Background: Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a secreted regulator of cell-surface low-density lipoprotein receptor (LDLR), acting mostly on liver cells. PCSK9 binds a specific region in the ectodomain of the LDLR, the Epidermal Growth Factor (EGF). PCSK9 plays a significant role in LDLR intracellular degradation and consequent elevation of plasma LDL levels (hypercholesterolemia). Current drug development efforts are directed at blocking PCSK9-LDLR interaction via antibodies to reduce LDL levels. The nature of PCSK9-LDLR interaction (flat interface) and the limited understanding of the molecular mechanism of action are hindering the opportunity to develop small molecular inhibitors.

Aim: Our aim was to explore therapeutic options beyond the use of antibodies against PCSK9. Our experiments set out to look at the ability of PCSK9’s C-terminal domain (CTD) to bind the LDLR and inhibit the PCSK9-mediated degradation.

Results: To study the effects of CTD on the levels of LDLR we transfected HepG2 cells (a human hepatoma line), which naturally secrete PCSK9, and HEK293T cells (human embryonic kidney), which do not secrete PCSK9, with increasing amounts of pro-ctDNA construct expressing CTD (40, 20, 40ag). Our results show that 20 and 40ag of CTD DNA caused an 11% and 27% increase in LDL levels, respectively, in LDLR protein in HepG2 cells, but not in HEK 293T cells. We purified its c-tide CTD from HEK293T media, and were able to show that CTD binds EGF AB repeats of LDLR in a pH-dependent manner with IC50 of 1529nM at pH 3.5, 6, and 7, and no effect at pH 4. Interestingly, full-length PCSK9 was able to compete CTD binding to the EGF-AB with Kd of 94nM at pH 6.3.

Conclusions: Our data suggests that full-length PCSK9 and CTD compete for the binding to the LDLR, thus CTD may serve as a therapeutic agent for treatment of hypercholesterolemia, by blocking LDLR degradation.

MATERIALS AND METHODS

Creating the CTD Plasmid: Take 100μL of E. coli bacteria and 30ng of pro-ctDNA1.1 construct of CTD, heat shock, add 1ml of LB broth, plate onto ampicillin (+ plate), incubate overnight at 37°C, then pick 1 colony place into 500ml LB broth (with 50μl of ampicillin) incubate overnight, perform a maxiprep, and isolate the plasmid.

Maintaining Cell Lines: Each respective cell line (HEK 293 T, HEP G2, HEP G2 + PCSK9, HEP G2 + D374Y) was grown and maintained with DMEM media (+ 10% FBS and 1% PST)

Transfection: Day 0: Plate 4 million cells per 10 cm plate. Day 1: While the cells are in exponential growth use the Calcium Phosphate Mammalian Transfection kit (Profection) to introduce CTD plasmid into each cell line. Day 2: Discard and replace the media. Day 3 & 4: Collect media (add 400 µl PMST to 40 ml of media), and replace the media. Day 4:

Protein Extraction: Discard the media, Wash the cells 3 times with PBS. Add 1 ml of RIPA Buffer (+150 µl Protease Inhibitor). Spin down the cells and collect the supernatant. Perform a Lowry assay to determine the protein concentration.

Western Blot: Proteins were separated via electrophoresis using a 4%-12% gradient SDS-PAGE gel. The gel was then transferred onto a nitrocellulose membrane and the proteins were detected using specific primary Antibodies to LDLR, LRP1, and GAPDH (the loading control). The bands were then quantified using Image J software.

Protein Purification: To determine that transfection occurred the media that was collected on Days 3 & 4 of transfection underwent protein purification. Protein purification was carried out at constant temperature of (4°C). A His tag column was used to ensure that CTD was the protein that was being collected. Elution buffer was prepared using increasing levels of 3M Imidazole. 10 fractions were collected then a WB was performed. One gel was stained with Coomassie Blue to determine the clearest fraction and the other gel was specifically probed for the CTD protein using PCSK9 Antibody.

Binding Assay: Using a Circulix Binding Assay Kit (see figure 5 for schematic representation) we were able to measure the binding of His Tagged PCSK9 and His tagged CTD binding to LDLR

DISCUSSION / CONCLUSIONS

Results from the experiment represented in Figure 1 are very promising. Fig 1c suggests that with a higher concentration of CTD (40ag), there is an increase in LDLR by 27% and no effect on LRP1. This preliminary result provides a good basis for future studies, where titrating increased protein concentrations in vitro, in cell lines that produce PCSK9 and then moving to in vivo experiments within mice. Previous literature concluded that with increasing concentrations of PCSK9 there is a reduction in LDLR. Figure 3 reinforces those conclusions. PCSK9 degrades LDLR and has no effect on LRP1 levels. In figure 3, HEP G2 (control) compared to Hep G2 + PCSK9 there is a 68% reduction in LDLR levels and comparing Hep G2 (control) to D374Y, a gain of function mutation, there is a 77% decrease in LDLR. The experiment represented by fig 2a uses a cell line, HEP 293T, that does not express PCSK9. Interestingly, fig 2b suggests that when CTD binds to LDLR in the absence of PCSK9 there is an 11% reduction in LRP1.

Although the carboxyl terminal was previously shown to be a target for binding to LDLR, our data in figure 6a suggests that the carboxyl-terminal ends specifically to the EGF (AB) repeats of the LDLR receptor, which is a novel discovery. Figure 5 suggests that CTD has preferential binding at a pH of 5.3 which is biologically significant to the pH level of lysosomes. The novel discovery of the CTD binding to the EGF (AB) segment will be further explored by determining the shortest module of CTD that can still bind to this specific portion. Currently, the only drugs being considered as therapeutic approaches to the effects of PCSK9 are antibodies that neutralize the effects of PCSK9. By using a smaller molecular weight inhibitor this could greatly benefit patients.

Our data in figs. 4a, 6a, also suggests that CTD binds in an order of magnitude lower than PCSK9. In figure 6b the His tagged CTD is being outcompeted by LRP1:PCSK9 demonstrating a similar curve to figure 4b. In future experiments, we'll test CTD tagged to LDLR will be made into a GST tagged CTD, which will allow us to use the Circulix binding assay to allow CTD to act as a competitive inhibitor to His tagged PCSK9.

Overall, CTD shows promising preliminary data as a competitive inhibitor of PCSK9. With further experiments CTD has potential to become a part of drug therapy to lower Hypercholesterolemia patients circulating plasma LDL levels, leading to a reduced risk of developing atherosclerosis.

REFERENCES