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Lidocaine impairs proliferative and biosynthetic functions of aged human dermal fibroblasts

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Abstract

Background—The aged are at increased risk of postoperative wound healing complications. Since local anesthetics are commonly infiltrated into the dermis of surgical wounds, we sought to determine if local anesthetics adversely affect proliferative and biosynthetic functions of dermal fibroblasts. We also evaluated the effect of local anesthetics on IGF-1 and TGF- β 1, growth factors that are important regulators of wound healing.

Methods—Human dermal fibroblasts from aged and young donors (HFB) were exposed to local anesthetic agents at clinically relevant concentrations. We screened the effects of lidocaine, bupivacaine, mepivacaine and ropivacaine on proliferation of HFB. Lidocaine was the most detrimental to proliferation in HFB. We then evaluated the effect of lidocaine on expression and function of the growth factors, IGF-1 and TGF- β 1. Lastly, concurrent exposure to lidocaine and IGF-1 or TGF- β 1 were evaluated for their effects on proliferation and expression of dermal collagens, respectively.

Results—Lidocaine and mepivacaine inhibited proliferation in aged HFB (for lidocaine 88% of control, 95% CI 80%-98%, $p=0.009$ and for mepivacaine 90% of control, 95% CI 81%-99%,

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p=0.032), but not in young HFB. Ropivacaine and bupivacaine did not inhibit proliferation. Due to the clinical utility of lidocaine relative to mepivacaine, we focused on lidocaine. Lidocaine decreased proliferation in aged HFB, which was abrogated by IGF-1. Lidocaine inhibited transcripts for IGF-1 and IGF1R in fibroblasts from aged donors (IGF-1, log2 fold-change -1.25 {42% of control, 95%CI 19%-92%, p=0.035} and IGF1R, log2 fold-change of -1.00 {50% of control, 95%CI 31%-81%, p=0.014}). In contrast, lidocaine did not effect the expression of IGF-1 or IGF1R transcripts in the young HFB. Transcripts for collagen III were decreased after lidocaine exposure in aged and young HFB (log2 fold-change -1.28 {41% of control, 95%CI 20%-83%, p=0.022} in aged HFB and log2 fold-change -1.60 {33% of control, 95%CI 15%-73%, p=0.019} in young HFB). Transcripts for collagen I were decreased in aged HFB (log2 fold-change -1.82 {28% of control 95%CI 14%-58%, p=0.006}), but were not in the young HFB. Similar to the transcripts, lidocaine also inhibited the protein expression of collagen III in young and aged HFB (log2 fold-change -1.79 {29% of control, 95%CI 18%-47%, p=0.003} in young HFB and log2 fold-change -1.76 {30% of control, 95%CI 9%-93%, p=0.043} in aged HFB). The effect of lidocaine on expression of collagen III protein was obviated by TGF- β 1 in both young and aged HFB.

Conclusions—Our results show that lidocaine inhibits processes relevant to dermal repair in aged HFB. The detrimental responses to lidocaine are due, in part, to interactions with IGF-1 and TGF- β 1.

Introduction

Disrupted surgical wound healing is common (about 500,000 cases annually in the United States) and leads to poor patient outcomes and excess costs (1). Impaired surgical wound healing is particularly a problem in the older population, who are at increased risk of wound dehiscence and infection (2). Dysregulation of growth factors, which is further compromised during the response to stress, is one of the features of poor healing in older hosts (3). For example, in an aged mouse model, deficits in growth factor expression contributed to delayed cutaneous wound healing (4). Although there are many cell types in the skin, fibroblasts are the primary cell responsible for both proliferation and biosynthesis of extracellular matrix (ECM) in the dermis. Dermal fibroblasts are commonly used to elucidate many of the mechanisms of impaired wound healing in aging. These cells are an accepted model for studies of dermal wound healing (5) and, specifically, of age-related changes in cutaneous wound healing (6). Cellular replication and deposition of ECM are often reduced in aged dermal fibroblasts when studied in vivo and in vitro (7). Punch biopsies obtained repeatedly over the life span of hamsters found that in vitro proliferative capacity of dermal fibroblasts mimicked in vivo dermal wound repair (8).

Proliferation and biosynthesis in the dermis are modulated by the expression and activity of growth factors that are altered with aging, such as Insulin like Growth Factor-1 (IGF-1) and Transforming Growth factor β 1 (TGF- β 1) (9). IGF-1 is a mitogenic factor for cells and promotes the interaction of dermal fibroblasts with the extracellular matrix during tissue injury and repair (10,11). Synthesis of IGF-1 (12), and the expression of its cognate receptor, IGF1R, by fibroblasts is an essential determinant of proliferation in the dermis (13). Decreased proliferative capacity of aged dermal fibroblasts is associated with reduced IGF-1

and IGF1R (Insulin like Growth Factor-1 Receptor) expression and activation (14). The fibrogenic growth factor TGF- β 1, is highly expressed in connective tissue, but its expression decreases with aging (15). A lack of TGF- β 1 results in less deposition of extracellular matrix proteins, such as the interstitial collagens III and I that comprise the majority of the dermal ECM.

Treatment of cutaneous pain by local anesthetic agents is used by many medical specialties. There is a wide spectrum of treatment regimens for local anesthetics; from short procedures using local infiltration to treatment of post-operative pain or complex pain syndromes in which skin patches and subcutaneous infusions are used for days (16,17). Although prolonged use of local anesthetics (lidocaine) dermal patches is indicated only on intact skin and the use of local anesthetic agents is considered relatively safe, in vitro and in vivo studies have suggested a potential detrimental effect on any wounds that are undergoing healing (18). Prior studies have shown that local anesthetic agents are toxic to neural and muscle tissues in a concentration and agent specific manner (19,20). It has been suggested that local anesthetics have an adverse effect on wound repair by delaying the synthesis of collagens (21) and/or by an anti-proliferative effect on mesenchymal cells (22).

In this study, we used primary dermal fibroblasts obtained from aged and young human donors to test the hypothesis that local anesthetics affect proliferative and biosynthetic functions that are important for cutaneous wound healing.

Methods

Cell lines

Based on our prior data (14) with respect to expected effect size and standard deviation of the outcome, using a power of 0.8 (β) and significance set at 0.05 (α , two-tailed), we needed 3-4 samples each of young and aged human dermal fibroblasts to find differences in proliferation. Early passage human dermal fibroblasts (HFB) lines from 4 healthy aged male donors (mean age=83yrs), AG04152 (83yrs), AG04383 (80yrs), AG04057 (81yrs), and AG04064 (92yrs) referred to as “aged HFB” and 4 healthy young male donors (mean age=28yrs), AG11747 (22yrs), AG11242 (30yrs), AG05415 (29yrs), and AG10046 (31yrs) referred to as “young HFB”, were obtained from the Coriell Institute (NIA Aging Cell Repository, Coriell Institute for Medical Research, Camden NJ) and cultured in DMEM with 5% fetal bovine serum (FBS) (Cellgro Manassa, VA). Unless otherwise noted, each cell line was studied individually and each experiment was repeated 3 times. All experiments were performed in DMEM with 1% FBS. (23). To establish their phenotype, all cell lines were examined for morphology, staining for senescence-associated beta galactosidase, and expression of the cell cycle inhibitors, p16 and p21. To determine if the cells’ phenotype corresponded to the chronological age of their donor, cell lines were ranked in order from highest to lowest for each marker of aging and each line was given an average rank score as we have previously described (14). Initial screenings of the four local anesthetics were done on 6 HFB cell lines (3 aged HFB and 3 young HFB); further experiments were performed on 8 HFB cell lines (4 aged HFB and 4 young HFB).

Local anesthetics

We used concentrations of local anesthetics similar to that previously published using human fibroblast cell lines (24). In a separate study, similar concentrations of local anesthetics were not cytotoxic to human oral fibroblasts (25). It should be noted that an important determinant of tissue concentration of local anesthetics is dermal blood flow (26), which is highly variable and results in contiguous dermal regions being exposed to a wide range of local anesthetic concentrations. In order to approximate the exposures that dermal fibroblasts typically see in vivo, we extrapolated from existing studies in animal models. As an example, a pharmacokinetic model in the rat suggests that the penetration of lidocaine is up to a depth of one centimeter below the skin and occurs predominantly during the first two hours (27). For the purpose of this study we assumed that due to systemic uptake and incomplete penetration, approximately 5% of the local anesthetic reaches the dermal fibroblasts. Therefore, we diluted the local anesthetics to expose the fibroblasts to a final concentration of 1/20 of clinically available local anesthetics. The final concentration of local anesthetics used in all the assays are: lidocaine 1% (Hospira Inc, Lake Forest, IL) and mepivacaine 1% (Fresenius Kabi USA, Schaumburg, IL) at a final concentration of 500ug/ml, bupivacaine 0.25% (Hospira Inc, Lake Forest, IL), and ropivacaine 0.5% (Fresenius Kabi USA, Schaumburg, IL) at a final concentration of 125ug/ml. Normal saline, at the same volume as the local anesthetic, served as the control in all experiments.

Cell proliferation assays

For screening of different local anesthetics, we chose six HFB cell lines; three young (AG11747, AG11242, AG10046) and three aged (AG04064, AG04383, AG04152) for individual experiments based on consistent alignment with a young and aged phenotype, respectively, as previously described (14). HFB cell lines were plated at subconfluence in 5% DMEM in triplicate in 96 well plates (5000 cells/well). Cells were allowed to adhere for 6 hours and were then serum deprived with DMEM 1%FBS overnight. The following day, cells were exposed to saline or a local anesthetic. 24 hours later, all media were changed to 1% DMEM and proliferation was measured after 48 hours using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI).

Expression of transcripts

Four young and four aged HFB cell lines were plated in 5% DMEM in 6 well plates (Corning, Tewksbury, MA) at 75% confluence for 6 hours. Media were then changed to 1% DMEM overnight. The next day cells were exposed to lidocaine (500ug/ml), Bupivacaine (125ug/ml) or normal saline (carrier) in 1% DMEM. RNA was isolated from cells after 3, 6 and 18 hours using Trizol (Invitrogen). RNA purity and integrity were assessed by spectrophotometric analysis. A total of 1 µg of RNA was reverse transcribed using an iScript kit (Bio-Rad Laboratories, Hercules, CA). RT-PCR was performed using an ABI 7900 RT-PCR instrument with SYBR Green Master Mix (Bio-Rad) for human insulin like Growth Factor-1 (IGF-1), insulin like Growth Factor-1 Receptor (IGF1R), collagen I (COL I), collagen III (COL III) and Transforming Growth factor β1 (TGF-β1). The following primer sequences were used:

IGF-1 F: GGACCGGAGACGCTCTGC

IGF-1 R: AGCTGACTTGGCAGGCTT

IGF1R F: CCATTCTCATGCCTTGGT

IGF1R R: TGCAAGTTCTGGTTGTCG

COL I F: GTGCTAAAGGTGCCAATGGT

COL I R: ACCAGGTTACCGCTGTTAC

COL III F: CCAGGAGCTAACGGTCTCAG

COL III R: CAGGGTTTCCATCTCTTCCA

TGF- β 1 F: TCCGGTTTGATCTTTCCAAG

TGF- β 1 R: ATCCGGAACGTCTCATTGTC

GAPDH F: GGCCTCCAAGGAGTAAGACC

GAPDH R: AGGGGTCTACATGGCAACTG

All experiments were performed in triplicate and normalized to GAPDH mRNA. Fluorescent signals were analyzed during each of the 40 cycles consisting of denaturation (95 °C, 15 s) and annealing (54 °C, 15 s). Relative quantitation was calculated using the comparative threshold cycle method and data is shown as average fold change for lidocaine from control.

IGF-1 stimulation

To determine the optimal concentration of IGF-1 to induce proliferation, preliminary experiments were performed on a subset of young and aged HFB. HFB were placed in low serum conditions (DMEM + 1% FBS) for 6 hours and were then exposed to recombinant human IGF-1 at 10ng/ml, 50ng/ml, and 100ng/ml (R&D systems) for 24 hours. The concentration of IGF-1 that was optimal for proliferation after 48 hour (as determined by the proliferation assay described above) was determined to be 50ng/ml. The effects of lidocaine with and without IGF-1 were performed on 4 young and 4 aged HFB.

Crystal violet staining

Representative young and aged HFB cell lines were plated in 5% DMEM on 10 well teflon masked microscope slides (5000 cells/well) (Creative Scientific Methods, Phoenix, AZ) for 6 hours and cells were serum deprived with 1% DMEM overnight. The following day, cells were exposed to lidocaine or saline with or without IGF-1 for 24 hours and media were changed to 1% DMEM. After 48 hours, cells were fixed in 10% NBF, stained with 0.1% crystal violet, and imaged using light microscopy.

TGF- β 1 stimulation

Aged and young HFB were placed in low serum conditions overnight (1% DMEM) and then exposed to saline or lidocaine with or without recombinant human TGF- β 1 (10ng/ml) (R&D

systems, Minneapolis, MN). The concentration of TGF- β 1 was based on prior work using these cell lines (28). Protein was isolated from the cells and western blotting completed as described below.

Western blots

Four young (AG11747, AG11242, AG10046, AG05415) and four aged (AG04064, AG04383, AG04152, AG4057) HFB cell lines that were used for the proliferation assay were plated in 5% DMEM in 6 well plates for 6 hours and then serum deprived overnight with 1% DMEM. The following day cells were exposed to saline or lidocaine with or without TGF- β 1. All media were changed to 1% DMEM after 24 hours of exposure and cell lysates were collected after 48 hours. Cells were lysed in NP40 buffer with protease and phosphatase inhibitors (Sigma, Saint Louis, MO). Lysates were prepared for electrophoresis as described (29). Equal amounts of protein were subjected to 10% SDS-PAGE, followed by transfer to nitrocellulose. After incubation with 5% milk, blots were probed with antibodies (1-5 μ g/ml) against COL III (ABCAM, Cambridge, MA), COL I (ABCAM, Cambridge, MA) and GAPDH (Millipore, Billerica, MA).

Statistics

As expected there was variability among cell lines within each group (young and aged donors). Accordingly, for each cell line, our results indicate differences relative to the cell line-specific controls. Within each age group we determined whether the active treatments differed from controls using a linear model on the log2-transformed outcomes with condition entered as a fixed effect and cell line as a random effect. Residuals were assessed and no obvious departures from Normality and variance homogeneity assumptions were noted. Within each age group and each outcome, multiple conditions were compared with controls and adjustments for possible type I error were included in the analyses. Dunnett's method for multiple comparisons was utilized in the analyses in which the design was completely balanced. When the design was imbalanced the Bonferroni method was used. Statistical significance was defined by $p < 0.05$ and biological significance was defined as a change of 10% or greater.

Results

Local anesthetics are variable in their effect on HFB proliferation

We tested the hypothesis that exposure to local anesthetics inhibits proliferation of dermal fibroblasts. Bupivacaine and ropivacaine did not inhibit proliferation of dermal fibroblasts in the aged HFB or in young HFB (Figure 1). Lidocaine and mepivacaine inhibited proliferation in aged HFB (for lidocaine 88% of control, 95% CI 80%-98%, $p=0.009$ and for mepivacaine 90% of control, 95% CI 81%-99%, $p=0.032$). Lidocaine did not inhibit proliferation in young HFB to a biologically significant degree (94% of control, 95% CI 90%-99%, $p=0.027$ in young HFB) and mepivacaine did not inhibit proliferation in young HFB (97% of control, 95% CI 92%-102%, $p=0.3$) (Figure 1). Further studies focused only on the specific effects of lidocaine for the following two reasons. First, lidocaine inhibition of proliferation had the largest effect on aged fibroblasts. Secondly, lidocaine is commonly used for both short procedures and prolonged infusions.

Lidocaine affects transcription in aged HFB to a greater extent than in young HFB

We sought to define the effect of lidocaine on the transcription of IGF-1 and IGF1R in dermal fibroblasts from aged and young donors. Each cell line was studied separately and we calculated the fold change induced by exposure to lidocaine at 500ug/ml. Our results show that 18 hours of exposure to lidocaine inhibits transcripts of IGF-1 and IGF1R in the fibroblasts from aged donors (Figure 2, Panel B) (IGF-1, log2 fold-change -1.25 {42% of control, 95%CI 19%-92%, p=0.035} and IGF1R, log2 fold-change -1.00 {50% of control, 95%CI 31%-81%, p=0.014}), but not in the young HFB (IGF-1, log2 fold-change -1.04 {49% of control, 95%CI 11%-216%, p=0.332} and IGF1R, log2 fold-change of -0.87 {55% of control, 95%CI 9%-331%, p=0.72}) (Figure 2, Panel A). Transcripts for collagen III and collagen I were decreased after lidocaine exposure for 18h in aged HFB (For collagen III: log2 fold-change -1.28 {41% of control, 95%CI 20%-83%, p=0.022}, and for collagen I: log2 fold-change -1.82 {28% of control 95%CI 14%-58%, p=0.006}). In young HFB lidocaine decreased transcripts for collagen III (log2 fold-change -1.60 {33% of control, 95%CI 15%-73%, p=0.019}). Our results did not indicate a statistically significant difference in young HFB with respect to collagen I (log2 fold-change -1.95 {26% of control 95%CI 5%-125%, p=0.076}). Shorter exposures to lidocaine (3 and 6 hours) did not result in significant changes in expression of IGF-1, IGF-1R, collagen I or collagen III transcripts (data not shown). Interestingly, lidocaine did not show evidence of change in the average mRNA levels of the pro-fibrotic growth factor TGF- β 1 in young or aged HFB (log2 fold-change 0.87 {183% of control 95%CI 83%-401%, p=0.12} for aged HFB, and log2 fold-change 0.84 {179% of control 95%CI 40%-805%, p=0.54} in young HFB), but there was a variable response among cell lines in each group. Exposure of aged HFB to lidocaine did not change the expression of transcripts for Epidermal Growth Factor, the Epidermal Growth Factor Receptor or basic Fibroblast Growth Factor (data not shown).

Lidocaine inhibits proliferation in aged HFB, an effect that is abrogated by IGF-1

We then tested the hypothesis that lidocaine exposure inhibits proliferation of HFB and that this effect is abrogated by treatment with IGF-1. As shown by representative crystal violet staining photographs of dermal fibroblasts from an aged donor AG04383 (Figure 3, Panel A), exposure to lidocaine reduces the number of viable cells. As expected, the pro-proliferative growth factor IGF-1 increased the number of cells such that proliferation was not visually different from control when IGF-1 was co-administered with lidocaine. For quantification of proliferation, we show the results from four separate young cell lines and four aged cell lines (Figure 3, Panel B). We found that lidocaine reduced proliferation in aged HFB (76% of control, 95%CI 66%-81%, p<0.0001 gray bars), to a greater extent than in young HFB (89% of control, 95%CI 75%-106%, p=0.25 black bars). Exposure to IGF-1 alone did not significantly increase proliferation, relative to control, in either young or aged HFB. When given concurrently, the addition of IGF-1 abrogated the inhibitory effect of lidocaine on proliferation in aged HFB.

Lidocaine inhibits the expression of collagen III in both young and aged HFB an effect that is abrogated by TGF- β 1

As noted above, lidocaine had a variable effect on transcripts for the pro-fibrotic growth factor, TGF- β 1. To test whether lidocaine inhibits the biosynthesis of key dermal structural proteins, we used RT-PCR and western blotting to assess the transcription and expression of collagen III and collagen I. Our results show that lidocaine significantly inhibits the transcription of collagen III in both young and aged HFB and inhibits collagen I in aged HFB (discussed above, Figure 2). Of note, exposure of aged HFB to lidocaine did not change the expression of the extracellular matrix protein fibronectin ($p>0.3$, data not shown). Figure 4, Panel A shows a representative western blot of an aged (AG04383) cell line for collagen III and collagen I. Densitometric analysis of individual experiments from four young (Figure 4, Panels B and C black bars) and four aged cell lines (Figure 4, Panels B and C gray bars), demonstrated that lidocaine inhibits the expression of collagen III in young and aged HFB (log2 fold-change -1.79 {29% of control, 95%CI 18%-47%, $p=0.003$ } in young HFB and log2 fold-change -1.76 {30% of control, 95%CI 9%-93%, $p=0.043$ } in aged HFB). The expression of collagen I was not affected by lidocaine in the young cell lines (log2 fold-change -1.33 {40% of control, 95%CI 2%-857%, $p=0.72$ }). Despite its effects on collagen I transcripts, lidocaine did not alter the expression of collagen I protein in aged HFB in a statistically significant fashion (log2 fold-change -1.45 {37% of control, 95%CI 12%-110%, $p=0.064$ }). As expected from other model systems (30), exposure to TGF- β 1 had little effect on the expression of collagen III and collagen I in both young and aged HFB. However, TGF- β 1 obviated the inhibitory effect of lidocaine on collagen III expression, in both young and aged HFB (Figure 4, Panel B).

Discussion

Proliferation and biosynthesis are key functions during all stages of wound healing. It is generally accepted that both processes are reduced in the dermis with aging and contribute to the 30-40% delay in wound healing that is seen in aged hosts (31). Reduced production of autocrine and paracrine growth factors contributes, in part, to delayed wound repair in aging (32). Indeed, manipulation of growth factors continues to be investigated as a treatment modality to improve wound healing in older persons (33). Local anesthetic agents have been shown to affect the viability and function of various cells and tissues including chondrocytes, nucleus pulposus cells, corneal endothelial cells, neurons and rodent wounds (19,34-36). In this study we hypothesized that local anesthetics, at concentrations that represent exposure of dermal fibroblasts during skin infiltration, could negatively impact cellular functions that are important for wound repair. It is understood that in vitro findings often form the basis for concerns of toxicity, but need to be confirmed in vivo (37).

Past work on the toxicity of local anesthetics has been studied primarily in neuronal cells. Mechanisms of toxicity of different local anesthetic agents are varied and include impacts on sodium channels, mitochondrial functions (38) and inflammatory responses (39,40). For screening purposes, we chose four agents with widespread utility in clinical practice. Our results suggest that lidocaine and mepivacaine inhibit proliferation in aged HFB, whereas bupivacaine and ropivacaine do not. Although the magnitude of the effect is relatively small

(about 10-25%), given that wound healing is reduced by 30-40% in aging, a decrease of 10-25% in proliferation could constitute a clinically significant contribution to age-associated deficits in dermal repair.

To our knowledge the connection between IGF-1 or TGF- β 1 and local anesthetic agents has not been previously examined. We hypothesized that the effect of lidocaine on aged HFB is mediated (at least in part) by regulating the expression of growth factors that are implicated in the aging process and participate in dermal repair, such as IGF-1 and TGF- β 1. The role of the IGF-1/IGF1R axis in aging and longevity is well established in humans, as well as in model systems from a range of biologic phyla (41). Up-regulation of IGF-1 is observed within days after various insults such as hypoxic-ischemic brain injury, brain contusion and penetrating trauma (42-44). Moreover, IGF-1 (produced mostly by dermal fibroblasts) is increased following skin injury (45) and implicated in animal models of wound healing (46). TGF- β 1 is the primary growth factor that regulates collagen deposition and has been implicated in both dermal healing and scarring. It is generally accepted that deposition and remodeling of collagen is slower in aged animals (47). However, when dermal HFB from aged donors were exposed to TGF- β 1 they showed similar biosynthetic and contractile properties as that of young HFB (28).

In this study, the reduction of proliferation in the aged donors was mediated (at least in part) by the IGF-1/IGF1R axis, as lidocaine exposure resulted in a reduction in IGF-1 and IGF1R mRNA transcripts only in the aged HFB. Furthermore, lidocaine-induced reduction of proliferation was abrogated by exposure to IGF-1. Notably, in a neuronal model in vitro, the cytotoxic effects of local anesthetics were not reversed by Nerve Growth Factor (48). The effect of lidocaine on key structural ECM components focused on transcription and expression of collagen III, which provides an early provisional matrix, and collagen I, which comprises the majority of the structural matrix that maintains dermal integrity (9). Lidocaine decreased transcripts for collagen III in both young and aged HFB, and collagen I in the aged HFB. Although lidocaine inhibited the transcription of both collagens in aged HFB, the negative impact on the protein accumulation was noted only for collagen III. The lack of change in collagen I protein likely reflects the high content of collagen I in dermal HFB in the control state, thereby limiting the detection of potential lidocaine-induced changes. The accumulation of collagen III protein (with its significantly shorter half-life than collagen I), would be more susceptible to lidocaine, irrespective of the phenotypic age of the cell line [4]. Similar to the effect of IGF-1 on proliferation, the lidocaine-induced reduction of biosynthesis in aged HFB was abrogated by exposure to TGF- β 1. It is noteworthy that although there is a trend to an increase in TGF- β 1, lidocaine did not significantly alter the transcription of this growth factor. This does not exclude the possibility of significant lidocaine effects on downstream mediators of TGF- β 1 activity, such as the SMADs (49). Similar reductions in collagen accumulation after local anesthetic infiltration were observed in aged mice and were accompanied by increased activity of the collagen-degrading enzyme matrix metalloproteinase-2 (50).

Limitations of this study include the observation that regardless of donor age, dermal fibroblasts lose their chronologic phenotype with respect to replicative capacity when proliferation is measured in vitro (51). In vitro simulation of aging is dependent on its

maintaining an aged phenotype in culture. To mitigate this concern, as noted above, we used only early passage cells and have previously confirmed that these cells maintain the chronologic phenotype of their donor (14). Moreover, the use of a single cell type (human dermal fibroblasts from young and aged male donors) as an in vitro model for dermal function is also a limitation. Wound healing is a complex system, both temporally and spatially, with numerous cell types and effectors. It is likely that lidocaine influences inflammation (52), angiogenesis (53) and other processes that are essential in wound healing, but could not be examined in this study. Although we screened four local anesthetic agents for their effect on fibroblast proliferation, our experiments focused on lidocaine for reasons noted above. Lastly, the dermal concentrations of local anesthetics have not been measured; we chose a hypothetical concentration for local anesthetic exposure that was similar for both young and aged HFB. Since there is a decrease in the dermal microcirculation in aged skin (54), it is likely that reduced blood flow increases the concentration of local anesthetics by decreasing uptake and subsequent clearance. We propose that age-related deficits in the dermal circulation enhance, rather than reduce, the effect of lidocaine on aged HFB.

In summary, we propose that lidocaine inhibits cellular functions in dermal HFB from aged donors, which could impact subsequent cutaneous wound healing. Our results suggest that age-related responses to lidocaine are due, in part, to interactions with IGF-1 and TGF- β 1. These key growth factors are altered during normal aging and are necessary for wound repair. It is important to note that the effect of local anesthetic agents on reducing the stress response and alleviating pain could benefit some wound healing responses (55), which might offset the potential deleterious consequences of local anesthetics. Determining the net impact of lidocaine on wound healing responses in aging requires further investigation, similar to research examining the effects of intrathecal lidocaine on neurological injury (56). We encourage additional studies to evaluate wound healing in the elderly after exposure to local anesthetics.

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Figure 1. Lidocaine and Mepivacaine, but not Bupivacaine and Ropivacaine, inhibit proliferation in aged HFB

Three young and three aged HFB cell lines were treated as follows (from left to right in each panel): control conditions; exposure to lidocaine 500ug/ml, mepivacaine 500ug/ml, bupivacaine 125ug/ml and ropivacaine at 125ug/ml. The left panel shows that in HFB from young donors none of the local anesthetics significantly inhibited proliferation. Right panel shows that in HFB from aged donors, both lidocaine and mepivacaine inhibited proliferation ($p=0.009$ and $p=0.032$ for lidocaine and mepivacaine respectively) (* indicates $p<0.05$). Bupivacaine and ropivacaine did not inhibit proliferation in young or aged HFB.

Figure 2. Lidocaine affects transcription in aged HFB to a greater extent than in young HFB

Four young and four aged HFB cell lines were exposed to lidocaine at 500ug/ml. RT-PCR was used to evaluate change in levels of transcripts for insulin like Growth Factor-1 (IGF-1), insulin like Growth Factor-1 Receptor (IGF1R), collagen III (COL III), collagen I (COL I) and Transforming Growth factor β 1 (TGF- β 1) in response to lidocaine exposure in dermal fibroblasts from young (Panel A) and aged (Panel B) donors. Data shown are average log2 fold change for lidocaine relative to saline control. Results indicate that lidocaine inhibits mRNA levels of IGF-1 and IGF1R in the aged HFB, but not young HFB. Lidocaine significantly inhibits the transcription of collagen III in both young and aged HFB, but only collagen I only in aged HFB. The effect of lidocaine on levels of TGF- β 1 was not statistically significant in young or aged HFB.

Figure 3. Lidocaine inhibits proliferation of aged HFB, which is abrogated by IGF-1

Four young and four aged HFB cell lines were treated with lidocaine 500ug/ml or saline, with or without IGF-1 (50ng/ml). Panel A shows a representative aged cell line (AG04383) that was stained with crystal violet under the four treatment conditions. From left to right: control conditions; exposure to lidocaine, which shows a marked reduction in the number of cells; exposure to IGF-1 shows a marked increase in the number of cells; and exposure to IGF-1 and lidocaine shows cell number is not visually different from control conditions. Panel B shows the effect of lidocaine on proliferation from 4 young HFB (black bars in each condition) and 4 aged HFB (gray bars in each condition). Lidocaine reduces proliferation significantly in the aged HFB (73%, $p<0.05$), but not in the young HFB. In aged HFB, treatment with IGF-1 abrogated the lidocaine-induced reduction in proliferation (* indicates $p<0.05$).

Figure 4. Lidocaine inhibits the expression of collagen III in both young and aged HFB, an effect that is abrogated by TGF- β 1

Four young and four aged HFB cell lines were treated with lidocaine 500 μ g/ml or saline, with or without TGF- β 1 (10ng/ml). Panel A shows a representative western blot of cell lysates from an aged HFB (AG04383) probed with specific antibodies against collagen III (COL III), collagen I (COL I), and GAPDH. Panels B and C are average densitometric analyses of four young HFB (black bars) and four aged HFB (gray bars) for collagen III and collagen I, respectively. Bar plots show that lidocaine inhibits the expression of collagen III in young and aged HFB (Panel B, black and gray bars, respectively). Notably, despite its effects on collagen I transcripts (Figure 2), lidocaine did not alter the expression of collagen I protein in aged HFB in a statistically significant fashion (log₂ fold-difference from control -1.76, 95%CI 3.41-0.10, $p=0.064$) (Panel C, gray bars) (*indicates $p<0.05$). Treatment by TGF- β 1 abrogated the lidocaine-induced reduction in collagen III expression in both young and aged HFB, and collagen I expression in aged HFB.