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Inhibition of Fas-Fas Ligand Interaction Attenuates Microvascular Hyperpermeability Following Hemorrhagic Shock

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Abstract

Hemorrhagic shock (HS) induced microvascular hyperpermeability poses a serious challenge in the management of trauma patients. Microvascular hyperpermeability occurs mainly due to the disruption of endothelial cell adherens junctions, where the 'intrinsic' apoptotic signaling plays a regulatory role. The purpose of this study was to understand the role of the 'extrinsic' apoptotic signaling molecules, particularly Fas-Fas ligand interaction in microvascular endothelial barrier integrity. Rat lung microvascular endothelial cells (RLMECs) were exposed to HS serum in the presence or absence of the Fas ligand inhibitor, FasFc. The effect of HS serum on Fas receptor and Fas ligand expression on RLMECs was determined by flow cytometry. Endothelial cell permeability was determined by monolayer permeability assay and the barrier integrity by β -catenin immunofluorescence. Mitochondrial ROS formation was determined using dihydrorhodamine 123 probe by fluorescent microscopy. Mitochondrial transmembrane potential (MTP) was studied by fluorescent microscopy as well as flow cytometry. Caspase-3 enzyme activity was assayed fluorometrically. RLMECs exposed to HS serum showed increase in Fas receptor and Fas ligand expression levels. FasFc treatment showed protection against HS serum induced disruption of the adherens junctions, and monolayer hyperpermeability ($p < 0.05$), in the endothelial cells. Pretreatment with FasFc also decreased HS serum induced increase in mitochondrial ROS formation, restored HS serum induced drop in MTP, and reduced HS serum induced caspase-3 activity in RLMECs. These findings open new avenues for drug development to manage HS induced microvascular hyperpermeability by targeting the Fas-Fas ligand mediated pathway.

INTRODUCTION

Hemorrhagic shock (HS) followed by resuscitation leads to the release of various extracellular cytokines such as tumor necrosis factor- α (TNF- α) and interleukins, as well as up-regulation of intracellular pro-apoptotic molecule, BAK (1-3). Previous work from our laboratory has shown involvement of 'intrinsic' or mitochondrial mediated apoptotic signaling in HS induced microvascular hyperpermeability (2).

Apoptosis is carried out by a cascade of caspases, which can be activated either by 'extrinsic' / receptor mediated or 'intrinsic' / mitochondria mediated pathways (4). The extrinsic or the receptor mediated apoptotic pathway gets initiated, when the cell surface receptors or death receptors of the TNF receptor superfamily such as Fas receptor, binds to

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their respective ligands namely Fas ligand (4-6). On activation by Fas ligand, Fas receptors show aggregation and recruitment of the adaptor molecule Fas-associated death domain (FADD) and procaspase-8 to form a complex known as the Death Inducing Signaling Complex (DISC) (4,6). Procaspase-8 on binding to FADD becomes active caspase-8 and initiates apoptosis by directly activating the downstream effector caspase-3 leading to cell death (4,6). The intrinsic or mitochondrial mediated apoptotic pathway begins when apoptotic signal directly approaches mitochondria resulting in increase in mitochondrial ROS formation, collapse in the mitochondrial transmembrane potential, and release of apoptogenic factor cytochrome *c*. The cytochrome *c* in turn triggers a caspase cascade resulting in activation of effector caspase-3 leading to cell death (2,4,7).

Fas ligand (CD95-L/APO-1L/CD178), a member of the TNF family of type 2 membrane proteins, is predominantly expressed by activated T lymphocytes, natural killer (NK) cells, and in immune privileged tissues such as the eyes and testicles (5,6). Fas receptor (TNFR6/CD95/APO-1) is a member of the TNF family of type I membrane receptors expressed on many tissues such as cardiac, kidney, lung, and liver as well as on vascular endothelial cells (8-13). The engagement of Fas receptor by Fas ligand is implicated in many physiological and pathological processes (6,8-15). Previous studies have shown that Fas receptor and Fas ligand (Fas-Fas ligand) are upregulated on cardiac myocytes during ischemia-reperfusion (I/R) injury (8). Overexpression of Fas-Fas ligand on lung epithelial cells has been shown to play a major role in the pathogenesis of acute respiratory distress syndrome (ARDS) (9). Fas-Fas ligand system also gets activated after blunt chest trauma giving rise to inflammatory response and lung contusion (10). Fas-Fas ligand is also involved in the apoptosis of renal microvascular endothelial cells during acute renal failure due to I/R injury (11). The objective of this study is twofold; first, to determine the role of Fas-Fas ligand in HS induced microvascular endothelial cell hyperpermeability, and secondly, to determine the effect of inhibition of Fas-Fas ligand interaction on HS induced microvascular endothelial cell hyperpermeability.

Recent studies have shown that Fas-Fas ligand mediated apoptosis may lead to endothelial cell dysfunction and loss of hepatic sinusoidal endothelial cells, as well (12-14). It is also known that when endothelial cells are exposed to TNF- α or oxidative stress (H_2O_2), they show upregulation of Fas-Fas ligand expression on the cell surface (14). However, the precise role of Fas-Fas ligand during HS induced microvascular hyperpermeability is not known. In this study we have used a recombinant Fas/TNFRSF6/CD95 Fc (FasFc) chimeric / fusion protein composed of the extracellular domain of Fas receptor linked to the Fc region of human immunoglobulin G subclass 1 (IgG1) (15). FasFc is similar to a well-known therapeutic drug etanercept, a TNF- α receptor blocker, in the mechanism by which it binds to its specific ligand. The drug etanercept is produced by linking the extracellular domain of TNF- α receptor 2 with Fc region of human IgG1 (16). Etanercept blocks the TNF- α receptor-ligand interaction by binding to the TNF- α ligand and so it is frequently used in the treatment of rheumatoid arthritis, ankylosing spondylitis, psoriasis and other autoimmune diseases (16). Similarly, FasFc competitively binds to the Fas ligand as a decoy receptor preventing Fas-Fas ligand interaction and subsequent apoptotic signal transduction (15,17). Fas ligand engagement by FasFc, stopped $CD4^+$ T cell proliferation and cytokine secretion in one study (17).

In the current study, we have postulated that FasFc would attenuate hemorrhagic shock serum induced microvascular endothelial cell hyperpermeability by blocking the Fas-Fas ligand system. HS serum has been implicated as an important mediator of HS induced microvascular hyperpermeability (18).

METHODS

Collection of serum from hemorrhagic shock/ sham rat model

Hemorrhagic shock (HS) serum was obtained from male Sprague-Dawley rats weighing 275-325 gms (Charles River Laboratories International, Inc, Wilmington, MA). The animals were anesthetized using urethane (1.5g/kg). The right internal jugular vein was cannulated using polyethylene tubing (PE-50, 0.58 mm ID) for fluid (normal saline; 2 ml/hr), right carotid artery was cannulated for withdrawing blood until mean arterial pressure (MAP) dropped to 40 mmHg from 90 mmHg to simulate stage IV HS condition and the left femoral artery was connected to a blood pressure (BP) analyzer for BP monitoring. The HS condition was maintained for 60 minutes followed by resuscitation for 60 minutes by its own blood and then blood was collected for separating the shock serum. For obtaining sham serum, rats were subjected to the same procedures above except inducing HS, followed by blood collection, and serum separation. *In vitro* experiments were performed using sham serum and HS serum collected from a hemorrhagic shock rat model as described above at dilution ratio of 1:2 with sterile PBS.

Monolayer permeability assay

Rat lung microvascular endothelial cells (RLMECs) (VEC-technologies, Rensselaer, NY), were grown as monolayers on fibronectin (Sigma-Aldrich, St. Louis, MO) coated Corning's Transwell plates using MCDB-131 complete media (VEC-technologies, Rensselaer, NY). Sixty minutes prior to the start of the experiments, the monolayers were exposed to fresh media without phenol red. All treatments and pre-treatments were carried out for 60 minutes each. The treatment of the monolayers were followed by adding fluorescein isothiocyanate-bovine albumin, (FITC-albumin) (5 mg/ml, Sigma-Aldrich, St. Louis, MO), to the luminal (upper) chamber of the Transwell and was allowed to equilibrate for 30 minutes. The samples (100 µl) collected from the abluminal (lower) chambers were analyzed for FITC-albumin fluorescent intensity using a fluorometric plate reader at excitation/emission 494 nm/520 nm.

Effect of hemorrhagic shock serum on Fas receptor-ligand expression levels on endothelial cells

To study the expression levels of Fas receptor and Fas ligand on RLMECs, a control or sham serum treatment group and HS serum treated group were utilized. Identical numbers of cells were incubated with primary antibody against Fas receptor and Fas ligand (Santa Cruz Biotechnology, Santa Cruz, CA), for 20 minutes at room temperature followed by incubation with respective FITC tagged secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), for 20 minutes at 37°C with 5% CO₂ in the dark and then evaluated by using a Becton Dickinson FACS Canto II flow cytometer. The flow data were obtained by using three replicates of stained and unstained samples from each group. The data were analyzed by using FlowJO flow cytometry analysis software.

Effect of Fas ligand on endothelial cell monolayer permeability

RLMECs were grown as monolayers on fibronectin coated Transwell plates. The following groups were studied: an untreated control group, recombinant Fas ligand (R&D system, Minneapolis, MN), treated groups at increasing concentrations of 5 ng/ml, 10 ng/ml, 25 ng/ml, 50 ng/ml and 100 ng/ml. Briefly, FITC-albumin (5 mg/ml) was added to the luminal (upper) chamber of the Transwell and was allowed to equilibrate for 30 minutes. The samples (100 µl) collected from the abluminal (lower) chambers were analyzed for FITC fluorescent intensity using a fluorometric plate reader at excitation/emission 494 nm/520 nm.

Effect of FasFc on Fas ligand induced increased in microvascular endothelial cell hyperpermeability

RLMEC monolayers grown on Transwell plates were used. The following groups were studied: an untreated control group, recombinant Fas ligand (10 ng/ml; 60 minutes) treated group, recombinant Fas ligand group pre-treated (60 minutes) with FasFc (50 ng/ml; 60 minutes) (R&D system, Minneapolis, MN), and FasFc (50 ng/ml; 60 minutes) alone group. Monolayer permeability was determined as described above.

Effect of caspase-3 inhibitor on Fas ligand induced increase in microvascular endothelial cell hyperpermeability

RLMEC monolayers grown on Transwell plates used. The following groups were studied: an untreated control group, recombinant Fas ligand (10 ng/ml) treated group, recombinant Fas ligand (10 ng/ml) group pre-treated with caspase-3 inhibitor Z-DEVD-FMK (100 μ M) (R&D system, Minneapolis, MN), and Z-DEVD-FMK (100 μ M) alone group. Sixty minutes prior to the experiments, the monolayers were exposed to fresh media without phenol red. Monolayer permeability was determined as described above.

Effect of FasFc on hemorrhagic shock serum induced microvascular endothelial cell hyperpermeability

RLMEC monolayers grown on Transwell plates used. The following groups were studied: a control or sham serum group, HS serum treated group, HS serum group pre-treated with FasFc (50 ng/ml) and FasFc (50 ng/ml) alone group. The monolayers were treated with FasFc (50 ng/ml), for 60 minutes followed by treatment with HS serum for 60 minutes. Monolayer permeability was determined as described above.

Effect of FasFc on hemorrhagic shock serum induced disruption of microvascular endothelial cell adherens junctions

RLMECs were grown as monolayers on chamber slides. The following groups were studied: a control or sham serum group, HS serum treated group, HS serum group pre-treated with FasFc (50 ng/ml) and FasFc (50 ng/ml) alone group. Cells were fixed in 4% paraformaldehyde and processed for immunofluorescence using a polyclonal antibody against β -catenin (Santa Cruz Biotechnology, Santa Cruz, CA), overnight at 4°C followed by an FITC-tagged secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were mounted using antifade reagent containing DAPI and visualized utilizing a confocal fluorescent microscope at 60X.

Effect of FasFc on hemorrhagic shock serum induced mitochondrial ROS formation

RLMECs were grown on chamber slides. The following groups were studied: a control or sham serum group, HS serum treated group, HS serum group pre-treated with FasFc (50 ng/ml) and FasFc (50 ng/ml) alone group. Cells were exposed to dihydrorhodamine 123 (Invitrogen, molecular probes, Carlsbad, CA), for 30 minutes. The cells were washed twice in PBS and observed under a fluorescent microscope at 40X.

Effect of FasFc on hemorrhagic shock serum induced decrease in mitochondrial transmembrane potential (MTP)

Fluorescent microscopy—To determine the changes in MTP, RLMECs were grown on chamber slides and the following groups were studied: a control or sham serum group, HS serum treated group, HS serum group pre-treated with FasFc (50 ng/ml) and FasFc (50 ng/ml) alone group. Cells were incubated with JC-1 (Cell Technology, Inc, Mountain View,

CA), for 15 minutes at 37°C, washed in PBS, and observed immediately under a fluorescent microscope at 40X.

Flow cytometry—Mitotraker Red CM-H₂XRos (Invitrogen, molecular probes, Carlsbad, CA), was applied as a mitochondrial specific fluorescent probe for detection of MTP using flow cytometric analysis. The following groups were studied: a control or sham serum group, HS serum treated group, HS serum group pre-treated with FasFc (50 ng/ml) and FasFc (50 ng/ml) alone group. Briefly, cells were incubated with mitotraker Red CM-H₂XRos (5 ng/μl) for 20 minutes at 37°C with 5% CO₂ in the dark and then evaluated by using a Becton Dickinson FACS Canto II flow cytometer using absorption at 578 nm and emission at 599 nm. The flow data were obtained by using three replicates of stained and unstained samples from each of the above four groups and were analyzed by using FlowJO flow cytometry analysis software.

Effect of FasFc on hemorrhagic shock serum induced caspase-3 activity

RLMECs were grown on fibronectin coated cell culture dishes in complete MCDB-131 media. The following groups were studied: a sham serum treated/control group, HS serum treated group, HS serum group pre-treated with FasFc and FasFc alone group. The RLMECs were lysed by adding caspase-3 sample lysis buffer provided in the assay kit (R&D system, Minneapolis, MN). The substrate conjugate provided in the assay kit was labeled with a fluorescent probe 7-amino-4-trifluoromethyl coumarin. The homogenates were used for protein estimation followed by treatment with the substrate conjugate for the caspase-3 assay. The resulting fluorescent intensity was measured in a fluorescent plate reader using excitation / emission wavelength at 400 nm and 505 nm respectively.

Statistical Analysis

All data are expressed as means ± SEs. The comparisons between groups were made using analysis of variance followed by Bonferroni's post-test for multiple comparisons. Student's *t*-test was also employed wherever required. A '*p*' value 0.05 was considered as statistically significant. Statistical analysis was performed using Prism Graphpad software.

RESULTS

Hemorrhagic shock serum increases Fas receptor-ligand expression levels on endothelial cell

RLMECs exposed to HS serum showed increase in expression of Fas receptor as well as Fas ligand compared to sham serum treated group. This was demonstrated in the graphical form where mean fluorescence value (MFI) of HS serum treated groups was more than sham serum treated group ($p < 0.05$; Figure 1).

Fas ligand increases endothelial cell monolayer permeability

RLMECs showed increased monolayer permeability following Fas ligand treatment compared to the untreated control RLMECs. The FITC albumin fluorescent intensity in the media from abluminal chamber was significantly higher in RLMECs treated with Fas ligand compared to the control group suggesting an increase in endothelial cell monolayer permeability ($p < 0.05$; Supplemental Digital Content: S1). However, among the Fas ligand treatment groups with 10 ng/ml, 25 ng/ml, 50 ng/ml and 100 ng/ml concentrations fluorescent intensity of FITC-albumin in the media from the abluminal chamber did not differ statistically (Supplemental Digital Content: S1).

FasFc attenuates Fas ligand induced increase in microvascular endothelial cell hyperpermeability

RLMECs showed increased monolayer permeability following Fas ligand treatment compared to the untreated control RLMECs. The FITC albumin fluorescent intensity in the media from the abluminal chamber was significantly higher in RLMECs treated with Fas ligand compared to the control group suggesting an increase in endothelial cell monolayer permeability. However, the monolayers pre-treated with FasFc showed significantly less fluorescent intensity of FITC-albumin in the media from the abluminal chamber ($p < 0.05$, Supplemental Digital Content: S2).

Caspase-3 inhibitor attenuates Fas ligand induced increase in microvascular endothelial cell hyperpermeability

RLMECs showed increased monolayer permeability following Fas ligand treatment compared to the untreated control RLMECs. The FITC albumin fluorescent intensity in the media from abluminal chamber was significantly higher in RLMEC treated with Fas ligand compared to the control group, suggesting an increase in endothelial cell monolayer permeability. However, the monolayers pre-treated with Z-DEVD-FMK showed significantly less fluorescent intensity of FITC-albumin in the media from the abluminal chamber ($p < 0.05$, Supplemental Digital Content: S3).

FasFc attenuates hemorrhagic shock serum induced increase in microvascular endothelial cell hyperpermeability

RLMECs showed increased monolayer permeability following HS serum treatment compared to the control or sham serum treated RLMECs. The FITC albumin fluorescent intensity in the media from abluminal chamber was significantly higher in RLMEC treated with HS serum compared to the control or sham group, suggesting an increase in endothelial cell monolayer permeability. However, the monolayers pre-treated with FasFc showed significantly less fluorescent intensity of FITC-albumin in the media from the abluminal chamber ($p < 0.05$, Figure 2A).

FasFc protects against hemorrhagic shock serum induced disruption of microvascular endothelial cell adherens junctions

RLMECs from the control or sham serum treated group showed continuous distribution of β -catenin at the adherens junctional complex. Following HS serum treatment the adherens junction was disrupted, evidenced by diffuse and punctate distribution of β -catenin and formation of intercellular gaps. HS serum exposed cells, when pre-treated with FasFc, prevented the disruption of the junctional complexes in the endothelial cells (Figure 2B).

FasFc decreases hemorrhagic shock serum induced mitochondrial ROS formation

In RLMECs, treatment of HS serum resulted in increased mitochondrial ROS formation as evidenced by the increase in red fluorescence shown by dihydrorhodamine 123 dye compared with control or sham serum endothelial cells. However, when the cells were pretreated with FasFc followed by HS serum treatment, there was a decrease in red fluorescence indicating that there was a decrease in mitochondrial ROS production (Figure 3A).

FasFc protects against hemorrhagic shock serum induced decrease in mitochondrial transmembrane potential

The cells from the control or sham serum treated group show more mitochondrial red fluorescence than cytoplasmic green fluorescence of JC-1 probe, indicating normal healthy mitochondria. After HS serum treatment, there was a decrease in mitochondrial red

fluorescence of J aggregates, indicating the decrease in mitochondrial transmembrane potential. However, when the cells were pretreated with FasFc followed by HS serum treatment, there was an increase in red fluorescence, indicating that FasFc had protected cells against an HS serum induced drop in their mitochondrial transmembrane potential (Figure 3B).

In the flow cytometric analysis, the control or the sham serum treatment group showed an increase in the red fluorescence of mitotracker red CM-H₂XRos dye compared to HS serum treated RLMECs. This result is represented in graphical form, where mean fluorescence index (MFI) of mitotracker Red CM-H₂XRos dye shows higher values in the control or sham serum treated group and the cells pretreated with FasFc compared to HS serum treated RLMECs ($p < 0.05$; Figure 4). The above finding affirms that cells pretreated with FasFc showed protection against HS serum induced decrease in mitochondrial transmembrane potential.

FasFc decreases hemorrhagic shock serum induced caspase-3 activity

HS serum treated RLMECs showed a significant increase in caspase-3 activity when compared with the sham serum treated endothelial cells ($p < 0.05$; Figure 5). FasFc pretreatment significantly reduced the HS serum induced increase in caspase-3 activity ($p < 0.05$; Figure 5).

DISCUSSION

During HS, due to I/R injury, upregulation of various cytokines takes place (1,3). The results from this study have also demonstrated that on exposure to HS serum, there is increased expression of Fas receptor and Fas ligand on RLMECs. In order to determine the effect of Fas ligand on endothelial cell permeability, RLMECs were exposed to recombinant Fas ligand. The results showed that recombinant Fas ligand induced microvascular endothelial cell hyperpermeability which was inhibited by the use of the Fas ligand blocker, FasFc. This was accomplished upstream in the extrinsic apoptotic pathway by preventing binding of Fas ligand to Fas receptor and thus interfering in signal transduction. Our results have also demonstrated that by inhibiting the downstream effector caspase-3 in the apoptotic pathway, with a caspase-3 specific inhibitor Z-DEVD-FMK, Fas ligand induced endothelial cell hyperpermeability could be attenuated. It is known from the literature that Fas-Fas ligand interaction initiates a receptor mediated 'extrinsic' apoptotic pathway, which results in cell death (4-6). In the present study, the results have demonstrated the role of mitochondrial pathway in the interaction of Fas-Fas ligand induced microvascular endothelial cell hyperpermeability. Recent studies from our laboratory have shown a positive correlation between mitochondrial mediated 'intrinsic' apoptotic signaling and HS induced microvascular hyperpermeability (2).

Mitochondria generate adenosine triphosphate (ATP) by phosphorylating adenosine diphosphate (ADP) using the energy released during electron transport while generating water from hydrogen and oxygen through the electron transport chain (ETC) (19,20). The ETC uses NADH, Succinate and FADH₂ to generate electrons which are transferred through mitochondrial complexes I-IV (NADH dehydrogenase, succinate dehydrogenase cytochrome c reductase, and cytochrome c oxidase, respectively) to oxygen which is converted to water by a reduction process (19,20). The ETC uses a series of electron donors as well as electron acceptors to transfer electrons finally to oxygen (19,20). This movement of electrons from electron donors to the most electronegative acceptors generates energy, which is released during the pumping of protons (H⁺) into the intermembrane space creating a proton gradient across the mitochondrial membrane (19,20). This energy is then used by ATP synthase (complex V) to generate ATP from ADP (19,20). A small number of

electrons do not complete the transfer process in the ETC and cause incomplete reduction of oxygen, resulting in the formation of the reactive oxygen species such as superoxide. This process creates oxidative stress in the mitochondria resulting in mitochondrial dysfunction (19,20).

In conditions such as HS, there is increased production of mitochondrial ROS (2,21). Also it has been demonstrated that during endothelial cell hyperpermeability, there is involvement of ETC complex III in increased production of mitochondrial ROS (21). Mitochondrial ROS can have a direct effect on mitochondrial transition pores on the inner mitochondrial membrane to open, or can activate sphingomyelinase to produce ceramide, which can then open the mitochondrial transition pores (21). Also, it has been shown that ROS leads to a drop in mitochondrial transmembrane potential, which results in the oxidation of mitochondrial transition pores and triggers their opening and subsequent release of cytochrome *c* to the cytosol by mitochondrial outer membrane permeabilization (22). Cytochrome *c* then causes aggregation of the adaptor molecule Apaf 1, to form apoptosome along with procaspase-9. The apoptosome then goes on to cleave procaspase-9 to active caspase-9. The active caspase-9 initiates the final step in the apoptotic cascade of activating effector caspase-3 (22). The active caspase-3 then either cleaves β -catenin or disrupts β -catenin and alters the composition and organization of the adherens junctional protein complex mitigating its barrier function leading to endothelial cell hyperpermeability (2,18,23,24).

In this study, we have conducted experiments showing that pre-treatment of endothelial cells with FasFc interferes with the above mentioned chain of events by attenuating HS serum induced increase in mitochondrial ROS production, decrease in mitochondrial transmembrane potential, and downstream activation of caspase-3. In summary, HS leads to an increase in microvascular endothelial cell permeability by overexpressing Fas ligand and Fas receptors on the endothelial cells. Inhibiting Fas-Fas ligand interaction has protective effects against HS mediated disruption of the adherens junctional protein complex and thereby attenuates microvascular endothelial cell hyperpermeability. The findings from this study may have clinical significance in developing newer therapies targeting Fas-Fas ligand interaction in the management of HS induced vascular hyperpermeability. However this statement warrants further *in vivo* and clinical studies to make these results work from bench to bed side.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Flow cytometric analysis demonstrating increase Fas receptor and Fas ligand expression levels on rat lung microvascular endothelial cells (RLMECs) following exposure to hemorrhagic shock (HS) serum compared to control or sham serum. **A)** Two-dimensional dot plots showing forward Scatter or (FSC) Vs. Side Scatter (SSC) with gates indicating percentage of live cells and those undergoing apoptosis among control / Sham and HS serum groups. And the mean fluorescence index (MFI) calculations were done based on the values obtained from the live cell gates. **B)** MFI of Fas receptor antibody showing higher values of fluorescence for HS serum treated RLMECs compare to control / sham serum treated cells, indicating increased expression levels of Fas receptor in HS serum treated RLMECs (* $p < 0.05$). **C)** MFI of Fas ligand antibody showing higher values of fluorescence for HS serum treated RLMECs compare to control / sham serum treated cells, indicating increased expression levels of Fas ligand in HS serum treated RLMECs (* $P < 0.05$).

Figure 2.

A) Recombinant human FasFc chimeric protein attenuates HS serum induced hyperpermeability in RLMECs. HS serum increases RLMEC permeability compared to control / sham serum treated cells ($*p < 0.05$; $n = 6$). HS serum treated monolayers when pre-treated with FasFc show significant decrease in permeability compared with HS serum treated monolayers ($^ap < 0.05$; $n = 6$). FasFc alone treated monolayers show no significant change in permeability compared to the control / sham serum treated monolayers. **B)** Recombinant human FasFc chimeric protein protects against HS serum induced adherens junction disruption in RLMECs. Immunofluorescence of β -catenin visualizes cell margins at cell-cell junctions. Control / Sham serum treated cells show continuity in β -catenin localization along the cell margins indicating an intact adherens junctional complex. HS serum treated RLMECs show discontinuity of β -catenin immunofluorescence at cell margins indicating disruption of the adherens junctional complex (Arrows). The RLMECs pretreated with FasFc prior HS serum treatment show protection against adherens junction disruption evidenced by the continuity of the β -catenin immunofluorescence at the cell-cell contacts. The nucleus is seen in blue color with DAPI staining.

Figure 3.

A) Recombinants human FasFc chimeric decreases HS serum induced increase in mitochondrial ROS formation in RLMECs. Following exposure to dihydrorhodamine 123, HS serum treated RLMECs show increased ROS formation evidenced by increased red fluorescence compared to control / sham serum treated cells. However cells treated with FasFc do not show an increase in red fluorescence demonstrating protection against HS serum induced increase in mitochondrial ROS formation. **B)** Recombinant human FasFc chimeric prevents HS serum induced decrease in mitochondrial transmembrane potential (MTP) in RLMECs. Cells, on exposure to JC-1 probe, emit green fluorescence indicating cytoplasmic accumulation of the dye whereas the red fluorescence indicates J-aggregates formation in normal and healthy mitochondria. In control / sham serum treated cells, JC-1 shows greater degree of red fluorescence, indicating intact mitochondria. After HS serum treatment, there is decrease in red fluorescence, indicating the loss of MTP. This drop in MTP is prevented by pretreating the cells with FasFc before exposing them to HS serum, which is demonstrated by restoring red fluorescence of J aggregates indicating normal functioning mitochondria.

Figure 4.

Flow cytometric analysis using the mitotracker Red CM-H₂XRos fluorescent probe determines MTP in RLMECs following HS serum and FasFc treatment. **A)** Two-dimensional dot plots showing forward Scatter or (FSC) Vs. Side Scatter (SSC) with gates indicating percentage of live cells, and those undergoing apoptosis among control / sham serum, HS serum, pretreatment with FasFc followed by HS serum and FasFc alone treatment group. **B)** MFI of mitotracker Red CM-H₂XRos probe showing lower values of fluorescence for HS serum treated RLMECs compare to control / sham serum treated cells, indicating a drop in MTP in HS serum treated RLMECs (* $p < 0.05$). Pretreating the cells with FasFc prevented HS serum induced drop in MTP of RLMECs (^a $p < 0.05$). The MFI calculations were done based on the values obtained from the live cell gates.

Figure 5.

HS serum induces caspase-3 activity in RLMECs. RLMECs exposed to HS serum showed an increase in caspase-3 activity compared to sham serum exposed or control RLMECs (* $p < 0.05$). Pre-treatment with FasFc decreases HS serum induced increase in caspase-3 activity significantly (^a $p < 0.05$).