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Effect of Local Anesthetics on Human Mesenchymal Stromal Cell Secretion

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Abstract

Anti-fibrotic and tissue regenerative mesenchymal stromal cell (MSC) properties are largely mediated by secreted cytokines and growth factors. MSCs are implanted to augment joint cartilage replacement and to treat diabetic ulcers and burn injuries simultaneously with local anesthetics, which reduce pain. However, the effect of anesthetics on therapeutic human MSC secretory function has not been evaluated. In order to assess the effect of local anesthetics on the MSC secretome, a panel of four anesthetics with different potencies — lidocaine, procaine, ropivacaine and bupivacaine — was evaluated. Since injured tissues secrete inflammatory cytokines, the effects of anesthetics on MSCs stimulated with tumor necrosis factor (TNF)- α and interferon (IFN)- γ were also measured. Dose dependent and anesthesia specific effects on cell viability, post exposure proliferation and secretory function were quantified using alamar blue reduction and immunoassays, respectively. Computational pathway analysis was performed to identify upstream regulators and molecular pathways likely associated with the effects of these chemicals on the MSC secretome. Our results indicated while neither lidocaine nor procaine greatly reduced unstimulated cell viability, ropivacaine and bupivacaine induced dose dependent viability decreases. This pattern was exaggerated in the simulated inflammatory environment. The reversibility of these effects after withdrawal of the anesthetics was attenuated for TNF- α /IFN- γ -stimulated MSCs exposed to ropivacaine and bupivacaine. In addition, secretome analysis indicated that constitutive secretion changes were clearly affected by both anesthetic alone and anesthetic plus TNF α /IFN γ cell stimulation, but the secretory pattern was drug specific and did not necessarily coincide with viability changes. Pathway analysis identified different intracellular regulators for stimulated and unstimulated MSCs. Within these groups, ropivacaine and bupivacaine appeared to act on MSCs similarly via the same regulatory mechanisms. Given the variable effect of local anesthetics on MSC viability and function, these studies underscore the need to evaluate MSC in the presence of medications, such as anesthetics, that are likely to accompany cell implantation.

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Keywords

Mesenchymal stromal cells; local anesthetics; secretome; pathway analysis

1. Introduction

Mesenchymal stromal cells (MSCs) are readily accessible and self-renewing cells that have been incorporated into a growing number of therapeutic tissue regenerative applications due to their mesodermal differentiation potential and cytoprotective function. Among these treatment applications are myocardial infarction,¹ colitis,² liver disease,³ kidney disease,⁴ Crohn's disease,⁵ central nervous system (CNS) trauma⁶ and many autoimmune diseases.^{7–9} Recently, MSC have also been utilized to augment joint cartilage replacement¹⁰ and to treat wounds resulting, for example, from diabetic ulcers¹¹ and burn injury^{12,13} While some MSC functions require direct cell contact, most depend upon MSC secretion of tissue regenerative and immunomodulatory soluble factors. MSC are very responsive to their extracellular milieu which, in fact, controls their secretory responses.¹⁴ Procedurally, local anesthetics may be applied both to alleviate pain and following surgical implantation of the MSC cell or cell/tissue constructs.^{15–19} However, the effect of local anesthetics in the extracellular environment on these tissue protective MSC properties has not been thoroughly investigated.

Since the targets of anesthetics are generally neuronal cells, many laboratories have investigated neuronal responses to anesthetics using hippocampal tissue and isolated cell culture models.^{20–24} Others have begun to characterize MSC viability and/or chondrocyte differentiation responses to local anesthetics, predominantly to evaluate the potential implications for joint repair and cartilage replacement^{15,18} Lucchinetti *et al.* characterized the local anesthetic effect on cell viability, proliferation and skin cell differentiation using a murine MSC and wound healing model, where graded ropivacaine doses inhibited cell proliferation and delayed wound closure.²⁵

Despite the fact that MSC are exposed to anesthetics during implantation and their regenerative effects are regulated predominantly by secretory function, few studies have quantified the effects of local anesthetics on MSC secretion. MSC secreted products can control fibrosis and determine ultimate wound healing outcomes in acute and chronic diabetic and traumatic wounds.^{11–13} Therefore, the current studies were designed to assess the effect of local anesthetics in combination with inflammatory cytokines on MSC viability and secretome changes. Our studies indicate that local anesthetics can alter the MSC secretome depending on anesthetic dose and potency, as well as the inflammatory milieu. These drugs may therefore play a significant role in MSC mediated regeneration.

2. Materials and Methods

2.1. Chemicals and reagents

All anesthetics (lidocaine, ropivacaine, procaine and bupivacaine) and other chemicals were purchased from Sigma Aldrich (Oakville, Ontario, Canada), unless otherwise stated. All cell

culture reagents were purchased from Life Technologies (Carlsbad, CA, USA), unless otherwise stated.

2.2. MSC culture

Human bone marrow-derived MSCs purchased from the Institute for Regenerative Medicine (Texas A&M College of Medicine, Temple, TX, USA) were thawed at passage 2 and plated as a monolayer culture at 4000 cells/cm² in a humidified 37°C, 5% CO₂ incubator. They were cultured in Minimum Essential Medium α containing no deoxy- or ribonucleosides, and supplemented with 10% fetal bovine serum (Atlanta Biologics, Flowery Branch, GA, USA), 2 mM L-glutamine, 1 ng/mL basic fibroblast growth factor, 100 U/mL penicillin and 100 mg/mL streptomycin. The cells were grown to 70% confluence, trypsinized, seeded into 96-well plates at 2000 cells/well (6250 cells/cm²), and allowed to attach overnight.

2.3. MSC treatment conditions

Cell culture medium was replaced with medium containing 0, 10, 100, 500, or 1000 μ M of lidocaine, ropivacaine, procaine, or bupivacaine, with or without tumor necrosis factor (TNF- α) (25 ng/mL) and interferon (IFN- γ) (25 ng/mL) (R&D Systems, Minneapolis, MN, USA) and incubated for 48 h at 37°C in a humidified 5% CO₂ incubator.

2.4. Assessment of cell viability/proliferation

After 48 h of culture in the presence of the local anesthetics, the culture supernatants were gently collected, stored at -20°C and replaced with fresh medium containing alamar blue reagent (alamar-Blue, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The cells were then returned to the incubator for continued culture. The fluorescence of the wells was read once an every hour for 5 h and then again after 24 h using a microplate reader (DTX 880 Multimode Detector, Beckman Coulter, Fullerton, CA, USA), returning the cells to the incubator between readings.

2.5. Cell counting

After 48 h of culture with the anesthetics and 24 h of culture in fresh medium containing alamar blue, supernatants were gently aspirated and replaced with 4% (w/v) paraformaldehyde (PF) for 20 min at room temperature to fix the cells. Enough 1X phosphate buffered saline (PBS) (Life Technologies, Carlsbad, CA, USA) was added to each well to achieve 1% (w/v) PF. The fixed cells were stored at 4°C until further use. Fixed cells were transitioned to room temperature, washed 3X with PBS for 5 min, and incubated with 100 μ L of Hoechst stain (Molecular Probes, Eugene, OR, USA) diluted 1:5000 in PBS for 30 min in the dark. Cells were then washed twice with PBS and maintained in 200 μ L of PBS for imaging. Stained nuclei were visualized at 4X using an inverted fluorescent microscope (IX81, Olympus, Tokyo, Japan) and counted using SlideBook software version 5.0 (Intelligent Imaging Innovations, Denver, CO, USA) image analysis software.

2.6. Cytokine measurement

The cell culture supernatants collected from MSCs after 48 h of culture in medium containing the local anesthetics with or without TNF- α /IFN- γ were thawed and analyzed

using enzyme linked immunosorbent assays for interleukin (IL)-6 (Biolegend, San Diego, CA, USA) and prostaglandin E2 (PGE2, Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions. Absorbances were recorded using a microplate reader (DTX 880 Multimode Detector, Beckman Coulter, Fullerton, CA, USA). A bead-based multiplex analysis (Bio-Plex Pro Human Cytokine Group I, Bio-Rad Laboratories Inc., Hercules, CA, USA) of 27 cytokines, chemokines and growth factors was also performed using these supernatants, according to the manufacturer's instructions. Data were obtained using a Bio-Plex 200 System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.7. Pathway analysis

Ropivacaine, bupivacaine and differentially secreted cytokines identified in the multiplex immunoassay were analyzed for overlapping and interacting molecular networks, pathways and cellular functions using Ingenuity Pathway Analysis software (IPA, QIAGEN, Redwood City, CA) with a significance level of $\alpha = 0.001$. The data were uploaded to Core Analysis, which extracts information on genomic and proteomic interactions from literature databases to identify upstream regulators, networks and biological functions associated with each molecule. Identified networks were assigned a score which represents the log probability that the network was found by random chance; a score better than 2 is usually attributed to valid network. Custom pathways were then established to link anesthetics and differentially secreted proteins by overlapping upstream regulators using My Pathways and Path Designer functions.

2.8. Statistical analysis

Data points represent the mean $\pm$ standard error of the mean (SEM) for the indicated number of independent observations (n). Viability/proliferation data are reported as fluorescence intensities. Cell counts and levels of secreted factors were normalized to their respective controls (medium containing no anesthetics, with or without TNF- α /IFN- γ); baseline levels of the controls are reported for reference. Statistical differences between the data were determined using Student's t -test (two groups) or analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) *post hoc* analysis in Kaleida-Graph software version 4.1 (Synergy Software, Reading, PA, USA). Differences between MSCs cultured without TNF- α /IFN- γ (unstimulated) were generally compared to those cultured with TNF- α /IFN- γ (stimulated) using Student's t -test (two groups), unless otherwise stated. Comparisons to respective unstimulated and stimulated controls (medium without anesthetics) were performed using ANOVA with Fisher's LSD *post hoc* analysis with a significance level of $\alpha = 0.05$.

3. Results

3.1. Effect of local anesthetics on MSC viability

The cytotoxic effects of lidocaine, bupivacaine, ropivacaine (amide anesthetics) and procaine (an ester anesthetic) on human bone marrow-derived MSCs were evaluated and compared. Since implanted MSC are exposed to tissue microenvironments which are likely to be inflammatory, we also assessed the effect of the anesthetics on MSCs stimulated with TNF- α and IFN- γ . Alamar blue, a metabolic indicator, was used to assess cell viability. As

indicated in Fig. 1, while neither lidocaine nor procaine had a major effect on unstimulated MSC reduction of alamar blue, both ropivacaine and bupivacaine had significant and dose dependent effects. We also observed that MSC viability was decreased by TNF- α /IFN- γ stimulation alone, and further decreased, in a dose dependent manner, for stimulated MSCs exposed to the more toxic and longer acting anesthetics, ropivacaine and bupivacaine.²⁶ Exposure to procaine in combination with the inflammatory cytokines resulted in lower viable cell numbers at the highest dose. There were also small but significant reductions in viability for stimulated MSCs treated with lidocaine at the highest dose tested compared to corresponding unstimulated conditions.

3.2. MSC recovery after anesthesia withdrawal

Since local anesthetics are absorbed and metabolized, their activity diminishes over time.^{23,26} We therefore evaluated the recovery of the MSC over several hours following the withdrawal of the anesthetic to determine the reversibility of the cytotoxic effects. As indicated in Fig. 2, both unstimulated and stimulated MSCs experienced some degree of recovery in cell proliferation once the anesthetics were withdrawn. After withdrawal of the anesthetics, the proliferation of unstimulated MSCs only differed from that of cells in the medium at the highest concentration (1000 μ M) of bupivacaine [Figs. 2(a)–2(d)]. For MSCs exposed to TNF- α /IFN- γ , recovery kinetics were slowed for cells that were exposed the highest concentration of ropivacaine and all concentrations of bupivacaine [Figs. 2(e)–2(h)].

To complement these observed effects of the anesthetics, we also quantified cell number 24 h after removal of the anesthetics using image-based cell counting. As indicated in Fig. 3, the previously observed effects of anesthesia exposure were also reflected in the cell number, with the largest reduction in cell number seen for ropivacaine and bupivacaine. Again, the changes for lidocaine and procaine were only noted by decreases in cell number for stimulated cells and at the highest dose tested.

3.3. Effect of local anesthetics on MSC secretion

Since most MSC therapeutic functions are attributed to secreted products, we looked at the effects of anesthesia exposure on cytokine secretion. We first examined IL-6 secretion, as this cytokine has been shown to be secreted by both stimulated and unstimulated MSC.²⁷ As indicated in Fig. 4, lidocaine did not have drastic effects on IL-6 secretion from unstimulated or TNF- α /IFN- γ stimulated cells relative to medium controls, although baseline MSC secretion increased dramatically following TNF- α /IFN- γ stimulation (0.78 ± 0.15 ng/mL versus 26.1 ± 1.7 ng/mL). Stimulated MSCs exposed to procaine had a very slight yet statistically significant decrease in IL-6 secretion. The ropivacaine and bupivacaine IL-6 secretion results were more variable. Although there was a slight decrease in IL-6 secretion from unstimulated cells exposed to these anesthetics, ropivacaine or bupivacaine exposure increased IL-6 secretion from stimulated cells (Fig. 4). This effect was most pronounced at the highest doses tested, which also substantially reduced MSC viability.

Several studies^{28,29} including our own³⁰ suggest that PGE2 is important in regulating MSC anti-inflammatory function. Therefore, we also examined the effect of both a lower (100

μM) and higher (1000 μM) dose anesthesia exposure on PGE2 secretion. Figure 5 indicates that the effect of the anesthetics on PGE2 was more variable than on IL-6 secretion. The PGE2 levels were dramatically higher for stimulated cells cultured in the absence of anesthetics (2.00 ± 0.02 ng/mL versus 27.4 ± 2.2 ng/mL). Low doses of anesthetic did not dramatically alter stimulated or unstimulated MSC secretion of PGE2. However, the high dose anesthetic effects were more pronounced. Unstimulated MSC PGE2 secretion decreased relative to control levels for all anesthetics. Activated MSCs generally resulted in higher relative PGE2 secretion for all anesthetics at 1000 μM compared to unstimulated MSCs, with the exception of procaine, which followed the same decreasing trend as the unstimulated cells. Therefore, while the differences were highest within the bupivacaine and ropivacaine groups where cell viability and IL-6 secretion were also affected, the PGE2 secretory trend did not necessarily correspond with viability changes. In fact, high doses of both lidocaine and procaine reduced PGE2 secretion from unstimulated MSCs despite the fact that these anesthetics did not greatly alter viability or IL-6 secretion profiles.

Given the fact that the PGE2 and IL-6 secretion responses exhibited different trends, we extended our secretome analysis using a multiplex antibody-conjugated bead immunoassay to quantify the measurement of a panel of 27 cytokines, chemokines and growth factors. We used this panel to screen for secretome changes induced by high doses (1000 μM) of ropivacaine and bupivacaine, as these conditions had the largest effects on viability and secretion. As depicted in Figs. 6(a) and 6(b), large decreases in IL-8 and monocyte chemoattractant protein (MCP)-1 were induced by both anesthetics in unstimulated cells, while IL-12 and vascular endothelial growth factor (VEGF) responses were unchanged (baseline levels are shown in Table 1). A larger panel of analytes was detectable from stimulated MSCs (Table 2). In combination with TNF- α /IFN- γ stimulation, ropivacaine and bupivacaine induced very large increases in IL-1 β and IL-9 secretion levels, but reduced levels of chemokine ligand 5 (RANTES) [Figs. 6(c) and 6(d)]. Exposure of stimulated MSCs to 1000 μM ropivacaine decreased IL-8 and increased VEGF and granulocyte colony-stimulating factor (GCSF) secretion, all of which remained relatively unchanged for bupivacaine treated cells. These results collectively indicate that anesthetics can significantly alter the pattern of the MSC secretome and is dependent on the anesthetic used as well as the state of the inflammatory milieu.

3.4. Pathway analysis

Due to the variations in secretion profile seen between unstimulated and stimulated MSCs exposed to the local anesthetics, IPA was used to examine potential upstream regulators of these differences as well as overlapping pathways and cell functions. Pathway analysis of the different experimental conditions resulted in identification of two sets of the most likely upstream regulators. These findings suggest that ropivacaine and bupivacaine affect the same regulatory patterns, which are dependent on the stimulation state of the MSCs. For unstimulated MSC secretome, adenosine receptor a2b (ADORA2B), interferon regulatory factor 3 (IRF3), TNF receptor superfamily member 18 (TNFRSF18), proteinase 3 (PRTN3) and Jun, a member of the activator protein 1 (AP-1) family, were predicted by the Core Analysis as the most likely upstream regulators. Different upstream regulators were identified for TNF- α /IFN- γ -stimulated MSCs. This group included IL1- β , nuclear factor

kappa-B1 (NF- κ B1), aryl hydrocarbon receptor (AHR) and TNF. Toll-like receptor (TLR)-4 was also identified in the literature database as being associated with changes in the secreted factors we measured. In our *in vitro* system, however, TLR-4 ligands are not present. Therefore, this upstream regulator is not considered here but may become relevant in a non-sterile pro-inflammatory environment. Cell-cell signaling and interaction was identified as the most relevant molecular and cellular function, with overlapping pathways for 12 molecules in the MSC secretome data. Additionally, 11 molecules were linked to cellular growth and proliferation, cell death and survival, and cellular development. Additionally, networks involved in cellular movement and immune cell trafficking were identified for the stimulated MSC secretome, overlapping with three of the secreted molecules (IL-8, IL-15 and IL-12).

Custom pathways were established between the most likely upstream regulators determined by the Core Analysis based on the unstimulated (Fig. 7) and stimulated (Fig. 8) secretome multiplex data using IPA's My Pathways and Path Designer functions. According to the Ingenuity knowledge base and current literature, both ropivacaine and bupivacaine exert changes in cellular function and viability in a variety of cell types by increasing reactive oxygen species (ROS) and stimulating MAPK signal transduction pathways, specifically the p38 MAPK and JNK pathways.^{25,31} In turn, these changes lead to stimulation of NF- κ B and AP-1 signaling which may cause profound changes in the transcriptome, including differential transcription of cytokines and chemokines such as IL-1 β and IL-8,^{32,33} which we observed were differentially secreted from the MSCs.

4. Discussion

Local anesthetics are likely to be used in conjunction with MSC implantation for a variety of wound healing and joint repair procedures. While differentiated MSCs may serve as a source of replacement cells in some applications,^{15,18,25} an increasing number of studies have focused on the anti-fibrotic and tissue regenerative MSC properties which are largely controlled by a plethora of secreted cytokines and growth factors.^{3,13,34–36} However, the effect of anesthetics on MSC secretory function has not been thoroughly assessed. Our studies were designed to evaluate the effect of a panel of local anesthetics on MSC survival, secretory function and recovery from anesthetic treatment alone and in a pro-inflammatory environment. The results of our studies indicate that (1) local anesthetics can affect human bone marrow-derived MSC viability, (2) the effect is drug specific and reversible, (3) in an inflammatory milieu, MSCs are more susceptible to adverse anesthetic viability effects and (4) secretory changes do not necessarily correspond to viability effects. Based on our predictive pathway analysis, anesthetics may interact with pro-inflammatory cytokines via regulation of ROS to alter the MSC secretome. These studies underscore the need to evaluate MSC and their functions in the presence of drugs, such as anesthetics, that are likely to accompany cell implantation.

While many studies have evaluated the effect of local and systemic anesthetics on neuronal cells or tissues, far fewer have studied the effect of anesthetics on MSCs or MSC-like cells. Rahnama *et al.* investigated the cytotoxic effect of 1 h exposures to the local anesthetics lidocaine, bupivacaine and ropivacaine on human bone marrow-derived MSCs at 24 h post-

exposure.¹⁸ In their studies, lidocaine reduced MSC numbers, whereas neither bupivacaine nor ropivacaine affected overall cell number significantly. Lucchinetti *et al.* evaluated the effect of 1–3 day exposure of the local anesthetics lidocaine, ropivacaine and bupivacaine on unstimulated murine bone marrow-derived MSC cell proliferation and osteogenic differentiation. They found that local anesthetics inhibited both of these cellular functions.²⁵ In addition, to simulate wound environment they exposed MSC to TNF- α and anesthetic for a 24 h period. While TNF- α increased NF- κ B and I κ B phosphorylation, the anesthetics reduced these regulatory processes and subsequent simulated wound healing. Breu *et al.* studied the effects of local anesthetics on human bone marrow-derived MSC viability both pre- and post-chondrogenic differentiation.¹⁵ The cells were exposed to bupivacaine, ropivacaine or mepivacaine for 1 h time periods. Significant reductions in viability were described in all cases. Finally, Girard *et al.* examined the effect of a 2 h lidocaine exposure on human adipose stem cell viability and secretion and found that both viability and IL-6 secretion were reduced.³⁷

Although most of these studies are consistent with ours, we did not detect significant viability reduction post-lidocaine incubation. However, it is important to note that our viability metric was alamar blue reduction, which describes the metabolic state of the cell population and does not evaluate either necrosis or apoptosis of individual cells. Decreased alamar blue reduction may be associated with either decreased metabolic activity per cell or fewer cells within the population. However, we also quantified post-exposure cell number, where we did detect lidocaine and procaine mediated reductions using higher concentrations, but only when cells were also stimulated with TNF- α /IFN- γ . Cell number reductions in response to ropivacaine and bupivacaine were more dramatic. Clinically, lidocaine and procaine are used at doses of 1–2% (10–20 mg/mL), equivalent to approximately 35–74 mM.^{26,38,39} Bupivacaine and ropivacaine have higher potencies and are typically used at lower concentrations of 0.25–0.5% and up to 1% (2.5–5 mg/mL and 10 mg/mL), respectively, equivalent to approximately 7–15 mM and 37 mM. These doses are higher than those used in our studies and in many other *in vitro* studies^{15,25} Upon injection, these doses may be diluted, but the final concentration is unclear. These anesthetics are also typically co-administered with molecules such as epinephrine to prolong action^{26,37} These factors make drawing direct parallels between *in vitro* and *in vivo* doses difficult. However, it is clear from our studies that exposure of MSCs to anesthetics has a significant effect on viability and secretion. Regardless, an expanded range of doses and formulations should be examined in future studies.

While many procedures require only one local anesthetic administration, post-surgical procedures have utilized multiple or continuous anesthetic applications for pain management.^{40,41} Since bupivacaine and ropivacaine are slower and longer acting than lidocaine and procaine, which are fast and short-lived, these anesthetics would generally be chosen, especially if only one application is administered.^{23,26,38,39} However, although they are relatively toxic at higher concentrations, lower dose administration may be used with continuous administration to reduce toxicity.²⁶ Our studies used 48 h anesthesia exposure to evaluate longer term anesthetic effects. By using longer incubation times we were able to monitor viability and functional changes following longer anesthesia exposure. However,

more acute effects of the shorter acting anesthetics may be missed by using this approach. Therefore, future studies will also include shorter exposure regimens.

Our studies were also designed to investigate MSC secretory function post-anesthesia exposure to determine whether there was a drug specific and dose responsive effect on secretion. In order to mimic an injured tissue inflammatory environment, the effects of anesthetics were also evaluated in conjunction with TNF- α /IFN- γ stimulation. Our results indicated that stimulation affected secretory changes and viability in the presence of anesthesia. With and without anesthesia, TNF- α /IFN- γ stimulation increased the baseline secretory response of MSC dramatically compared to unstimulated cells. While IL-6 secretion generally followed the inverse of stimulated cell viability change changes, this was not the case with PGE2 secretion, which was effected more variably. The results of our multiplex analysis further suggested that depending on anesthetic used, as well as the inflammatory milieu, some secreted factor levels may increase and others may be reduced. Amide local anesthetics have previously been implicated in the reduction of the expression of TNF- α -induced pro-inflammatory cytokines and chemokines such as IL-1 β , IL-8, RANTES (CXCL5) and MCP-1 in epithelial cells and murine MSCs.⁴² In our studies, both ropivacaine and bupivacaine decreased TNF- α /IFN- γ -induced secretion of IL-8. However, in contrast to the results reported by Lahav *et al.* on the effects of bupivacaine, we observed an increase in IL-1 β secretion and no significant change in IL-1RA secretion from MSCs in response to TNF- α /IFN- γ in the presence of either anesthetic, suggesting upregulation of pro-inflammatory functions. The increases in IL-9 secretion we observed, however, speak to anti-inflammatory MSC functions (stimulation of proliferation and anti-apoptosis). Therefore, further experimental analysis will include co-culture with immune system cells such as macrophages, to determine how these secretome changes ultimately affect intercellular regulation. Given the fact that our previous studies demonstrated that macrophage conversion from pro- to anti-inflammatory phenotypes is directly dependent on increased MSC PGE2 levels,^{30,43} it is unclear if ropivacaine and bupivacaine will have detrimental or beneficial effects on this regulatory cellular function.

To begin to investigate the mechanisms underlying the interaction of anesthetics and pro-inflammatory cytokines on the MSC secretome, we utilized IPA to interpret the secretome data in the context of biological processes, pathways, upstream regulators and molecular networks. Cellular functions of stimulated MSCs extended beyond cell survival to include functions typically involved in initiation and regulation of an immune/inflammatory response (migration and immune cell recruitment).⁴³ This result is in line with the expected response of MSCs to a pro-inflammatory environment. However, the decreases in secretion of some chemokines (IL-8 and RANTES) suggest that MSC-mediated recruitment of immune cells may be attenuated in a pro-inflammatory microenvironment by the presence of ropivacaine. The connection between the action of the local anesthetics and the predicted upstream regulators could be attributed to the formation of ROS and their effects on cell function.²⁵ ROS are known to stimulate a number of pathways that lead to cell death, including MAPK signal transduction pathways. Generally, p38 MAPK is preferentially stimulated by environmental stress and inflammatory cytokines^{44,45} Huang *et al.* showed that bupivacaine induces ROS and p38 MAPK, up-regulates the expression of the

transcription factor c-Jun (AP-1) and significantly increases NF- κ B presence in the nucleus in neuronal cells. However, the stimulation or inhibition of these pathways by local anesthetics has been shown to be cell and system dependent. A recent study performed on TNF- α stimulated mouse derived MSC treated with ropivacaine and bupivacaine showed inhibition of NF- κ B pathway.²⁵ In this study, both bupivacaine and ropivacaine-mediated increase in intracellular ROS caused inhibition of TNF- α induced I κ B phosphorylation and abolished translocation of NF- κ B to the nucleus. Therefore, further studies with human derived MSC are required to elucidate the mechanisms underlying the interaction of local anesthetics on cellular function and secretion.

5. Conclusions

We have investigated the effect of a panel of local anesthetics on unstimulated and stimulated MSC viability and secretion patterns. We detected potency differences within this panel, which were evident in both viability changes and cell recovery post-exposure; these responses were dependent on TNF- α /IFN- γ mediated MSC stimulation. In addition, we examined the secretory responses of anesthesia-exposed MSC and found that while there were dose dependent and anesthesia specific effects, these effects appeared to be secreted-factor specific and variable. For example, the less potent anesthetics, lidocaine and procaine, affected PGE2 but not IL-6 secretion, suggesting differential secretion regulation in the presence of both stimulation and specific anesthetics. The effects of exposure to both anesthetics and inflammatory environments are predicted to be regulated by changes in ROS production in the MSCs. These studies will ultimately assist in the development of local anesthetic administration protocols that are compatible with MSC therapeutic functions in wound healing and other applications.

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Fig. 1.

Cytotoxic effects of local anesthetics on MSCs. The bar heights represent the relative fluorescence intensities (RFI) of reduced alamar blue reagent at 4 h of incubation on unstimulated (gray bars) or $\text{TNF-}\alpha/\text{IFN-}\gamma$ -stimulated (25 ng/mL each, white bars) MSCs treated for 48 h with basal medium (0 μM) or medium containing 10 μM , 100 μM , 500 μM or 1000 μM of lidocaine, ropivacaine, procaine or bupivacaine. The data are the mean $\pm$ SEM of $n = 11$ –15 independent observations. *Statistically different ($p < 0.05$) from the respective basal medium control (0 μM). †Statistically significant difference ($p < 0.05$) between corresponding stimulated and unstimulated conditions.

Fig. 2.

Recovery of MSC proliferation after removal of local anesthetics. The RFI of reduced alamar blue reagent at 1–4 h and 24 h after the removal of the 48 h treatment conditions: basal medium, medium containing (a) 10 μM, (b) 100 μM, (c) 500 μM or (d) 1000 μM of the individual anesthetics, or medium with TNF-α/IFN-γ (25 ng/mL each) and (e) 10 μM, (f) 100 μM, (g) 500 μM or (h) 1000 μM of the individual anesthetics. Each data point is the mean ± SEM of $n = 11$ –15 independent observations. *Statistically different ($p < 0.05$) from the respective basal medium control (0 μM).

Fig. 3.

Effects of local anesthetics on the number of MSCs. The number Hoechst-stained nuclei present after treatment unstimulated (gray bars) or TNF- α /IFN- γ -stimulated (25 ng/mL each, white bars) MSCs for 48 h with medium containing 10 μ M, 100 μ M, 500 μ M or 1000 μ M of lidocaine, ropivacaine, procaine or bupivacaine were normalized to corresponding basal medium controls (0 μ M). The data are the mean $\pm$ SEM of $n = 11$ independent observations. The baseline cell counts in the medium control unstimulated and stimulated images were 947 ± 69 and 474 ± 27 , respectively. *Statistically different ($p < 0.05$) from the respective basal medium control (0 μ M). †Statistically significant difference ($p < 0.05$) between corresponding stimulated and unstimulated conditions.

Fig. 4.

Effects of local anesthetics on MSC secretion of IL-6. Levels of IL-6 quantified in cell culture supernatants collected from unstimulated (gray bars) or TNF- α /IFN- γ -stimulated (25 ng/mL each, white bars) MSCs treated for 48 h with medium containing 10 μ M, 100 μ M, 500 μ M or 1000 μ M of lidocaine, ropivacaine, procaine or bupivacaine were normalized to corresponding basal medium controls (0 μ M). The data are the mean $\pm$ SEM of $n = 11$ independent observations. The baseline IL-6 levels in the control unstimulated and stimulated conditions were 0.78 ± 0.15 ng/mL and 26.1 ± 1.7 ng/mL, respectively.

*Statistically different ($p < 0.05$) from respective the medium control (0 μ M). †Statistically significant difference ($p < 0.05$) between corresponding stimulated and unstimulated conditions.

Fig. 5.

Effects of local anesthetics on MSC secretion of PGE2. Levels of PGE2 quantified in cell culture supernatants collected from unstimulated (gray bars) or TNF- α /IFN- γ -stimulated (25 ng/mL each, white bars) MSCs treated for 48 h with medium containing 10 μ M or 1000 μ M of lidocaine, ropivacaine, procaine or bupivacaine were normalized to corresponding basal medium controls (0 μ M). Each bar is the mean $\pm$ SEM of $n = 11$ independent observations. The baseline PGE2 levels in the control unstimulated and stimulated images were 2.00 ± 0.02 ng/mL and 27.4μ 2.2 ng/mL, respectively. *Statistically different ($p < 0.05$) from the medium control (0 μ M). †Statistically significant difference ($p < 0.05$) between corresponding stimulated and unstimulated conditions.

Fig. 6.

Multiplex bead analysis of effects of local anesthetics on MSC secretion. The levels of secreted proteins that were measurable in the supernatants of (a) (b) unstimulated or (c) (d) TNF- α /IFN- γ -stimulated (25 ng/mL each) MSCs treated for 48 h with medium containing 1000 μ M of ropivacaine or bupivacaine were normalized to corresponding basal medium controls (0 μ M). Each bar is the mean $\pm$ SEM for $n = 8-11$ independent observations of each analyte in the assay. *Statistically different ($p < 0.05$) from the medium control.

Fig. 7.

Custom pathway established for the predicted upstream regulators in unstimulated MSC and ropivacaine/bupivacaine action. Tumor necrosis factor receptor superfamily, member 18 (TNFRSF18), adenosine A2b receptor (ADORA2B), proteinase 3 (PRTN3), jun proto-oncogene/activator protein 1 (JUN, AP-1) and IRF3 were identified by IPA Core Analysis function as the most likely upstream regulators for unstimulated MSC in the presence of local anesthetics. JNK and P38 MAPK were identified as mediators of local anesthetic actions by using the Path Explorer function which establishes relationships between nodes (gene products/molecules) by the action of mediators using the Ingenuity Knowledge Base. Solid lines indicate a direct relationship between nodes (gene products/molecules) and dashed lines indicate indirect relationships. Letters beside each line indicate the type of interaction between the nodes: **A**-Activation, **E**-Expression (includes metabolism/synthesis for chemicals), **I**-Inhibition, **LO**-Localization, **P**-Phosphorylation/dephosphorylation, **PP**-Protein-protein interactions and **T**-Transcription. Relationships legend can be found at <http://ingenuity.force.com/ipa/IPATutorials#>.

Fig. 8.

Custom pathway established for the predicted upstream regulators in TNF- α /IFN- γ -stimulated MSC and ropivacaine/bupivacaine action. Interleukin 1 beta (IL-1 β), TNF, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and AHR were identified by IPA Core Analysis function as the most likely upstream regulators for stimulated MSC in the presence of local anesthetics. Solid lines indicate direct relationship between nodes (gene products/molecules) and dashed lines indicate indirect relationships. Letters beside each line indicate the type of interaction between the nodes: **A**-Activation, **E**-Expression (includes metabolism/synthesis for chemicals), **I**-Inhibition, **LO**-Localization, **M**-Biochemical Modification, **P**-Phosphorylation/dephosphorylation, **PD**-Protein-DNA binding, **PP**-Protein-protein interactions, **RB**-Regulation of Binding, **T**-Transcription, **TR**-Translocation and **UB**-Ubiquitination. Relationships legend can be found at <http://ingenuity.force.com/ipa/IPATutorials#>.

Table 1

Baseline secretion of detectable factors from MSCs cultured for 48 h in medium (no stimulation), as measured in a multiplex immunoassay.

Analyte	Concentration (pg/mL)
IL-8	114 ± 10
IL-12(p70)	34.8 ± 5.0
MCP-1	402 ± 41
VEGF	1,135 ± 266

Table 2

Baseline secretion of detectable factors from MSCs cultured for 48 h in pro-inflammatory medium (TNF- α /IFN- γ , 25 ng/mL each), as measured in a multiplex immunoassay.

Analyte	Concentration (pg/mL)
IL-1RA	304 $\pm$ 15
IL-4	9.8 $\pm$ 0.3
IL-1 β	17.7 $\pm$ 1.0
IL-2	28.1 $\pm$ 1.2
IL-9	8.8 $\pm$ 1.3
IL-12(p70)	22.3 $\pm$ 2.5
IL-15	41.3 $\pm$ 1.7
IL-17	48.8 $\pm$ 4.0
VEGF	450 $\pm$ 48
G-CSF	115 $\pm$ 5
GM-CSF	20.3 $\pm$ 4.7
IL-8	63,334 $\pm$ 8,889
RANTES	17,899 $\pm$ 4,460