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Mechanical loading promotes mast cell degranulation via RGD-integrin dependent pathways

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Abstract

Mast cells are known to respond to a number of stimuli, such as IgE antibody–antigen complexes, pathogens, chemical compounds, and physical stimulation, resulting in the activation of these cells and subsequent release of cytokines, inflammatory mediators and granules which can influence the pathophysiology of neighboring cells. Although different forms of physical stimulation (i.e. shear stress and acupuncture) have been investigated, the effect of cyclic tensile loading on mast cell activation has not. To characterize the response of mast cells to tensile loading, RBL-2H3 cells were embedded in a 3-dimensional fibrin construct and subjected to 24 h of cyclic loading at 0%, 5% or 10% peak tensile strain. Mechanical loading significantly increased RBL-2H3 cell secretion of β -hexosaminidase (2.1- to 2.3-fold, respectively) in a load- and time-dependent manner when compared to the controls. Furthermore, no evidence of load-induced cell death or alterations in cell proliferation was observed. To determine if RGD-dependent integrins mediated the degranulation of mast cells during mechanical loading, cell–matrix interactions were inhibited by treating the cells with echistatin, a disintegrin that binds RGD-dependent integrins. Treatment with echistatin significantly attenuated load-induced degranulation without compromising cell viability. These results suggest a novel mechanism through which mechanical loading induces mast cell activation via RGD binding integrins.

Keywords

Mast cells; Mechanical load; Extracellular matrix; Integrin; Fibrin; 3D culture

1. Introduction

Classical mast cell activation occurs through the aggregation and cross-linking of the high affinity Fc ϵ R1 receptor by antigen-bound IgE antibodies, leading to degranulation and release of inflammatory mediators (Bischoff, 2007; Galli et al., 2008). Other studies have found mast cells to be activated by environmental (e.g., bacterial toxins, chemical agents), biochemical (e.g., complement component 5, endothelin-1) and physical (osmotic pressure, shear stress) stimuli (Cochrane and Douglas, 1973; Murray et al., 2004; Yang et al., 2007). Studies have also illustrated changes in mast cell accumulation in response to alterations in the mechanical environment in vivo. For example, increased mast cell density has been observed within hypertrophic dermal scars of mice subjected to mechanical stress (Aarabi et al., 2007) and within the soleus muscles of rats subjected to hindlimb unloading (Dumont et

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Conflict of interest

None of the authors have a conflict of interest.

al., 2007). Furthermore, many cardiovascular diseases (e.g. volume overload, hypertension, and myocardial infarctions) exhibit altered mechanical loading and impaired contractile function (Gupta and Grande-Allen, 2006; Abraham et al., 2007), followed by an increase in mast cell activation and density. To date, the cellular and molecular mechanisms mediating mast cell response to physical stimuli have received little attention.

The mechanical loads applied to extracellular matrices (ECM) are essential in maintaining tissue homeostasis (Riser et al., 1992; Wang et al., 1993; Gupta and Grande-Allen, 2006); however deviations in matrix mechanics can activate pathological signaling pathways and/or induce cellular stress responses (O'Donnell et al., 1988; Duranti et al., 1995; Gupta and Grande-Allen, 2006). Numerous pathways have been identified through which mechanical forces are transduced into biological signals (Hamill and Martinac, 2001). Cell surface adhesion molecules, including integrins, serve critical roles in transmitting mechanical forces from the ECM to cells (Wang et al., 1993; Wilson et al., 1995; Ingber, 2006). Integrins are heterodimeric transmembrane glycoproteins composed of α and β subunits (Arnaout et al., 2005), and members of the $\beta 1$ and $\beta 3$ subfamilies bind ECM proteins containing an arginine-glycine-aspartic acid (RGD) sequence (e.g. fibronectin, laminin and fibrin). These receptor–ligand interactions mediate outside-in signaling (e.g. mechanotransduction), thus allowing cells to perceive changes in the deformation and stiffness of the ECM (Gao et al., 2007; Kock et al., 2009). Although mast cells express members of the RGD-integrin family, including $\alpha 5 \beta 1$, $\alpha \nu \beta 3$ and $\alpha_{IIb} \beta 3$ (Ra et al., 1994; Yasuda et al., 1995), and most of the *in vivo* studies previously mentioned correlate changes in mast cell number with alterations in mechanical load, the role of integrins in the response of mast cells to mechanical loading have not been investigated.

The present studies utilized a novel 3-dimensional model system to evaluate the effects of uniaxial cyclic mechanical load on mast cell degranulation to elucidate the role of integrin–ECM interactions in modulating these effects. By varying the peak strains applied to the constructs, load-dependent cell degranulation was observed. The mechanism mediating this load-dependent degranulation was investigated using a competitive inhibitor of RGD-integrins. Our results suggest a novel mechanism by which mechanical loading induces mast cell degranulation via RGD-integrins.

2. Materials and methods

All chemicals were purchased from Sigma (Sigma Chemical Co., St. Louis, MO) except where noted.

2.1. Cell culture

RBL-2H3 cells, a rat basophilic leukemia cell line commonly used as a model system for studying mast cell degranulation, were a gift from Dr. David Holowka (Cornell University; Ithaca, NY) and cultured in Dulbecco's Modified Eagles Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% newborn bovine serum, 5% fetal bovine serum, 100 U/mL penicillin G, 100 μ g/mL streptomycin, and 10 μ g/mL gentamicin (Invitrogen) at 5% CO₂ and 37 °C. Prior to suspension in 3D fibrin gels, cells were rinsed with Moscona's saline buffer (1.4 M NaCl, 390 mM KCl, 120 mM NaHCO₃, 94 mM glucose, 2 mM NaH₂PO₄), trypsinized [0.25% trypsin, 1 mM ethylenediaminetetraacetic acid (EDTA); Invitrogen] and counted. Fibrin was chosen as the 3D matrix for use in these studies because of its durability, biodegradability, increasing popularity as a tissue engineering scaffold and it is an endogenous ECM component that mast cells encounter (e.g. it is part of the provisional ECM deposited after a myocardial infarction and during wound healing).

2.2. Mechanical loading studies

A device for applying cyclic tension to 3D cell-fibrin constructs was adapted from a previously published system (Vanderploeg, 2004). Two rigs, each capable of loading up to 12 constructs simultaneously, were independently controlled by a single motion controller (Newmark Systems, Mission Viejo, CA) and a PC (Fig. 1, A). Each rig consisted of a polysulfone/polyether ether ketone “rake” attached to an integrated stepper motor and linear stage (Newmark Systems) and a polysulfone culture tray. A rotary encoder permitted high-resolution ($\sim 0.02 \mu\text{m}$) control of stage position. Trays were protected from contamination by a polycarbonate shield and both rigs fit into a standard tissue culture incubator.

Cell-fibrin constructs were attached to the posts via porous polyethylene (PE) endblocks (hydrophilic, 45–90 μm pore size; Porex, Fairburn, GA), and were prepared by casting a cell-fibrinogen suspension in rectangular molds with the two endblocks (Fig. 1, B). To characterize the mechanics of fibrin construct deformation, 2D strain fields were generated via particle tracking and image correlation using Vic2D software (Correlated Solutions, Columbia, SC). Particles were dispersed on the surface of a construct and the construct subjected to stepwise increases in nominal strain (strain calculated from prescribed displacement of the linear stage). Digital images of the construct were captured at each step, and a subset of the construct surface was analyzed for strains in the x - y plane. Measured strains along the axis of stretch (ϵ_{xx}) were near-uniform in the central portion of the constructs and consistent with the nominal strains (within 1–4% over the strains examined). Measured strains in the y -axis (ϵ_{yy}) were negative (indicative of compression) due to Poisson’s effect and nearly uniform along the length of the construct. In regions nearest the endblocks, both ϵ_{xx} and ϵ_{yy} appeared to depart from central-region values. Shear strains in the x - y plane (ϵ_{xy}) were consistently low (< 0.006) across the area of analysis. Strains in the third dimension (ϵ_{zz}) were not measured in this experiment, but would be expected to exhibit a Poisson effect similar to that observed in ϵ_{yy} . Collectively, these data demonstrate that the loading rigs are capable of generating controlled, well-defined tensile strains in 3D fibrin constructs.

To prepare 3D fibrin constructs, autoclaved PE endblocks were seated in casting molds pre-coated with bovine serum albumin (BSA). RBL-2H3 cells were suspended at a density of 3×10^6 cells/mL in phenol red free/serum free media (PSF with 100 U/mL penicillin G, 100 $\mu\text{g/mL}$ streptomycin). Fibrinogen (14.1 mg/mL) and thrombin (20 U/mL; 10% final volume; Millipore, Billerica, MA) were added to the cell suspension to produce a fibrin gel construct of approximately 10 mg/mL clottable fibrin. For inhibition studies, RBL-2H3 cells were incubated for 30 min at room temperature with 0 nM, 0.05 nM, 0.1 nM, or 0.2 nM echistatin and resuspended in PSF media, fibrinogen and thrombin as noted above. The cell-fibrinogen suspension was pipetted into the casting wells and allowed to polymerize for 1 h. After polymerization, fibrin gels were detached and floated in PSF culture medium for 24 h prior to loading. The 3D fibrin constructs were attached to the loading apparatus (See Fig. 1) and incubated in PSF medium or PSF-echistatin while subjected to continuous cyclic loading at 5% or 10% peak tensile strain and a frequency of 1.2 Hz for 24 or 48 h in a tissue culture incubator. Control constructs were cultured under identical conditions in the absence of loading (denoted as 0% strain). Culture media was collected every 4, 12, 24 and 48 h and replaced with fresh media every 24 h.

2.3. RBL-2H3 integrin expression

To assess RBL-2H3 integrin expression, culture dishes were coated with collagen type 1 (2.4 mg/mL), fibrinogen (28 mg/mL), or fibrin (10 mg/mL) before being seeded with RBL-2H3 cells. To extract protein, cell lysis buffer (25 mM Tris (Biospectra, Inc, Stroudsburg, PA), 22 mM NaCl, 25 mM NaF, 5% glycerol (FisherScientific, Pittsburgh,

PA), 0.5% nonidet P40, 0.025% sodium deoxycholate) was added to each dish and cells were lysed by scraping. Lysates were centrifuged and total protein concentration determined using a BCA assay (BioRad). Equal amounts of protein (30 μ g/lane) were loaded onto 8% SDS-PAGE gels (Thermo-Scientific; Rockford, IL) and subjected to electrophoresis followed by western blotting using a rabbit α -rat β 1 integrin (1:1000; N-20; Santa Cruz, Santa Cruz, CA) and mouse α -rat β 3 integrin (1:1000; H-96; Santa Cruz). Protein bands were detected using donkey α -rabbit (NA034V; GE Healthcare, Little Chalfont Buckinghamshire, UK) or sheep α -mouse (NXA931; GE Healthcare) HRP conjugated secondary antibodies and West Femto (ThermoScientific) chemiluminescent reagent.

2.4. Measuring RBL-2H3 cell activation by β -hexosaminidase activity

RBL-2H3 degranulation was assessed by the release of β -hexosaminidase into culture media (Schwartz et al., 1979; Serebriiskii and Golemis, 2000). Media (50 μ L) was mixed with 1 mM p-Nitrophenyl-N-acetyl- β -D-glucosaminide (NA β G; EMD Biosciences, La Jolla, CA), a β -hexosaminidase substrate, and incubated at 37 $^{\circ}$ C for 1 h. The reaction was quenched with 0.05 M sodium bicarbonate and absorbance read at 405 nm using a Benchmark Plus (Biorad) platereader.

2.5. Toluidine blue stain

To visualize RBL-2H3 morphology, cell-fibrin constructs were fixed in 4% paraformaldehyde and cut into thin sections. Sliced gels were stained with Toluidine Blue (Fisher) in 70% ethanol, 1% sodium chloride (Fisher). After washing, slices were dehydrated quickly in 95% and 100% ethanol and cleared in xylene before being mounted and imaged on a ME600 light microscope (Nikon, Japan).

2.6. Proliferation assay

Cellular proliferation was assessed using 5-Bromo-2'-deoxy Uridine (BrdU) Labeling Detection Kit (Roche; Indianapolis, IN) according to the manufacturer's directions. Briefly, 3D fibrin-RBL-2H3 constructs were prepared as described above, placed in the loading apparatus and incubated with PSF media containing BrdU labeling reagent. After 24 and 48 h, gels were washed and fixed in 50 mM glycine:ethanol solution. For staining, constructs were permeabilized with 0.01% Triton X-100/0.1 M glycine/PBS, blocked with 5% BSA/PBS and stained with a mouse anti-BrdU antibody. Incorporation of BrdU was detected using a fluorescein anti-mouse IgG and nuclei were visualized by staining with 6-diamidino-2-phenylindole (DAPI). Stained gels were imaged on a 510LSM confocal laser scanning microscope (Carl Zeiss Microimaging, Thornwood, NY). The percent cell proliferation was calculated as the number of FITC positive cells divided by the total number of cells as determined by DAPI staining.

2.7. Cell viability

RBL-2H3 viability was examined using a Live:Dead Assay kit (Marker Gene Technologies, Inc) according to the manufacturers' instructions. Before removing constructs from the loading apparatus fluorescent reagents (4 mM carboxyfluorescein diacetate and 2 mM propidium iodide) were added to the media and incubated for 40 min. Stained gels were imaged on a 510LSM confocal laser scanning microscope (Carl Zeiss) and percent cell viability calculated as the number of viable cells divided by the total number of cells (viable and dead).

2.8. Statistical analysis

All assays were carried out on 3 independent experiments (per time point and per loading condition), with 3 replicates in each experiment except where noted. For analysis of

confocal images, five random fields were chosen from each image. Data was analyzed by ANOVA and Tukey's method of multiple comparisons. Differences were deemed significant for $p < 0.05$.

3. Results

3.1. Mechanical loading induces degranulation of RBL-2H3 cells

RBL-2H3 cells undergo degranulation and release of pro-inflammatory mediators when stimulated. To investigate whether mechanical loading can induce degranulation, RBL-2H3 cells were seeded in 3D fibrin scaffolds, subjected to 1.2 Hz cyclic loading at 5% or 10% peak strain and 0% (static control) for 4, 12 and 24 h (Fig. 1), and assayed for degranulation via β -hexosaminidase release (Fig. 2). RBL-2H3-fibrin constructs deformed to peak tensile strains of 5% or 10% demonstrated significant ($p < 0.01$) time-dependent increases in β -hexosaminidase release (Fig. 2A). The release of β -hexosaminidase from loaded constructs was statistically similar to static controls for 4 h and 12 h durations of loading, but by 24 h loaded constructs exhibited significantly greater (50% and 43%, respectively) release of this degranulation marker than the static controls ($p < 0.01$). For comparison, RBL-2H3 cells were treated with antigen-bound IgE, representing classical, physiological mast cell activation and degranulation, and the release of β -hexosaminidase measured. RBL-2H3 cells subjected to mechanical loading released similar levels of β -hexosaminidase compared to IgE stimulated cells (data not shown), suggesting that mechanical environment may be as effective as the classical activation pathway in stimulating mast cell degranulation.

The morphology of RBL-2H3 cells was examined by Toluidine Blue staining (Fig. 2B). After 24 h, RBL-2H3 cells in 0% control constructs were uniformly circular (Fig. 2B-I). In contrast, cells subjected to cyclic loading at peak strains of 5% and 10% appeared irregularly shaped (Fig. 2B-II and B-III respectively), with granules, stained deep blue, observed at the periphery of these cells (indicated by arrows). In static controls, granules were confined to the intracellular compartment, suggesting that mechanical stimulation elicits release of granules from the cell.

3.2. Mechanical loading does not alter RBL-2H3 viability or proliferation

To confirm that increased degranulation was due to a specific cellular response to the mechanical stimuli and not caused by alterations in cell number RBL-2H3 viability and proliferation were evaluated. Fig. 3A shows representative confocal micrographs assessing RBL-2H3 viability after 0%, 5% or 10% mechanical loading for 24 and 48 h. Approximately 70% of RBL cells were viable after 24 h and 48 h of loading (Fig. 3B), with no significant differences between loading conditions. Fig. 4A shows representative confocal micrographs of BrdU positive RBL-2H3 cells subjected to 0%, 5% or 10% mechanical loading for 24 and 48 h. Examination of BrdU labeling indicated that there were no loading-dependent alterations in RBL cell proliferation, with 30–35% of cells proliferating in all loading conditions (Fig. 4B). Taken together, these results indicate that mechanical loading does not alter the viability or proliferation of RBL-2H3 cells.

3.3. Integrins as mediators of load-induced degranulation

Western blotting revealed that RBL-2H3 cells express integrins β_3 and β_1 (Fig. 5A). To determine if these integrins were involved in transmitting mechanical signals mediating degranulation, RBL-2H3 cells were treated with the RGD inhibitor, echistatin. Echistatin competitively binds $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ integrins, inhibiting integrin–matrix interactions (Marcinkiewicz et al., 1996; Wierzbicka-Patynowski et al., 1999). Cell–fibrin constructs were treated with varying concentrations of echistatin (0, 0.05, 0.1 or 0.2 nM) and subjected to mechanical loading for 4 or 24 h followed by analysis of β -hexosaminidase activity (Fig.

5). As observed previously, untreated samples (0 nM echistatin) cyclically loaded to peak strains of 5% (Fig. 5C) or 10% (Fig. 5D) for 24 h exhibited significant ($p < 0.05$) increases in β -hexosaminidase activity compared to static controls (Fig. 5B). Static controls (Fig. 5B) showed no differences in release of β -hexosaminidase over time or with increasing doses of echistatin. For constructs subjected to 24 h of loading, statistically significant dose-dependent decreases in β -hexosaminidase activity were observed (Fig. 5C and D). At the highest concentration of echistatin tested, 0.2 nM, degranulation was reduced to levels observed in static controls. This observed decrease in degranulation was not due to echistatin cytotoxicity, as cell viability assays conducted on constructs treated with 0.2 nM echistatin showed no significant difference in cell viability compared to non-treated (0 nM) controls (data not shown). These results demonstrate that disrupting RGD-integrin-fibrin interactions significantly impaired RBL-2H3 degranulation in response to mechanical stimulation.

4. Discussion

Several studies have illustrated that mechanical stresses, such as compression or tension, can elicit cellular responses (Carver et al., 1991; Smith et al., 1997; MacKenna et al., 1998; Bishop and Lindahl, 1999; Li et al., 2003; Barbee et al., 1994). The goal of this study was to determine if mechanical loading stimulates activation and degranulation of mast cells. Our results demonstrate that RBL-2H3 cells degranulate when subjected to 5% or 10% strain, and although there were no detectable differences in activation between these strains, it remains possible that lower strain magnitudes could reveal a step or dose-like response to mechanical load. Our data also demonstrated that treatment with 0.2 nM echistatin reduced load-induced degranulation of RBL-2H3 cells to static control levels, suggesting that the cells' response to loading is mediated exclusively by RGD-binding integrins.

No significant differences were observed in cell viability between loaded and static groups in the presence or absence of echistatin indicating that mechanical loading nor echistatin treatments promoted membrane leakage or rupture, and therefore did not influence the observed load-dependent increases in degranulation. Although a typical consequence of compromised cell-ECM interactions is cell death, RBL cells have been shown to be viable in non-adherent conditions (Grodzki et al., 2003). This is a result that is consistent with our findings. Proliferation in loaded constructs was comparable to that in the static controls, also suggesting that the load-induced increase in β -hexosaminidase release was not due to changes in overall cell number.

This study is the first to investigate the effects of cyclic loading on RBL-2H3 cells however various studies have examined how other forms of mechanical stress stimulate mast cell activation. Mast cells undergoing micropipette aspiration exhibited transient calcium increase and cytoskeletal reorganization (Horoyan et al., 1990), while cells subjected to low power laser irradiation, shear stress and excessive heating demonstrated increased intracellular Ca^{2+} levels and histamine release (Yang et al., 2007; Zhang et al., 2008). In our studies it was not possible to examine cytoplasmic Ca^{2+} changes due to the design of our loading system and the thickness of the 3D fibrin constructs. However, a number of mediators are released upon mast cell degranulation, including β -hexosaminidase (Kalinin, 1987; Ohno et al., 1990), which is a commonly used and well-accepted marker of degranulation (Schwartz et al., 1979; Serebriiskii and Golemis, 2000).

The peak strains used in this study were selected within ranges of previously published data that specifically used uniaxial mechanical loading. (Sadoshima et al., 1992; Carver et al., 1991; Vanderploeg, 2004). Furthermore, the peak strains and frequency used in our experiments are similar to those experienced by cells within the cardiac wall (~10% strain in

magnitude) (Lee and McCulloch, 1997; Gupta and Grande-Allen, 2006); therefore it is possible for mast cells to encounter these loading strains and frequency in vivo. By extending these studies to include tissue-specific loading regimens (e.g., alternative frequencies and duty cycles), multiple ECM components (such as collagen), and partner cell types (e.g., fibroblasts or myocytes), we will gain substantial insight into the microenvironmental cues that regulate mast cell degranulation.

One limitation of our model system is that due to the material properties of the fibrin scaffold and the uniaxial loading mode, the mechanical stimuli delivered to the cells in these studies were effectively multiaxial and rather complex. In calibration experiments in which we mapped steady-state strains in 2D, the measured shear strains were generally low ($\epsilon_{xy} \sim 0.6\%$ for nominal $\epsilon_{xx} = 10\%$). However, consistent with the Poisson effect, we observed considerable compressive strains in a direction transverse to the primary axis of applied (tensile) deformation. Poisson's ratio (ν) for fibrin hydrogels has been reported to be in the range of 0.25–0.3, and we measured a mean transverse strain of -3.7% for a measured $\epsilon_{xx} = 9.6\%$, yielding a measured $\nu = 0.39$. With these characteristics of the applied displacements, we cannot determine whether the loading-induced degranulation is a response to compressive, tensile, or the combination of tensile and compressive strains.

Our results suggest a novel mechanism for mast cell activation in which changes in a tissue's mechanical environment can result in degranulation. While the β integrin subunits involved in mediating RBL cell mechanotransduction have been identified, further studies will delineate any differences in RGD-integrin specificity or RBL cell mechanotransduction response within alternate 3D matrices (i.e. collagen, fibronectin). Mast cells are increasingly associated with pathophysiological conditions, such as cardiovascular (Kelley et al., 2000; Brower and Janicki, 2005; Levick et al., 2009) and pulmonary diseases (O'Donnell et al., 1988; Duranti et al., 1995); therefore identifying and understanding alternative pathways involved in their activation may further define the role of mast cells in disease.

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Fig. 1.

Description and characterization of devices for applying cyclic tension to 3D cell-fibrin constructs. A: Two custom-made rigs (c) were independently controlled by a Newmark Systems NSC-M (a) and a PC (b). Each rig consisted of a Newmark Systems Microslide linear stage (d), a polysulfone "rake" (e), and a polysulfone culture tray (f) with 12 slots. The stage and tray were mounted on a polycarbonate base and a polycarbonate cover (c), visible in the top panel, was added to prevent contamination. Constructs were loaded between fixed posts (g) in the tray and polyetheretherketone posts (h) in the mobile rake. B: Constructs (2 cm × 1 cm × 0.635 cm) were prepared by polymerizing the fibrin in a mold with porous polyethylene endblocks. Holes in the endblocks permit rigid fixation to fixed and mobile posts. Strains in the x and y directions were determined by tracking particles on the construct surface with Vic2D image correlation software. Images of the undeformed (reference) and deformed construct are shown in the left column. The middle column shows the magnitudes of ϵ_{xx} (denoted by color, superimposed on the reference image) for each step in applied strain. The right column similarly shows the magnitudes of strain in the y direction (ϵ_{yy}) and indicates a stepwise increase in compression along the y -axis due to Poisson's effect. C: Measured strains along the axis of deformation agreed well with the nominal strains (within 1–4% for strain magnitudes of 0.1–0.25), and transverse strains (ϵ_{yy}) increased in magnitude with applied tensile strains. Error bars indicate the standard deviation of measured strains within the analyzed region. Dashed blue line indicates measured=nominal. (D) Schematic of prescribed sawtooth waveforms for loading studies. Constructs were loaded dynamically at 1.2 Hz to peak strains of 0%, 5%, or 10%.

Fig. 2.

Mechanical loading induced RBL-2H3 degranulation. A: Significant increases in RBL-2H3 degranulation were detected upon 5% and 10% peak strain when compared to constructs subjected to 0% strain at 24 h ($*p=0.002$). In addition, a time-dependent increase in β -Hexosaminidase activity with 5% and 10% peak tensile strain was also observed (black line above bars). Data are mean values $\pm$ SD from three independent runs. Statistical significance ($p < 0.05$) was determined using ANOVA and Tukey post-hoc test. Error bars represent the standard deviation in the samples. B: Toluidine blue stains of RBL-2H3 cells in fibrin constructs show degranulation. There is a noticeable difference in the abundance of granules (indicated by arrows) found around the cell periphery and the cellular structure of the RBL-2H3 cells seeded in constructs subjected to 0% (I) cyclic tension when compared to 5% (II) or 10% peak strain (III). These images further suggest mast cell degranulation can be induced by cyclic tension. Images were taken at $50\times$ objective on a Nikon Eclipse ME600 light microscope and scale bars are $20\text{ }\mu\text{m}$.

Fig. 3.

Mechanical loading does not compromise cell viability. A: Representative confocal images of RBL-2H3 cells stained during cyclic loading. RBL-2H3 cells were stained with carboxyfluorescein diacetate (green) to visualize viable cells and propidium iodide (red) to visualize non-viable cells (arrow). Constructs subjected to 5% (II and V) and 10% (III and VI) peak strain did not exhibit increased apoptosis when compared to the controls (I and IV). B: Quantitative analysis of cell viability assay showed no significant difference at 24 h and 48 h in cell viability (just under 70%) between the RBL-2H3 seeded constructs subjected to 0%, 5% and 10% peak strain, implying that mechanical load-induced cell degranulation is not due to cell death. Data are mean values $\pm$ SD from three independent

runs. Statistical significance ($p < 0.05$) was determined using ANOVA and Tukey post-hoc test. Error bars represent the standard deviation in the samples and scale bars is 100 μm .

Fig. 4.

Mechanical loading does not induce RBL-2H3 proliferation. A: Representative confocal images of proliferating RBL-2H3 cells during cyclic loading. RBL-2H3 cells were stained with an anti-BrdU antibody (green) and a nuclear stain, DAPI (blue), to visualize proliferation. Constructs subjected to 5% (II and V) and 10% (III and VI) peak strain showed no change in proliferation when compared to the controls (I and IV); arrows depict proliferating cells. B: Quantitative analysis of proliferation assay showed no significant difference in proliferation between the RBL-2H3 seeded constructs subjected to 0%, 5% and 10% peak strain; therefore the observed increased in degranulation is likely not associated with alterations in proliferation. Data are mean values $\pm$ SD from three independent runs.

Statistical significance ($p < 0.05$) was determined using ANOVA and Tukey post-hoc test. Error bars represent the standard deviation in the samples and scale bars is 50 μm .

Fig. 5.

Time and dose-dependent inhibition of RBL-2H3 cell degranulation via RGD-integrins by Echistatin during cyclic tension. (A) RBL-2H3 cells were plated on fibrin (10 mg/mL; FBN), fibrinogen (28 mg/mL; FBGN) or collagen type 1 (2.4 mg/mL; COL 1) and mouse α -rat $\beta 3$ integrin and rabbit α -rat $\beta 1$ integrin antibodies were used to identify $\beta 1$ and $\beta 3$ integrin protein. (B, C and D) β -Hexosaminidase assay with echistatin treatment at 0% (B), 5% (C) and 10% (D) peak tensile strain. Significant increases in RBL-2H3 degranulation were detected between 4 and 24 h from constructs treated with 0 nM, 0.05 nM and 0.1 nM Echistatin ($p < 0.05$; represented by *). At 24 h, significant decreases in degranulation were observed as Echistatin concentration increased when compared to the 0 nM treated constructs ($p < 0.05$; represented by #). Data are mean values $\pm$ SD from three independent runs. Statistical significance ($p < 0.05$) was determined using ANOVA and Tukey post-hoc test. Error bars represent the standard deviation in the samples.