Research Article

Healing Cells in the Kidney of the Adult Rat

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ABSTRACT

Chronic kidney disease is an increasing public health issue. Prevalence has been estimated to be 8-16% worldwide. Complications include increased mortality including cardiovascular mortality, progression of kidney disease, acute kidney injury, cognitive decline, anemia, disorders of mineral and bone, and fractures. Two types healing cells, pluripotent stem cells and totipotent stem cells, have been located, isolated and characterized from skeletal muscle, adipose tissue, bone marrow, dermis, and blood of adult animals, including humans. The current study was undertaken to determine whether these two populations of healing cells were present in the kidney of the adult rat. Adult rats were euthanized following the guidelines of Mercer University’s IACUC. The kidneys were harvested, fixed, cryosectioned and stained with two antibodies diagnostic for these healing cells, i.e., stage-specific embryonic antigen-4 (SSEA-4) for pluripotent stem cells and carcinoembryonic antigen-cell adhesion molecule-1 (CEA-CAM-1) for totipotent stem cells. Cells staining for SSEA-4 were located amongst the tubules in the medulla of the kidney, whereas cells staining for CEA-CAM-1 were located among the glomeruli in the cortex of the kidney and among the tubules in the medulla of the kidney. This is the first report of native populations of both pluripotent and totipotent healing cells in the adult rat kidney. Studies are ongoing to address their functional significance during normal kidney maintenance and repair.

Keywords
Healing cells, Totipotent stem cells, Pluripotent stem cells, SSEA, CEA-CAM-1, Adult rat, Kidney, Renal, Immunocytochemistry, ELICA-fixative.

Introduction

Chronic kidney disease is defined by a reduction in glomerular filtration rate, an increase in urinary albumin excretion, or both. Chronic kidney disease is an increasing public health issue. Prevalence has been estimated to be 8-16% worldwide. Complications include increased mortality including cardiovascular mortality, progression of kidney disease, acute kidney injury, cognitive decline, anemia, disorders of mineral and bone, and fractures [1].

McCullough, et al. [2] reviewed 59 studies and chose six of the highest quality for further examination. They estimated the prevalence from the six studies measuring impaired kidney function using estimated glomerular filtration rate in community screening samples. These studies reported a prevalence ranging from 1.7% in a Chinese study to 8.1% in a U.S. study, with four studies reporting an estimated prevalence of 3.2-5.6%. Thus it was estimated that chronic kidney disease is common in the general population with a prevalence similar to that of diabetes mellitus, indicating the substantial health challenge of chronic kidney disease.
For the United States, the prevalence of chronic kidney disease of all forms increased from 10.0% in 1988-1994 to 13.1% in 1999-2004. The increase was attributed partly to the increasing prevalence of diabetes mellitus and hypertension, raising concerns about future increases in the incidence of kidney failure and other complications of chronic kidney disease [3].

Renal failure is a devastating and debilitating disease. According to the Center for Disease Control, kidney disease (nephritis, nephrotic syndrome, nephrosis) was the ninth leading cause of death in 2015 [4]. Currently, limited treatment options are available for those with end stage renal disease (ESRD). In regards to quality of life, transplantation of a non-diseased kidney is often the best choice for treatment of ESRD; however, transplantation requires either death of a donor or critical short-term and long-term consequences experienced by an altruistic living donor. Dialysis is another alternative and is responsible for increasing longevity in persons with ESRD. However, patients on dialysis suffer from comorbidities, in particular depression, arteriosclerosis, uremic osteodystrophy and β2-microglobulin-associated amyloidosis with complicating spinal stenosis, carpal tunnel syndrome, bone cysts and arthropathy [5,6]. Discovery of resident healing cells, such as totipotent stem cells and pluripotent stem cells, in the kidneys of adult rats might offer hope for future treatment options of ESRD in humans.

Materials and Methods

Animal Use
The use of animals in this study complied with the guidelines of Mercer University. These guidelines reflect the criteria for humane animal care of the National Research Council as outlined in “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (National Academy Press, 1996).

Tissue Harvest
Adult male Sprague-Dawley rats (n=10) were humanely euthanized using carbon dioxide inhalation, as per Mercer University IACUC protocols. Once euthanized, the rats were placed on crushed ice. A midline incision was made in the abdomen, the kidneys dissected from the surrounding tissue, bisected, and then placed into ELICA fixative. The ELICA fixative consisted of aqueous 0.4% v/v glutaraldehyde, 2% w/v paraformaldehyde, and 1% w/v glucose, Ph 7.4, with an osmolality 1.0 [7]. The kidneys were fixed for two weeks at ambient temperature. After fixation, the kidneys were transferred and stored in Dulbecco’s Phosphate Buffered Saline (DPBS, Invitrogen, GIBCO, Grand Island, NY) at pH 7.4 at ambient temperature [7].

Cryosectioning
The kidneys were embedded in Tissue Tek OCT Compound 4583 (Miles Laboratory, Ames Division, Elkhart, IN) and then frozen at -20°C. The kidneys were sectioned on a Tissue Tek Cryostat II (GMI, Ramsey, MN) to a thickness of 7 microns. The sections were applied to positively charged slides (Mercedes Medical, Sarasota, FL) and stored at -20°C until stained [7].

Immunocytochemistry
Immunocytochemical staining was performed following established procedures for ELICA analysis [7,8]. Seven-micron tissue sections were incubated with 95% ethanol to remove the OTC cryostat embedding medium and then washed under running water for five minutes. The tissue sections were incubated with 5.0% (w/v) sodium azide (Sigma, St. Louis, MO) in DPBS for 60 minutes. They were then washed in running water for five minutes, and incubated with 30% hydrogen peroxide (Sigma, St. Louis, MO) for 60 minutes to irreversibly inhibit endogenous peroxidases [9]. Tissue sections were rinsed with running water for five minutes and incubated for 60 minutes with blocking agent (Vectastain ABC Reagent Kit, Vector Laboratories Inc., Burlingame, CA) in DPBS [8]. The blocking agent was removed and the sections rinsed with running water for five minutes. They were then incubated with primary antibody for 60 minutes. The primary antibodies consisted of 0.005% (v/v) carcinoembryonic antigen cell adhesion molecule-1 (CEA-CAM-1) in DPBS for totipotent stem cells [10]; 1 µg per ml of stage-specific embryonic antigen-4 for pluripotent stem cells (SSEA-4, Developmental Studies Hybridoma Bank, Iowa City, IA) in DPBS [11]; and smooth muscle alpha-actin (1A4, Sigma Chemical Co., St Louis, MO) in DPBS [7,12]. The primary antibody was removed. The sections were rinsed with running water for five minutes, and incubated with secondary antibody for 60 minutes. The secondary antibody consisted of 0.005% (v/v) biotinylated affinity purified, rat adsorbed anti-mouse immunoglobulin G (H + L) (BA-2001, Vector Laboratories) in DPBS [11]. The secondary antibody was removed. The sections were rinsed with running water for five minutes, and then incubated with avidin-HRP for 60 minutes. The avidin-HRP consisted of 10 ml of 0.1% (v/v) Tween-20 (ChemPure, Curtain Matheson Scientific, Houston, TX) containing 2 drops reagent-A and 2 drops reagent-B (Peroxidase Standard PK-4000 Vectastain ABC Reagent Kit, Vector Laboratories) in DPBS [7]. The avidin-HRP was removed. The sections were rinsed with running water for five minutes, and incubated with AEC substrate (Sigma) for 60 minutes. The AEC substrate was prepared as directed by the manufacturer. The substrate solution was removed. The sections were rinsed with running water for 10 minutes and then coverslipped with Aqua-mount (Vector Laboratories) [7].

Positive and negative controls were included to assure the validity of the immunocytochemical staining [7]. The positive controls consisted of adult-derived totipotent stem cells (positive for CEA-CAM-1) [7,10], pluripotent stem cells (positive for SSEA-4) [7,11], and smooth muscle surrounding blood vessels within the tissue (positive for IA4) [7,10]. The negative controls consisted of the staining protocol with DPBS alone (no antibodies or substrate), without primary antibodies (CEA-CAM-1, SSEA-4, or IA4), without secondary antibody (biotintylated anti-mouse IgG), without avidin-HRP, and without substrate (AEC) [7].

Visual Analysis
Stained sections were visualized using a Nikon TMS phase contrast microscope with bright field microscopy at 40x, 100x, and 200x. Photographs were taken with a Nikon CoolPix 995 digital camera.
Animal model systems were utilized to determine the potential to form any somatic cell of the body [10,11,20]. Both populations of healing cells have shown the capacity in vitro for extended self-renewal and the of post-natal animals [20]. Young and colleagues reported the presence of similar healing cells staining for SSEA-4 were located in the medulla. CEA-CAM-1 were located in both the cortex and medulla, while with their specific antibodies [7,10,11]. As noted, cells staining for pluripotent stem cells (staining for SSEA-4), based on staining with their specific antibodies [7,10,11]. As noted, cells staining for totipotent stem cells (staining for CEA-CAM-1) and putative pluripotent stem cells were identified among the glomeruli in the cortex and among the tubules in the medulla of the adult rat kidney (Figure 1B). The positive procedural controls, smooth muscle alpha-actin staining in the tunica media of the vasculature, confirmed the specificity of the staining process (Figure 1C). All four negative procedural controls demonstrated no staining of any kind within the tissue (Figure 1D, representative picture).

Results

The staining process resulted in SSEA-4 staining of cells (putative pluripotent stem cells) being located among the tubules in the medulla of the adult rat kidney (Figure 1A). Cells staining for CEA-CAM-1 (putative totipotent stem cells) were identified among the glomeruli in the cortex and among the tubules in the medulla of the adult rat kidney (Figure 1B). The positive procedural controls, smooth muscle alpha-actin staining in the tunica media of the vasculature, confirmed the specificity of the staining process (Figure 1C). All four negative procedural controls demonstrated no staining of any kind within the tissue (Figure 1D, representative picture).

Discussion

This study was conducted to identify putative healing cells, i.e., totipotent stem cells, pluripotent stem cells, and mesodermal stem cells, in individuals with Parkinson disease [21-23] and individuals with interstitial pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD) [17]. In an Institutional Review Board (IRB)-approved clinical trial for Parkinson disease, totipotent stem cells were delivered by intra-nasal infusion and pluripotent stem cells and mesodermal stem cells were delivered by intravenous infusion. One hundred percent of the individuals showed marked improvement two months after their single autologous treatment. From that point forward, 75% of the participants were either stable or showed marked improvement at 7 months and 14 months after treatment. The 25% that began to decline two months after their single autologous treatment declined at a slower rate and still maintained a better quality of life than before their treatment began through these same time periods of 7 months and 14 months post-treatment [19].

We have also tested autologous healing cells, i.e., totipotent stem cells, pluripotent stem cells, and mesodermal stem cells, in individuals with Parkinson disease [21-23] and individuals with interstitial pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD) [17]. In our type-I diabetes model, totipotent stem cells and pluripotent stem cells from a recipient were combined with decellularized porcine pancreatic matrices and pancreatic islets from the donor. The resulting pancreatic islet organoids secreted significantly more species-specific insulin per ng DNA compared to native pancreatic islets by themselves [14].

Two individuals volunteered for our safety and efficacy pulmonary disease trial utilizing autologous stem cells. One individual was diagnosed with severe interstitial pulmonary fibrosis (IPF) while the other was diagnosed with chronic obstructive pulmonary disease (COPD). In this trial, healing cells were harvested by venipuncture, separated from the blood cells, segregated into totipotent stem cells, pluripotent stem cells, and mesodermal stem cells, and activated. All the totipotent stem cells and half of the pluripotent stem cells were nebulized into the lungs, whereas the other half of the pluripotent stem cells and all of the mesodermal stem cells were transfused into the blood stream by intravenous infusion. Forced expiratory volume in one second (FEV1) [24] was measured before and one month after stem cell treatment in

![Figure 1: Immunocytochemistry of cryostat sectioned adult rat kidney.](image-url)
both individuals to ascertain whether their single autologous stem cell treatment had a positive or negative affect on their respective pulmonary function. The individual with IPF (interstitial pulmonary fibrosis) had an initial pre-treatment FEV1 of 14% and a post-treatment FEV1 of 27%, demonstrating a 93% increase in the FEV1. The individual with COPD (chronic obstructive pulmonary disease) had an initial pre-treatment of 30% and a post-treatment FEV1 of 46%, demonstrating an increase in their FEV1 of 53% [17]. Both individuals are still alive, six years after their initial treatment. These results suggested that autologous healing cells could be safely used as a treatment modality for pulmonary diseases.

While these safety and efficacy studies of healing cells as a potential treatment modality for Parkinson Disease, Interstitial Pulmonary Fibrosis, and Chronic Obstructive Pulmonary Disease did not address the issue of whether autologous healing cells would be an effective treatment for those individuals with end stage renal disease, it does suggest that possibility. Future studies will need to address this issue directly.

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