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5 Hydrodynamic Delivery of siRNA in a Mouse Model of Sepsis Doreen E. Wesche-Soldato, Joanne Lomas-Neira, Mario Perl, Chun-Shiang Chung, and Alfred Ayala.

Abstract Although studies blocking the Fas pathway indicate it can decrease organ damage while improving septic cecal ligation and puncture, CLP mouse survival, little is known about how Fas-Fas ligand FasL interactions mediate this protection at the tissue level. Thus, although truncating Fas-FasL signaling ameliorates many untoward effects of sepsis, the pathological mode of action is distinct at the tissue level. Sepsis is a major cause of morbidity and mortality in patients in the intensive care unit, with approximately a third of the , annually reported cases resulting in death. With respect to organ damage and mortality associated with sepsis in mouse models, our laboratory and others have reported that it is at least in part attributable to the activation of the Fas-FasL signaling pathway and not TLR4. Clearly, any cell that takes up Fas siRNA or is affected by it, even after CLP, would be considered a potential target and therefore may be involved in septic morbidity. Sepsis induces extensive apoptosis of lymphocytes, and this has been suggested to contribute to immunosuppression and mortality. By the same token, we also sought to establish cell types that express FasL and their contribution to Fas-FasL signaling in the liver and spleen after sepsis. Here, we report that in accordance with previous work, Fas is up-regulated in the liver and the spleen after sepsis, particularly in hepatocytes and lymphocytes, respectively. The studies described here were performed in accordance with the National Institutes of Health and The Guide for the Care and Use of Laboratory Animals, and were approved by the Brown University and Rhode Island Hospital institutional animal care and use committees. Model of Sepsis The surgical procedure to generate sepsis was performed as previously described. A midline incision 1. The cecum was isolated, ligated, punctured twice with a gauge needle, and was gently compressed to extrude a small amount of cecal material. The cecum was returned to the abdomen, and the muscle and skin incisions were closed with Ethilon suture material Ethicon, Inc. Before suturing the skin 2 to 3 drops of lidocaine Abbott Laboratories was administered to the wound for analgesia. The mice were subsequently resuscitated with 1. Sham controls were subjected to the same surgical laparotomy and cecal isolation, but the cecum was neither ligated nor punctured. The target sequences used for Fas is as follows: NPCs in the top layer were then washed and used for flow cytometry phenotyping or further cell isolations. Typically, just under cells were resuspended in degassed running buffer consisting of 0. Viable hepatocyte populations were isolated using the two-step perfusion method as previously described. The liver was then perfused with warm modified L medium containing glucose and collagenase IV for 15 minutes. However, before these studies, we initially attempted to establish the nature of the homing of these isolated cells to certain tissues. To do this, 1. Twenty-four hours after injection, the liver, thymus, spleen, heart, and lung were harvested from the recipients and flash-frozen in OCT. Histological changes in these sections were determined by a pathologist who was blinded to the treatment identity of the samples. The liver was subsequently perfusion-fixed with 2. Ultrathin sections 50 to 60 nm were prepared, retrieved onto mesh copper grids, and contrasted with uranyl acetate and lead citrate. Blotting procedures, chemiluminescent detection, and densitometric analysis were performed as previously described by our laboratory. Assessment of Apoptosis For annexin V staining, 1. Cells were then analyzed by flow cytometry. Sections were incubated with anti-active caspase 3 antibody BD Pharmingen for 1 hour and then blocked with a peroxidase-blocking solution. Sections were then incubated with anti-rabbit horseradish peroxidase secondary antibody. Hepatocytes were plated at a concentration of 2. Cultures were left overnight and were subsequently analyzed to establish the percent cytotoxicity as measured by release of lactate dehydrogenase LDH. Percent cytotoxicity was calculated as: The liver was perfused and NPCs isolated as described above. Kupffer cells were enriched by adherence on plastic. Statistics The data are presented as a mean and SE of the mean for each group. Results T-Cell Populations Increase in the Liver after CLP Although we have previously shown that in vivo Fas siRNA administration maintains suppression of Fas protein in the

liver and spleen up to 10 days, while concomitantly improving survival after CLP, it was not clear which cell types were in fact the target of this treatment. Numbers of these cells followed the same trend, indicating the change in percentage was not attributable to influx or loss of other cell types in the population. Of note, the influx of liver T cells was not associated with a change in Mig or IP levels because no difference was seen in septic or sham mouse liver expression data not shown.

Chapter 2 : Publications Authored by Joanne Lomas-Neira | PubFacts

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The authors have declared that no competing interest exists. This is an open-access article distributed under the terms of the Creative Commons License http: Reproduction is permitted for personal, noncommercial use, provided that the article is in whole, unmodified, and properly cited. Abstract siRNA small interfering RNA interference represents an exciting new technology that could have therapeutic applications for the treatment of viral infections. However, a major challenge in the use of siRNA as a therapeutic agent is the development of a suitable delivery system. Persistent HCV infection is a leading cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma 1 - 4. HCV belongs to the family Flaviviridae, and its genome is a positive-strand 9. Introduction of siRNA into mammalian cells leads to mRNA degradation with exquisite sequence specificity without activating an interferon response. Thus, RNA interference represents an exciting new technology that could have therapeutic applications for the treatment of viral infections 10 , Unfortunately, small RNAs have not been applied in treating diseases. One of the reasons is that there is no suitable delivery system for effectively transferring siRNA in vivo 10 - A major challenge in the use of siRNA small interfering RNA as a therapeutic agent is the development of a suitable delivery system. Because the main goal of in vivo delivery is to have active siRNA oligos in the target cells, the stability of siRNA oligos in the extracellular and intracellular environments after systemic administration is the most challenging issue The first hurdle is the size of the nucleotide double-stranded siRNA oligos: Second, the double-stranded siRNA oligos are relatively unstable in the serum environment and they can be degraded by RNase activity within a short period of time. DNA is relatively stable, but still can be degraded by DNase and it is difficult to introduce naked plasmids into cells. Therefore, suitable carriers are required for this purpose. Currently, there are two major methods by which siRNAs are delivered into hepatocytes in experimental animals 19 , One is the hydrodynamic method, in which siRNA expressing plasmids in a solution that is injected into the blood by the tail vein. This solution causes high pressure in blood circulation so that siRNA expressing plasmids enter into hepatocytes. The stability and efficiency of this method have been variable. Another method is the use of viral vectors. Their transfection efficiency is high, but there are many disadvantages such as immune response, integration, and mutations which pose obstacles for clinical use. Many non-viral carriers have been used in the delivery of DNA, which has a great advantage for its application in drug target validation and also allows multiple administrations of siRNA, which are crucial for siRNA therapeutic applications 21 - Several proteins as non-viral vectors have been studied using positive charge of the protein to bind with DNA to form complexes in cells Histone is a potential protein because of its excellent DNA-assembling ability. Our idea was to use HPhA which comes from the hyperthermophile Pyrococcus horikoshii OT3 which has the same basic structure of eukaryotic histones. Its molecular weight is smaller than eukaryotic histones, and it has good stability and DNA-assembling ability. The resultant plasmids psh, psh, psh, and psh were used in the experiments. Scrambled siRNA control cloned into the same vector was used as a negative control in all of the experiments. HphA or Lipofectamine TM was added at various concentrations and allowed to incubate for 24 h. The cytotoxicity was measured by the reduction of methylthiazol tetrazolium MTT; Sigma, USA observed in mitochondria at 24 h after the initial treatment. The inhibition rate was calculated for each experimental group by comparison with MTT results from non-treated medium alone cells Western Blotting Cells were harvested using sodium dodecyl sulfate sample buffer in 72 h after transfection. Proteins were visualized using an enhanced-chemiluminescence detection kit Amersham Pharmacia, Piscataway, NJ Luciferase activity measuring Cells in well cluster plates were digested by trypsogen 72 h after transfection. Cells were agitated for 15 sec, then centrifuged for 15 sec. Luciferase activity was measured by a FLX luminometer Intracellular RNA purification and analysis HL cells were harvested by trypsin digestion 72 h after transfection and washed

three times with phosphate buffered saline PBS, pH 7. Statistical analysis Statistical analyses were performed on SAS software version 8. A P-value less than 0. Results Cytotoxicity assays In order to evaluate toxicity, the number of surviving HL cells was monitored after 24 h incubation with various concentrations of either the HPhA or Lipofectamine TM

Chapter 3 : Lomas-Neira, Joanne

The use of siRNA in vivo as well as in animal models has become more widespread in recent years, leading to further questions as to the best mode of delivery that will achieve optimal knockdown.

Int J Med Sci ; 10 8: How to cite this article: However, a major challenge in the use of siRNA as a therapeutic agent is the development of a suitable delivery system. Persistent HCV infection is a leading cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1 - 4]. HCV belongs to the family Flaviviridae, and its genome is a positive-strand 9. Introduction of siRNA into mammalian cells leads to mRNA degradation with exquisite sequence specificity without activating an interferon response. Thus, RNA interference represents an exciting new technology that could have therapeutic applications for the treatment of viral infections [10 , 11]. Unfortunately, small RNAs have not been applied in treating diseases. One of the reasons is that there is no suitable delivery system for effectively transferring siRNA in vivo [10 - 15]. A major challenge in the use of siRNA small interfering RNA as a therapeutic agent is the development of a suitable delivery system. Because the main goal of in vivo delivery is to have active siRNA oligos in the target cells, the stability of siRNA oligos in the extracellular and intracellular environments after systemic administration is the most challenging issue [16]. The first hurdle is the size of the nucleotide double-stranded siRNA oligos: Second, the double-stranded siRNA oligos are relatively unstable in the serum environment and they can be degraded by RNase activity within a short period of time. DNA is relatively stable, but still can be degraded by DNase and it is difficult to introduce naked plasmids into cells. Therefore, suitable carriers are required for this purpose. Currently, there are two major methods by which siRNAs are delivered into hepatocytes in experimental animals [19 , 20]. One is the hydrodynamic method, in which siRNA expressing plasmids in a solution that is injected into the blood by the tail vein. This solution causes high pressure in blood circulation so that siRNA expressing plasmids enter into hepatocytes. The stability and efficiency of this method have been variable. Another method is the use of viral vectors. Their transfection efficiency is high, but there are many disadvantages such as immune response, integration, and mutations which pose obstacles for clinical use. Many non-viral carriers have been used in the delivery of DNA, which has a great advantage for its application in drug target validation and also allows multiple administrations of siRNA, which are crucial for siRNA therapeutic applications [21 - 24]. Several proteins as non-viral vectors have been studied using positive charge of the protein to bind with DNA to form complexes in cells [25]. Histone is a potential protein because of its excellent DNA-assembling ability. Our idea was to use HPhA which comes from the hyperthermophile Pyrococcus horikoshii OT3 which has the same basic structure of eukaryotic histones. Its molecular weight is smaller than eukaryotic histones, and it has good stability and DNA-assembling ability. The resultant plasmids psh, psh, psh, and psh were used in the experiments. Scrambled siRNA control cloned into the same vector was used as a negative control in all of the experiments. HphA or Lipofectamine TM was added at various concentrations and allowed to incubate for 24 h. The cytotoxicity was measured by the reduction of methylthiazol tetrazolium MTT; Sigma, USA observed in mitochondria at 24 h after the initial treatment. The inhibition rate was calculated for each experimental group by comparison with MTT results from non-treated medium alone cells [27]. Western Blotting Cells were harvested using sodium dodecyl sulfate sample buffer in 72 h after transfection. Proteins were visualized using an enhanced-chemiluminescence detection kit Amersham Pharmacia, Piscataway, NJ [11]. Luciferase activity measuring Cells in well cluster plates were digested by trypsogen 72 h after transfection. Cells were agitated for 15 sec, then centrifuged for 15 sec. Luciferase activity was measured by a FLX luminometer [11]. Intracellular RNA purification and analysis HL cells were harvested by trypsin digestion 72 h after transfection and washed three times with phosphate buffered saline PBS, pH 7. Statistical analysis Statistical analyses were performed on SAS software version 8. A P-value less than 0. Results Cytotoxicity assays In order to evaluate toxicity, the number of surviving HL cells was monitored after 24 h incubation with various

concentrations of either the HPhA or Lipofectamine TM Click on the image to enlarge. Luciferase activity was measured 72 h after transfection. However, expression of luciferase was clearly shown when HphA was used to transfect. The inhibition rates were different for the various targeting sites Table 1. This replicon contains a full-length genome of an HCV genotype 1a virus.

Chapter 4 : - NLM Catalog Result

The use of siRNA in vivo as well as in animal models has become more widespread in recent years, leading to further questions as to the best mode of delivery that will achieve optimal knockdown. While the exact mechanism of siRNA uptake at a cellular level has yet to be fully elucidated, various.

Chapter 5 : Publications Authored by Chun-Shiang Chung | PubFacts

[et al.] -- Hydrodynamic delivery of siRNA in a mouse model of sepsis / Doreen E. Wesche-Soldato [et al.] -- Nasal delivery of siRNA / Vira Bitko and Sailen Barik.