An *In Vitro* System for Investigation of Sepsis-Induced Acute Kidney Injury: Identification of IGFBP7 Expression in Isolated Human Proximal Tubule Epithelial Cells

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Abstract #62

Purpose: IGFBP7 has recently been discovered as an early biomarker for Acute Kidney Injury (AKI), but little information is available regarding its role, if any, in the pathogenesis of this disease. To investigate AKI mechanisms, we have established and characterized a primary cell culture model of isolated human primary proximal tubule epithelial cells. **Methods:** Human kidney samples were obtained from the Center for Organ Recovery and Education (CORE). Cortex tissue was isolated and dissociated with collagenase and sieving. The resultant slurry was cultured until viable cells reached confluence. Proximal tubule epithelial cells (PTECs) were immunoaffinity-isolated with an antibody against the proximal tubule specific protein CD13 (Aminopeptidase N) using the Dyanbead pan-mouse IgG system. After culture on transwell permeable supports in varying media, CD13+ PTECs and CD13- cells were characterized by immunoblot and immunofluorescence for expression of the proximal tubule markers CD13, Gamma-glutamyl transpeptidase (GGT), and Aquaporin 1(AQP1), and for the distal tubule markers E-cadherin and the sodium chloride co-transporter (NCC). IGFBP7 expression was assessed from the media of cultured cells by immunoblot analysis. **Summary:** CD13 isolation resulted in cells that were positive for CD13, GGT, and AQP1, yet negative for E-cadherin and NCC, providing evidence from multiple markers that these cells are of proximal tubule origin. Immunoblot analysis of the media from CD13+ cells demonstrates that indeed IGFBP7 is produced by these cells. We have also identified that IGFBP7 is preferentially expressed in the CD13+ PTECs compared to the CD13- cells, suggesting that PTECs may be a primary source of IGFBP7 expression in the kidney. Lastly, we identified the ability to modulate IGFBP7 expression by adjusting serum, insulin, and glucose concentrations, where decreasing serum or insulin decreased IGFBP7 expression, and decreasing glucose increased IGFBP7 expression. **Conclusion:** We have successfully developed a viable human cell culture model system for cellular and molecular analysis of AKI, in which we can isolate PTECs at high purity. Using this system, we confirmed that the early biomarker IGFBP7 is produced by PTECs. This system and knowledge will now allow for investigation of the potential role of IGFBP7 in the molecular etiology of AKI.

Purpose

With Acute Kidney Injury (AKI) on the rise in the ICU, the search for better diagnostic markers and a better understanding of the molecular and cellular etiology of the disease has increased. This group recently reported the discovery and validation of two early superior biomarkers, IGFBP7 and TIMP2, which has led to development of the Nephrocheck® test for AKI risk assessment. While these markers are superior to any other current diagnostic for the detection of AKI, little is known about any potential biological role for either of these markers in the disease. To study potential molecular and cellular effects of these molecules, we have developed an *in vitro* model system of primary human proximal tubule epithelial cells.

Methods

SAMPLE PROCUREMENT: Human kidneys rejected for transplant were obtained from the Center for Organ Recovery and Education (CORE) through a protocol approved by the University of Pittsburgh Committee for Oversight of Research and Clinical Training Involving Decedents (CORID). All samples were perfused and stored in HTK, and packed in ice.

SAMPLE PROCESSING: Each whole kidney was processed to generate and archive samples for immunohistochemistry (IHC), immunoblot analysis, RNA/DNA analysis and primary cell culture. For cell culture, the cortex was separated from the medulla, and both tissues were processed separately. Samples were minced and digested for 1 hour at 37C in HBSS with Collagenase IV and DNAse. Following digestion, the slurries were subjected to mechanical dissociation by sieving through 250um sieves to remove undigested connective tissue. The resultant slurry was fractioned for passage zero (P0) frozen stocks and culture until viable cells reached confluence.

CELL CULTURE: For propagation and isolation of primary cells, the cortex dissociation slurry was cultured on rat tail collagen 1 coated culture plates in DMEM/F12 supplemented with 5% FBS, insulin, selenium, transferrin, penicillin, and streptomycin, in a humidified 37 C incubator with 5% CO2. For experimentation, primary and HK2 cells were cultured in various medias on transwell permeable supports with the same temperature, humidity, and CO2 conditions.

PROXIMAL TUBULE EPITHELIAL CELL (PTEC) ISOLATION: PTECs were immunoaffinity-isolated from confluent cortex tissue cultures (passage 1-3) with an antibody against the proximal tubule specific protein CD13 (Aminopeptidase N) using the Dyanbead pan-mouse IgG system per manufacturer instructions. All experimentation was performed from cell culture passages 2 to 6. The remaining non-isolated cells (CD13-) were retained and used for comparison.

CELL CHARACTERIZATION AND IGFBP7 EXPRESSION: After culture on transwell permeable supports, CD13+ PTEC, CD13-, and HK2 cells and cell lysates were characterized by immunoblot and immunofluorescence for expression of the proximal tubule markers CD13, Gamma-glutamyl transpeptidase (GGT), and Aquaporin 1(AQP1), and for the distal tubule marker E-cadherin. IGFBP7 expression was assessed by immunoblot analysis of cell lysate and media from cultured cells.

DATA ANALYSIS: Immunoblots were quantitated using ImageJ software. Antibody signals from lysate analysis were normalized to GAPDH signal, and antibody signals from media were normalized to lysate protein assay or cell number. Statistics and graphing were performed using Excel.

Summary

- Number of samples received 11
- Number of kidneys received 20
- Subject age range 5mo - 67yr
- Median age 50
- Male/Female ratio 4/7
- Cold ischemia time range 3-51 hours
- Successful culture 100%
- Archived samples for culture ~300

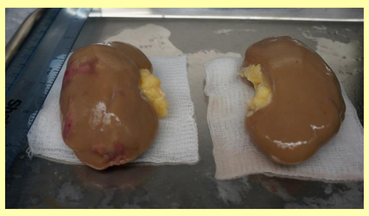
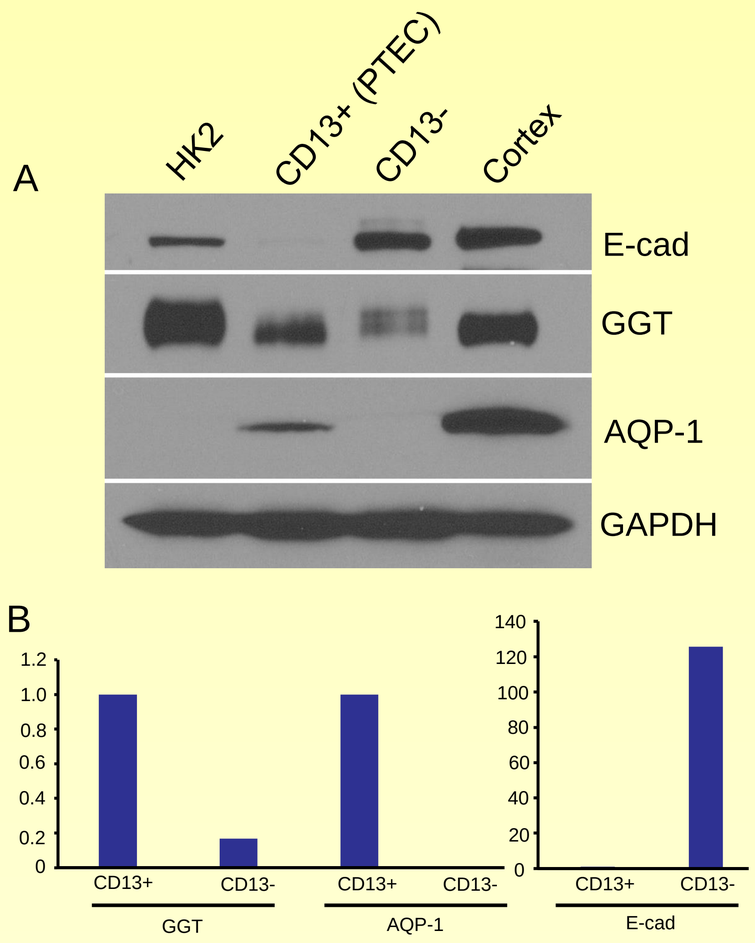


Figure 1: Human kidney sample procurement and processing. Since July of 2014, we have received 11 kidney samples from CORE . For each kidney, sections were fixed in paraformaldehyde for IHC, cortex and medulla samples were snap frozen for immunoblot and nucleic acid analysis, and separate cortex and medulla samples were dissociated for cell culture and storage. Listed are some demographic and experimental data.



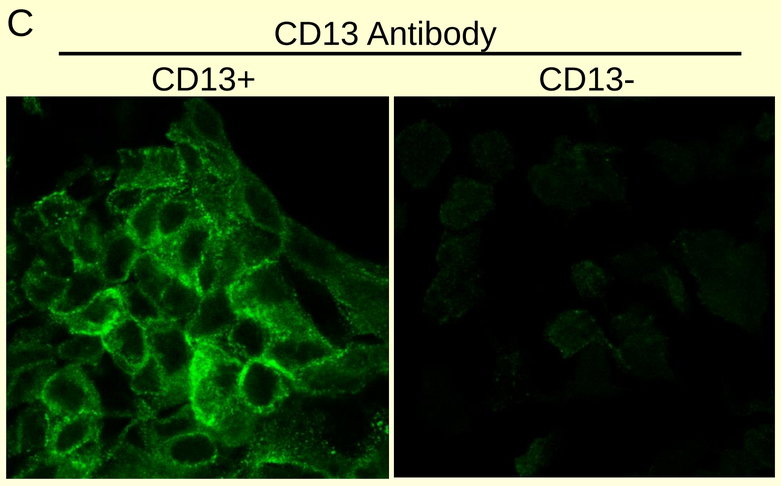


FIGURE 2: Characterization of immunoaffinity isolated primary cells (PTECs). After Immunoaffinity isolation, CD13 positive (CD13+ (PTEC)) and CD13 negative (CD13-) cells were subjected to immunoblot and immunofluorescence analysis for characterization. A) CD13+ and CD13- cells, along with HK2 cells (HK2) and tissue lysate from a cortex sample of the primary kidney sample HAK3 (Cortex), were subjected to immunoblot analysis for expression of the proximal tubule markers Gamma glutamyl transpeptidase (GGT) and Aquaporin-1 (AQP-1), the distal tubule marker E-cadherin (E-cad), and GAPDH for a loading control. B) Signals from each antibody staining was normalized to the GAPDH signal and graphed. C) CD13+ and CD13- cells were subjected to immunofluorescence analysis using anti-CD13, the antibody used for the isolation process.

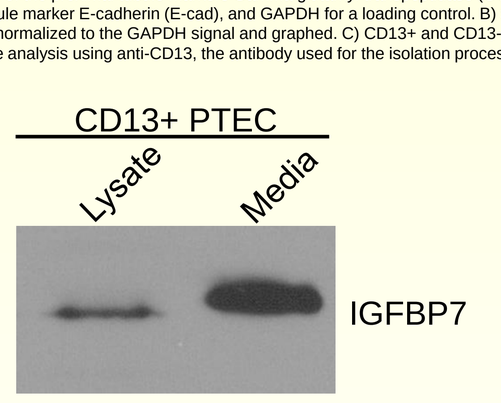
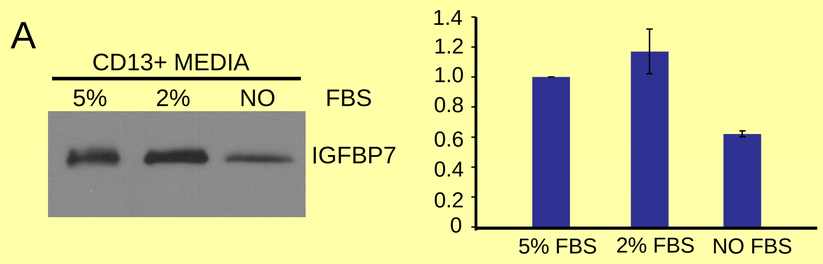
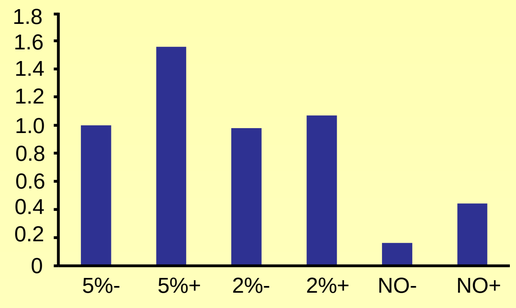


FIGURE 3: IGFBP7 expression by CD13+ PTEC cells in culture. CD13+ cells were cultured on transwell permeable supports and fed every day for 1 week. A fraction of media was removed, protected with 5x sample buffer and frozen. Lysate was made from the cells, and the lysate (50ug) and media (40ul) were subjected to immunoblot analysis for IGFBP7 expression.





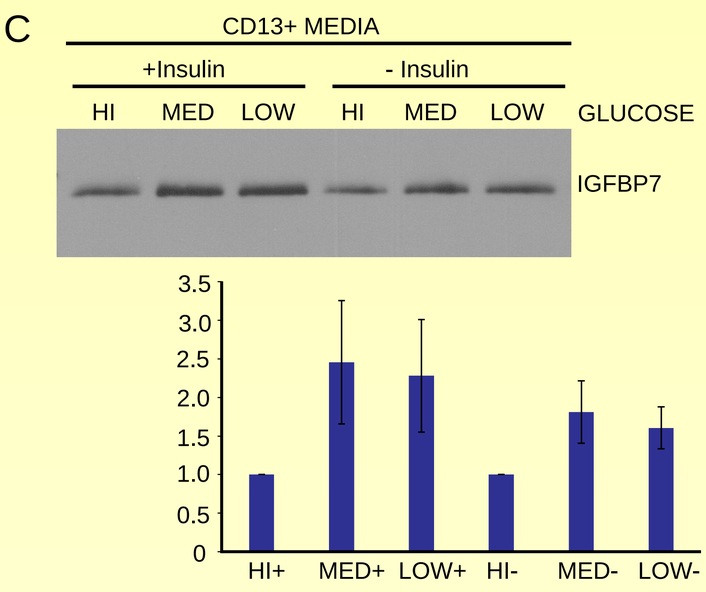


FIGURE 4: Effects of serum, insulin, and glucose on IGFBP7 expression. CD13+ PTEC cells were cultured on transwell permeable supports and fed every day for 1 week in medias with differing base media and differing serum, insulin, and glucose concentrations. A fraction of media cultured for 24 hours was subjected to immunoblot analysis for detection of IGFBP7. A) Culture media described in the methods was supplemented with either 5%, 2% or no fetal bovine serum (FBS). Error bars = standard deviation. B) Cells were cultured in the media used above further modified to supplement (+) or remove (-) insulin. MO= Media that was not exposed to any cells. C) Media with high glucose levels (HI-DMEM/F12), medium glucose levels (MED-DMEM+F12), or low glucose levels (LOW-DMEM alone), were either supplemented with insulin (+) or not (-). Error bars = standard deviation.

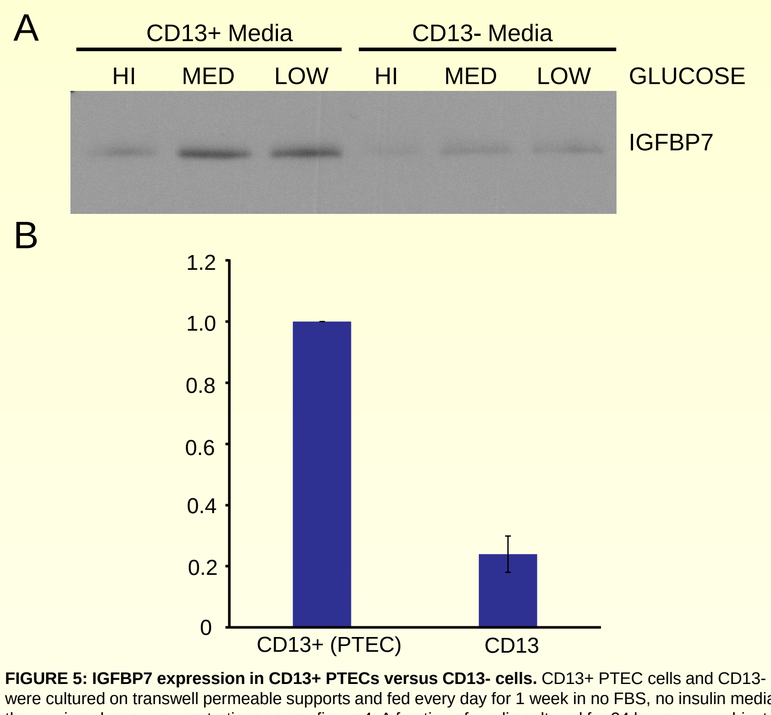


FIGURE 5: IGFBP7 expression in CD13+ PTECs versus CD13- cells. CD13+ PTEC cells and CD13- cells were cultured on transwell permeable supports and fed every day for 1 week in no FBS, no insulin medias with the varying glucose concentrations as per figure 4. A fraction of media cultured for 24 hours was subjected to immunoblot analysis for detection of IGFBP7. A) Example immunoblot of results. B) Graphical representation of the level of IGFBP7 expression of CD13+ and CD13- cells from multiple passages and platings. Error bars = standard deviation.

Conclusion

- **We have established a viable human cell culture model system in which we can isolate PTECs at high purity**
- **Confirmed that the early biomarker IGFBP7 can be produced by primary PTECs**
- **PTECs may be the primary source of IGFBP7 expression in the kidney**