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BIOLOGY

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Normally, the kidneys filter wastes and help balance water, salt, and electrolyte levels in the body. When the kidneys stop working, waste products, fluids, and electrolytes accumulate. Acute kidney injury (AKI) is one class of renal injury that can result from renal ischemia, acute drug, or toxicant exposure [10]. AKI leads to the breakdown of the epithelium of the proximal tubule of the kidney, which is integral to the reabsorption and balancing of salts and electrolytes filtered in the kidney. Morphologically, AKI appears as a denuded epithelium and casts formed within blood vessels. These casts are made up of various proteins and dead or dying cells that have sloughed off of the epithelium. Renal ischemia, the primary cause of AKI in humans, is a decrease in the blood supply of the kidney caused by constriction or obstruction of the blood vessels, in part due to the cast formation. In animal models of AKI, renal ischemia has been shown to alter levels of various growth factors. Growth factor release from the injured regions of the kidney is thought to induce the repair process in the epithelium. We hypothesize that hepatocyte growth factor (HGF), a growth factor that may be involved in renal repair, is released from ischemic tissue and may induce the migration of reparative cells to the injured region.

INTRODUCTION

Recent findings suggest that in animal models of AKI, native kidney cells are the primary source of reparative cells. These reparative cells are believed to be either mature epithelial cells or daughter progenitor cells, two different modes of cellular repair of the kidneys that have been suggested to occur [2], [1]. One characteristic of reparative cells is the capacity to migrate, whether progenitor cells or mature epithelial cells. The recruitment of these reparative cells may be controlled by regulatory signal molecules including growth factors. The kidney is a known site of synthesis for several growth factors [9], and several have been suggested to be involved in the repair process in the kidney [3], [4], [5]. Uniquely, HGF has been shown to have ameliorative effects even when administered after the acute insult [8].

Past studies suggest that in AKI animal models, HGF exerts strong mitogenic and morphogenic effects for renal epithelial cells and HGF mRNA and blood HGF level are markedly induced after AKI [6]. When recombinant HGF was injected into during a four-week period, DNA synthesis of tubular epithelial cells was found to be higher than in control mice without the HGF injection, suggesting that tubular cell expansion is promoted by HGF [11]. Additionally, in human patients with AKI, elevated urine HGF levels are described as consistent with a role for HGF in promoting tubular cell proliferation, whereas, patients with chronic glomerular or polycystic disease, as well as patients with advanced chronic renal insufficiency and healthy controls, show detectable but low urine HGF levels [11]. All of this evidence suggests that HGF may play an active role in promoting renal regeneration in AKI.

HGF is a pleiotropic growth factor synthesized in the kidney that affects growth, motility, differentiation, and morphogenesis of its target cells. HGF can be secreted from cells into the extracellular environment by cleavage of signal peptides. The receptor for HGF is c-Met tyrosine kinase, a proto-oncogene protein, which has two disulfide-linked subunits, and an intracellular tyrosine kinase domain. Once HGF is released, it stimulates the c-Met receptor on its target cell in order to activate certain signal transduction cascades, and signaling via binding of HGF to the c-Met receptor induced by the tyrosine kinase activity results in the subsequent phosphorylation of the c-terminal tyrosine residues [7].

There are still many aspects of HGF that are not fully

understood in renal tissue regeneration. Whether HGF prevents injury or facilitates repair following AKI remains to be clarified. Molecules involved in regulation of HGF and how HGF is involved in regulation of other growth factors also needs further attention. The current study uses an *in vitro* model used that allows an examination of the release of HGF in ischemia, which may be an important aspect of the repair process following AKI. Such research is significant because it can facilitate the discovery of therapeutic treatments for patients with many types of renal injury, including AKI.

MATERIALS AND METHODS

A. AKI Model

Mice were subjected to ischemia, induced by uninephrectomy and contralateral renal ischemia, and then allowed to recover for 1-7 days. Specifically, 12-week-old male C57BL/6J mice were anesthetized using 2.5% isoflurane by inhalation. Under aseptic precautions, a right nephrectomy was performed via a right loin incision. A similar incision was made in the left loin, and the left renal pedicle was exposed, secured and clamped with a micro-serrefine vascular clamp for 30 minutes. Blanching of the entire kidney was observed and ensured loss of blood flow. During this period, the kidney was kept moist using sterile gauze soaked in saline. At the end of ischemia, the clamp was removed to allow reperfusion, which was confirmed visually, and the kidney was returned to the abdominal cavity in its original position. The incision was closed with 4-0 prolene sutures and the animals were allowed to recover.

B. Transwell in vitro *System*

Kidneys were removed from the AKI mice at 1, 2, 4 or 7 days after the ischemia-reperfusion injury. Using a Transwell *in vitro* System (Figure 1), injured kidneys from one mouse were minced and placed in the lower chamber containing DMEM/F12 culture media. As a control, uninjured C57BL/6 mouse kidneys were used in the lower chamber. The upper chamber of the Transwell contained DMEM/F12 with 1% fetal bovine serum added and the kidney tissue of a donor mouse which expresses GFP ubiquitously. The media of the lower chamber ("conditioned media") was collected after 24 hours and particulate material was removed by centrifugation at 4°C at 1300 rpms for 5 minutes. The conditioned media was kept at -20°C until use. The porous filter was removed from the Transwell, the upper surface was cleared by wiping with a cotton tip, and the filter was washed in phosphate buffered saline (PBS). After fixation, the filter was mounted on slides and examined by fluorescence microscopy for migration of cells in response to renal injury (Figure 2).

C. Bradford Protein Assay

Protein content was analyzed using a Bradford Protein Assay using the manufacturer's protocol. In a microtiter plate, bovine serum albumin (BSA) standard was loaded in a ½ series dilution to generate a standard curve. Conditioned media samples, in duplicate, were also analyzed. Bradford Reagent, a diluted dye reagent, was added to each well and the plate was read in a Universal Microplate Reader (BioTek) at an absorption spectrum of 595 nm. The optical density readings from the BSA curve were used to calculate a regression curve, which was then used to determine the protein concentrations of the conditioned media samples.

D. SDS-PAGE

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Figure 4) was performed, according to standard protocols. A polyacrylamide gel, consisting of a 7.5% running gel and a 4.3% stacking gel, was used. The conditioned media samples were prepared in Laemmli sample buffer at a concentration 10 ug/ml and loaded in the wells of the gel. The gel was run for approximately one hour, fixed with 50% methanol: 7% acetic acid, and then stained overnight with SyproRuby. After washing, the gel was imaged on a VersaDoc 3000 (BioRad).

E. Western Blot

A Western Blot (Figure 5) was performed according to standard protocols. A polyacrylamide gel was prepared as described above and SDS-PAGE was run. The protein was transferred onto a PVDF membrane. The membrane was blocked with 5% dry milk in tris-buffered saline (TBS) containing 0.1% Tween-20. A 1/1000 dilution (0.1 µg/ml) of antibody to HGF (R&D Systems, goat anti-human HGF) was incubated with the membrane overnight. The specific antibody solution was removed, and the membrane was washed. The membrane was then incubated with 1/30,000 dilution of a peroxidase-conjugated secondary antibody (Pierce, rabbit anti-goat IgG) for 3 hours. The HGF protein labeling was developed by incubation of the membrane with SuperSignal West Dura Extended Duration Substrate (Pierce) and imaged on a Konica Minolta SRX-101-A.

RESULTS

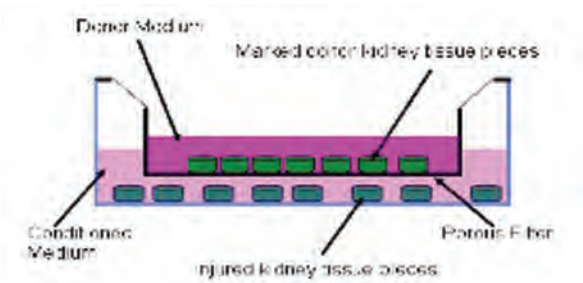

Figure 1: Diagram of the Transwell in vitro System Slices of healthy donor kidney (upper chamber) and injured recipient kidney from ischemic mice (lower chamber) were placed in separate chambers. After 24 hour incubation, conditioned media was collected from the lower chamber and the porous filter was examined for the presence of migrated cells.

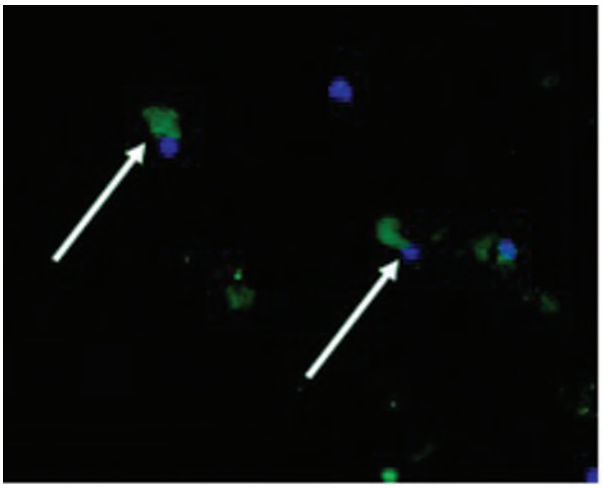

Figure 2: Cellular Response to Renal Injury
Representative image of porous filter with cells that have migrated in response to renal injury. White arrows indicate GFP+ cells (green) with DAPI-labeled nuclei (blue).

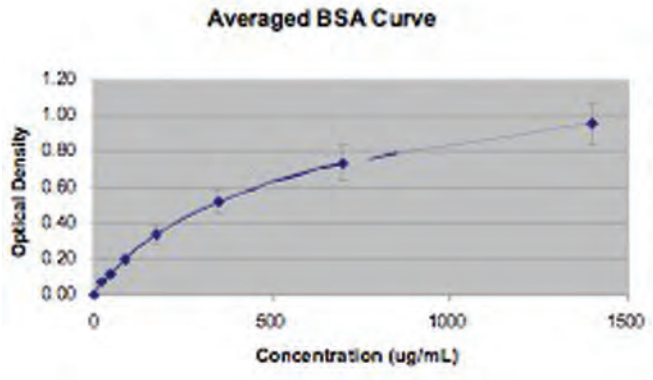

Figure 3: Averaged BSA Curve
Optical density readings at each concentration of BSA used in the standard curves were averaged and a standard deviation was calculated. The small standard deviations at each point demonstrate the consistency of the protein analysis across assays.

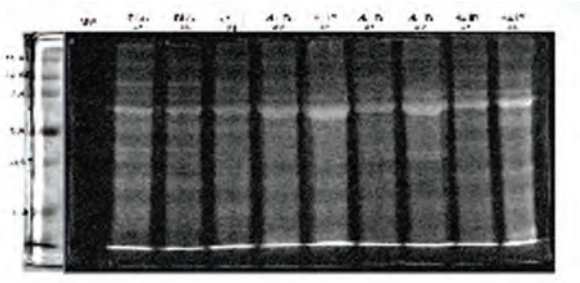

Figure 4: SDS-PAGE Gel of Total Protein
SDS-PAGE was run on conditioned media samples of injured renal tissue, and the gel was stained with SyproRuby to visualize total protein content of each sample. Each lane contains conditioned media prepared from uninjured, control kidney (BL/6) or from kidneys extracted at different times post-renal injury (dx IR, day post-injury at which kidney sample was obtained). No protein bands were seen to be grossly amplified in any of the lanes. Similar banding intensity throughout the samples provides confirmation of the accuracy of the calculated protein concentrations.

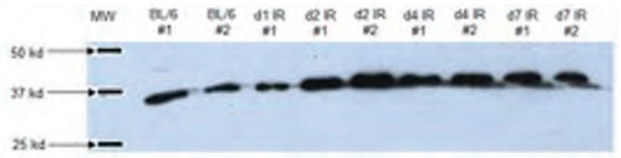

Figure 5: Western Blot of HGF
A Western Blot was performed using conditioned media samples of injured renal tissue to identify the presence of hepatocyte growth factor (HGF). A single band was seen at approximately 37 kD, near the reported molecular weight of the α chain of HGF. The release of HGF into conditioned media appears to be greater from kidneys obtained at 2 days post-injury than that seen in the control kidneys (BL6) or in kidneys obtained at 1 day post-injury. This increase in the level of HGF appears to be sustained in kidneys obtained through 7 days post-injury.

CONCLUSIONS

Inter-assay BSA standard curves used to analyze protein content of experimental samples were found to be highly consistent. HGF was present in the conditioned media of the injured renal tissue and appeared to be released from injured tissue in a time-dependent manner. Whether HGF prevents injury or facilitates repair following AKI remains to be clarified. Future efforts will focus on demonstration of a direct correlation between the release of HGF from injured kidney and the migration of reparative cells *in vitro* and *in vivo*.

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