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TITLE: Liposomes and Liposome-Annexin Complexes as Adjunctive Therapy for Reperfusion Injury and Hemorrhagic Shock

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14. ABSTRACT: Previous studies by ourselves and others have shown that natural antibodies recognize epitopes on ischemic tissue and catalyze the initiation and subsequent development of reperfusion injury. Prior to the beginning of these studies, my laboratory had developed monoclonal antibodies from wild type mice that were able to individually transfer the capacity of immunoglobulin deficient Rag-/- mice to develop intestinal ischemia-reperfusion injury. We found that these disease-causing natural antibodies recognized either phospholipids or annexin-4. The overall goal of the studies has been to test the concept that a therapeutic which targets the very first steps of reperfusion injury by blocking the effects of antibodies to phospholipids and annexin-4 could be developed. This approach could be used in conjunction with fluids in order to provide a major benefit and substantially decrease the morbidity and mortality associated with battlefield injuries. Studies we performed in collaboration with Dr. George Tsokos at USUHS showed that phospholipid-bearing liposomes that we made protected mice he studied in intestinal ischemia-reperfusion injury models. Our studies at the University of Colorado Health Sciences Center in the past year have focused on developing annexin-4 as a recombinant protein therapeutic and testing the ability of it to block ischemia-reperfusion injury. We report the successful creation of the recombinant protein and positive proof-of-concept data with annexin-4 in intestinalischemia-reperfusioninjury.

15. SUBJECT TERMS
Ischemia-reperfusion injury, Inhibitors, Liposomes, Annexin-4, Phospholipids

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INTRODUCTION:
Previous studies have shown that natural antibodies recognize epitopes on ischemic tissue and catalyze the initiation and subsequent development of reperfusion injury. The nature of these neo-epitopes has been uncertain. We had previously developed monoclonal antibodies from wild type mice that were able to individually transfer the capacity of immunoglobulin deficient Rag-/- mice to develop intestinal ischemia-reperfusion injury. We found that these hybridomas, all derived from B cells of naïve wild type mice secreting natural antibodies, recognized either phospholipids or annexin-4. The overall goal of the studies has been to test the concept that a therapeutic that targets the very first steps of reperfusion injury by blocking the effects of natural antibodies binding to phospholipids and annexin-4 revealed during reperfusion could be developed and would be effective. Our funded studies at the University of Colorado in the past year have focused on developing annexin-4 as a recombinant protein therapeutic. We have then tested the ability of this potential therapeutic reagent to block ischemia-reperfusion injury, in collaboration with Drs. George Tsokos and Steve Tomlinson. We report herein the successful creation of the recombinant protein and positive proof-of-concept data with annexin-4.

BODY:
The statement of work included:
1. **Prepare and deliver liposomes that vary in composition and size for in vivo testing of biologic effects on ischemia-reperfusion and hemorrhagic shock models.**
Data that we accumulated prior to the initiation of the funding period demonstrated the ability of liposomes bearing cholesterol and the phospholipids phosphatidyl-choline and phosphoglycerol to block intestinal ischemia-reperfusion injury. Data accumulated in the funding period demonstrated that the same liposome composition blocked the development of stroke injury in a murine model of this condition, administered 30 minutes after the onset of reperfusion injury. These results, performed in collaboration with Dr. Steve Tomlinson of the Medical University of South Carolina, have broadened the potential for this therapeutic approach into other conditions. We have not to date performed experiments in which we have altered the composition and size of liposomes, focusing instead to this point on studies outlined below in Statement #2.

2. Prepare and deliver recombinant annexin-4 in sufficient quantities for in vivo testing of biologic effects.
In order to be able to test the ability of recombinant annexin-4 to block ischemia-reperfusion injury, we first needed to develop strategies to express recombinant protein. We used strategies to make both bacterial and mammalian-derived recombinant protein. For bacterial expression, the cDNA sequence of mouse annexin-4 was taken from Invitrogen clone # 4947415. cDNA encoding for annexin-4 was amplified by PCR. The forward primer used was: 5′- GGT ATT GAG GGT CGC ATG GAA GCC AAA GGA GGA AC -3′. The reverse primer was: 5′- AGA GGA GAG TTA GAG CCT TAA TCA TCT CCT CCA CAG AGA

ATG -3′. Ligation-independent cloning (LIC) was done in pETXa/LIC vector (Figure 1). The vector adds a Trx-Tag, His-Tag, and S-Tag to the N-terminus of the protein. These tags are cleavable with Factor Xa leaving a native version of the N-terminus.

To express annexin-4 in mammalian cells, the forward and reverse primers were 5' CTG GTA CCA GCA TGG AAG CCA AAG GAG -3`and 5' TCT CGA GAA TCA TCT CCT CCA CAG AGA ATG-3` respectively. The primers added restriction sites KpnI to the start of annexin-4 and Xho to the end. The KpnI/XhoI fragment was amplified by PCR from genomic DNA. The KpnI/XhoI fragment was subsequently cloned into pSecTag2/Hygro B expression vector (Figure 1), and expression of the protein was done in the F-293 cell line. The vector adds an Ig k-chain leader sequence to the N-terminus of the protein for secretion. This is cleaved during translation, leaving approximately 15 amino acids on the N-terminus of annexin-4. The vector also adds a myc epitope and a His-Tag to the C-terminus of the protein.

To synthesize bacterial annexin-4 and then purify it to a high level for *in vivo* work, several methods were evaluated over the first several months of funding. The final approach developed started with the expression vector pETXa/LIC being transformed in BL21 bacteria. Bacterial expression cultures were incubated at 37°C in LB medium containing ampicillin (50µg/mL) until an A_{600} of 0.6 was reached. Recombinant protein expression was induced by the addition of IPTG (Sanland-chem, USA) to a final concentration of 0.3 mM. Following 6 hours of incubation at 32°C, bacteria were harvested by centrifugation at 10,000×*g* for 10 minutes at 4°C. After harvesting the cells, they were resuspended in PBS with Complete, EDTA-Free Protease Inhibitor Cocktail Tablets (Roche). Bacteria were lysed by 4 freeze-thaw cycles. The cell lysate was then incubated with DNase and RNase for 30 minutes. The material was then centrifuged at 100,000×*g* for 40 minutes, and the cell pellet was resuspended in 6M urea for 30 minutes. Centrifugation at 100,000 ×*g* for 40 minutes was used to remove undissolved debris. The pre-cleared supernatant was then diluted (2:1) with PBS and its pH adjusted to approximately 7.6 with NaOH. Material was then applied to a TALON^{TM} resin column (Clontech) that had been equilibrated with 4M urea. The protein bound to Co^{2+} through the His(6) tag was refolded on the column by use of a discontinuous gradient from 4M to 0.25M urea, starting with the equilibration buffer and finishing with a buffer containing 10 mM imidazole in DPBS pH 7 (Figure 2). The refolded protein was eluted with a buffer containing 38 mM, 75 mM, and 150 mM imidazole. The presence of protein and the purity of it in fractions were confirmed by SDS gel and staining with Coomassie blue.

To synthesize mammalian annexin-4 as an alternative approach and then purify it to a high level for evaluation and potential *in vivo* work, several methods were evaluated over the first several months of funding. The final approach developed utilized FreeStyle^{TM} 293 cells (Invitrogen) that were transfected with pSecTaq2/Hygro (Invitrogen) vector expressing annexin-4 using the

manufacturer's protocol. Cells were harvested after 48 hours. Lysis was performed by incubating the cells in lysis buffer (0.02M Tris pH 8.0, 0.5% Triton, 0.5% Chaps) for 20 minutes on ice. The lysate was collected after the cell debris was removed by centrifugation, and was then diluted 1:5 with DPBS with EDTA-Free Protease Inhibitor Cocktail Tablets (Roche). The sample was then run through a column with TALON^{TM} beads which had been equilibrated with DPBS. The column was then washed with DPBS pH 7.6, then DPBS pH 7.0. Afterwards, the column was washed with buffer containing 10 mM imidazole. Elution was done in discontinues gradient of 38 mM, 75 mM, and 150 mM imidazole in DPBS. The fractions were run on 10% Bis-Tris gels and stained with Coomassie blue. Selected fractions were concentrated using Centricon Plus 20 (Millipore) for further use.

3. Prepare and deliver liposome-annexin 4 complexes for in vivo testing of biologic effect.

One major additional question that was first addressed was whether natural antibodies to annexin-4 could be detected in wild type mice. Annexin-4 produced in bacteria was used as the antigen, as this protein was able to be made in large quantities and purified as above, and murine serum samples were tested by ELISA for IgM and IgG antibodies to the antigen. Reactivity was clearly demonstrated in both the IgM and IgG fractions, consistent with the proposed role of this natural antibody system where pre-formed antibody would be present against this neo-antigen exposed during the reperfusion phase.

The data showing that mice have natural antibodies to annexin-4, similarly to natural antibodies to phospholipids, supported the next experiments where bacteria-derived recombinant annexin-4 was tested to determine whether it could block intestinal ischemia-reperfusion injury in wild type mice. These experiments were performed in the laboratory of Dr. George Tsokos at USUHS with material we provided. Therein, recombinant annexin-4 was injected into wild type mice undergoing intestinal ischemia-reperfusion injury. Intestines from mice without annexin-4 treatment and mice with annexin-4 injected 30 minutes after the beginning of the reperfusion phase were analyzed for the level of injury. Results showed that the injection of the annexin-4 significantly reduced injury to the level what sham operated animals had.

Further mechanism studies were performed in large part in this laboratory and included: 1) the demonstration that annexin-4 was present diffusely in the cytoplasm at baseline but could be detected in conjunction with anti-annexin-4 natural antibody in reperfused tissue, and 2) the levels of natural anti-annexin-4 natural antibodies were diminished in mice that had an altered natural antibody repertoire and that we have previously shown were protected from ischemia-reperfusion injury because of that altered repertoire. Finally, in collaboration with Dr. Steve Tomlinson at the Medical University of South Carolina, we showed that the same annexin-4 which protected mice from intestinal ischemia-reperfusion

injury blocked experimental stroke injury *in vivo*. These results again broadened the potential therapeutic use of this approach to other medical conditions.

To date we have not yet been able to perform experiments with both annexin-4 and liposomes together. Initial experiments need to be performed to determine how to stably associate these proteins and under what conditions they could be delivered in vivo and remain in that configuration.

4. Determine the pharmacokinetics and in vivo efficacy of liposomes, annexin 4, and liposome-annexin 4 complexes in ischemia-reperfusion and hemorrhagic shock models.
To date we have not yet performed experiments using dose-response approaches, or evaluated pharmacokinetics.

KEY RESEARCH ACCOMPLISHMENTS:

- Liposomes containing the phospholipids phosphatidyl-choline and phosphoglycerol on a cholesterol core block the development of intestinal ischemia-reperfusion injury in wild type mice given after the onset of reperfusion.
- Liposomes containing the phospholipids phosphatidyl-choline and phosphoglycerol on a cholesterol core block the development of experimental stroke injury in wild type mice given after the onset of reperfusion.
- Annexin-4 can be made in high quantities in bacteria, refolded and purified.
- Bacterial annexin-4 blocks the development of intestinal ischemia-reperfusion injury in wild type mice.
- Bacterial annexin-4 blocks the development of experimental stroke injury in wild type mice.

REPORTABLE OUTCOMES:

- Creation of recombinant annexin-4 and liposomes with therapeutic potential as outlined above.
- Patent application submission (March 2007): Prevention and Treatment of Ischemia-Reperfusion Injury and Related Conditions. Co-inventors: Dr. V. Michael Holers, UCDHSC, Dr. Steve Tomlinson (Medical University of South Carolina), Dr. George Tsokos (USUHS). See appendix below.
- Manuscript in preparation describing results.

CONCLUSION:
These experiments have sought to expand the therapeutic options for ischemia-reperfusion injury and hemmorhagic shock to include inhibition of the inflammatory response by the development of proof-of-concept data for use of adjuvant therapy with liposomes, annexin-4 or liposome-annexin-4 complexes. Initial data have been positive in a pre-clinical model, and sufficient patent protection has been sought so that the concepts and therapeutics are

appropriately protected and can be commercialized for application to military and civilian indications. Successful development of adjuvant therapy using our approach with phospholipid-bearing liposomes and/or annexin-4 to fluid resuscitation, a method that can be given at the same time using the same intravenous delivery devices, should provide substantial clinical benefit.

REFERENCES:
Not applicable.

PERSONNEL RECEIVING SUPPORT:
Kuan Chen, B.S.
Liudmila Kulik, Ph.D.

APPENDICES:
Page 1 of Patent Application (follows).

Prevention and Treatment of Ischemia-Reperfusion Injury and Related Conditions

Field of the Invention

This invention generally relates to the use of lipids, annexin, and lipid-annexin complexes for the prevention and/or treatment of ischemia-reperfusion injury and reperfusion injury associated with a variety of diseases and conditions, as well as therapeutic targets and compositions for the prevention and treatment of such diseases.

Background of the Invention

Ischemia-reperfusion (I-R) injury refers to damage to a tissue caused when the blood supply returns to the tissue after a period of ischemia (restriction in blood supply). The absence of oxygen and nutrients from the blood creates a condition in which the restoration of circulation results in inflammation and oxidative damage, rather than restoration of normal function. Ischemia-reperfusion injury can be associated with traumatic injury, including hemorrhagic shock, as well as many other medical conditions such as stroke or large vessel occlusion, and is a major medical problem. More particularly, ischemia-reperfusion injury is important in heart attacks, stroke, kidney failure following vascular surgery, post-transplantation injury and chronic rejection, as well as in various types of traumatic injury, where hemorrhage will lead to organ hypoperfusion, and then subsequent reperfusion injury during fluid resuscitation. Ischemia-reperfusion injury, or an injury due to reperfusion and ischemic events, is also

observed in a variety of autoimmune and inflammatory diseases. Independently of other factors, ischemia-reperfusion injury leads to increased mortality.

Previous studies by the present inventors and colleagues have shown that certain types of natural antibodies recognize epitopes on ischemic tissue and catalyze the initiation and subsequent development of ischemia-reperfusion injury (1,2). Ischemia-reperfusion injury, as well as hypovolemic shock and subsequent tissue damage, is known to be caused by complement and Fc receptor activation and the recruitment and activation of neutrophils and other inflammatory cells (2). However, despite this understanding of the "downstream" mechanisms of tissue injury, the specific mechanism by which these pathogenic processes are initiated has, prior to the present invention, remained obscure.

SUPPORTING DATA: Figures:

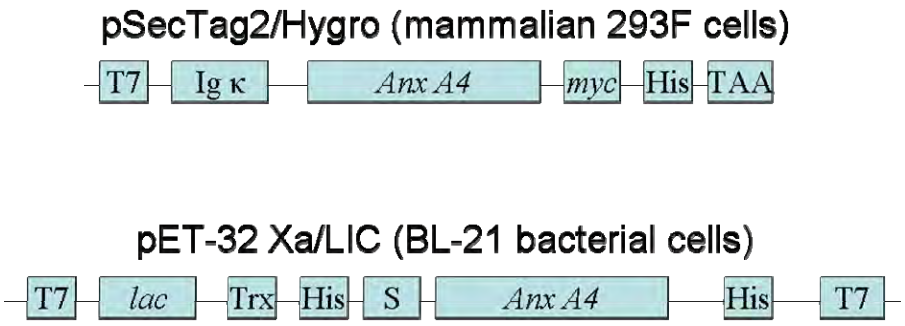

Figure 1. Production of recombinant annexin-4 for *in vivo* inhibition experiments: constructs created are shown.

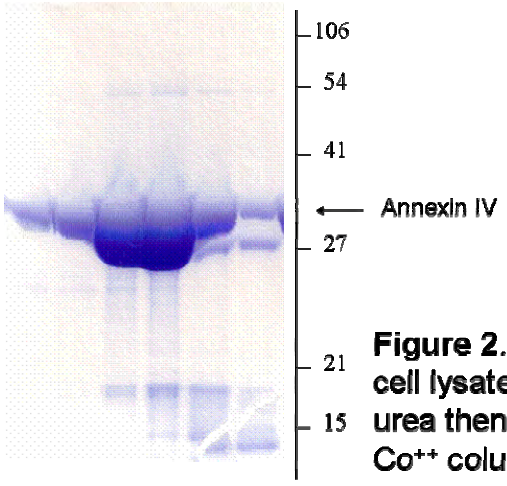

Figure 2. BL-21 bacterial cell lysate treated with urea then purified with Co^{++} column.