessive local and systemic release of growth factors needed during surgery and for wound healing may promote tumor recurrence both locally and at distant sites. Epidermal growth factor and transforming growth factor-β levels are increased, as is vascular endothelial growth factor. In addition, anti-angiogenic factors, such as angiostatin and endostatin, may be reduced by surgery [38]. Both the neuroendocrine

and cytokine stress response to surgery results in perioperative immunosuppression, including the cellular immune system [39]. Major surgery suppresses cellular immunity for several days[31]. There is a measurable decrease in the production of cytokines that favor cellular-mediated immunity such as interleukin (IL)-2, IL-12, and interferon-γ, and an increase in the production of cytokines that interfere with cell-mediated immunity, such as IL-10. There is a decrease in the number of circulating NK cells, cytotoxic T lymphocytes, dendritic cells, and T-helper cells [39]. A peak in immunosuppression is said to occur at day 3 [40], and this may be a window of opportunity during which minimal residual disease can grow and spread. Cell-mediated immunity can reduce the likelihood of metastasis even if it had not prevented the primary tumor. Conceptual progress in the last decade has added two emerging hallmarks of potential generality to this list-reprogramming of energy metabolism and evading immune destruction [41].

Increased surgical stress augments cancer metastasis via surgical stress-induced expression of proteinases in the target organ of metastasis in a mouse model [42]. Less immunosuppressive, such as laparoscopic surgery, has been shown to be associated with a longer disease-free survival and time to recurrence when compared with open resection [43, 44].

These effects of surgery begin simultaneously, and may act in synergy. For example, during the immediate postoperative period, preexisting micrometastases may grow because of decreased levels of antiangiogenic factors and increased levels of growth factors. Similarly, dissemination of single tumor cells, combined with systemic suppression of antitumor immunity, may enable the seeding of metastases in distant locations.

Effect of anesthesia on cancer recurrence

Isoflurane and halothane inhibit IFN stimulation of NK cell cytotoxicity in mice [45]. Multiple studies have demonstrated in vitro effects that may have some relevance in the cancer setting. For instance, sevoflurane alters the release of cytokines (IL-1β and TNF-α, but not IL-2) by NK and NK-like cells in vitro [46].

One large retrospective analysis found that general anesthesia for excision of primary melanoma was associated with a decrease in the survival rate (relative risk of 1.46) compared with local anesthesia [47]. Paravertebral anesthesia and analgesia for breast cancer surgery reduces the risk of recurrence or metastasis during the initial years of follow-up [48]. The difference was attributed to the use of general anesthetic agents. General anesthesia decreases circulating NK cells in patients undergoing elective orthopedic surgery [49]. Neutrophil, macrophage, dendritic, and T-cell function are also impaired [50].

Opioid administration has been shown to suppress cell-mediated and humoral immunity which including NK cell activity, production of immune-stimulating cytokines, phagocytic activity, and antibody production [51]. Morphine suppresses rat NK cell cytotoxicity in a dose-dependent manner [52]. The suppression is naloxone-sensitive. Morphine at clinically relevant doses increases angiogenesis and promotes breast tumor growth in mice [53]. Opioids also suppress postoperative NK cell cytotoxicity in humans [54]. One group in this study received high-dose fentanyl (75–100 μg kg^{-1}) and the second group received lower dose fentanyl (up to 6 μg kg^{-1}). At 24 h after operation, both groups had similar suppression of NK cell cytotoxicity (~20%). This suppression was more prolonged in the high-dose fentanyl group in which it lasted beyond the second postoperative day. The same study looked at the in vitro effect of human recombinant IL-2, IFN-α, and IFN-β. The NK cell suppression seen in this study was fully reversed by IL-2 and partially reversed by IFN-α and IFN-β. This

may be a potential target for immunotherapy. This opioid effect may be mediated by the neuroendocrine response they elicit. Healthy volunteers have also been shown to have components of their cell-mediated immunity, including NK cell cytotoxicity, suppressed by a morphine infusion [55]. Morphine administration, at doses within the range of analgesic use, can cause measurable suppression of some components of the human cellular immune system.

Role of local anesthetics in breast cancer surgery

In addition to the well established action of analgesia and antiarrhythmia, studies have demonstrated that local anesthetics have a broad spectrum of effects including anti-inflammatory and antimicrobial properties. Local anesthetics have a broad spectrum of pharmacological actions that go beyond the familiar pain relief and anti-arrhythmic effects [56, 57]. Recent observations of their chondrotoxicity call for caution in the clinical use of intra-articular injections [58]. Furthermore, there is growing evidence that local anesthetics might exert beneficial actions in the treatment of cancer by inhibition of cell proliferation, invasion, and migration [21, 59, 60]. These effects appear unrelated to their modulation of sodium channels [61]. Previous studies suggest that neuronal apoptosis induced by local anesthetics is mediated, at least in part, by mitogen-activated protein kinase (MAPK) pathway [62, 63]. However, little is known whether similar mechanisms apply to local anesthetic-induced cytotoxicity in cancer cells. The most commonly used anesthetic, lidocaine, effectively inhibits the invasiveness of cancer cells at concentrations used in surgical procedures [64]. This anti-invasive effect seems unrelated to its anesthetic activity (sodium channel blockade). Other local anesthetics have been shown to trigger apoptosis in a variety of human cells [65, 66]. The mechanisms underlying these effects are not yet fully understood.

Recent evidence indicates that wound infiltration with local anesthetics reduces postoperative pain after breast surgery [67, 68]. The apoptosis-inducing activity of local anesthetics may thus might provide additional benefits to their use that could have substantial clinical implications.

Role of local anesthetics in thyroid cancer surgery

The treatment options for persistent or recurrent disease include additional surgery and radioactive iodine therapy. Compartmental neck dissection for locoregional recurrence is recommended by the American Thyroid Association guidelines [69]. Another alternative treatment with promising results is ultrasound-guided percutaneous ethanol injection and radiofrequency ablation [70-72]. During the procedure, lidocaine infiltration to the puncture site and soft tissue around the recurrent tumor is a simple and effective method of pain control [73]. No adverse effects were reported except for that ultrasound image quality may be compromised with a large amount of lidocaine [72].

AIMS OF THE STUDY

While breast cancer management has made great strides, important questions remain that have critical therapeutic and clinical implications for patients. It is hoped that rethinking adding local anesthetics on procedure of surgical intervention will provide valuable perspectives on the current state of breast cancer management, especially as innovative treatment options are introduced that could considerably impact clinical outcomes.

Given that local anesthetics are frequently used in fine-needle aspiration of thyroid lesions and locoregional control of persistent or recurrent thyroid cancer, it would be interesting to find out whether local anesthetics have an antiproliferative effect on thyroid cancer cells in clinically relevant concentrations.

The specific aims of the study were:

- To examine the effects of two common local anesthetics, lidocaine and bupivacaine, on breast tumor cells in vitro and in a murine xenograft model.
- To find out whether local anesthetics have an antiproliferative effect on thyroid cancer cells.
- To investigate the signaling pathways involved in local anesthetics' effects in thyroid cancer cells.

MATERIALS AND METHODS

Reagents

Lidocaine and bupivacaine were purchased from Sigma (St. Louis, MO). Specific pan-caspase inhibitor (z-VAD-fmk), caspase-8 inhibitor (z-IETD-fmk), and caspase-9 inhibitor (z-LEHD-fmk) were obtained from BD Biosciences (San Jose, CA).

Cell lines and culture conditions

The human thyroid carcinoma cell lines 8505C and K1 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and the European Collection of Cell Cultures (ECACC, Salisbury, United Kingdom), respectively. 8505C cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in RPMI 1640 medium (Invitrogen/Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). K1 cells were cultured in Dulbecco's modified Eagle's medium (Gibco) mixed with Ham's F12 (Gibco) and MCDB 105 (Sigma, St. Louis, MO) medium in 2:1:1 proportions, supplemented with 10% FBS and 2 mM L-glutamine. Both 8505C and K1 have been authenticated to be unique thyroid cancer cell lines [74]. Lidocaine, bupivacaine, procaine, PD98059, SB203580, and SP600125 were obtained from Sigma. Osmolality of the culture media for experiments was analyzed by the freezing point depression method using an Advanced 3320 Micro Osmometer (Advanced Instruments, Norwood, MA).

Human breast tumor cell lines MCF-7 and MCF-10A were purchased from the American Type Culture Collection. The MCF-7 cell line is derived from malignant pleural effusions of breast adenocarcinoma. MCF-10A is a spontaneously immortalized but non-transformed human mammary epithelial cell line derived from breast tissue

with fibrocystic changes. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (SAFC Biosciences, Brooklyn, Vic., Australia), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. MCF-10A cells were cultured in DMEM/F12 supplemented with 5% horse serum (Invitrogen/Gibco, Carlsbad, CA), 20 ng/mL epidermal growth factor, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 µg/mL insulin, 100 U/mL penicillin, and 100 µg/mL streptomycin. Both cell lines were incubated in an atmosphere of humidified 5% CO2 at 37°C.

Cell viability assay and drug combination analysis

Thyroid cancer cell viability assay was performed in triplicate for each experiment as previously described [75]. Briefly, 8505C and K1 cells were seeded into 96-well plates one day before lidocaine and bupivacaine (individually or in combinations) was added at the indicated concentrations. For the thiazolyl blue tetrazolium bromide (MTT) colorimetric assay, 100 µl of MTT reagent (5 mg/ml; Sigma) was added to the cell culture and cells were incubated at 37 °C for 4 h. The formazan crystals converted from tetrazolium salts by viable cells were solubilized with acidified isopropanol. The absorbance was measured with Varioskan Flash (Thermo Fisher Scientific, Waltham, MA) at wavelength of 570 nm. The absorbance of control cells (incubated without drugs) was defined as 100%.

Breast tumor cells were plated 5 x 10^3/well in 96-well tissue culture plates and treated with lidocaine or bupivacaine (individually or in combinations) at indicated concentrations for 6, 24, or 48 hr. To determine the cell viability, 200 µL of the MTS reagent (0.2 mg/mL, Promega, Madison, WI) was added to the cells, which were then incubated in the dark at 37°C for 1.5 hours. The absorbance was measured at 490 nm using Varioskan Flash (Thermo Fisher Scientific, Waltham, MA). The median effective

dose (ED_{50}) values were calculated using the probit method of Miller and Tainter. Drug synergy was determined by the CI and isobologram analyses according to the median effect methods described by Chou and Talalay [76]. On the basis of the dose-response curves from MTT assays, the CI values were calculated. Synergism, additivity and antagonism are defined as CI < 1, CI = 1 and CI > 1, respectively. In the isobologram graph, combination data points that fall on, above, and beneath the oblique line represent an additive, antagonistic, and synergistic effect, respectively.

DNA fragmentation analysis

DNA was extracted using QIAamp DNA mini kit (51306; Qiagen, Valencia, CA) according to the instructions of the manufacturer. After DNA quality was verified with spectrophotometric measurements, 1 μg of extracted DNA was electrophoresed on a 1.5% agarose gel, visualized by EZ-vision dye staining (Amresco, Solon, OH) under ultraviolet illumination, and photographed using Digital Image Stocker (DS-30, FAS III, Toyobo, Osaka, Japan). Apoptotic DNA fragmentation of samples corresponding to less than 1500 bp DNA was densitometrically determined.

Annexin V apoptosis assay

The Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Kit (BioVision) was used to determine whether cell death was due to apoptosis. Cells were plated on coverslips in 24-well culture plates. After treatment with lidocaine or bupivacaine at indicated concentrations for 4 hours, cells were stained with 2.5 μg/mL Annexin V-FITC and 4 μg/mL Hoechst 33285 (Sigma) in binding buffer (10 mM HEPES/NaOH [pH 7.4], 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2) for 20 min at room temperature in the dark. Cells were then fixed with 2% formaldehyde in binding buffer for 5 min. After washing with binding buffer, cells were mounted and observed with a

fluorescence microscope (Axio Imager A1, Carl Zeiss AG, Oberkochen, Germany). For quantitative analysis, the fraction of FITC-positive cells (apoptotic cells) in each group was determined based on calculations from 10 randomly selected fields under the fluorescence microscope.

Analysis of cell apoptosis

Cell apoptosis was analyzed using the annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences Pharmingen, San Diego, CA) according to the instructions of the manufacturer. Thyroid cancer cells were treated with the indicated concentrations of lidocaine and bupivacaine for 24 to 72 h. Both floating and attached cells were harvested and washed, followed by incubation with 5 μl of annexin V-FITC and 5 μl of PI for 15 min at room temperature in the dark. After the incubation, 400 μl of binding buffer solution was added, and flow cytometry analysis was performed.

Colony formation assay

For colony formation assay, 400 cells/well were seeded into six-well plates, allowed to adhere for 24 h, and treated with the indicated concentrations of lidocaine and bupivacaine from day 2. After 8 to 15 days, colonies were stained with 3% crystal violet and colonies containing >50 cells were counted.

Cell cycle analysis

The effect of lidocaine and bupivacaine on the cell cycle was analyzed by PI staining and flow cytometry [77]. After treatment with lidocaine and bupivacaine for 6, 24, and 48 h, thyroid cancer cells were harvested, gently washed, and fixed in 70% cold ethanol at 4 °C overnight. The fixed cells (1x106) were incubated with RNase A for 30

min at room temperature, and stained with PI solution using the BD Cycletest Plus DNA reagent kit (BD Biosciences, San Jose, CA) in the dark. Subsequently, the cells were analyzed on a FAS Calibur flow cytometer (BD Biosciences) equipped with Cell Quest Pro software. The distribution of cells in G0/G1, S and G2/M phases of cell cycle was estimated using the ModFit LT software (Verity Software House, Topsham, ME). As an estimate of the proportion of apoptotic cells, the percentage of hypodiploid cells was calculated in the DNA histogram.

Determination of mitochondrial membrane potential (ΔΨm)

Mitochondrial membrane potential was determined by flow cytometry using the ΔΨm-dependent fluorescent dye JC-1 (CS0390; Sigma). JC-1 is a lipophilic, cationic dye that that can selectively enter into mitochondria, and undergoes a reversible change in fluorescence emission according to the ΔΨm. Healthy cells with high ΔΨm will form JC-1 aggregates and fluoresce red, whereas those apoptotic cells with low ΔΨm will contain monomeric JC-1 and fluoresce green. After treatment with lidocaine and bupivacaine for the indicated time, thyroid cancer cells were harvested and incubated with JC-1 for 20 min at 37 °C according to the manufacturer's instructions. The samples were then subject to flow cytometry.

Western blot assay

Total breast cellular proteins were extracted using M-PER lysis buffer (Thermo). Lysates were centrifuged and proteins were heat-denatured. Protein concentration was determined using the Bradford assay kit (Bio-Rad, Hercules, CA). Total proteins (30 μg) were separated by 10-15% SDS-PAGE and then transferred onto a nitrocellulase membrane (Amersham Biosciences/GE Healthcare, Piscataway, NJ). Membranes were blocked in 5% (w/v) non-fat milk and immunblotted with primary antibodies as

indicated. Antibodies were diluted 1:1000 for anti-caspase 8 (ALX-804-429; Enzo, Farmingdale, NY), anti-caspase 9 (9508; Cell Signaling, Danvers, MA), anti-caspase 7 (9494; Cell Signaling), and anti-poly ADP-ribose polymerase (PARP) (P248; Sigma) and 1:10 000 for anti-β-actin (A5441; Sigma) and α-tubulin (T5168; Sigma). Immunoreactive proteins were detected using horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) and the enhanced chemiluminescence system (ECL plus; Merck Millipore, Darmstadt, Germany).

Total thyroid cellular proteins were extracted, quantified, and subjected to gel electrophoresis according to standard procedures as we described previously [78]. The antibodies used in our study, including anti-caspase 3 (#9662), anti-cleaved caspase 3 (#9661), anti-caspase 7 (#9494), anti-poly(ADP-ribose) polymerase (PARP) (P248; Sigma), anti-Bax (#2774), anti-Bcl-2 (#2872), anti-phospho-ERK (#9101), anti-ERK (#9102), anti-phospho-p38 (#4511), anti-p38 (#9212), anti-phospho-JNK (#9255), anti-JNK (#9252), and anti-actin (A5441; Sigma). Antibodies were obtained from Cell Signaling, Danvers, MA unless specified otherwise. The antigen-antibody complexes were visualized with by chemiluminescence with the Amersham ECL detection system (GE Healthcare, Piscataway, NJ).

Measurement of cytochrome c release

To determine the release of cytochrome c from mitochondria to cytoplasm, preparations of cytosolic extracts were carried out with the Mitochondria Isolation Kit (#89874; Thermo Scientific/Pierce, Rockford, IL) according to the manufacturer's instructions. In brief, thyroid cancer cells (2x107) were harvested after treatment with lidocaine and bupivacaine for the indicated time periods, washed, and incubated with cytosol extraction buffer in ice for 10 min. The supernatant was collected through

centrifugation at 700 x g for 10 min at 4 °C. The cytosolic fraction was obtained through centrifugation again at 12,000 x g for 15 min at 4 °C, and was analyzed by Western blotting using anti-cytochrome c antibody (#4272; Cell Signaling) as described above.

Assay for caspase 3 activity

The caspase 3 activity was assayed using the ApoTarget Caspase-3 Colorimetric Protease Assay Kit (KHZ0022; Invitrogen) according to the manufacturer's protocol. Briefly, 5 x 106 thyroid cancer cells were treated with lidocaine and bupivacaine with or without specific inhibitors for the indicated time periods. The cells were harvested using trypsinization and cell lysates prepared as described above. Samples of the cell lysates (100 μg protein per sample) were mixed with reaction buffer and 200 μM substrate (DEVD-pNA) and incubated for 2 hours at 37 °C in the dark. The absorbance was then measured at 405 nm and the sample readings calculated by subtracting the absorbance of blank samples.

Global gene expression analysis

8505C cells were treated with lidocaine (12 mM), bupivacaine (4 mM), or left untreated for 24 hours. Cells were harvested and total RNA was isolated using the TRI Reagent (Sigma). After RNA integrity was verified, cDNA was synthesized using the Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen), subsequently labeled using the One-Color DNA Labeling Kit (Roche NimbleGen, Madison, WI), and hybridized to a Human HG18 expression array (12x135K) using the NimbleGen Hybridization System. Arrays were scanned and chip images were collected on a NimbleGen MS200 Microarray Scanner. Following the acquisition and initial quantification of array images, raw array data were normalized per chip and per gene and filtered based on raw signal intensity and detection call. Genes with an expression

fold change of ≥ 2 between a treatment and a control were considered to be significant. To determine the potential mechanistic network, transcripts with differential expression were studied using the MetaCore pathway analysis suite (GeneGo-Thomson Reuters, New York, NY) and Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA).

In vivo xenograft model

The xenograft model was established and modified as previously described.[9] In brief, 17-estradiol was implanted into thirty female BALB/c nude mice. Twenty-four hours later, 1×10^7 MCF-7 cells in 100 μL mixture of PBS and growth factor–reduced matrigel were injected subcutaneously into the trunks of mice. Tumor growth was determined by caliper measurements. Tumor volume was calculated as ½ × length × width^2 to approximate an ellipsoid volume. When the tumor volume reached 100 mm^3, mice were treated with peritumoral injections of lidocaine, bupivacaine, or saline (n = 10 per group, sacrificed at two time points). To ensure precise delivery of local anesthetic solutions or saline, we used a short-needle, 30-gauge, 0.3-mL insulin syringe. A total of 0.1 mL of 0.5% (w/v) lidocaine (21.3 mM), 0.125% (w/v) bupivacaine (4.3 mM), or 0.9% (w/v) saline were infiltrated to the normal tissue abutting the tumor by 5 separate injections (0.02 mL x 5). After 24 and 48 hours, five mice from each group were sacrificed, and the tumor tissues were harvested. Proteins were extracted from the tumor tissues and subjected to Western blot analysis. Tissue samples were also fixed in 10% buffered formalin, embedded in paraffin, and stained with the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay.

TUNEL assay

The fluorescein-FragEL DNA fragmentation detection kit (QIA39; Calbiochem,

Merck Millipore) was used to detect apoptosis in paraffin-embedded mouse tumor sections according to the manufacturer's protocol. After deparaffinization and rehydration, the sections were incubated with 20 μg/mL proteinase K at room temperature for 20 min for permeabilization, followed by rinsing with substituting tris-buffered saline, and then incubated with equilibration buffer at room temperature for 30 min. After equilibration, the sections were incubated with TdT labeling reaction mixture at 37 ˚C for 1.5 h. During this labeling reaction, TdT labels the exposed 3′-OH ends of DNA fragments with fluorescein-labeled deoxynucleotides and catalyzes the addition of fluorescein-labeled and unlabeled deoxynucleotides. Finally, the sections were embedded in fluorescein-FragEL mounting medium and examined by fluorescent microscope (Olympus, IX71, Japan).

Statistical analysis

All experiments for breast tumor cells were performed at least in triplicate. When three separate experiments yielded consistently reproducible results, no more duplicate experiments were carried out. Otherwise, more experiments were repeated until convincingly consistent results were obtained. The number of independently performed experiments was not determined based on statistical power analysis. Results are expressed as mean ± standard error of the mean. For statistical comparisons, Student's t-test and one-way analysis of variance (ANOVA) followed by post hoc Dunnett's tests were used. All analyses were performed using the SPSS program version 17.0 (SPSS Inc., Chicago, IL). A *P* value < .01 was considered statistically significant. Results from thyroid cancer cells were expressed as means ± SEM. Comparisons between groups were performed using a two-tailed Student's t test. Values of $P < 0.05$ were considered significant.

RESULTS

Effects of lidocaine and bupivacaine on breast tumor cell viability

The viability of MCF-7 and MCF-10A cells was determined after incubation with lidocaine or bupivacaine at serially diluted concentrations for 6, 24, and 48 hours. Lidocaine and bupivacaine inhibited the growth of both breast tumor cell lines in a dose- and time-dependent manner (Fig. 1, all $P < .001$). The ED50 values of lidocaine and bupivacaine were significantly lower in MCF-7 cells than in MCF-10A cells ($P < .001$). For MCF-7 cells, the ED50 of lidocaine was 4.5 ± 0.26 mM and that of bupivacaine was 1.3 ± 0.11 mM at 24 hours.

Drug synergy was determined using the combination index (CI) and isobologram analyses according to the median effect methods. In the isobologram graph, combination data points that fall on, above, and beneath the oblique line represent additive, antagonistic, and synergistic effects, respectively. As shown in Fig. 2, co-treatment with lidocaine and bupivacaine for 48 hours had a slight antagonistic effect (CI, 1.26 ± 0.12) in MCF-7 cells and a nearly additive effect (CI, 1.05 ± 0.03) in MCF-10A cell

Induction of breast cancer cell apoptosis

We investigated the mechanism underlying the reduced viability of cells treated with lidocaine and bupivacaine. Apoptosis is characterized by the degradation of nuclear DNA in response to various apoptotic stimuli in a wide variety of cell types. DNA gel electrophoresis revealed that treatment of MCF-7 and MCF-10A cells with lidocaine and bupivacaine for 24 hours caused oligonucleosomal DNA fragmentation in a dose-dependent fashion (Fig. 3, all $P < .001$ except for $P = .002$ for MCF-10A cells

treated with lidocaine).

During apoptosis, translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer leaflet is common. Based on its affinity for PS, annexin V can be used as a sensitive probe for cell surface changes. MCF-7 and MCF-10A cells were treated with lidocaine or bupivacaine for 4 hours, fixed, and detected using fluorescence microscopy. As shown in Fig. 4, the fraction of cells in an early state of apoptosis was determined by staining cells with annexin V. The mean percentage of MCF-7 cells that were apoptotic after treatment with lidocaine (7.4 mM) or bupivacaine (2.6 mM) was 74% and 81%, respectively. In the non-tumorigenic cell line MCF-10A, treatment with lidocaine (7.4 mM) or bupivacaine (2.6 mM) resulted in apoptosis of 8% and 19% of the cells, respectively ($P < .001$).

These results are in concordance with our cell viability data indicating that lidocaine and bupivacaine exhibit higher cytotoxicities in MCF-7 than in MCF-10A cells.

Caspase activation induced by lidocaine and bupivacaine

Apoptosis is largely controlled by a family of intracellular cysteine proteases known as caspases. Caspases can be grouped into initiators (caspase 2, 8, 9, and 10) and effectors (caspase 3, 6, and 7). PARP, an enzyme involved in DNA damage and repair, is cleaved by caspase 3 and caspase 7 during apoptosis. This cleavage inactivates PARP and contributes to a cell's commitment to undergo apoptosis. Previous studies have shown that MCF-7 cells do not express caspase 3 or caspase 10 [79, 80]. As such, caspase 7 might compensate for the lack of caspase 3, while caspase-3–deficient MCF-7 cells are still sensitive to apoptotic cell death. As shown in Fig. 5, lidocaine and bupivacaine induced proteolytic activation of caspase 7 in a dose- and time-dependent manner ($P < .001$). Similarly, increased PARP cleavage was observed in MCF-7 cells

treated with lidocaine and bupivacaine (Fig. 6, P < .001). Compared with the control group, lidocaine treatment of 4.5 mM and bupivacaine treatment of 1.3 mM showed significantly increased cleavage of caspase 7 and PARP approximately 2 to 6 hours after treatment.

Caspases are activated by two major signaling routes, the extrinsic death receptor and the intrinsic mitochondrial pathway. The binding of death receptor ligands to their respective receptors activate the initiator caspase 8, while the intrinsic (mitochondrial) pathway is mediated by the release of apoptogenic proteins which result in the activation of caspase 9. In MCF-7 cells treated with lidocaine and bupivacaine, both caspase 8 and caspase 9 were cleaved and activated about 6 hours after treatment (Fig. 7, P = .006 and .009 for caspase 8, P < .001 for caspase 9). These results suggest that apoptosis induced by local anesthetics involves both the extrinsic and intrinsic pathways.

Effects of caspase inhibitors on breast tumor cells

To further confirm the involvement of the extrinsic and intrinsic pathways in this process, MCF-7 cells were pretreated with a caspase 8 inhibitor, caspase 9 inhibitor, or a pan-caspase inhibitor before incubation with lidocaine or bupivacaine for 24 hours. All caspase inhibitors reduced the proteolytic activation of caspase 7 (Fig. 8, P < .001) and the downstream cleavage of PARP (Fig. 9, P < .001) induced by lidocaine and bupivacaine. Collectively, these data indicate that local anesthetics induce apoptosis in MCF-7 cells through activation of the caspase-dependent extrinsic and intrinsic apoptosis pathways.

Effects in xenograft breast tumors

The effects of lidocaine and bupivacaine were evaluated in a xenograft model

to determine whether local anesthetics could induce apoptosis in vivo. Clinical concentrations of lidocaine (21.3 mM) and bupivacaine (4.3 mM) were infiltrated around xenograft tumors. The tumors that were treated with local anesthetics had higher expression of cleaved caspase 7 than did those treated with saline (Fig. 10, P = .003 at 24 h and P = .008 at 48 h). Identification of apoptotic cells by DNA fragmentation assays revealed the presence of a multitude of DNA strand breaks in treated tumor cells (Fig. 11). These findings indicate that lidocaine and bupivacaine also induce apoptosis of breast tumor cells in vivo.

Inhibition of thyroid tumor cell growth and colony formation by local anesthetics

Cell viability of 8505C and K1 thyroid cancer cells was determined by incubating with lidocaine and bupivacaine at serially diluted concentrations for 24 and 48 hours. Lidocaine and bupivacaine inhibited the growth of both thyroid cancer cells in a dose-dependent manner (Fig. 12). At 24 hours, the median effective dose (ED50) of lidocaine was 7.3 mM for 8505C cells and 6.8 mM for K1 cells, respectively. These were lower than the commonly used concentration of 1% (w/v) lidocaine (42.67 mM). Similarly, the ED50 at 24 hours of bupivacaine was 3.1 mM for 8505C cells and 1.3 mM for K1 cells. Both were much lower than the clinical concentration of 0.5% (w/v) bupivacaine (17.34 mM).

Drug synergy was determined by the combination index (CI) and isobologram analyses according to the median effect methods (Fig. 13). The CI was 1.17 ± 0.03 for 8505C cells and 1.14 ± 0.06 for K1 cells. These results suggest that combination of lidocaine and bupivacaine yielded slight antagonism.

Furthermore, the effects of local anesthetics on tumor cell growth were determined by clonogenic assay. The results are shown in Fig. 14. A significant dose-dependent

reduction in colonies was observed in 8505C and K1 cells. Thyroid cancer cells did not form colonies following treatment with lidocaine > 4 mM or bupivacaine > 0.8 mM.

To examine whether the cytotoxic effect is restricted to amide-type local anesthetics, thyroid cancer cells were also treated with an ester-type local anesthetics, procaine. The ED50 was 39.6 mM and 30.9 mM, respectively, suggesting that the effect correlated with the potency, but not the class, of local anesthetics. Furthermore, to exclude the possibility that changes in pH or osmolality would account for the cytotoxic effects, pH and osmolality of the culture media for experiments were determined. There were no significant variations in pH or osmolality (Fig. 15), indicating that the observed effects were pharmacological in nature.

Induction of thyroid cancer cell apoptosis by local anesthetics

To determine the basis of reduced cell viability, cell cycle analysis was performed. There was no cell cycle arrest in thyroid cancer cells treated with lidocaine and bupivacaine for 6, 24, and 48 hours (data not shown). However, we observed a dose-dependent increase in the sub-G1 (hypodiploid) fraction following treatment with lidocaine and bupivacaine (Fig. 16). We also used annexin V/propidium iodide (PI) dual staining for further confirmation of local anesthetics-induced apoptosis in thyroid cancer cells. As shown in Fig. 17, treatment with lidocaine and bupivacaine resulted in apoptosis in a dose-dependent manner. Necrosis was also observed in high concentrations of lidocaine and bupivacaine. Taken together, these results suggest that growth suppression by local anesthetics in thyroid cancer cells involves induction of apoptosis.

Assessment of mitochondrial dysfunction

A decrease in mitochondrial membrane potential is one of the earliest events in

apoptosis. Mitochondrial membrane integrity was evaluated using the cationic dye JC-1, a highly specific probe for detecting changes in mitochondrial ΔΨm. JC-1 forms red aggregates in intact mitochondria, while green fluorescence is due to the formation of JC-1 monomers at low mitochondrial membrane potential. As shown in Fig. 18, lidocaine and bupivacaine significantly increased the formation of JC-1 monomers in 8505C and K1 cells in a dose-dependent way. The percentage of cells with low ΔΨm was slightly lower than the percentage of apoptotic cells treated with local anesthetics at the same concentrations.

We next sought to determine whether cytochrome c was released from mitochondria to the cytosol. As expected, a higher level of cytochrome c was measured in cytosol in both cell lines after treatment with lidocaine and bupivacaine (Fig. 19). Collectively, these data suggest that treatment of thyroid cancer cells with local anesthetics leads to activation of the mitochondrial apoptotic pathway.

Activation of caspases by local anesthetics

Release of cytochrome c has been shown to activate the downstream caspases that are ultimately required to induce apoptosis. We therefore examined whether caspase activation was involved in the induction of apoptosis by local anesthetics. Treatment with lidocaine and bupivacaine resulted in the activation of caspase 3 and caspase 7, and cleavage of PARP in 8505C and K1 cells dose-dependently (Fig. 20).

Furthermore, caspase colorimetric substrate assay showed that caspase 3 activity increased in a time-dependent manner in 8505C cells after treatment with lidocaine and bupivacaine (Fig. 21). These results clearly indicate that caspase activation plays an important role in thyroid cancer cell apoptosis induced by local anesthetics.

The proteins of the Bcl-2 family participate in the apoptotic process by

functioning as promoters (e.g., Bax) or inhibitors (e.g., Bcl-2). To activate the mitochondrial apoptotic pathway, activated Bax forms an oligomeric pore and results in the permeabilization of the mitochondrial outer membrane. We found that treatment with lidocaine and bupivacaine lead to a reduction in the Bcl-2 levels with a concomitant increase in the Bax levels (Fig. 20). Therefore, local anesthetics alter the protein levels of key members of the Bcl-2 family in a manner that favors an increase in the ratio of Bax/Bcl-2, which may contribute to the susceptibility of thyroid cancer cells to apoptosis.

Analysis of gene expression signatures affected by local anesthetics

To identify gene expression signatures that are associated with biological functions of local anesthetics, microarray and pathway enrichment analysis was carried out to compare expression patterns in 8505C cells treated with lidocaine and bupivacaine. The top ten pathways identified by the pathway enrichment analysis are listed in Tables 1 and 2. It is noteworthy that the most prominent transcriptional change in thyroid cancer cells treated with local anesthetics is apoptosis. Other pathways common to lidocaine and bupivacaine treatment include cytoskeletal remodeling and myeloid differentiation. Furthermore, we used in silico tools from Ingenuity to identify pathways reportedly involving molecular mechanism of cancer. Integrating microarray data from cells treated with lidocaine and bupivacaine, pathway analysis suggested that extracellular signal-regulated kinase (ERK) 1/2 and p38 MAPK are significantly up- or down-regulated in response to treatment with local anesthetics (Fig. 22).

Modulation of mitogen-activated protein kinase signaling

Our microarray and pathway enrichment analysis indicates that MAPK pathways are probably involved in the mechanisms mediating action of local

anesthetics on thyroid cancer cells. To examine this possible relationship, total cellular proteins were extracted from 8505C and K1 cells treated with lidocaine (12 mM) and bupivacaine (4 mM) for various time periods, and lysates were immunoblotted with specific antibodies against phosphorylated and total ERK1/2, p38, and c-Jun N-terminal kinase (JNK), respectively. As shown in Fig. 23, a decreased phosphorylation of ERK1/2 was observed. Lidocaine and bupivacaine significantly increased phosphorylation of p38 and JNK. Lidocaine and bupivacaine had no remarkable effect on total p38 and JNK protein level.

Effects of inhibition of mitogen-activated protein kinases

Subsequently, we would like to know whether the modulations of ERK1/2, p38 MAPK, and JNK signaling pathways account for apoptosis induced by local anesthetics. To this end, we examined the effects of specific inhibitors on caspase activation and PARP cleavage. PD98059 is an inhibitor of MAPK/ERK kinase (MEK), thereby inhibiting the phosphorylation and the activation of MAPK. SB203580 is a selective, reversible, and ATP-competitive inhibitor of p38 MAPK, whereas SP600125 is an anthrapyrazolone JNK inhibitor that competes with ATP to inhibit the phosphorylation of c-Jun. As shown in Fig. 24, PD98059 and SB203580, but not SP600125, reduced the activation of caspase 3 and cleavage of PARP. These results were confirmed by caspase 3 activity assay (Fig. 25). Cotreatment with PD98059 or SB203580 suppressed caspase 3 activity, whereas SP600125 paradoxically increased caspase 3 activity following treatment with local anesthetics. These data are accordant with the results of pathway enrichment analysis (Fig. 22) that ERK1/2 and p38 MAPK signaling pathways are involved in apoptosis induced by local anesthetics.

DISCUSSION

Our data demonstrate that lidocaine and bupivacaine induce apoptosis of breast tumor cells at clinically relevant concentrations. It is noteworthy that the apoptotic action of local anesthetics is more pronounced in malignant breast cancer cells than in mammary epithelial cells. Furthermore, our results suggest that the *in vitro* apoptotic effects of local anesthetics are reproducible *in vivo*.

Local anesthetics may directly or indirectly influence the oncologic outcomes of breast cancer. The direct effects of local anesthetics on tumor cells include induction of apoptosis [81], inhibition of invasion [64], and suppression of metastatic efficiency [82, 83]. Indirect actions of local anesthesia include attenuation of the neuroendocrine response to surgery, followed by improved preservation of immunocompetence [84]. Moreover, local anesthetics can render tumor cells more sensitive to the effects of chemotherapy [85] and systemic hyperthermia [86, 87]. In breast tumor cells, we found that local anesthetics induce apoptosis more effectively in breast cancer MCF-7 cells than in non-tumorigenic mammary epithelial MCF-10A cells. These results are encouraging from a clinical perspective, because a lower level of cytotoxicity toward normal breast epithelial cells may minimize collateral damage during treatment.

The mechanisms of cytotoxicity appear to be unrelated to the primary action of local anesthetics [88]. Lidocaine has been shown to directly inhibit the tyrosine kinase activity of epidermal growth factor receptor (EGFR) in corneal epithelial cells [89] and in human tongue cancer cells [60]. A previous study reported that MCF-7 cells have higher levels of cell surface and cytoplasmic EGFR expression than do MCF-10A cells [90]. Therefore, one plausible explanation for our results is that apoptosis induced by local anesthetics is associated with inhibition of EGFR tyrosine kinase activity.

Another potential mechanism whereby local anesthetics may influence tumor growth is by interaction with the tumor epigenome [91]. Genome stability and normal gene expression are maintained by a fixed and predetermined pattern of DNA methylation. Increased methylation frequently leads to downregulation of tumor suppressor genes, favoring tumor progression [91]. Procaine, the prototypic ester-type local anesthetic, has been shown to demethylate DNA and inhibit tumor growth in breast cancer cells [92]. Similar results have been obtained for lidocaine, the prototypic amide-type local anesthetic [93].

Caspase 8, an initiator capsase, is the key mediator of the extrinsic pathway [94]. This pathway involves the activation of caspase 8 through dimerization and autoproteolytic cleavage; the activated protein then processes the downstream effector caspase 7, which subsequently cleaves specific substrates, resulting in cell death. Activated caspase 9 also initiates a caspase cascade involving the downstream effector caspase 7, ultimately resulting in cell death. In the present study, we found that caspases 8 and 9 both mediate apoptotic signal pathways, leading to apoptosis of breast tumor cells treated with local anesthetics. In addition, a slight antagonism was observed with combination of lidocaine and bupivacaine. It is therefore likely that lidocaine and bupivacaine share a similar mechanism of apoptosis-inducing activity. These findings are reminders that our understanding of the mechanisms underlying local anesthetic function is far from complete.

Despite concerns that surgery may inhibit host defenses and facilitate the development of metastases [84], surgical tumor removal remains a highly relevant treatment option for cancer patients. Surgical trauma, stress, anesthetics and other drugs can interact with the cellular immune system and affect long-term outcomes. Regional anesthetic procedures and intravenous administration of local anesthetics have both been shown to reduce perioperative surgical stress [95, 96]. Experimental studies in

murine models of breast cancer have shown that regional anesthesia may also reduce the metastatic burden [97, 98]. Apart from the use of lidocaine as a regional anesthetic, its administration via intravenous infusion during the perioperative period is increasing. Perioperative lidocaine infusion reduces postoperative pain, decreases the need for opioids, and reduces nausea/vomiting [99]. Our results suggest that the apoptotic effects of local anesthetics deserve further clinical evaluation for use in breast cancer surgery. The peripheral continuous infiltration of local anesthetics or intravenous infusion of lidocaine is an effective analgesic technique [67, 100] that, because of its simplicity, may prove to be an important instrument in our armamentarium for breast cancer surgery.

It has been more than a century since the first synthetic local anesthetic was introduced in the early 1900s. Local anesthetics bind reversibly to a specific receptor site within the sodium channels and block ion movement. It was believed that the effects are reversible with recovery of nerve function and without damage to neuronal cells. However, reports of permanent neurologic injury have generated concern about the neurotoxicity of local anesthetics [101]. Accumulating evidence suggests that local anesthetics can cause rapid neuronal death through triggering apoptosis and necrosis [66, 102]. Moreover, a number of studies have reported local anesthetic toxicity on other cell types [89, 103, 104]. In the present study, we firstly demonstrated the direct effect of lidocaine and bupivacaine inhibiting cell growth and colony formation of thyroid cancer cells.

The mechanisms of cell toxicity from local anesthetics have not been fully elucidated. In U937 histiocytic lymphoma cells, lidocaine induced apoptosis at concentrations below 12 mM and induced necrosis at concentrations above 15 mM [105]. Similar observations have been reported by others [66, 102, 106]. In this study, both apoptosis and necrosis participate in cell death induced by local anesthetics.

Intriguingly, a slight antagonism was identified with combination of lidocaine and bupivacaine. It seems likely that lidocaine and bupivacaine induce cell death by a similar mechanism. When a cell is unable to die by apoptosis, it may undergo necrotic cell death. It is well known that ATP levels are a determinant of manifestation of cell death [107, 108]. Lidocaine and bupivacaine were shown to decrease ATP levels and viability of melanoma cells [109]. The mode of cell death likely depends on the duration of exposure and concentrations of local anesthetics.

Mitochondrial energetics affected by local anesthetics may account for the decrease in the ATP levels. Local anesthetics can reach mitochondria and this effect is highly dependent on the lipid-solubility of the local anesthetic [110]. Clinical and experimental myopathy induced by bupivacaine has been reported to result from mitochondrial dysfunction and reduced oxidative energy metabolism [111, 112]. Specifically, bupivacaine suppressed cell respiration through inhibition of complexes I and III, accompanied with production of reactive oxygen species [113]. Irwin *et al.* have shown that bupivacaine myotoxicity relied on active oxidative metabolism because highly glycolytic extensor digitorum longus were resistant to bupivacaine-induced toxicity [111]. Nonetheless, tumor cells predominantly produce energy through glycolysis (Warburg effect) [114]. Our findings are in agreement with those of a few reports demonstrating that mitochondrial dysfunction could also be elicited by local anesthetics in malignant tumor cells [105, 106, 115].

Mitochondria are essential modulators of apoptosis, necrosis and autophagy [108]. The intrinsic (mitochondrial) pathway of apoptosis is mediated by the release of pro-, and anti-apoptotic proteins, including cytochrome c. In our experiments, local anesthetics increased pro-apoptotic Bax expression and downregulated Bcl-2 expression, leading to a higher ratio between pro-apoptotic and anti-apoptotic Bcl-2 family proteins. That increased Bax/Bcl-2 ratio facilitates the release of mitochondrial

cytochrome c, which subsequently induces activations of caspase cascade that cleave PARP into inactive fragments. However, lidocaine and bupivacaine promoted mitochondrial injury to an extent less than that of apoptosis induced by the same concentrations of the local anesthetic. This suggests that a mitochondria-independent process may also play a role. This hypothesis is supported by the results of pathway enrichment analysis (Fig. 22), in which transcriptional alterations in the death receptor pathway components were involved.

Cellular microarrays are powerful experimental tools for identification of novel drug targets and pharmacogenomics. Unami and colleagues firstly reported that apoptosis-related genes were transcriptionally regulated by bupivacaine according to microarray analysis [115]. Recently, Lucchinetti *et al.* demonstrated that multiple transcriptional programs related to cell differentiation, tumorigenesis, and metastasis were negatively affected by ropivacaine [116]. The authors postulate that this means novel rationales for the perioperative use of local anesthetics in patients with cancer. Our data are in concordance with these results and showed a strong association between local anesthetics-induced alterations in gene expression and apoptosis. Furthermore, we identified that MAPK pathway is involved in the molecular mechanisms of apoptosis induced by local anesthetics.

In primary neuron cultures, bupivacaine and ropivacaine were found to activate p38 MAPK and JNK but not ERK1/2 [62]. Furthermore, pharmacological inhibition of these kinases attenuates neurotoxicity *in vitro*. The authors noted that axonal degeneration induced by lidocaine is prevented by p38 MAPK inhibitor but not by caspase inhibition [117]. Harato *et al.* also demonstrated that p38 MAPK inhibitor treatment attenuated the caspase 3 activity and cell death induced by bupivacaine [118]. In another study, p38 MAPK pathway is implicated in bupivacaine-induced apoptosis in human neuroblastoma SH-SY5Y cells [63]. In line with the findings in neuronal

apoptosis, we found that p38 MAPK activation plays a crucial role in local anesthetics-induced apoptosis in thyroid cancer cells. Negative regulation of proliferation is a highly conserved and important function of p38α in various cell types [119]. Interestingly, upregulated p38α activity has been reported in papillary and follicular thyroid cancer [120]. The notion is supported by the basal expression of phospho-p38 proteins in this study (Fig. 23). The dual role for p38 MAPK in thyroid cancer biology remains a virtually untapped area in spite of Ras-Raf-ERK pathway having been extensively studied in thyroid cancer.

In the present study, ERK1/2 activity was suppressed by lidocaine and bupivacaine. Joo *et al.* demonstrated that lidocaine significantly suppressed the increased ERK1/2 response in a rat model of neuropathic pain [121]. More recently, lithium was shown to protect against bupivacaine-induced neuronal injury through reversing the suppression of ERK1/2 signaling [122]. There is increasing evidence for a crosstalk between MAPK pathways. For example, p38 and JNK pathway activation in the induction of apoptosis may negatively regulate the ERK pathway [123]. It is also worth noting that there is a paradoxical decrease in caspase activation following PD98059 treatment. Many genetic alterations have been implicated in thyroid cancer, and the aberrant activation of the Ras-Raf-ERK pathway is most frequent [124]. Inhibition of the MAPK pathway arrests thyroid cancer cells in G1 phase [125]. Therefore, a plausible explanation for our results is that ERK1/2 inhibition increased susceptibility of thyroid cancer cells to other mechanisms of growth inhibition like cell cycle arrest and necrosis, which do not involve caspase activation.

Conclusions

In summary, this study is the first to find that clinically relevant concentrations of lidocaine and bupivacaine effectively induce apoptosis of human breast and thyroid

tumor cells. Local anesthetics are more cytotoxic for malignant than nonmalignant cells, offering a compelling rationale for exploiting these agents in breast cancer surgery. Emerging evidence indicating a relationship between local failure and outcome in breast cancer emphasizes the importance of the surgeon's role in reducing local recurrence and systemic failure after surgery. Administration of local anesthetics at the wound site is a rational approach to reducing the afferent nociceptive barrage. Our results demonstrate previously unrecognized benefits of local anesthetics and suggest that lidocaine and bupivacaine might be ideal infiltration anesthetics for breast cancer surgery.

Our study also deciphered the molecular mechanisms responsible for the cytotoxicity induced by local anesthetics in thyroid cancer cells, presenting the involvement of MPAK signaling pathways and suggesting potential benefits of the use of local anesthetics in clinical practice.

Future perspective

The importance of cellular immunity in long-term outcome after cancer surgery has been well demonstrated. Animal models and human studies both point to NK cell activity in the perioperative period as being a critical factor in determining outcome after potentially curative surgery. Other components of host immunity play important roles. The possible interaction between factors under the control of the anesthetist, such as anesthetic technique, and cellular immunity is becoming increasingly clear. For instance, there are multiple reports of specific drugs effecting NK cell activity.

The next step must be studies that look not only at the effect of anesthetic technique and other perioperative factors on markers such as NK cell activity, but also at their effects on long-term cancer outcome. Retrospective analyses have already shown a potential benefit on cancer outcome with regional techniques for breast and

prostate cancer surgery, and prospective randomized controlled trials in this area are underway. Other areas for future research would include the possible effect that different opiates may have on cancer outcome. As mentioned previously, tramadol may have unique benefits. COX-2 inhibitors also warrant further investigation, as a single preoperative dose has demonstrated anti-tumor effects in mice. The other factors discussed above, such as perioperative anxiety and postoperative pain, are also areas for future research. Interventions here may improve patient quality of life and survival.

In the meantime, this is a rapidly evolving and exciting area, but not one that is completely new. More than 30 years ago, it was observed that patients who received ether anesthesia had worse survival rates than patients who received halothane anesthesia for their primary breast cancer surgery [126]. This was attributed to the effect of the anesthetic on the 'pituitary–adrenal system' and the 'role of immunity in tumor cell implantation and growth of metastases'.

Although a number of fundamental questions concerning the basic nature of carcinoma metastasis remain incompletely understood, recent research has succeeded in implicating specific molecules in the regulation of discrete cell-biological aspects of the invasion-metastasis cascade. Moreover, the roles played by stromal cells during each step of the metastatic process are rapidly beginning to be appreciated. In many instances, this work has revealed unanticipated complexities and forced revision of established conceptual frameworks.

Looking ahead, we envision that technological advances will continue to revolutionize cancer biology and the study of metastasis. In light of the dire clinical realities associated with metastatic disease, we cannot overstate the importance of ensuring that this impressive pace of discovery continues and, additionally, is accompanied by the rapid translation of these basic research findings to the oncology clinic.

Surgical excision is the mainstay of treatment for potentially curable solid tumors. It is increasingly recognized that anesthetic technique and other perioperative factors have the potential to effect long-term outcome after cancer surgery. Surgery can inhibit important host defenses and promote the development of metastases. There is particular interest at present in the effect of regional anesthesia, which appears to be beneficial. Retrospective analyses have shown an outcome benefit for paravertebral analgesia for breast cancer surgery and epidural analgesia for prostatectomy. Interestingly, propofol conjugates (propofol-docosahexaenoate and propofol-eicosapentaenoate) have been looked at as treatments for breast cancer, as they have been shown to inhibit cellular adhesion, migration, and apoptosis in breast cancer cells [127].

The study represents a small piece in the cancer puzzle. My preliminary study showed that potential beneficial actions of lidocaine infiltration per se in anticancer effect and suggest that local block method might be an ideal anesthetic model for breast and thyroid cancer operations.

Local anesthesia mastectomy can be safely performed. The most obvious reason is to employ it during the critical perioperative period to eradicate residual disease or to prevent immunosuppression may yield a better cancer control outcome. Except for oncological benefit to using local anesthesia for cancer surgery, in vitro and animal studies showed that inhaled anesthetic drugs can promote amyloid beta oligomerization and impair memory. Population-based study, using the Taiwan National Health Insurance Research Database, provides statistically sound evidence for the association of dementia with anesthesia and surgery. Patients who undergo anesthesia and surgery may be at increased risk of dementia [124]. It also can result in markedly reduced health care costs without incurring additional morbidity or mortality. As importantly, less nausea and less pain infer better quality of cancer care and overall satisfactions of

patients. Overall satisfaction with all aspects of care in each of the previous surveys seems to be related to the satisfaction with and participation in the initial surgical care. Positive surgical experiences reduce negative perceptions associated with chemotherapy or radiation. The result satisfies the need to control spiraling healthcare costs while providing indirect benefits from compliance of further necessary cancer therapy.

These findings and our study results deserve further investigation and our future woks will focus on:

1. Investigate effects of local anesthesia combined with propofol sedation on breast cancer surgery and to evaluate the cost-effectiveness.
2. Study quality of life after breast surgery
3. Examine inflammatory and metabolic stress response to breast and thyroid cancer surgery by local anesthetic technique.
4. Check the effects of local anesthesia on cytokines profile that may be important in inflammatory response, tumor cell dissemination, deposition, and propagation in the early postoperative period.
5. Survival analysis to prove our hypothesis

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TABLES

Table 1.

Top ten pathways identified by pathway enrichment analysis of differentially expressed genes of 8505C human thyroid cancer cells following treatment with lidocaine (12 mM) for 24 hours. FDR, false discovery rate.

Pathway	P value	FDR
Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling	8.467E-10	5.92707E-07
Apoptosis and survival_FAS signaling cascades	5.344E-08	1.87052E-05
Regulation of lipid metabolism_RXR-dependent regulation of lipid metabolism via PPAR, RAR and VDR	1.674E-07	3.90509E-05
Blood coagulation_Blood coagulation	1.958E-07	0.000129219
Development_Hedgehog signaling	7.555E-07	0.000132204
Development_Role of HDAC and calcium/calmodulin-dependent kinase (CaMK) in control of skeletal myogenesis	1.032E-06	0.000144549
Development_G-CSF-induced myeloid differentiation	1.316E-06	0.00043431
Neurophysiological process_Receptor-mediated axon growth repulsion	2.335E-06	0.00024149
Signal transduction_cAMP signaling	2.415E-06	0.00024149
Immune response_HMGB1/RAGE signaling pathway	2.982E-06	0.000252541

Table 2.

Top ten pathways identified by pathway enrichment analysis of differentially expressed genes of 8505C human thyroid cancer cells following treatment with bupivacaine (4 mM) for 24 hours. FDR, false discovery rate.

Pathway	P value	FDR
Protein folding and maturation_POMC processing	1.843E-10	1.2825E-07
Immune response_IL-17 signaling pathways	2.347E-07	0.000101697
Development_G-CSF-induced myeloid differentiation	3.077E-07	0.000101697
Protein folding and maturation_Bradykinin / Kallidin maturation	7.610E-07	0.000167678
DNA damage_Role of Brca1 and Brca2 in DNA repair	1.150E-06	0.000400323
Immune response_IFN alpha/beta signaling pathway	1.874E-06	0.000434714
Apoptosis and survival_FAS signaling cascades	3.884E-06	0.000675776
Immune response_MIF-mediated glucocorticoid regulation	4.375E-06	0.000722955
Protein folding and maturation_Angiotensin system maturation \ Human version	6.366E-06	0.000841555
Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling	8.117E-06	0.000953231

FIGURES

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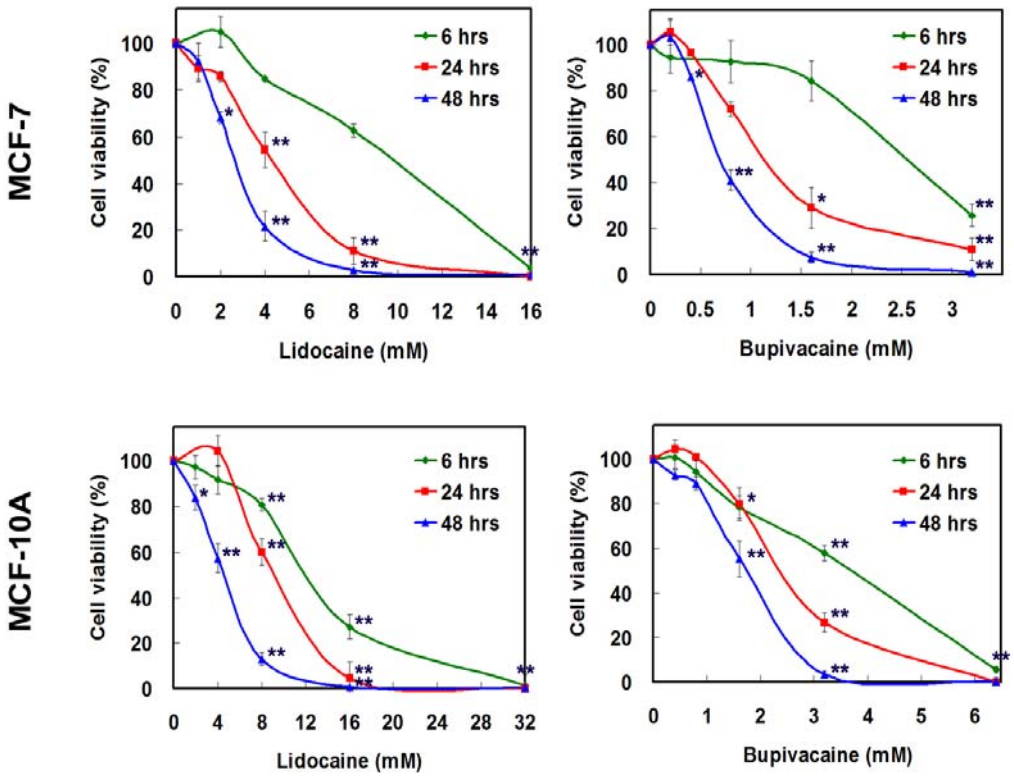


Figure 1. Effects of local anesthetics on the growth of breast tumor cells.

Cell viability was quantified by MTS assay in MCF-7 and MCF-10A cells treated with serial dilutions of lidocaine and bupivacaine for 6, 24, and 48 h. All values are expressed as mean ± standard error of the mean (ANOVA: all P < .001; Dunnett's test: * P < .01, ** *P* < .001 vs. control).

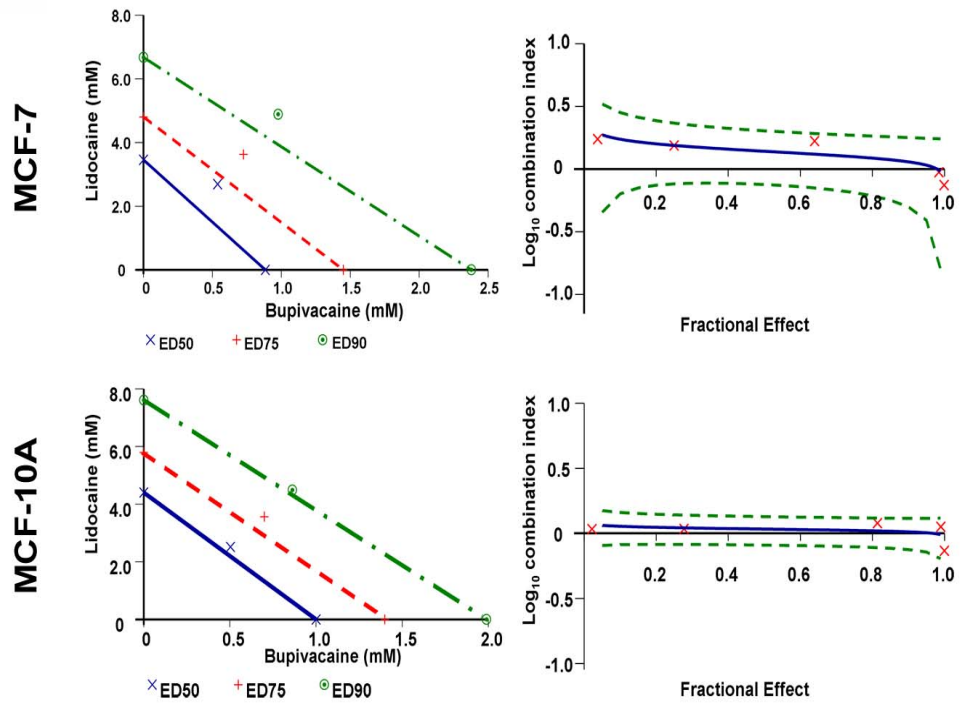


Figure 2. Effect of the combination of lidocaine and bupivacaine on breast tumor cell viability

Isobolograms and combination index plots were constructed using data from MCF-7 and MCF-10A cells treated with lidocaine and bupivacaine, individually or in the indicated combinations, with fixed molar ratios

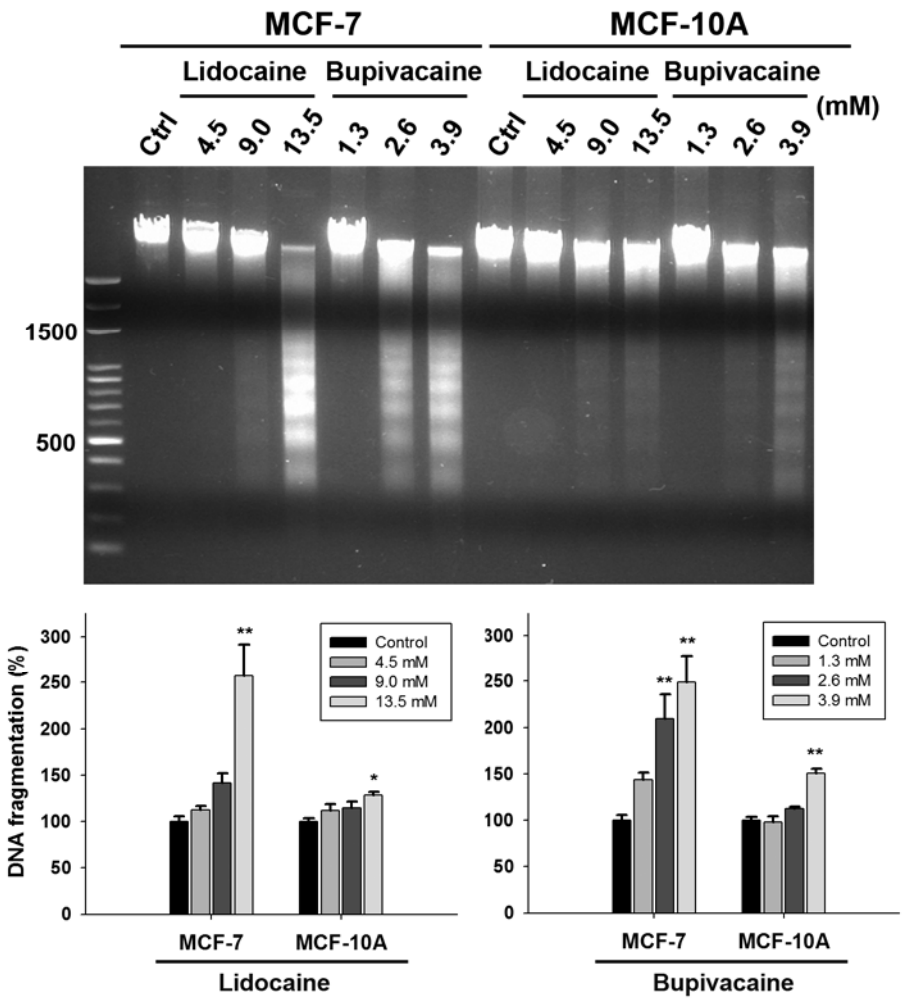


Figure 3. DNA fragmentation induced by local anesthetics in breast tumor cells.

DNA fragmentation was detected in MCF-7 and MCF-10A cells treated with the indicated concentrations of lidocaine and bupivacaine for 24 h. Representative photograph of agarose gel containing electrophoretically separated low-molecular-weight DNA fragments is shown. Banding patterns were densitometrically quantified (ANOVA: $P < .001$ except for $P = .002$ for MCF-10A cells treated with lidocaine; Dunnett's test: * $P < .01$, ** $P < .001$ vs. control).

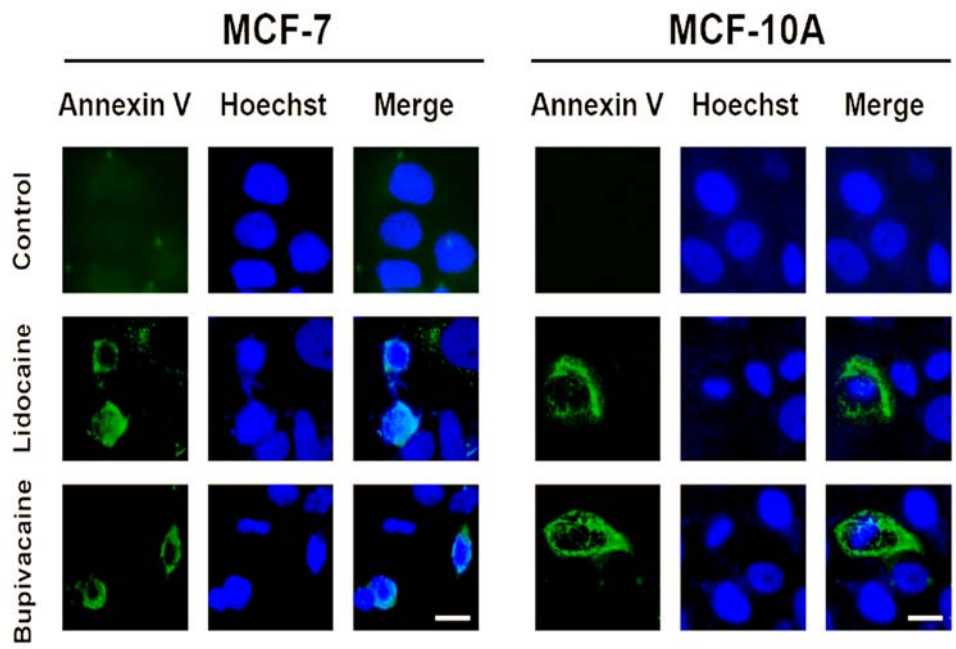


Figure 4. Apoptosis induced by local anesthetics in breast tumor cells.

MCF-7 and MCF-10A cells were treated with lidocaine (7.4 mM) and bupivacaine (2.6 mM) for 4 h. Representative fluorescence microscopy images of immunofluorescence staining of annexin V-FITC and Hoechst 33285 are shown. Scale bar, 25 μm.

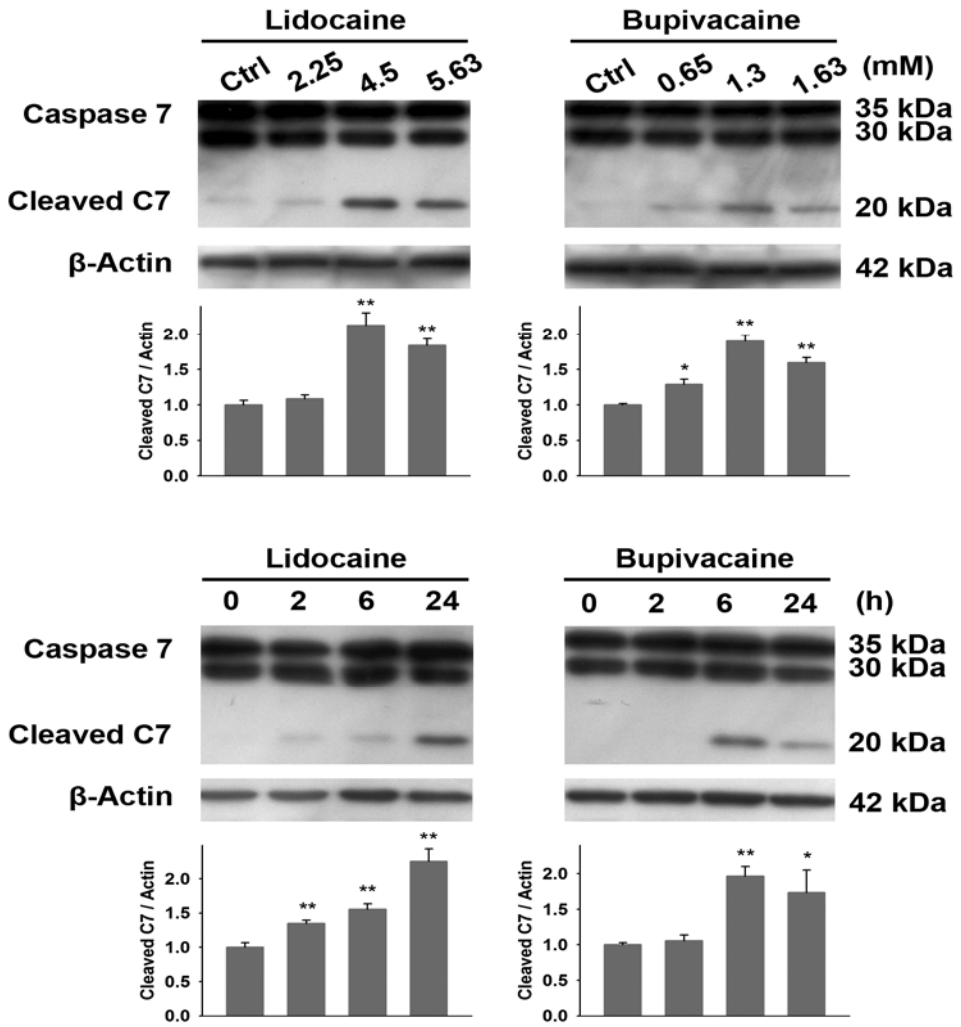


Figure 5. Expression of apoptotic proteins (caspase 7) in breast tumor cells treated with local anesthetics.

MCF-7 cells were treated with varying concentrations of lidocaine and bupivacaine for 24 h or were treated with lidocaine (4.5 mM) and bupivacaine (1.3 mM) for various times. Cells were harvested and samples were prepared for Western blot analysis of caspase 7 as detailed in Methods. The blot signals were quantified by densitometry and normalized to β-actin (ANOVA: all $P < .001$; Dunnett's test: * $P < .01$, ** $P < .001$ vs. control).

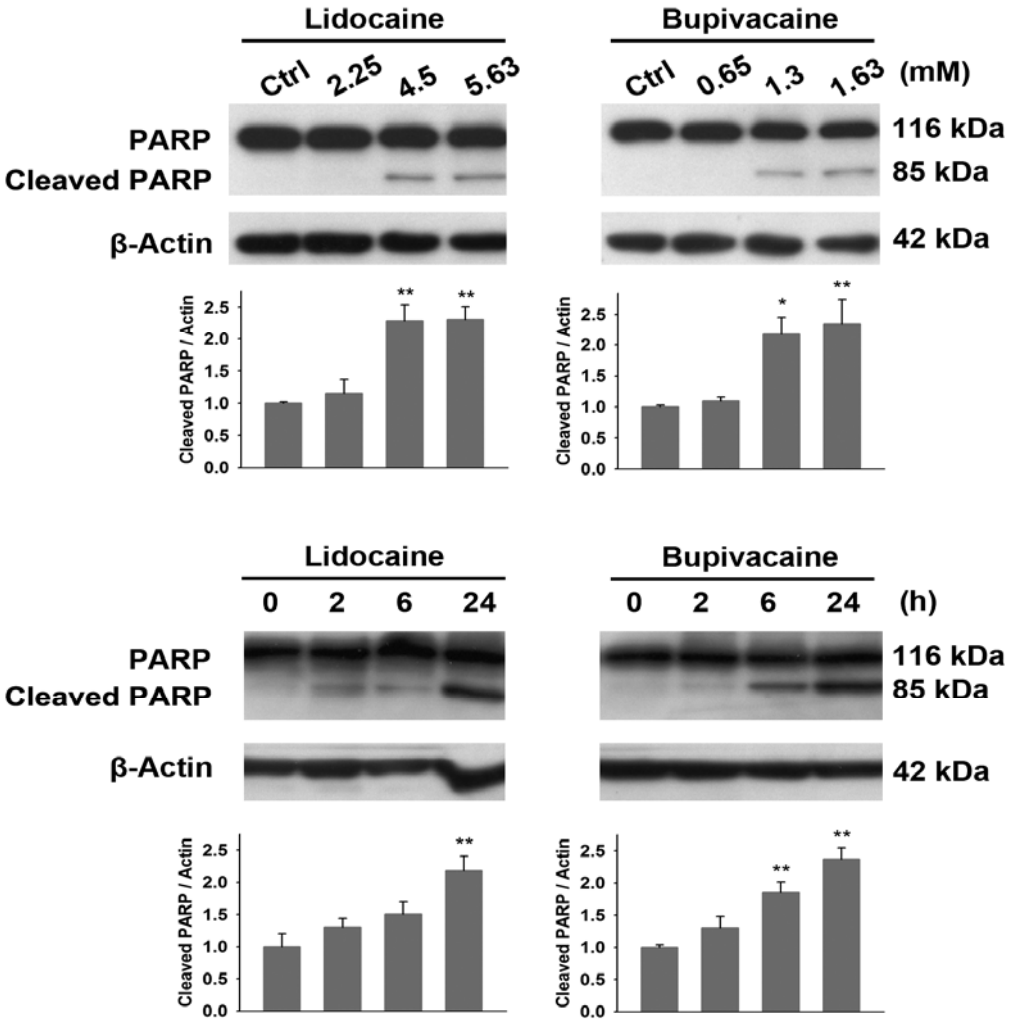


Figure 6. Expression of PARP in breast tumor cells treated with local anesthetics.

MCF-7 cells were treated with varying concentrations of lidocaine and bupivacaine for 24 h or were treated with lidocaine (4.5 mM) and bupivacaine (1.3 mM) for various times. Cells were harvested and samples were prepared for Western blot analysis of PARP cleavage as detailed in Methods. The blot signals were quantified by densitometry and normalized to β-actin (ANOVA: all $P < .001$; Dunnett's test: * $P < .01$, ** $P < .001$ vs. control).

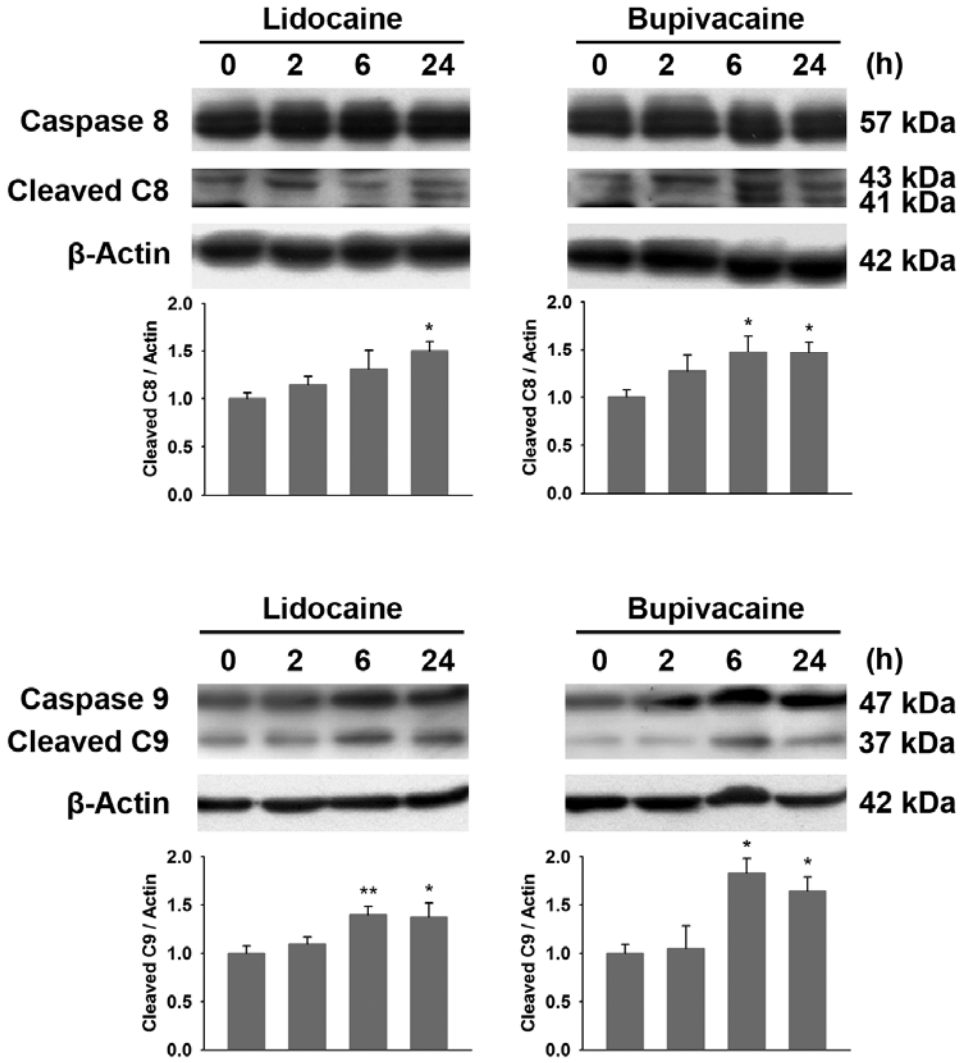


Figure 7. Expression of apoptotic proteins (caspase 8,9) in breast tumor cells treated with local anesthetics.

Treatment of MCF-7 cells with lidocaine (4.5 mM) and bupivacaine (1.3 mM) resulted in a significant increase in the cleavage of caspase 8 and 9 (ANOVA: P = .006 and .009 for caspase 8, both P < .001 for caspase 9; Dunnett's test: * P < .01, ** P < .001 vs. control).

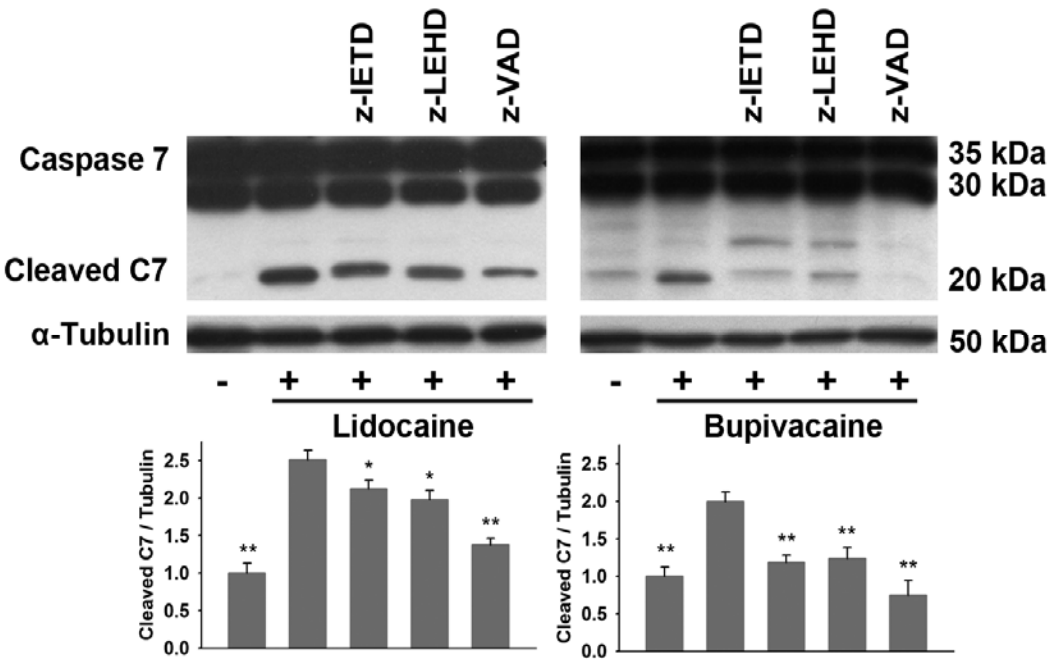


Figure 8. Effects of caspase inhibitors on anesthetic-induced apoptosis in breast tumor cells.

MCF-7 cells were pretreated with caspase 8 inhibitor (z-IETD-fmk, 20 μM), caspase 9 inhibitor (z-LEHD-fmk, 20 μM), or pan-caspase inhibitor (z-VAD-fmk, 20 μM) for 60 min before co-incubation with lidocaine (4.5 mM) or bupivacaine (1.3 mM) for 24 h. Cells were lysed and proteins were immunoblotted with antibodies against caspase 7. Band intensity was quantitatively measured by densitometry reading of the immunoblot (ANOVA: all $P < .001$; Dunnett's test: * $P < .01$, ** $P < .001$ vs. local anesthetic only).

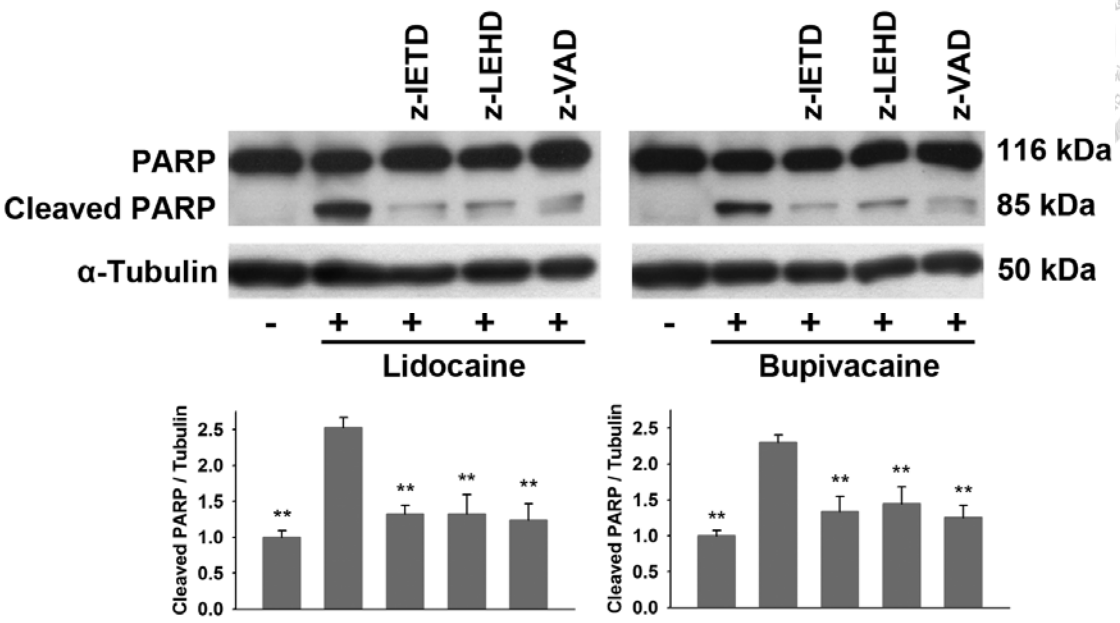


Figure 9. Effects of caspase inhibitor on anesthetic-induced PARP in breast tumor cells.

MCF-7 cells were pretreated with caspase 8 inhibitor (z-IETD-fmk, 20 μM), caspase 9 inhibitor (z-LEHD-fmk, 20 μM), or pan-caspase inhibitor (z-VAD-fmk, 20 μM) for 60 min before co-incubation with lidocaine (4.5 mM) or bupivacaine (1.3 mM) for 24 h. Cells were lysed and proteins were immunoblotted with antibodies against PARP. Band intensity was quantitatively measured by densitometry reading of the immunoblot (ANOVA: all $P < .001$; Dunnett's test: * $P < .01$, ** $P < .001$ vs. local anesthetic only).

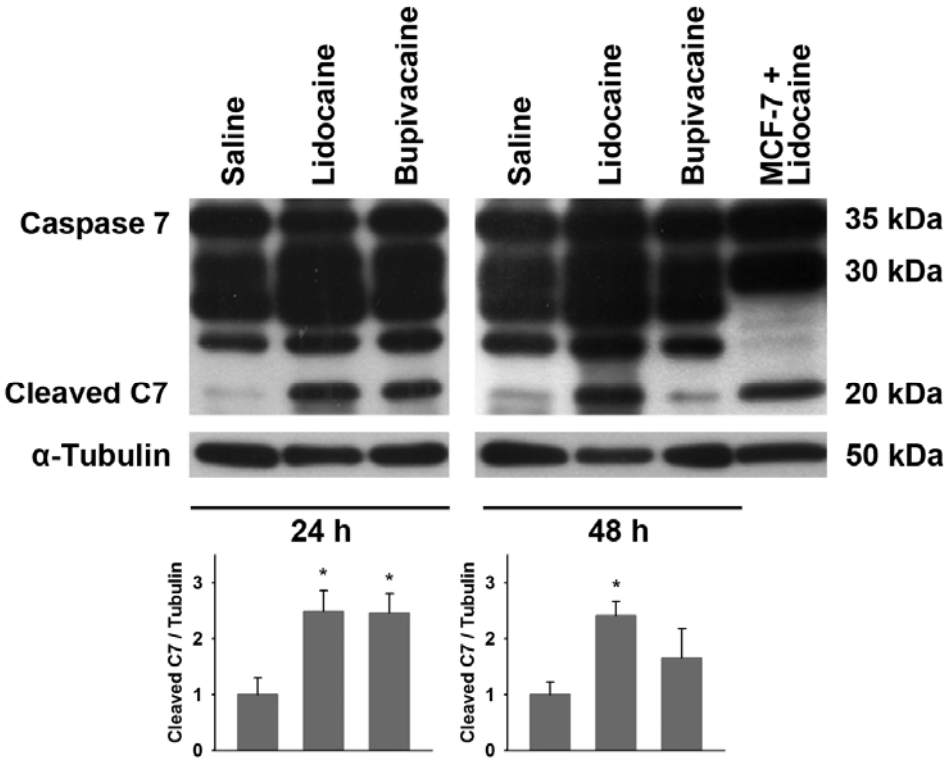


Figure 10. Expression of apoptotic proteins induced by local anesthetics in a xenograft model of breast cancer.

Nude mice were injected sc with 1 x 10^7 MCF-7 cells. After the tumor volume reached 100 mm^3, mice were treated with peritumoral injections of lidocaine (21.3 mM), bupivacaine (4.3 mM), or saline. Mice were sacrificed 24 or 48 h after treatment. Whole cell lysates from xenograft tumors were prepared. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies against caspase 7 and α-tubulin. A sample from MCF-7 cells treated with 4.5 mM lidocaine for 24 h *in vitro* was used as positive control. The blot signals were quantified by densitometry and normalized to α-tubulin (ANOVA: P = .003 for 24 h and P = .008 for 48 h; Dunnett's test: * P < .01 vs. saline).

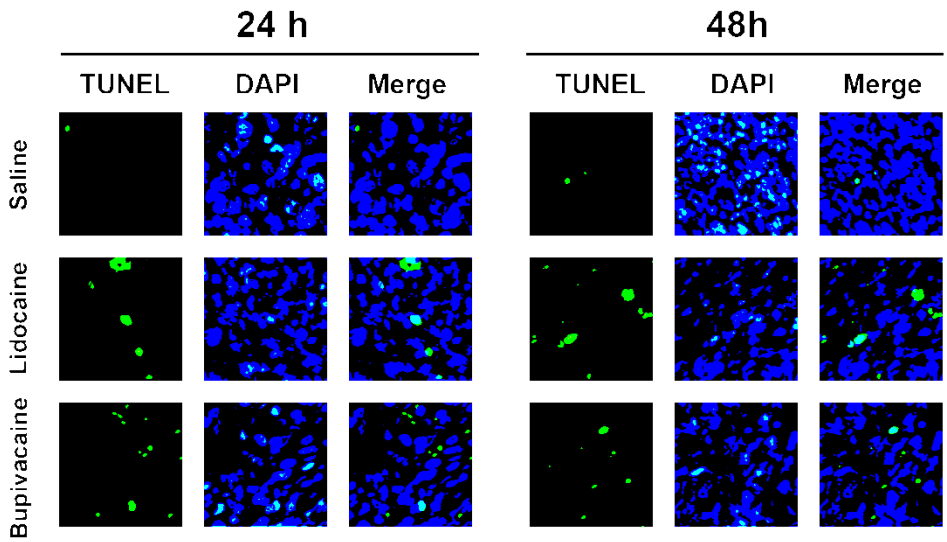


Figure 11. Apoptosis induced by local anesthetics in a xenograft model of breast cancer.

Nude mice were injected sc with 1 x 10^7 MCF-7 cells. After the tumor volume reached 100 mm^3, mice were treated with peritumoral injections of lidocaine (21.3 mM), bupivacaine (4.3 mM), or saline. Mice were sacrificed 24 or 48 h after treatment. Tumor tissues were fixed and the TUNEL staining was performed as described in Methods. Representative images of TUNEL staining 24 and 48 hours after local anesthetic infiltration are shown.

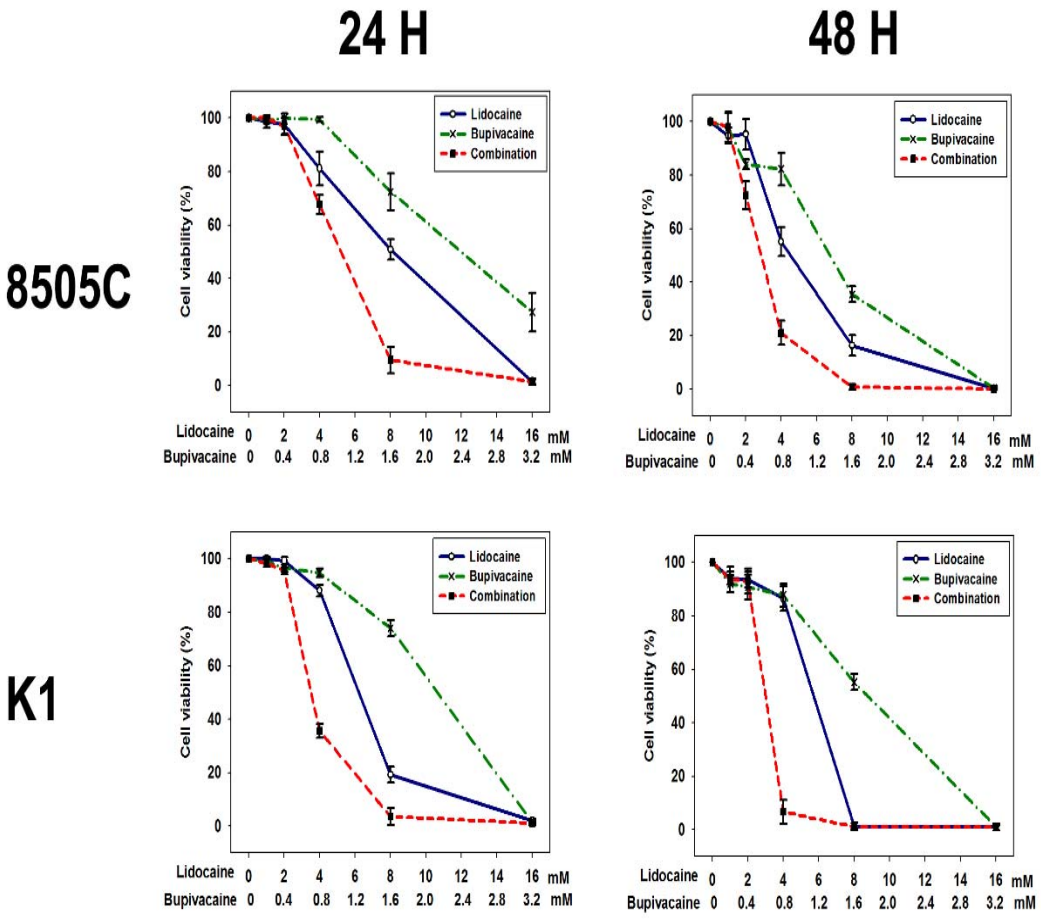


Figure 12. Effects of local anesthetics on cell growth of human thyroid cancer cells.

8505C and K1 cells were treated with serial dilutions of lidocaine and bupivacaine, individually or in combinations, for 24 and 48 h. Error bars represent standard error of the mean.

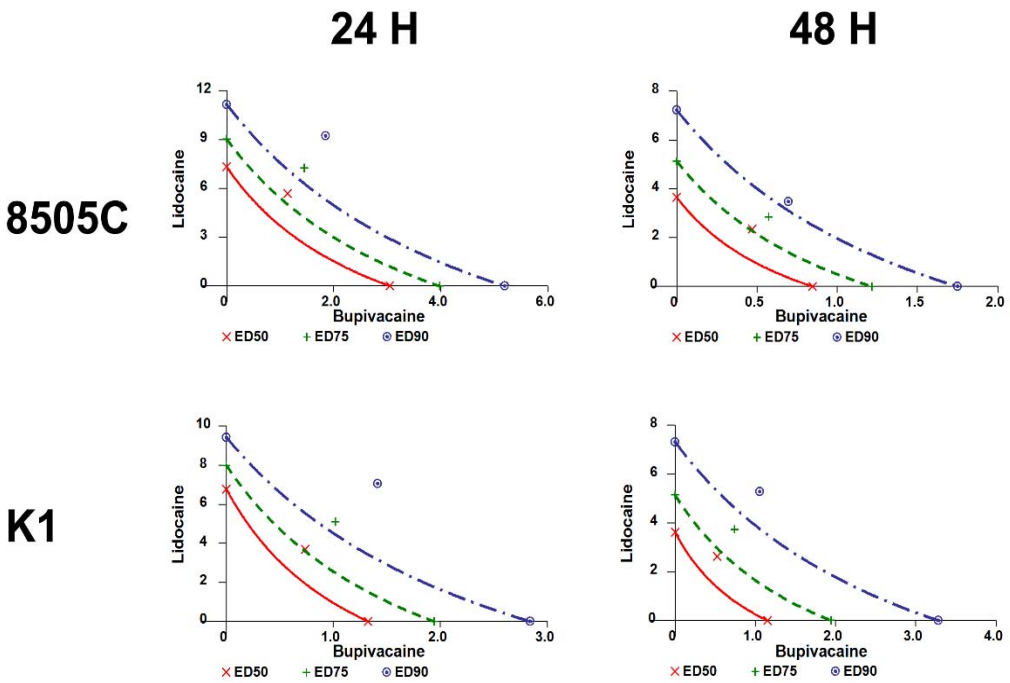


Figure13. Effect of the combination of lidocaine and bupivacaine on thyroid tumor cell viability

A conservative isobologram demonstrates that lidocaine and bupivacaine acts antagonistically to inhibit the cell growth of thyroid cancer cells. ED indicates effective dose. The ED50 (red X), ED75 (green crosses), and ED90 (blue circles) are graphed.

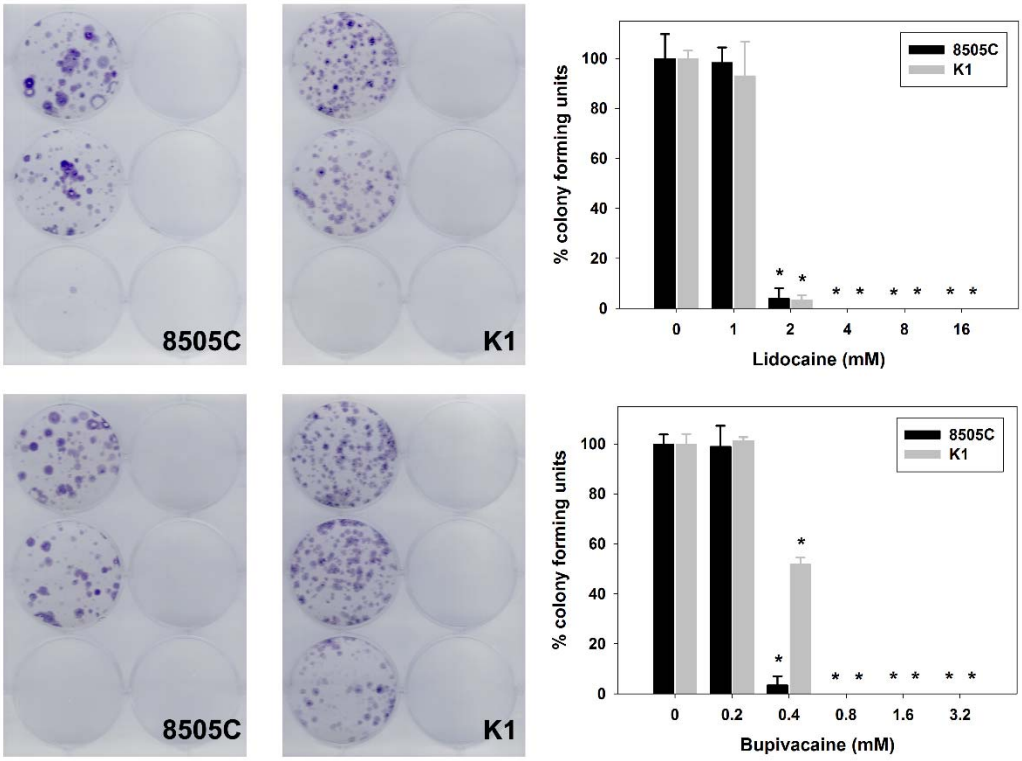


Figure 14. Effects of local anesthetics on colony formation of human thyroid cancer cells.

Treatment with lidocaine and bupivacaine resulted in reduction of colony formation of the 8505C and K1 cell lines. Error bars represent standard error of the mean. *, $P < 0.01$ versus control.

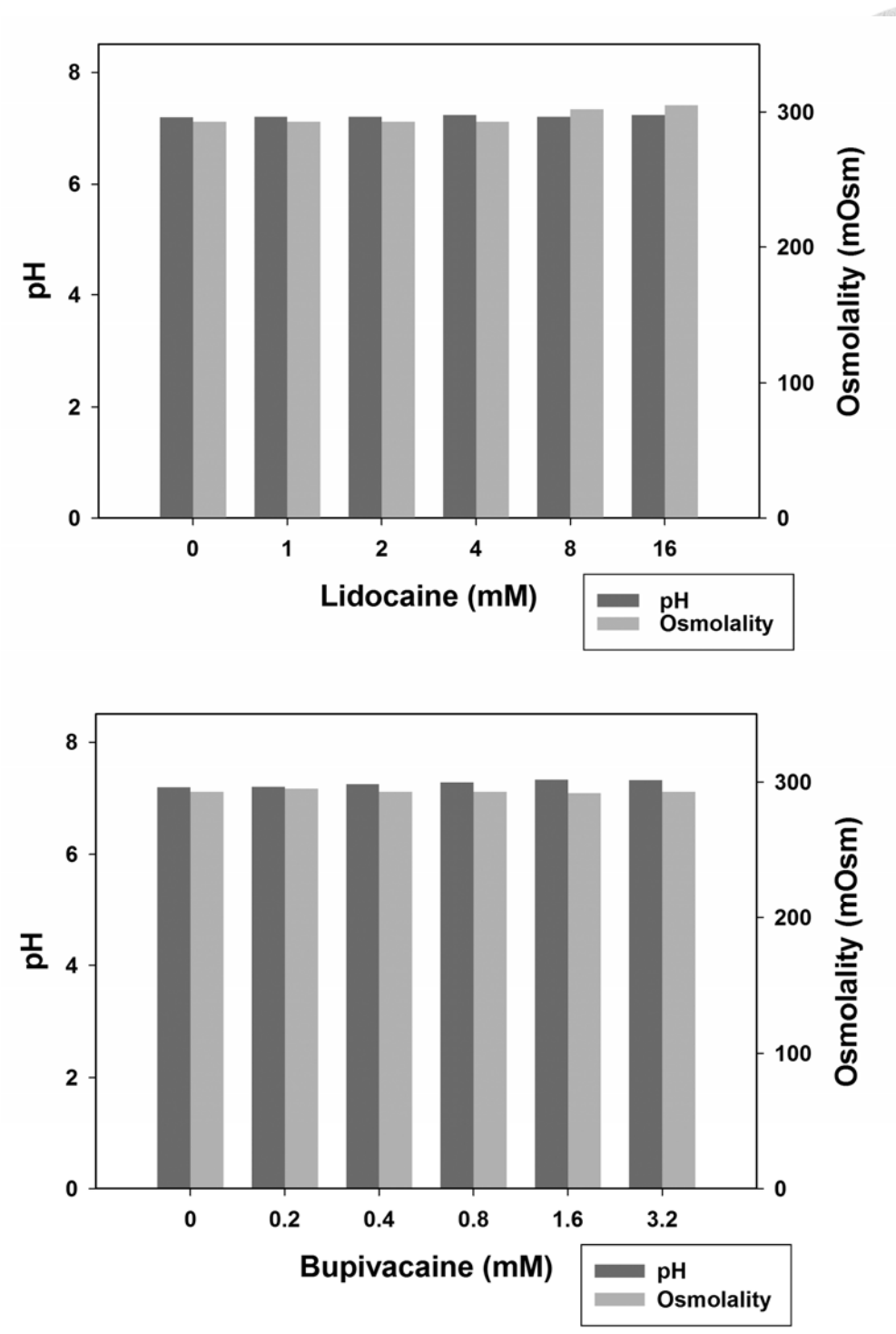


Figure 15. Cytotoxic effects of changes in pH or osmolality of the culture media for experiments.

Final pH and osmolality of the culture media for experiments.

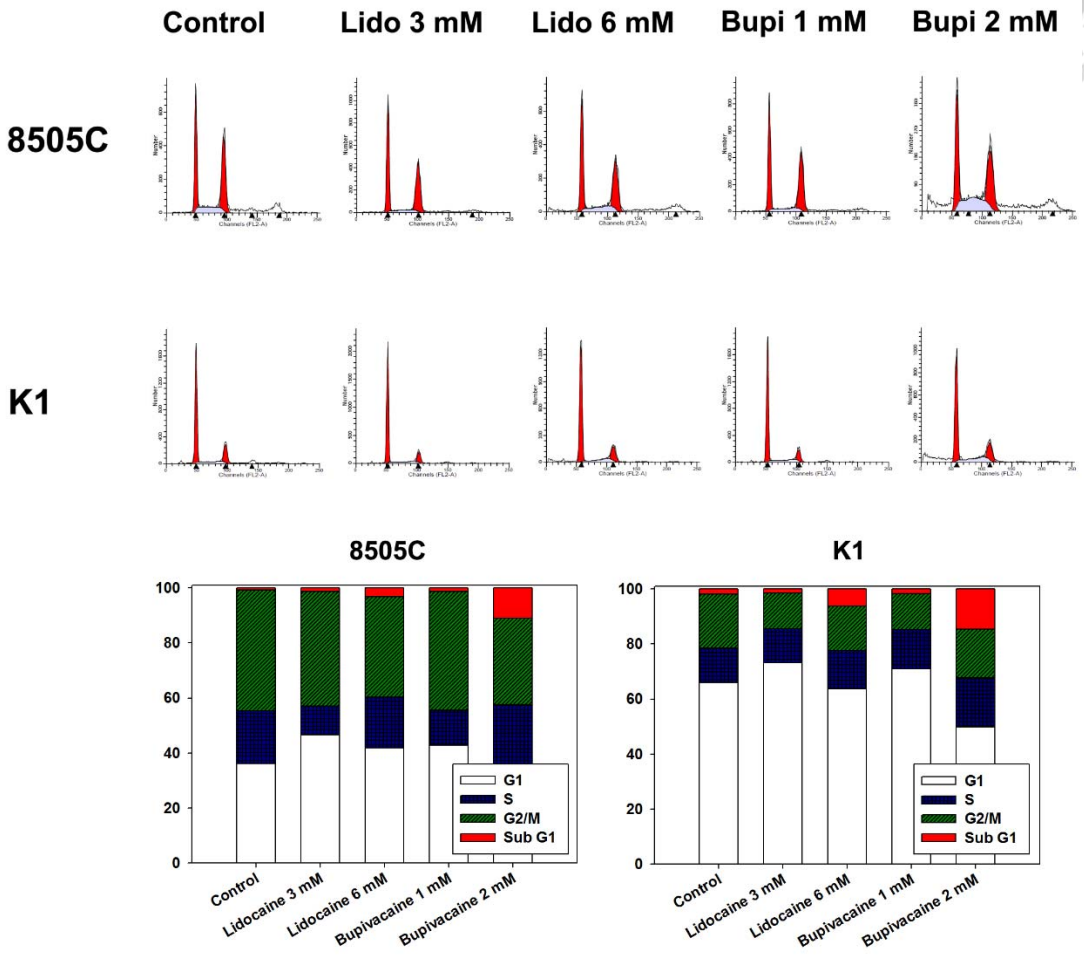


Figure 16. Effects of local anesthetics on cell cycle in human thyroid cancer cells

8505C and K1 cells were treated with the indicated concentrations of lidocaine and bupivacaine for 24 h. Thereafter, the cells were washed, fixed, and stained with propidium iodide (PI) and were analyzed for DNA content in different phases of the cell cycle by flow cytometry.

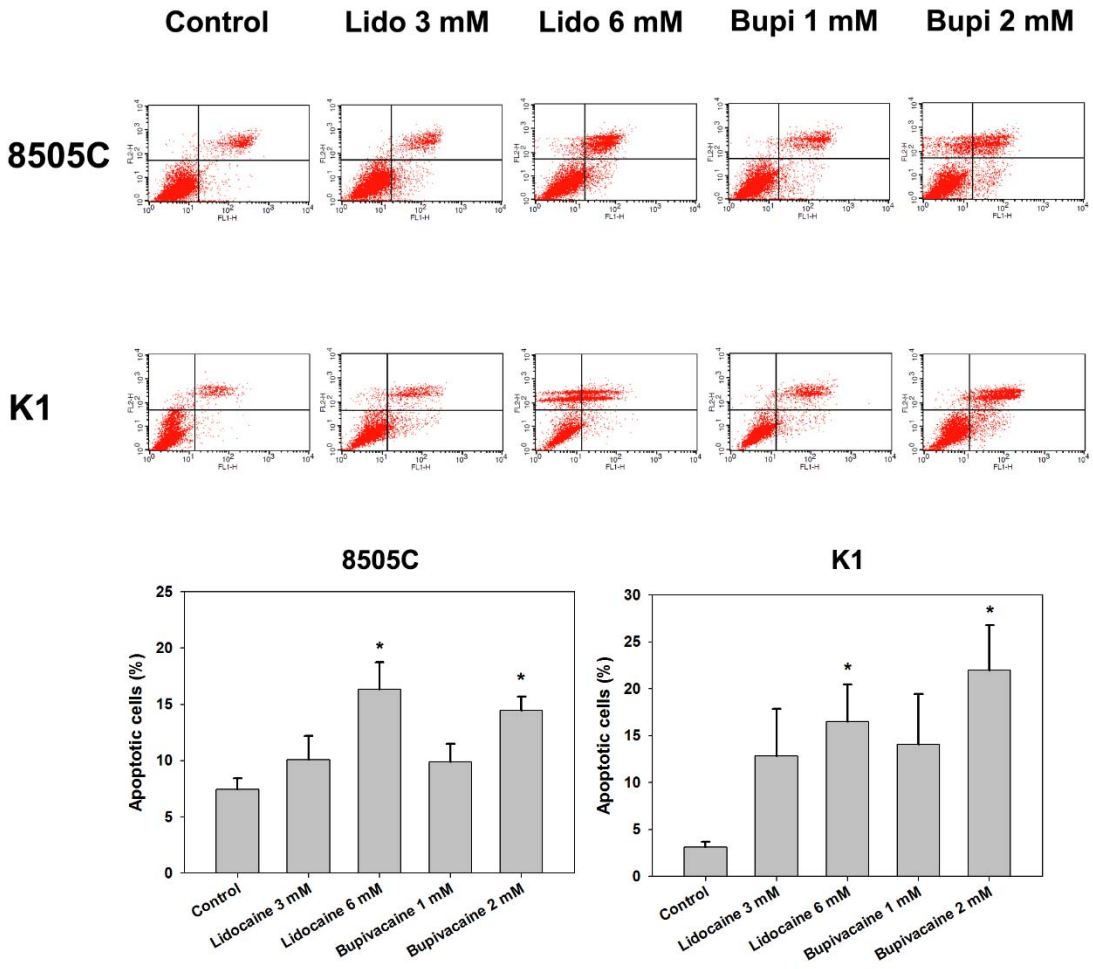


Figure 17. Effects of local anesthetics on cell apoptosis in human thyroid cancer cells

Thyroid cancer cells were treated with lidocaine and bupivacaine for 48 h, and cells were subsequently stained with fluorescein-conjugated annexin V and PI and analyzed by flow cytometry. Error bars represent standard error of the mean. *, $P < 0.05$ *versus* control.

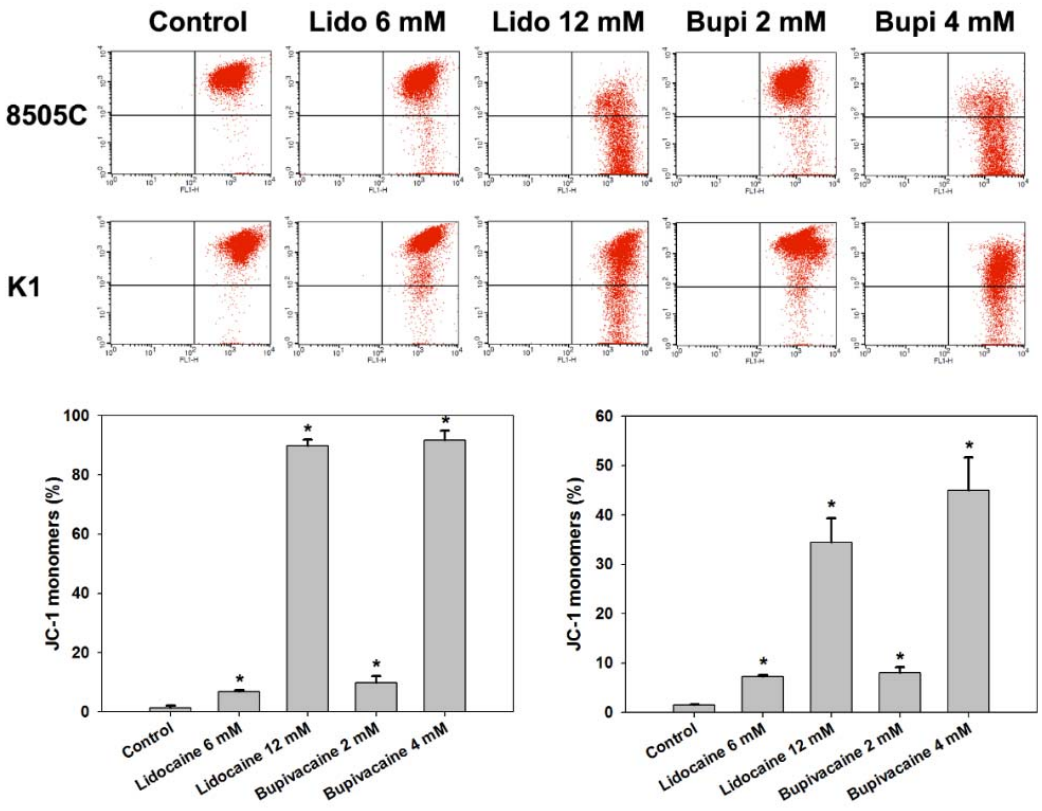


Figure 18. Change in mitochondrial membrane potential (ΔΨm) induced by local anesthetics in human thyroid cancer cells.

8505C and K1 cells were treated with the indicated concentrations of lidocaine and bupivacaine for 16 h. $\Delta\Psi_m$ change was monitored by loading with JC-1 and was analyzed by flow cytometry. Error bars represent standard error of the mean. *, $P < 0.05$ *versus* control.

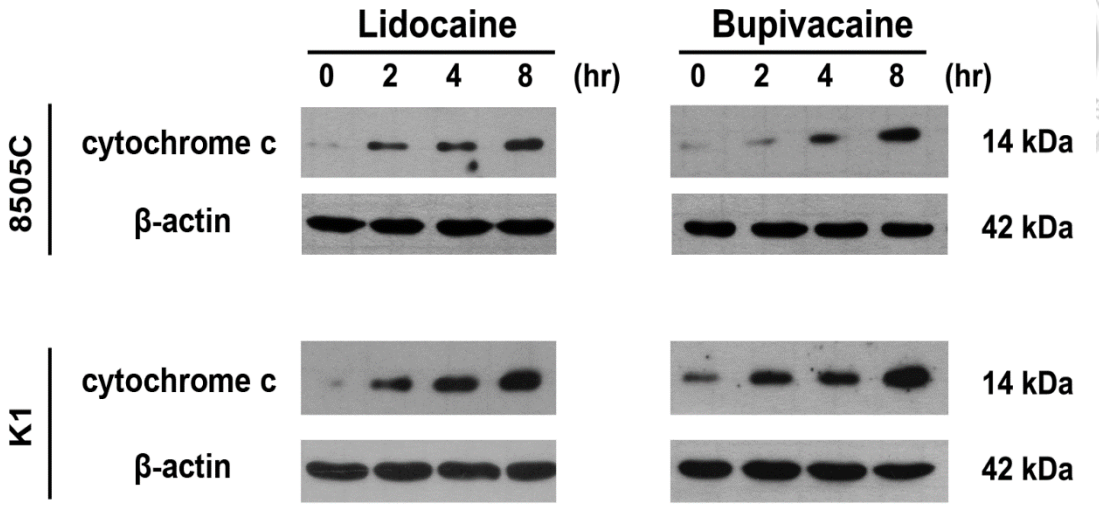


Figure 19. Cytochrome c release induced by local anesthetics in human thyroid cancer cells.

Thyroid cancer cells were treated with lidocaine (6 mM) and bupivacaine (2 mM) for the indicated periods of time. Cytochrome c release from mitochondria to cytosol was determined by Western blotting. The blots were stripped and reprobed with an antibody against actin for equal loading.

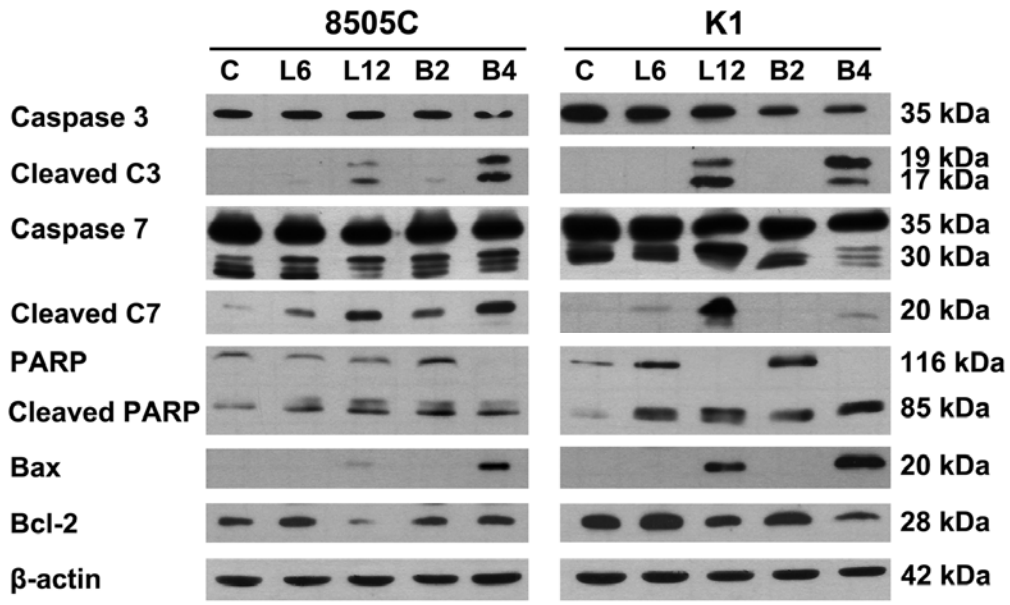


Figure 20. Expression of apoptotic proteins in human thyroid cancer cells treated with local anesthetics.

8505C and K1 cells were treated with the indicated concentrations of lidocaine (L6, 6 mM; L12, 12 mM) and bupivacaine (B2, 2 mM; B4, 4 mM) for 16 h. Cells were harvested and samples were prepared for Western blot analysis. C, control. PARP, poly(ADP-ribose) polymerase.

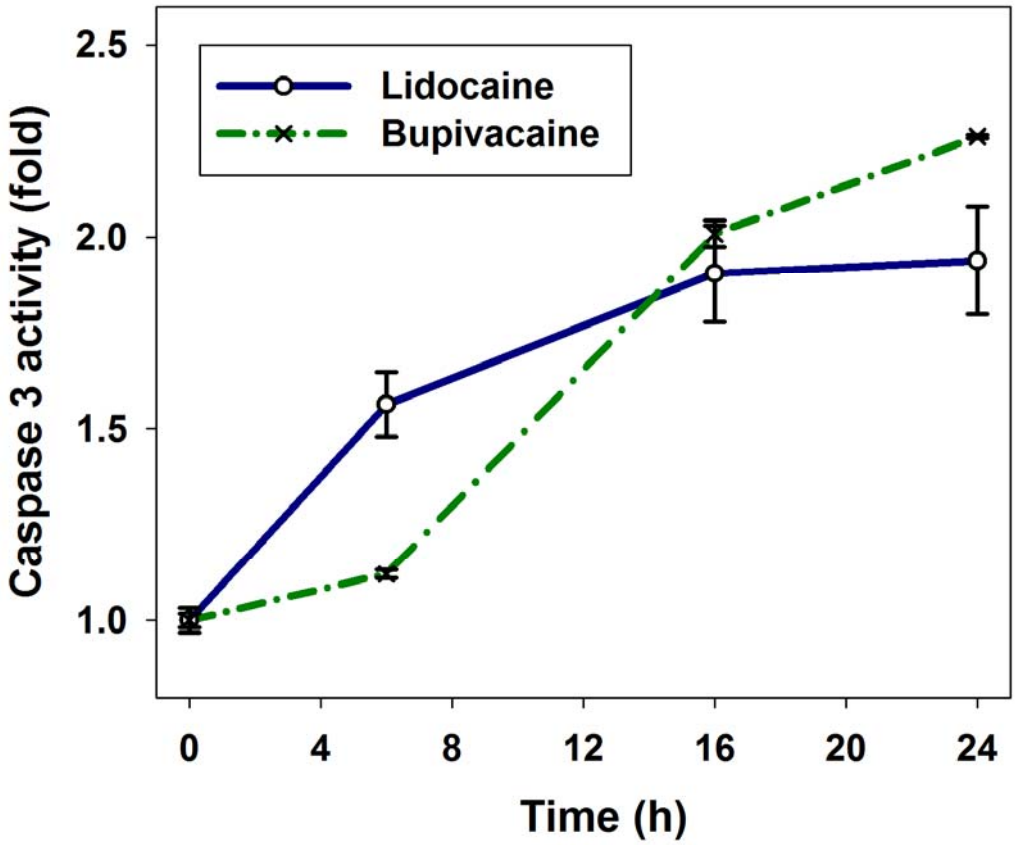


Figure 21. Caspase 3 activities in human thyroid cancer cells treated with local anesthetics.

Activity of caspase 3 in cell lysates from 8505C cells treated with lidocaine (12 mM) and bupivacaine (4 mM) for different time periods was determined using a colorimetric protease assay. Error bars represent standard error of the mean.

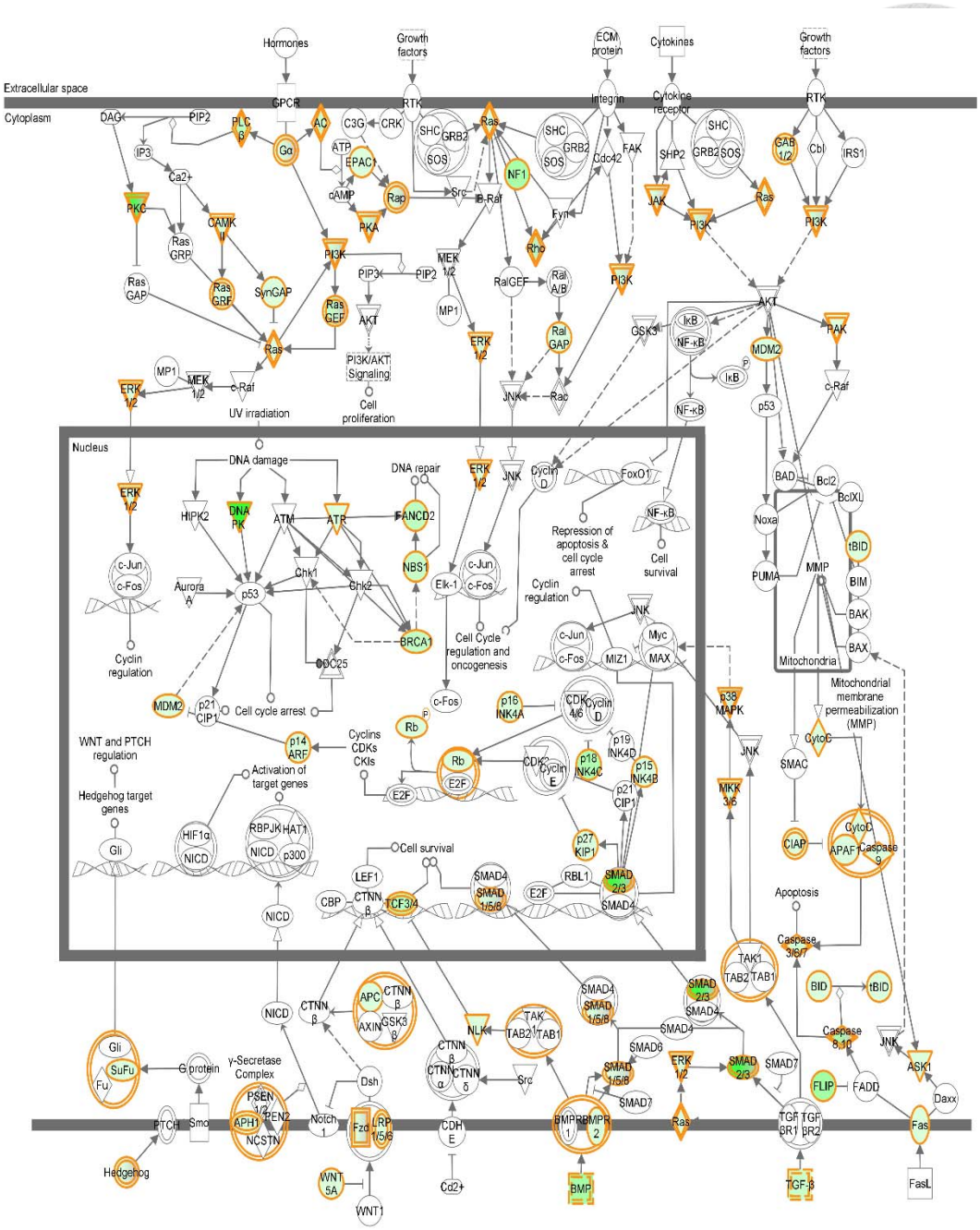


Figure 22. Molecular mechanism of cancer from Ingenuity Pathways Analysis.

In silico tools was used from Ingenuity to identify pathways reportedly involving molecular mechanism of cancer.

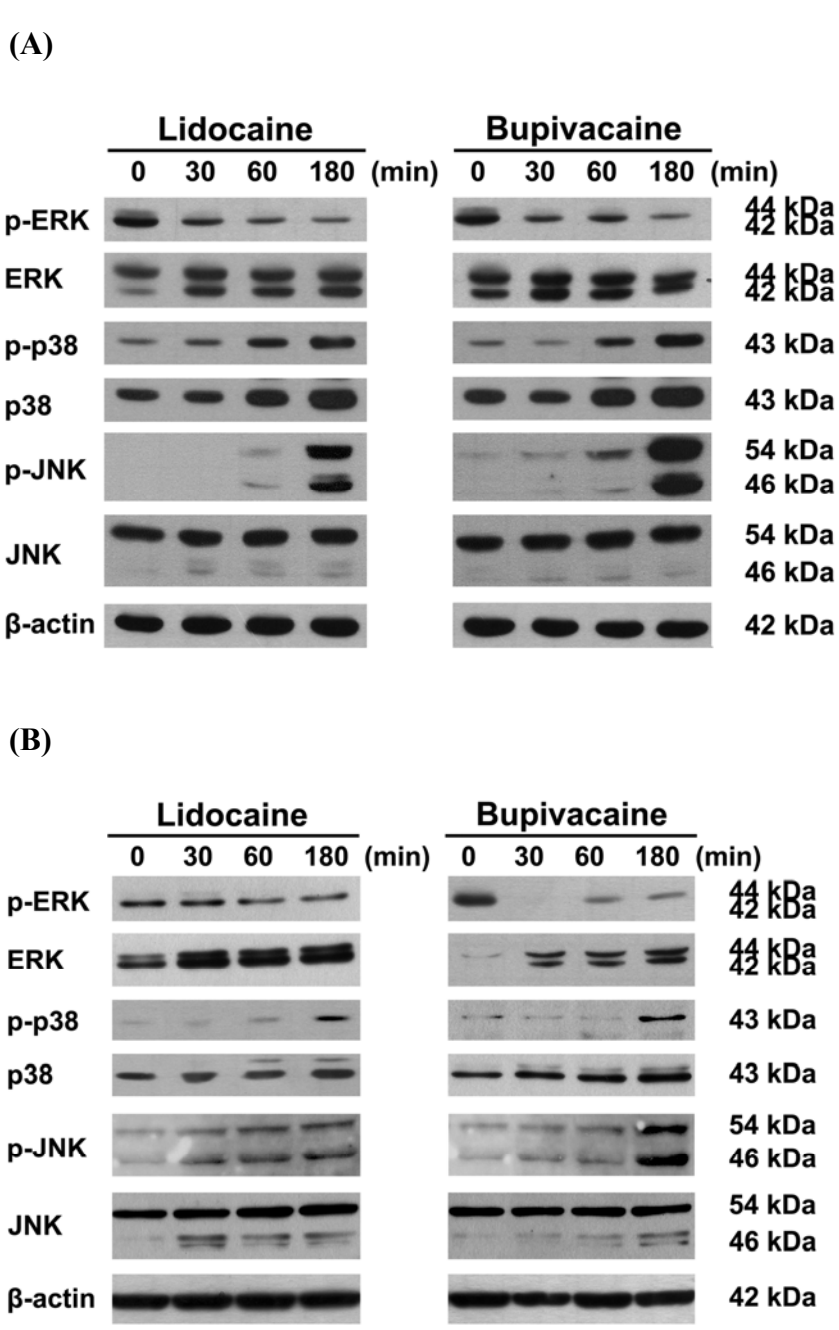
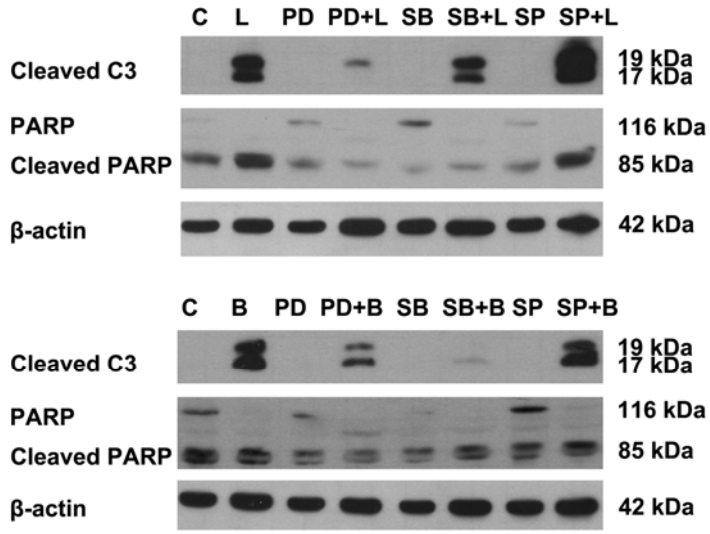


Figure 23. Mitogen-activated protein kinase signaling in human thyroid cancer cells treated with local anesthetics.

8505C **(A)** and K1 **(B)** cells were treated with lidocaine (12 mM) and bupivacaine (4 mM) for various periods of time, and activities of ERK, p38, and JNK were examined by Western blot analysis using phospho-specific antibodies. The total protein levels of ERK, p38, and JNK were also measured.

(A)



(B)

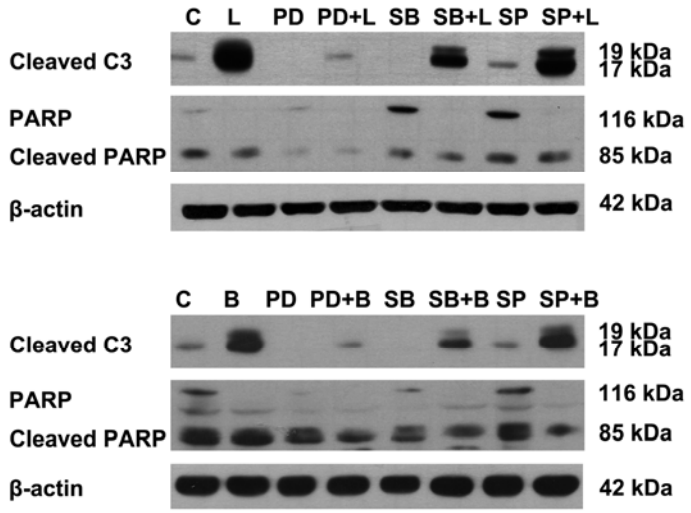


Figure 24. Effects of inhibition of mitogen-activated protein kinase signaling on apoptosis in human thyroid cancer cells treated with local anesthetics.

8505C cells **(A)** and K1 cells **(B)** were cotreated with 30 μM PD98059 (PD), 30 μM SB203580 (SB), or 30 μM SP600125 (SP) in addition to 12 mM lidocaine (L) or 4 mM bupivacaine (B) for 16 h. Cells were harvested and samples were prepared for Western blot analysis. C, control. C3, caspase 3. PARP, poly (ADP-ribose) polymerase.

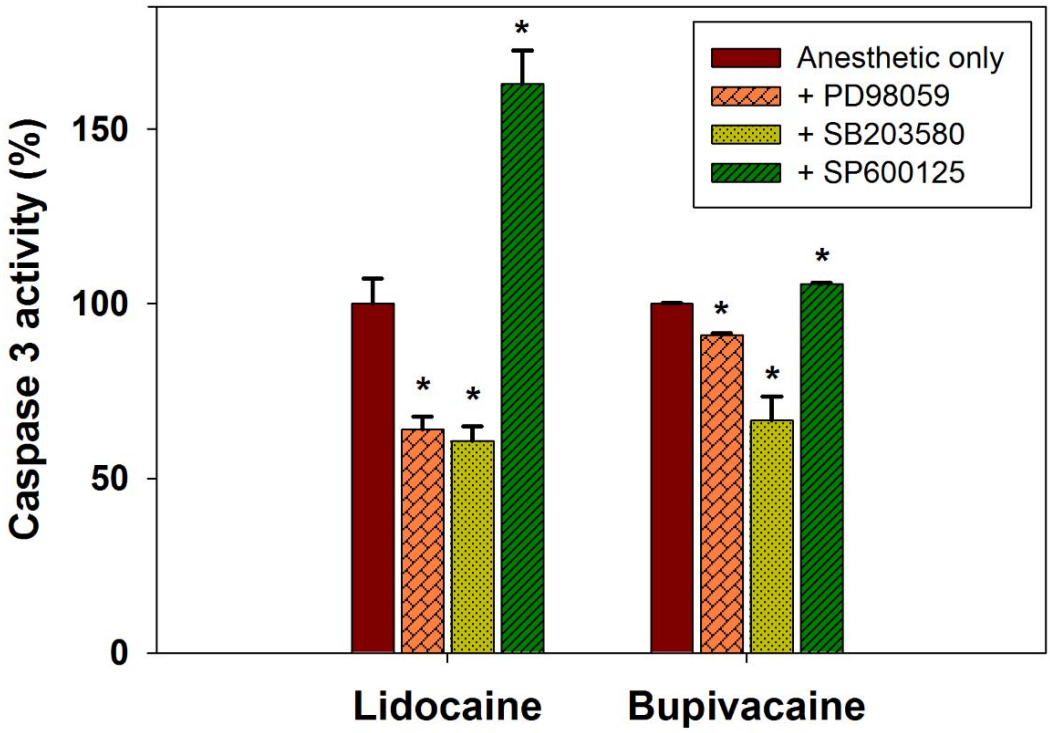


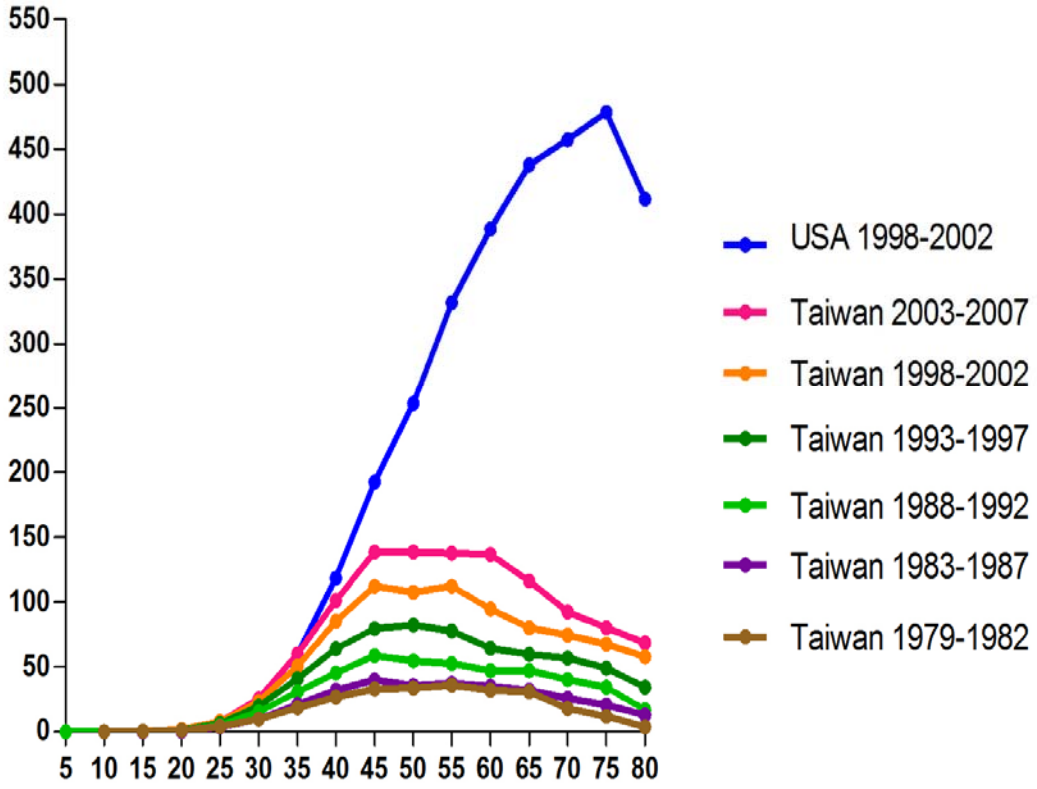
Figure 25. Effects of inhibition of mitogen-activated protein kinase signaling on activity of caspase 3 in human thyroid cancer cells treated with local anesthetics.

Activity of caspase 3 in cell lysates from 8505C cells treated with lidocaine (12 mM) and bupivacaine (4 mM) with or without specific inhibitors for 24 h was determined using a colorimetric protease assay. Error bars represent standard error of the mean. *, $P < 0.01$ *versus* control.

Supporting information

Supplemental Figure S1.

Incidence of invasive breast cancer in Taiwan



APPENDIX

Part of the results of this thesis has been published as follows:

1. **Yuan-Ching Chang,** Yi-Chiung Hsu, Chien-Liang Liu, Shih-Yuan Huang, Meng-Chun Hu, Shih-Ping Cheng Local Anesthetics Induce Apoptosis in Human Thyroid Cancer Cells through the Mitogen-Activated Protein Kinase Pathway. PLOS ONE (2014) DOI: 10.1371/journal.pone.0089563.
2. **Yuan-Ching Chang,** Chien-Liang Liu, Ming-Jen Chen, Yung-Wei Hsu, Shan-Na Chen, Chi-Hsin Lin, Chin-Man Chen, Feng-Ming Yang, Meng-Chun Hu, .Local Anesthetics Induce Apoptosis in Human Breast Tumor Cells Anesthesia and Analgesia. (2014) 118 (1): 116-24.

Other representative works

1. CS Chiang, SH Hu, B-J Liao, **Yuan-Ching Chang**, SY Chen Enhancement of cancer therapy efficacy by trastuzumab-conjugated and pH-sensitive nanocapsules with the simultaneous encapsulation of hydrophilic and hydrophobic compounds. Nanomedicine: Nanotechnology, Biology and Medicine. (2014) 118:116–24.
2. Chiachen Chen, **Yuan-Ching Chang**, Michael S. Lan, Mary Breslin Leptin stimulates ovarian cancer cell growth and inhibits apoptosis by increasing cyclin D1 and Mcl-1 expression via the activation of the MEK/ERK1/2 and PI3K/Akt signaling pathways. Int J of Oncol (2013) 42: 1113-1119.
3. Shih-Ping Cheng, Chien-Liang Liu, Yi-Chiung Hsu, **Yuan-Ching Chang**, Shih-Yuan Huang, Jie-Jen Lee, Expression and Biologic Significance of Adiponectin Receptors in Papillary Thyroid Carcinoma. Cell Biochem Biophys (2013) 65:203–210.

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5. Cheng SP, Yin PH, **Yuan-Ching Chang**, Lee CH, Huang SY, Chi CW. Differential roles of leptin in regulating cell migration in thyroid cancer cells. Oncology Reports (2010) 23:1721-7.
6. Shih-Ping Cheng, Gerard M. Doherty, **Yuan-Ching Chang** , Chien-Liang Liu,Leptin: The link between overweight and primary hyperparathyroidism? Medical Hypotheses (2010) 76: 94–96.
7. Chung-Hsin Tsai, Yung-Wei Hsu, Shih-Ping Cheng, ***Yuan-Ching Chang** et. al., Local anesthesia to improved postoperative outcome for breast cancer surgery: preliminary results. J Chinese Oncol. Soc. (2008) 24 (1): 3-9. *corresponding author
8. Hsiu-Chen Lin, Shin-Hui Tsai, Chien-Shu Chen, **Yuan-Ching Chang**, Chi-Ming Lee, Zhi-Yang Lai, Chun-Mao Lin Structure–activity relationship of coumarin derivatives on xanthine oxidase-inhibiting and free radical-scavenging activities. Biochemical Pharmacology (2008) 75: 1416–1425.
9. **Yuan-Ching Chang**, Fu-Wei Lee, Chien-Shu Chen, et. Al., Structure-activity relationship of C6-C3 phenylpropanoids on xanthine oxidase-inhibiting and free radical-scavenging activities. Free Radical Biology & Medicine (2007) 43: 1541–1551.