Stem cells from foreign body granulation tissue accelerate recovery from acute kidney injury

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Abstract

Background. In previous studies, we obtained mesenchymal stem cells called granulation tissue stem cells (GTSC) from a regenerating granulation tissue created by placing a foreign body in the subcutaneous tissue of rats. Here, we used GTSC to ameliorate ischemia/reperfusion-induced acute kidney injury (AKI) in rats.

Methods. In two groups of Fischer rats, we induced ischemia/reperfusion injury. Group 1 (treated rats) received one intravenous injection of GTSC 3 h after injury; Group 2 (control rats) received vehicle. Both groups were subsequently studied by renal function tests, kidney histology and immunohistochemistry.

Results. At 24 and 48 h after injury, plasma creatinine and blood urea nitrogen were significantly lower in the treated rats as compared to control rats. The levels remained low and declined to near baseline levels by Day 4 in the treated group. At the cortico-medullary region, the treated rats showed significantly higher renal tubular cell proliferation and less tubular cell apoptosis. Histological analysis of the kidney for tubular dilatation, necrosis, congestion and casts was not significantly different in the two groups. To understand the mechanism of the GTSC effect, messenger RNA levels of several growth and immune modulatory factors were quantified in cultured GTSC and compared with those in cultured glomerular epithelial cell (GEC; a non-stem cell line). GTSC had 2- to 8-fold higher expression of FGF2, HGF, IGF-1, vascular endothelial growth factor (growth factors) and IL-4, IL-6 (anti-inflammatory factors) than GEC.

Conclusions. Administration of GTSC accelerates recovery in rats with ischemia/reperfusion-induced AKI. This effect may be mediated by the paracrine action of growth and immune-suppressive factors secreted by these cells.

Keywords: acute kidney injury; cytokines; granulation tissue stem cells; growth factors; ischemia/reperfusion

Introduction

When a perforated polyvinyl tube is placed in the subcutaneous tissue of rats, it rapidly induces the formation of a well-vascularized regenerating granulation tissue that contains cells expressing markers of stem cells [1, 2] and characterized on culture as being of mesenchymal origin [3]. These granulation tissue-derived stem cells (GTSC) express stem cell markers, secrete high levels of vascular endothelial growth factor (VEGF) in media and transdifferentiate to different phenotypes in vitro [3]. Based on these properties, we hypothesized that foreign body-induced granulation tissue could serve as a novel source of mesenchymal stem cells that could be used to repair damaged organs. Here, we test the efficacy of cultured GTSC to ameliorate ischemic acute kidney injury (AKI) in rats.

Materials and methods

Construction of the polyvinyl tube for subcutaneous placement

A piece of polyvinyl chloride (PVC) tubing (L = 25 mm, internal diameter = 7 mm) (PVC 180; Nalgene Nunc International, Rochester, NY) was sealed by applying heat at both ends to create an enclosed chamber (inside volume ~0.7 mL) (Figure 1A). Eight holes (diameter 0.5 mm) were drilled around the chamber to allow diffusion between the tube contents and the surrounding tissue. The tubes were stored in 70% alcohol for sterility, then washed vigorously before placement with sterile saline and air-dried.

Surgical placement of the polyvinyl tube in rats

All the animal experiments were approved by the Institutional Animal Care and Use Committee of the John H. Stroger, Jr. Hospital of Cook County. Fischer 344 (F344) syngeneic males rats (225–250 g) were anesthetized with intraperitoneal sodium nembutal (5 mg/100 g weight), their backs shaved and cleaned with alcohol and povidone and two 1-cm incisions made on either side of the lumbar region. Two subcutaneous pockets were made by blunt dissection around the incision into which the polyvinyl tubes were inserted (two per rat) (Figure 1B). The incisions were closed with silk sutures, and the animals were allowed to recover and form granulation tissue around the tube.

Culture of stem cells isolated from the subcutaneous granulation tissue

The tissue encapsulating the polyvinyl tubes was harvested aseptically at Day 4 (Figure 1C and D). It was gently chopped and treated with 1 mg/mL collagenase (Sigma–Aldrich, St. Louis, MO) made in phosphate-buffered...
saline (PBS; 10 mM phosphate, 125 mM NaCl pH 7.4) four times each for 20 min with gentle shaking. Each time after collagenase treatment, the supernatant was centrifuged at 2000 rpm (1500 g) and the cell pellet washed several times with sterile RPMI-1640 medium. The pellet of cells was finally suspended in fresh mesenchymal stem cell growth medium supplemented with growth factors, antibiotics (penicillin 100 U/mL and streptomycin 100 mg/mL) and L-glutamine (4 mmol/L) as supplied by the manufacturer (Lonza, Walkersville, MD). The cultures were incubated in a 5% CO2–95% air environment at 37 °C and 4–5 days in culture without changing the medium. After reaching ~80% confluence, the cells were removed from the flasks by trypsin treatment and re-cultured on fresh flasks (Passage 1; split ratio 1:3). These cultured cells, called granulation tissue stem cells (GTSC), were characterized as mesenchymal stem cells by immune-staining and fluorescence-activated cell sorting (FACS) analysis for markers of stem cells, differentiation into adipocytes, osteocytes and chondrocytes and secretion of high levels of VEGF in the culture medium as described by us in earlier work [3]. The cells maintained similar mesenchymal stem cell properties in all cultured passages (up to 10 passages tested). In this study, Passages 3–5 cells were used.

**Induction of AKI in rats by ischemia–reperfusion**

Fischer 344 syngeneic male rats (250–300 g) were anesthetized with intraperitoneal sodium nembutal (5 mg/100 g weight) and the right kidney was removed through a midline laparotomy. The left kidney was exposed and the renal pedicle clamped for 45 min, following which the clamp was removed and kidney reperfusion was confirmed by visual inspection. This injury model produces severe AKI, auto-reversible by 1 week.

**Treatment of AKI rats with granulation tissue stem cells (GTSC)**

Three hours after the injury, Group 1 (treated rats; \( n = 8 \)) rats were given one intravenous injection of GTSC (Passages 3–5; \( 2 \times 10^6 \) cells in 0.7 mL volume of serum-free medium) and Group 2 (control rats; \( n = 8 \)) rats received 0.7 mL of vehicle. The rats were maintained on normal rat chow and water ad libitum and sacrificed either at 24, 48 h or 1 week. Blood was drawn daily for 7 days for plasma creatinine and blood urea nitrogen (BUN) measurements. Plasma creatinine was measured by colorimetric Jaffe reaction (Sigma Diagnostics, Inc., St. Louis, MO) and BUN levels by colorimetric improved Jaffe method that utilizes a chromogenic reagent, which forms a colored complex with urea measurable at \( A_{520} \) (BioChain Institute, Inc., Hayward, CA) [4]. At sacrifice, kidney tissues were collected for histological and immunohistochemistry examination.

**Fluorescent labeling of cultured GTSC for testing migration to injured kidney**

Passage 3 GTSC were incubated with 10 µg/mL of the fluorescent dye (acetoxyethyl ester also known as calcin AM; Molecular Probes, Inc., Eugene, OR) for 1 h at 37 °C. The labeled cells were washed with RPMI-1640 medium and re-suspended in fresh medium for injection. Cells were counted in a hemocytometer and the labeling was confirmed by visualizing the cells in a fluorescence microscope before injecting into rats. Fischer 344 syngeneic male rats were laparotomized and AKI was induced as described above. Three hours after inducing AKI, 2–3 million-labeled cells were injected intravenously and the rats were sacrificed after 24 and 48 h of injury. Samples of kidney, heart, liver and lung tissues were harvested, surrounded with generous amounts of OCT compound and snap frozen in dry ice. Cryostat sections (5-µm thick) of the tissues were briefly washed with PBS containing 1% Tween-20 solution (PBS-T), counterstained with 10 µg/mL of ethidium bromide for 10 min (for nuclear staining), washed again with PBS-T, mounted with PBS:glycerol (1:1) and examined under an epifluorescent microscope (Nikon, New York, NY).

**Kidney histology and immunohistochemistry**

At 24 h, 48 h or 1 week after the injury, kidney tissues were harvested, fixed in 4% formaldehyde and paraffin embedded. Kidney sections of 4-µm thickness were stained with hematoxylin and eosin and periodic acid–Schiff stains and analyzed by light microscopy for tubular injury (tubular cell vacuolization, tubular dilatation, congestion and casts). Tubular injury score was graded from five different low-power cortico-medullary fields of control and treated rats as follows: 0, normal; 1, area of tubular cell vacuolization, tubular dilatation, congestion and casts (lesion) involving <25% of the field; 2, lesion involving between 25 and 50% of the field; 3, lesion involving 50–75% of the field and 4, lesion area involving >75% of the field. Index of tubular injury score was calculated by averaging the grades.

**Proliferating cell nuclear antigen staining**

Slides from 24 to 48 h kidney sections were immune stained for proliferating cell nuclear antigen (PCNA) by primary incubating with primary anti-mouse PCNA antibody (Chemicon International Inc., Billerica, MA) followed by secondary antibody (anti-mouse IgG) conjugated with fluorescein isothiocyanate. The slides were then examined and photographed under epifluorescence (Nikon). PCNA score was calculated by counting PCNA-positive nuclei from five different low-power cortico-medullary fields of control and treated rats.

**TUNEL staining**

Apoptotic cells were determined by labeling DNA strand breaks by the TUNEL method using ApoTag® plus peroxidase (in situ apoptosis detection kit; Chemicon International Inc.). In brief,
DNA fragments on the slides were first enzymatically labeled with digoxigenin nucleotides followed by treatment with peroxidase-linked anti-digoxigenin antibody and finally developing the reaction with diaminobenzidine-H2O2 reaction (brown color). The sections were counterstained with methyl green and then examined and photographed under microscope (Nikon). Apoptotic score was quantified by counting apoptotic-positive nuclei (brown stained) in the kidney tissue from eight randomly examined high-power cortico-medullary fields for both control and treated rats.

**Messenger RNA levels of growth and immune modulating factors in cultured GTSC by complementary DNA array**

Microtiter (96-well) plate-based hybridization profiling analysis using reverse transcription of messenger RNA (mRNA) into complementary DNA (cDNA) (Signosis Inc., Sunnyvale, CA) was used to measure the expression of several genes in GTSC (Blank, B-actin, FGF2, HGF, IGF-1, VEGF, IL-4, IL-6, IL-10 and IL-1B). As an example of a non-stem cell, we chose to compare the GTSC with glomerular epithelial cells (GEC), a well-characterized rat kidney-derived cell line commonly used in experimental studies [5]. Briefly, total RNA was isolated from GTSC and GEC and cDNA copies of mRNA were synthesized using reverse transcriptase enzyme in the presence of biotin-dUTP. cDNA samples were introduced on the array plate wells, which were pre-coated with gene-specific oligonucleotides. The targeted genes were then specifically captured onto individual wells on the plate. The captured cDNAs were detected with streptavidin-HRP. A chemiluminescent substrate was added and the chemiluminescence was quantified by a luminometer (MLX microplate luminometer, DYNEX Technologies, Inc., Chantilly, VA). The results were quantitated as a ratio of signal corresponding to the gene of interest/signal corresponding to B-actin in the same sample.

**Statistical analyses**

The data are expressed as mean ± SEM. Data from different groups of rats were analyzed by either the Student’s t-test or analysis of variance. The level of statistical significance was set at P < 0.05.

**Results**

**Culture and morphologic characterization of GTSC**

Four days after inserting the polyvinyl tube, the granulation tissue was harvested and placed in culture. As previously described, GTSC started to attach within 2–3 h, reaching 80% confluency within 4–5 days [3]. In culture, the cells showed smooth muscle-like mesenchymal morphology (Figure 1E). On subsequent subcultures, the cells grew faster than the Passage 0 cells and were maintained successfully in culture until Passage 10 tested. As previously described [3], cultured GTSC expressed markers of both embryonic pluripotent cells (Oct-4 and Nanog) and adult stem cells (CXCR4 and Thy1.1). Furthermore, by FACS analysis, GTSC were positive for mesenchymal stem cell surface markers (CD90, CD59 and CD44) and negative for hematopoietic stem cell surface marker (CD45). In culture, GTSC secreted VEGF at a high rate (50–100 times higher than that observed in fibroblasts, primary mesangial cells and GEC), which was maintained for up to the 10 passages tested. When incubated in specific differentiation medium, these cells differentiated into adipogenic, osteogenic and chondrogenic lineages, indicating that they were multipotent cells.

**Administration of GTSC accelerated recovery in AKI rats**

It is well established in ischemia/reperfusion-induced AKI that plasma creatinine and BUN levels in the untreated AKI rats rise to a peak level at 24 h post-ischemia and then gradually decrease to near baseline by 1 week. In our study plasma creatinine and BUN levels in control AKI rats also reached peak levels at 24 h after injury as expected. However, at 24 h after injury, GTSC-treated rats had significantly lower levels of plasma creatinine and BUN than controls (plasma creatinine; 1.0 ± 0.04 versus 1.6 ± 0.12 mg/dL in controls; BUN: 71 ± 5.89 versus 138 ± 7.14 mg/dL in controls) (P < 0.05) (Figures 2 and 3). Furthermore, after 48 h of injury, plasma creatinine and BUN levels in the treated rats continued to remain lower as compared to control rats (P < 0.05) (Figures 2 and 3). The plasma creatinine and BUN levels remained low and declined to near baseline levels by Day 4 in the treated group but not until Day 7 in controls (Figures 2 and 3).

**Migration of GTSC to injured kidney**

Fluorescently labeled cells were injected intravenously into AKI rats in order to determine whether they migrate to the site of injury. At 24 h after injection, a few labeled cells appeared to be attached to the tubules, but there were none in the glomeruli or elsewhere in the injured kidney or in other internal organs (heart, liver and lung) (data not shown). By 48 h, the labeled cells initially present in the injured kidney became undetectable.

**Kidney histology**

Histology showed the typical histology of the kidney with diffuse tubular injury in the cortico-medullary area (tubular cell vacuolization, tubular dilatation, congestion and casts) in control rats at Day 1 after inducing AKI (data not shown). The extent of tubular damage between treated and control rats (by tubular injury scoring) at Days 1 and
levels were compared to those in GEC (control cell line). We found that of the many factors tested, GTSC expressed higher levels of FGF2, HGF, IGF-1, VEGF, IL-4 and IL-6 (2- to 8-fold) compared to levels expressed by GEC (Figure 6); albeit only FGF2 and IL-6 levels in GTSC were statistically higher when compared to levels in GEC.

Discussion

Despite decades of intensive research, AKI continues to exhibit high mortality not altered by pharmacological interventions [6, 7]. This lack of progress has led investigators to consider other forms of therapy, notably the use of stem cells harvested from the bone marrow [8–11]. In our laboratory, we have developed and previously described alternate and possibly easier means of obtaining stem cells; from the activated omentum [12] as well as from the induced granulation tissue called granulation tissue-derived stem cells (GTSC) [3] used in this study.

Previously, we have shown that GTSC express stem cell markers, secrete high levels of VEGF in media and trans-differentiate to different phenotypes in vitro [3]. In this study, we injected GTSC 3 hours after ischemic AKI in rats and found a significant beneficial effect. In the GTSC-treated rats, plasma creatinine and BUN levels were significantly lower at 24 and 48 h after injury as compared to controls and remained lower for the 7 days of follow-up. On histological examination of the cortico-medullary region, there was enhanced cell proliferation (as assessed by PCNA staining) and reduced tubular cell apoptosis (as assessed by TUNEL staining) in the GTSC-treated rats, suggesting increased cell renewal in the region of the kidney that is most sensitive to an ischemic insult [13, 14].

But, how do stem cell injections in ischemic AKI exert a beneficial effect? Do the cells persist in the kidney or do they act by a different mechanism? In our study, injected fluorescently labeled GTSC were found to be localized to the kidney tubules at 24 h but were undetectable 48 h after injury, suggesting that even a brief encounter of stem cells with injured tubules could lessen the severity of the injury and facilitate recovery. These findings are in agreement with the work of Tögel et al. [8], who used bone marrow-derived mesenchymal stem cells to accelerate recovery from AKI and likewise found that the injected cells localized only transiently in the injured kidney. By contrast, however, in other similar studies (cisplatin and glycerol models), injected bone marrow stem cells localized in the injured kidney and remained there much longer [15–17].

Could localizing injected stem cells improve renal function by transdifferentiating into renal tubular cells? Though suggested by a few studies using bone marrow cells [15–17], such a mechanism would seem unlikely in our study considering the level of cell localization and their transient persistence. A beneficial effect due to secretion of growth factors and immune-suppressive cytokines, as seen in our model, is more likely. Several studies have shown that growth factors (FGF2, HGF, IGF-1 and VEGF) promote angiogenesis, enhance cellular proliferation and inhibit apoptosis [18–21], while IL-4, IL-6 and IL-10 act as anti-inflammatory cytokines [22]. Also, supporting a paracrine

Fig. 3. BUN levels (mg/dL) in AKI rats treated with GTSC as compared to controls. Cells (2–3 million) were administered 3 h after inducing injury. Data are expressed as mean ± SEM. *Denotes statistically significant difference compared to all time points in both groups; #significant difference compared to all time points except Day 1 of treated group; †significant difference compared to Day 0 and Day 1 of control at P < 0.05. n = 8 in each group. While the control rats showed peak levels of BUN at 24 h after injury as expected, the peak levels in treated rats were significantly lower than in controls. After 48 h of injury, BUN levels in the treated rats continued to remain lower as compared to control rats.
Fig. 4. Immune staining of kidney tissue for PCNA at Day 2 after inducing AKI in rats. (A) PCNA-positive tubular cells in treated rats stained green (arrows). Inset: PCNA-positive tubular cells at higher magnification. (B) Lower number of PCNA-positive tubular cells in control rats (arrows). Inset shows PCNA-positive tubular cells at higher magnification. Magnification of A and B: ×100. Magnification of insets: ×300. (C): PCNA scores in AKI rats treated with GTSC as compared to control rats. The scores are expressed as average number of PCNA-positive nuclei per low power cortico-medullary field. Data are expressed as mean ± SEM. Total of five different fields were examined in each rat tissue. *Denotes statistically significant difference compared to control rats (P < 0.05).

Fig. 5. Immune staining of kidney tissue for apoptosis at Day 1 after inducing AKI in rats. (A) Apoptotic tubular cells in treated rats stained brown (arrows). Inset shows apoptotic-positive tubular cells at higher magnification. (B) Higher number of apoptotic tubular cells in control rats (arrows). Inset shows apoptotic-positive tubular cells at higher magnification. Magnification of A and B: ×200. Magnification of insets ×400. (C) Apoptosis scores in AKI rats treated with GTSC as compared to control rats. The score is expressed as percentage of apoptotic cells per high-power cortico-medullary field. Data are expressed as mean ± SEM. Total of eight different fields were examined in each rat tissue. *Denotes statistically significant difference compared to control rats (P < 0.05).
mechanism are studies showing (i) improvement following the injection of growth factors alone [23–25], (ii) secretion of growth and immune-suppressive factors by other adult-derived stem cells [8, 10, 11, 26], (iii) loss of beneficial effect when one of the key growth factors (IGF-1 and VEGF) is knocked down (by small interfering RNA) in the injected cells [27, 28] and (iv) the ameliorating effect of injecting conditioned medium collected from cultured stem cells in the cisplatin model of AKI [26].

Based on the paracrine hypothesis, the use of growth factors to treat AKI has been explored in humans [29]. The results of IGF-I injection have been equivocal; beneficial in experimental rat studies [23] but not in humans [29], perhaps because the human disease is more complex than that of the rat. Possibly, a mixture of several growth factors might be more effective than a single growth factor in treating human AKI. This might explain why injections of live stem cells that elaborate a myriad of growth factors have been more successful in treating AKI than single growth factor injections.

In conclusion, we used mesenchymal stem cells obtained from a foreign body-induced granulation tissue to ameliorate AKI in rats. Although bone marrow mesenchymal stem cells are currently undergoing clinical trials in humans, it is important to develop alternate sources of stem cells, such as from adipose and granulation tissues, because of their easier accessibility and abundance compared to cells from the bone marrow.

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