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PRINCIPAL INVESTIGATOR: Stephen Tomlinson, Ph.D.

CONTRACTING ORGANIZATION: Medical University of South Carolina
Charleston, SC 29425

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14. ABSTRACT The complement system is an important effector mechanism of innate and humoral immunity. Tumor cells are protected from complement by the expression of complement inhibitory proteins on their surface, and the downregulation or blockade of these inhibitory proteins may enhance anti-tumor antibody and T cell responses. The concept that we proposed to investigate was that siRNA mediated downregulation of a complement inhibitor on lung tumor cells will sensitize the tumor cells to complement and induce a protective immune response. We proposed to investigate an approach utilizing lung delivery of nanoparticles coated with an anti-MUC1 antibody to target delivery of complement inhibitor (Crry) siRNA to tumor cells. We prepared and characterized both nanoparticles and liposomes containing Crry siRNA that were also coated with anti-MUC1 mAb targeting antibody and a cell penetrating peptide. We demonstrated that the nanoparticles, but not liposomes, effectively downregulated Crry expression on lung tumor cells and increased complement deposition in vitro. However, the strategy failed to work in an in vivo model, and did not improve therapeutic outcome in a murine lung cancer model.					
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FINAL REPORT

Introduction

The concept that we proposed to investigate was that siRNA mediated downregulation of a complement inhibitor on lung tumor cells will sensitize the tumor cells to complement and induce a protective immune response. We proposed to investigate an innovative yet clinically relevant approach utilizing lung delivery of nanoparticles (NP) coated with an anti-MUC1 antibody to target delivery of complement inhibitor (Crry) siRNA to tumor cells. MUC1 is a glycoprotein normally expressed on the apical surface of ductal epithelial cells, but in many types of cancer, including lung cancer, MUC1 is highly overexpressed with a loss of polarized apical expression. In addition, cancer associated MUC1 is underglycosylated revealing internal sugar units and peptide sequences that are not normally exposed. It is the differences in expression of MUC1 on cancer cells that distinguish it as tumor specific. The anti-MUC1 mAb we will use for targeting in these studies (BCP8) recognizes the peptide backbone of tumor-expressed underglycosylated MUC1. The objectives of these studies were to:

1. Prepare nanoparticles containing Crry (inhibitor of C3 activation) siRNA and coated with anti-MUC1 mAb targeting antibody and cell penetrating peptide (CPP).
2. Optimize strategy for delivering targeted nanoparticles to lung tumor cells.
3. Determine effect of nanoparticle therapy on tumor cell complement inhibitor (Crry) expression, complement activation and deposition on tumor cells, anti-tumor immune response and therapeutic outcome.

Body

ADDITIONAL TASK

Based on data obtained after submission of grant, we also prepared liposome particles as delivery vehicles for siRNA, and characterized these alongside nanoparticles.

Task 1. Months 0-4. Preparation of nanoparticles (and liposomes)

NANOPARTICLES

PLGA-PEI (1:1) nanoparticles were synthesized by oil/water emulsion method. Oil phase consisted of 15 ml of acetone with 100 mg of PLGA and 100 mg of PEI. It was added dropwise to the water phase 50 ml with 20 mg/ml Pluronic F-68 under the ultrasonic bath sonication. After mixing sonication was continued for additional another 15 min. After preparation, nanoparticles were purified by triple centrifugation/resuspension in MilliQ water. PEGylation was achieved by reaction with NHS-PEG(3400)-OMe. Excess of NHS-PEG(3400)-OMe was removed by double centrifugation/resuspension in 20 mM HEPES buffer. Proteins (transferring and anti-MUC1 antibody) were then physically adsorbed on the NPs from 1 mg/ml protein solutions. Excessive protein was again removed by double centrifugation/resuspension in 20 mM HEPES buffer. Final volume of the suspension was 2 ml. Purified NPs were sterilized by filtering through 0.22 µm filter and frozen at -80°C in 20 mM HEPES with 10 wt./v. % of sucrose. After freezing for 24 h, NPs were transferred into lyophilizer and freeze dried overnight. NPs were

characterized by dynamic light scattering (DLS) and Z-potential measurement. Their size ranged between 90 and 110 nm; larger sizes were observed for antibody-coated NPs (see Table below). Zeta potentials were highly positive leading to high RNA binding capacities. No unbound RNA was found in supernatants if initial amount of RNA was less than the binding capacity (100% RNA binding yield).

LIPOSOMES:

Liposomes were prepared by lipid film method. Lipid film was prepared by evaporation of the lipids' solution in chloroform in a rotary evaporator in vacuum. The film was then suspended in 2 ml of 20 mM HEPES buffer and dispersed using an ultrasound probe sonicator. Liposomes consisted of 50% (mol.) DOTAP, 45% (mol.) Cholesterol and 5% (mol.) DSPE-PEG-oMe. Final concentration of lipids in liposomes was 10 mg/ml. Liposomes were purified by filtration through a 0.22 μ m filter and lyophilized in 10 wt. % sucrose 20 mM HEPES buffer. Protein attachment was performed using physical adsorption, as in the case of nanoparticles.

siRNA attachment to nanoparticles and liposomes

- Nanoparticles/liposomes were mixed with Crry siRNA and incubated for 30 mins with shaking
- Nanoparticles/liposomes were centrifuged at 4000/20000g, respectively for 15 minutes and OD₂₆₀ (as well as spectrum 200-350nm) of supernatant was measured.
- From OD₂₆₀ drop the percentage of siRNA bond to NPs was determined. Solution of siRNA in DI water was used as a standard.

We prepared: 1. particles containing Crry siRNA and coated with targeting antibody and cell penetrating peptide, 2. Particles containing scrambled siRNA and coated with targeting antibody and cell penetrating peptide, 3. Particles containing Crry siRNA and cell penetrating peptide, but without targeting antibody. The table below shows data from analysis of a typical preparation each of liposomes and nanoparticles.

Legend, Size, Z-potential, B.E. of proteins

	Liposomes				Nanoparticles			
	A1	A2	B1	B2	C1	C2	D1	D2
MUC1	+++		+++		+++		+++	
Transferrin	+++	+++	+++	+++	+++	+++	+++	+++
Rhodamine dye			+++	+++			+++	+++

	A1	A2	B1	B2	C1	C2	D1	D2
Size of carrier, nm	49±9	49±9	62±6	62±6	---	---	---	---
Size of conjugates, nm	81±17	75±7	76±9	91±20	203±33	189±22	228±26	213±24
Z of carrier, mV	52±5	52±5	32±2	32±2	---	---	---	---
Z of conjugates, mV	41±2	24±3	47±4	51±3	58±5	44±4	41±3	32±3
Transferrin loading µg/mL	138	132	~135		292	304	~300	
MUC1 loading, µg/mL	319	---	305	---	356	---	298	---
CRRY siRNA binding,mg/mL	---	---	---	---	0.61	0.77	0.85	0.87

*Binding efficiency of transferrin and antibody determined by change in OD₂₈₀ and fluorescent labeling of transferrin with AlexaFluor594

Table 1. Representative analyses of nanoparticle and liposome preparations.

Task 2. Months 1-4. Optimize strategy for delivering targeted nanoparticles to lung tumor cells.

and

Task 3. Months 4-6. Determine effect of nanoparticle therapy on expression of Crry and on complement deposition on lung tumor cells and normal cells in orthotopic model.

Preparations of nanoparticles and liposomes, with or without targeting anti-MUC1 mAb, and with or without (scrambled) anti-Crry siRNA were characterized for their ability to downregulate Crry expression on MUC1 expressing LLC1 tumor cells in vitro. The data shown below are for optimum amounts of liposomes and nanoparticles.

We first prepared stably transfected LLC/MUC1+ cells by transfection followed by rounds of cell sorting for selection. Figure 1 below shows flow cytometric analysis of LLC cells stably transfected with MUC1 or vector alone (control).

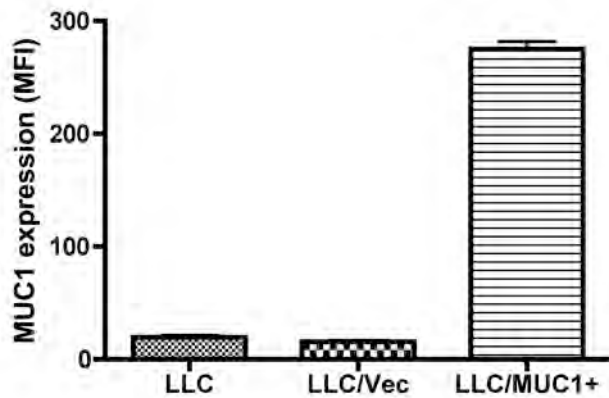


Figure 1. Stable transfection of LLC cells with MUC1. LLC cells were stably transfected in vitro with phCMV-MUC1 (LLC/MUC1+) or phCMV vector control (LLC/Vec) using lipofectamine transfection reagent. Expression of MUC1 by LLC/MUC1+ was confirmed by flow cytometry.

We next characterized the particles for downregulation of Crry on the LLC1/MUC1 tumor cells in vitro (Figs 2-4) and for effect on C3 deposition (Fig 5):

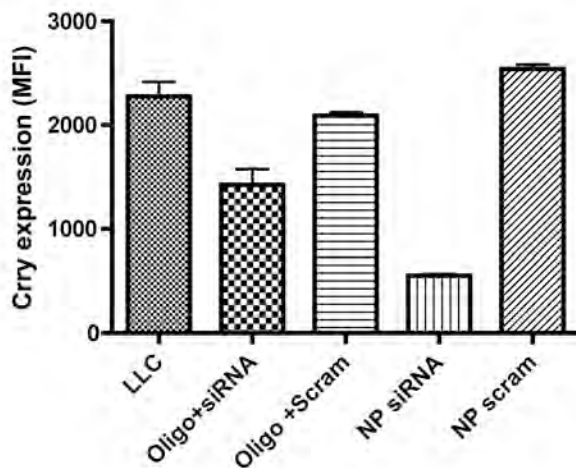


Figure 2. Anti-Crry loaded nanoparticles downregulate the expression of Crry in vitro. 8×10^4 LLC cells were plated and 24 hours later 300pmols of anti-Crry (NP siRNA) or scramble (NP scram) loaded nanoparticles were added to the media. Seventy-two hours after transfection the cells were analyzed for Crry expression by flow cytometry. As a control the cells were transfected with anti-Crry siRNA (Oligo+siRNA) or scramble siRNA (Oligo+Scram) using oligofectamine as the delivery reagent in place of the nanoparticles. The NP siRNA downregulated Crry better than Oligo+siRNA, while NP scram had no effect on the expression of Crry. A representative experiment is shown from multiple analyses using different preparations.

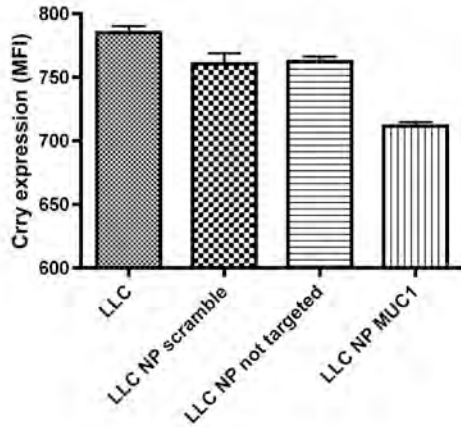


Figure 3. MUC1 antibody (BCP8) positive nanoparticles target LLC/MUC1 cells and downregulate Crry better than non-targeted nanoparticles in vitro.

8×10^4 LLC cells were plated and 24 hours later MUC1 antibody positive (NP MUC1 targeted) or MUC1 antibody negative (NP not targeted) nanoparticles were loaded with 300 pmols of anti-Crry siRNA (LLC NP not targeted and LLC NP MUC1 targeted) or scramble siRNA (LLC NP scramble) and added to the plated cells media. Seventy-two hours after transfection the cells were analyzed for Crry expression by flow cytometry. The anti-Crry siRNA loaded MUC1⁺ nanoparticles (targeted) downregulated Crry expression better than the non-targeted nanoparticles in vitro. A representative experiment is shown from multiple analyses using different preparations.

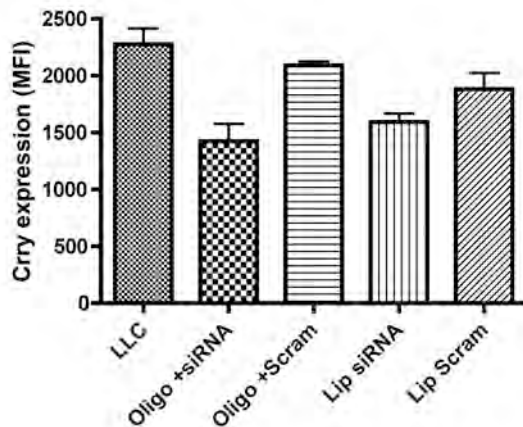


Figure 4. Anti-Crry loaded liposomes do not downregulate the expression of Crry in vitro. 8×10^4 LLC cells were plated and 24 hours later 300pmols of anti-Crry (Lip siRNA) or scramble (Lip scram) loaded liposomes were added to the media. Seventy-two hours after transfection the cells were analyzed for Crry expression by flow cytometry. As a control the cells were transfected with anti-Crry siRNA (Oligo+siRNA) or scramble siRNA (Oligo+Scram) using oligofectamine as the delivery reagent in place of the liposomes. The Lip siRNA only had a small effect on Crry expression. Therefore, for in vivo experiments we chose to use the nanoparticle constructs. A representative experiment is shown from multiple analyses using different preparations.

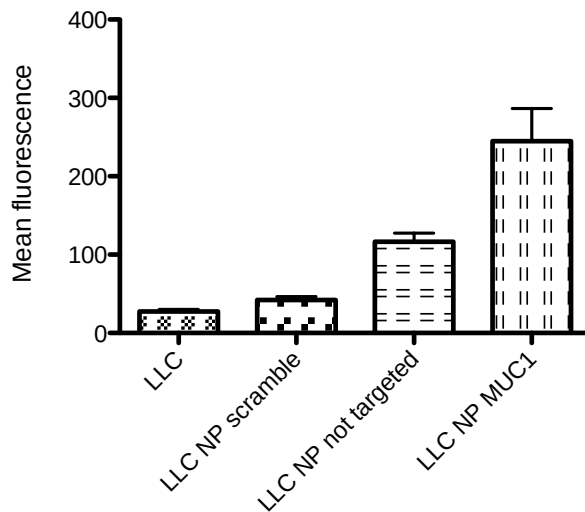


Figure 5. Complement (C3) deposition is increased on LLC/MUC1 cells after targeted delivery of anti-Crry siRNA. 8×10^4 LLC cells were plated and 24 hours later MUC1 antibody positive (NP MUC1 targeted) or MUC1 antibody negative (NP not targeted) nanoparticles were loaded with 300 pmols of anti-Crry siRNA (LLC NP not targeted and LLC NP MUC1 targeted) or scramble siRNA (LLC NP scramble) and added to the plated cells media. Seventy-two hours after transfection the cells were washed, incubated with anti-MUC1 mAb (BCP8) and C6 deficient mouse serum added (C6 deficiency prevents complement mediated lysis, but allow complement activation and C3 deposition). After 1 hour at 37 C, cells were detached and analyzed by anti-C3 flow cytometry. Mean $\pm$ SD.

Task 4. Months 6-12. Determine effect of targeted nanoparticle (and control) therapy on anti-cancer immune response.

and

Task 5. Months 6-12. Determine effect of targeted nanoparticle therapy on determinants of therapeutic outcome (lung nodule size and number, survival).

For these studies, we proposed to use Lewis lung carcinoma (LLC) cells stably transfected with human MUC1 (see preparation above) in a MUC1 transgenic mouse on a C57BL/6 background. These mice express the transgene in a pattern and level consistent with that seen in humans. The MUC1 transgenic mouse will enable us to use a syngeneic model expressing a relevant human tumor-associated antigen and will allow the study of immunogenic and immunotherapeutic strategies within the context of tolerance and autoimmunity.

Effect of MUC1-targeted nanoparticle delivery of Crry siRNA on therapeutic outcome in orthotopic model of lung cancer.

MUC1 transgenic mice were challenged i.v. with 1×10^6 LLC MUC1+ cells. Seven days after tumor cell challenge when lung tumors are evident, mice were treated intranasally with either MUC1 targeted nanoparticles loaded with 200 pmols anti-Crry siRNA or with

PBS. Mice were sacrificed 14 days after therapy and analyzed for lung tumors. Group size was 6. We isolated lungs, stained by H&E and analyzed for tumor growth. We saw no difference in tumor growth/metastases between the test and control group.

These studies were held up because of difficulties with the MUC1 transgenic breeding colony and the supply of MUC1 transgenic mice. However, we are currently repeating the above experiment and will analyze for tumor growth and Crry expression. If we obtain the same result, this will confirm a negative result showing that our strategy to target a therapeutic molecule to lung cancer cells in vivo failed. In this case, further studies analyzing anti-tumor immune response (task 4), and additional control experiments with different nanoparticles would not be worthwhile. Nevertheless, although our in vivo data was not promising, we plan to pursue these studies further if data from a repeat experiment is more promising.

Key Research accomplishments

Prepared nanoparticles and liposomes for targeted delivery of siRNA

Determined that nanoparticles can effectively deliver siRNA to a tumor cell in vitro and downregulate the expression of a complement inhibitor

Determined that a nanoparticle targeted to a tumor specific MUC1 antigen is more effective at delivering payload (siRNA) to a tumor cell in vitro than an untargeted nanoparticle.

Nanoparticles were superior to liposomes at delivering siRNA and downregulating expression of a complement inhibitor in vitro than liposomes.

Targeted delivery of siRNA for the downregulation of a complement inhibitor via a nanoparticle delivery system did not improve outcome in an orthotopic model of lung cancer.

Reportable outcomes

Nanoparticles targeted to MUC1 expressing cells and coated with a cell penetrating peptide for delivery of siRNA

Liposomes targeted to MUC1 expressing cells and coated with a cell penetrating peptide for delivery of siRNA

LLC cell line stably expressing human MUC1.

Conclusions

The overall goal of this project failed in that we were unsuccessful in modulating lung cancer growth in a murine model by our strategy for the targeted downregulation of a complement inhibitor. We are, however, progressing with some additional studies beyond the grant period to confirm (or otherwise) these findings. Nevertheless, the project did result in the generation of some reagents that effectively downregulate complement inhibitor expression in vitro. These reagents will be useful for the targeted

downregulation of complement inhibitors for in vitro studies, and may prove effective for different in vivo model applications.

List of personnel receiving pay from research effort.

Stephen Tomlinson, PhD (PI)
Carl Atkisson, PhD (co-I)
Emily Paulling (Technician)