Effect of Stem Cell Therapy on Adriamycin Induced Tubulointerstitial Injury

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Background and Objectives: It was postulated that adriamycin (ADR) induce renal tubulointerstitial injury. Clinicians are faced with a challenge in producing response in renal patients and slowing or halting the evolution towards kidney failure. The present study aimed at investigating the relation between the possible therapeutic effect of human mesenchymal stem cells (HMSCs), isolated from cord blood on tubular renal damage and their distribution by using ADR induced nephrotoxicity as a model in albino rat.

Methods and Results: Thirty three male albino rats were divided into control group, ADR group where rats were given single intraperitoneal (IP) injection of 5 mg/kg adriamycin. The rats were sacrificed 10, 20 and 30 days following confirmation of tubular injury. In stem cell therapy group, rats were injected with HMSCs following confirmation of renal injury and sacrificed 10, 20 and 30 days after HMSCs therapy. Kidney sections were exposed to histological, histochemical, immunohistochemical, morphometric and serological studies. In response to SC therapy, vacuolated cytoplasm, dark nuclei, detached epithelial lining and desquamated nuclei were noticed in few collecting tubules (CT). 10, 20 and 30 days following therapy. The mean count of CT showing desquamated nuclei and mean value of serum creatinine revealed significant difference in ADR group. The mean area% of Prussian blue+ve cells and that of CD105 +ve cells measured in subgroup S1 denoted a significant increase compared to subgroups S2 and S3.

Conclusions: ADR induced tubulointerstitial damage that regressed in response to cord blood HMSC therapy.

Keywords: Mesenchymal stem cells, Cord blood, Tubular damage, Adriamycin

Introduction

Drug induced tubular injury was reported (1). It was postulated that adriamycin (ADR) induce tubulointerstitial injury (2). Clinicians are faced with a challenge in producing response in renal patients and slowing or halting the evolution towards kidney failure (3).

Mesenchymal stem cells (MSCs) are stromal cells that have the ability to self-renew and exhibit multilineage

differentiation. MSCs can be isolated from a variety of tissues, such as umbilical cord, bone marrow and adipose tissue. The multipotent properties of MSCs make them an attractive choice for possible development of clinical applications (4). Cord blood transplant (CBT) has been widely used as an alternative source of mesenchymal cell support for stem cell transplant patients (5).

The present study aimed at investigating the relation between the possible therapeutic effect of HMSCs, isolated from cord blood on collecting tubular damage and their distribution. This was accomplished by using ADR induced nephrotoxicity as a model in albino rat.

Materials and Methods

Thirty three male albino rats weighing 150~200 g were used and divided into 3 groups placed in separate cages

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in the Animal House of Kasr El Aini. The rats were treated in accordance with guidelines approved by the Animal Use Committee of Cairo University.

Control group: 2 control rats, 1 for each experimental group. Each animal received single IP injection of distilled water.

Group A (ADR group): 16 rats, each received single (6) IP injection (7) of 5 mg/kg (8) ADR (Pharmacia Italia SPA, Nerviano, Italy) dissolved in distilled water. One rat was sacrificed thirty days following the day of injection for confirmation of tubular damage. The remaining rats were subdivided into: Subgroups (A1), (A2) and (A3), in each subgroup 5 animals were sacrificed 10, 20 and 30 days following the confirmation of tubular damage respectively.

Group S (SC therapy group): 15 rats received ADR by the same route, at the same frequency of administration and at the same dose as in the previous group. They were injected with 0.5 ml of cultured and labeled HMSCs suspended in phosphate buffer saline (PBS) in the tail vein (9). The injection was performed on two successive days following confirmation of tubular damage. Stem cells were isolated from cord blood (10). Cord blood collection was performed at the Gynaecology Department, Faculty of Medicine, Cairo University. Stem cell isolation, culture and labeling were performed at Hematology Unit, New Kasr El Aini Teaching Hospital.

The rats were subdivided into: Subgroups S1, S2 and S3, in each subgroup 5 rats were sacrificed 10, 20 and 30 days following SC therapy.

Before sacrifice, blood was collected from eyes of animals using capillary tubes for assessment of serum creatinine in Clinical Pathology Department, Faculty of Medicine Cairo University.

Cord blood collection (11)

The storage and transport temperature was 15~22°C, transport time was 8~24 hours, sample volume was 65~250 ml, and no sample had signs of coagulation or hemolysis.

Mononuclear cell fraction isolation (11)

The mononuclear cell fraction (MNCf) was isolated by carefully loading 30 ml of whole blood onto 10 ml of Ficoll density media (Healthcare Bio-Sciences) in 50 ml polypropylene tubes. Centrifuge for 30 minutes at room temperature at 450×g and the interphase collected after aspirating and discarding the supernatant. The interphase was washed with 20 ml PBS and centrifuged at 150×g for 5 minutes at room temperature. The supernatant was aspi-

rated and the cells were washed with PBS a second time. The cells were re-suspended in the isolation media to prevent adherence of monocytic cells. The isolation media was low-glucose DMEM (Dulbecco's modified Eagles medium) (Cambrex Bio Science, Minnesota, USA), penicillin (100 IU/ml) (Invitrogen), streptomycin (0.1 mg/ml) (Invitrogen), and ultraglutamine (2 mM) (Cambrex Bio-Science). Incubation was at 38.5°C in humidified atmosphere containing 5% CO₂.

Culture (11)

The isolation media were replaced after overnight incubation (12~18 hours) in order to remove non-adherent cells. The media were replaced every 3 days until MSC colonies were noted. The cultures were inspected daily for formation of adherent spindle-shaped fibroblastoid cell colonies. Sub-culturing was done by chemical detachment using 0.04% trypsin. Later, when cell numbers allowed, expansion was done in 25 cm² or 75 cm² tissue culture flasks.

Labeling (12)

Mesenchymal stem cells were labeled by incubation with ferumoxides injectable solution (25microgramFe/ml, Feridex, Berlex laboratories, Montville, New Jersey, USA) in culture medium for 24 hours with 375 nanogram/ml poly L lysine added 1 hour before cell incubation. Labeling was histologically assessed using Prussian blue. Feridex labeled MSCs were washed in PBS, trypsinized, washed and resuspended in 0.01 Mol/L PBS at concentration of 1×1,000,000 cells/ml.

Cell viability analysis

Cell viability was done using trypan blue dye exclusion test. This method is based on the principle that viable cells do not take up certain dyes, whereas dead cells do.

Flow cytometry (13)

Flow cytometric analyses were performed on a Fluorescence Activated Cell Sorter (FACS) flow cytometer (Coulter Epics Elite, Miami, FL, USA). HMSC were trypsinized and washed twice with PBS. A total number of 1×10⁵ HMSC were used for each run. To evaluate the HMSC marker profile, cells were incubated in 100 µl of PBS with 3 µl of CD105-FITC for 20 min at room temperature. Antibody concentration was 0.1 mg ml⁻¹. Cells were washed twice with PBS and finally diluted in 200 µl of PBS. The expression of surface marker was assessed by the mean fluorescence. CD105 (mesenchymal stem cell marker), CD133 (early hematopoietic & endothe-

lial progenitor stem cell marker) and CD45 (panleucocytic marker) were also used. The percentage of cells positive for CD 105 was determined by subtracting the percentage of cells stained non-specifically with isotype control antibodies.

The rats were sacrificed using lethal dose of ether. Median abdominal incision was performed, both kidneys were excised and fixed in 10% formal saline for 24 hours. Paraffin blocks were prepared and 5 μ m thick sections were subjected to the following studies:

Histological study

Hematoxylin and eosin (H&E) stain (14).

Histochemical study

Prussian blue (Pb) stain (15) for demonstration of iron oxide labeled therapeutic stem cells.

Immunohistochemical study

CD105 immunostaining (16) the marker for HMSCs. 0.1 ml prediluted primary antibody (CD105) rabbit polyclonal Ab (ab27422) and incubate at room temperature in moist chamber for 30~60 minutes. Tonsil used as positive control specimens. Cellular localization is the cell membrane. On the other hand, one of the lung sections was used as a negative control by passing the step of applying the primary antibody.

Morphometric study

Using Leica Qwin 500 LTD image analysis, assessment of the glomerular area using interactive measurements menu was done in 10 low power fields. The area% of Pb+ve cells and that of CD105+ve cells were estimated

in the renal cortex. The measurements were done in 10 high power fields.

Statistical analysis (17)

Quantitative data were summarized as means and standard deviations and compared using one-way analysis-of-variance (ANOVA). p-values <0.05 were considered statistically significant. Calculations were made on SPSS software.

Results

Haematoxylin and eosin (H&E) stained sections

Control sections revealed normal structure of CT (Fig. 1). In subgroup A1, vacuolated cytoplasm and dark nuclei of multiple cells lining multiple CT and desquamated nuclei were occasionally detected in the lumen of CT. In addition, mononuclear cellular infiltration was noticed between the CT (Fig. 2). In subgroup A2, detached epithelial lining of some CT was noticed. In addition, desquamated nuclei were found in the lumen of some other tubules (Fig. 3). Subgroup A3 demonstrated detached epithelial lining in some CT and marked congestion of peritubular capillaries (Fig. 4). In some other fields, desquamated cells exhibiting dark nuclei were found in the lumen of multiple CT surrounded by obviously congested peritubular capillaries (Fig. 5). In subgroup S1, vacuolated cytoplasm and dark nuclei were seen in cells lining fewer CT, compared to subgroup A1. Congestion of some peritubular capillaries was detected (Fig. 6). In subgroup S1, desquamated nuclei were seen in the lumen of few CT in

Fig. 1. CT (c) in control group (renal medulla; H&E, $\times 200$).

Fig. 2. Vacuolated cytoplasm and dark nuclei of multiple cells lining multiple CT (thin arrows). Desquamated nuclei are seen in the lumen of a CT (*). Note mononuclear infiltration (arrowhead) in subgroup A1 (renal medulla; H&E, $\times 200$).

Fig. 3. Detached epithelial lining of some CT (arrows) and desquamated nuclei in the lumen of some other CT (*) in subgroup A2 (renal medulla; H&E, $\times 200$).

Fig. 5. Desquamated cells exhibiting dark nuclei (*) in the lumen of multiple CT. Note congestion of peritubular capillaries (c) in subgroup A3 (renal medulla; H&E, $\times 200$).

Fig. 4. Detached epithelial lining of some CT (arrows) and marked congestion of peritubular capillaries (c) in subgroup A3 (renal medulla; H&E, $\times 200$).

Fig. 6. Vacuolated cytoplasm and dark nuclei (thin arrows) of the cells lining fewer CT, compared to Fig. 2. Note congestion of some peritubular capillaries (c) in subgroup S1 (renal medulla; H&E, $\times 200$).

comparison to subgroup A2 (Fig. 7). In subgroup S3, detached epithelial lining of few CT, desquamated nuclei in the lumen of other few tubules and less congested peritubular capillaries were detected in comparison to subgroup A3 (Fig. 8).

Prussian blue stained sections

Subgroup S1 revealed multiple spindle and few cuboidal Pb positive (+ve) cells in some peritubular capillaries and among the epithelial lining of the CT (Fig. 9). While, subgroup S2 recruited some spindle and few cuboidal +ve cells in few peritubular capillaries and among the CT (Fig. 10). Subgroup S3 showed fewer spindle positive cells compared to subgroups S1 and S2. The spindle cells were

commonly detected at CT with desquamated nuclei in the lumen (Fig. 11).

CD105 immunostained sections

Subgroup S1 showed +ve CD105 immunoexpression in multiple CT. The reaction appeared granular in spindle cells (Figs. 12, 13). Subgroup S2 demonstrated +ve immunostaining in fewer tubules compared to subgroup S1 (Fig. 14). Subgroup S3 revealed +ve reaction in fewer tubules compared to subgroups S1 and S2 (Fig. 15).

Morphometric results

The mean count of CT exhibiting desquamated nuclei denoted a significant difference ($p < 0.05$) in subgroups A2

Fig. 7. Desquamated nuclei in the lumen of two CT (*) compared to Fig. 3 in subgroup S2 (renal medulla; H&E, $\times 200$).

Fig. 8. Detached epithelial lining in few CT (arrows), desquamated nuclei in the lumen of other few tubules (*) and less congested peritubular capillaries (c) compared to Figs. 4 and 5 in subgroup S3 (renal medulla; H&E, $\times 200$).

and A3 compared to control and other experimental subgroups (Table 1, Fig. 16). Statistical analysis indicated a significant increase ($p < 0.05$) in the mean area% of PB +ve and CD105 +ve cells in subgroup S1 compared to S2 and S3. A significant difference ($p < 0.05$) was also recorded between subgroups S2 and S3 (Table 1, Figs. 17, 18).

Serological results

A significant increase ($p < 0.05$) in the mean value of serum creatinine was detected in ADR subgroups in comparison to control and SC therapy subgroups (Table 1, Fig. 19).

Fig. 9. Multiple spindle (s) and few cuboidal (cu) Pb+ve cells in peritubular capillaries and among the epithelial lining of CT in subgroup S1 (renal medulla; Prussian blue, $\times 400$).

Fig. 10. Some spindle (s) and few cuboidal (cu) +ve cells in a peritubular capillary and among the CT in subgroup S2 (renal medulla; Prussian blue, $\times 400$).

Discussion

The current study demonstrated ameliorating effect of cord blood HMSC therapy on ADR induced interstitial tubular injury. ADR being a commonly used chemotherapeutic drug, as evidenced by Dai et al. (18) was used as a model for induced nephropathy in albino rat. This was evidenced by histological, histochemical, immunohistochemical, morphometric and serological studies.

The changes in the collecting tubules (CT) in subgroup A1 showed vacuolated cytoplasm and dark nuclei of multiple cells lining multiple CT. It was postulated that these changes could be related to reactive oxygen species (ROS) production during ADR-induced nephrosis (19). Apopto-

Fig. 11. Fewer spindle (s) +ve cells at the epithelial lining and in the lumen of a CT with desquamated nuclei in the lumen (*) in subgroup S3 (renal medulla; Prussian blue, $\times 400$).

Fig. 13. Granular reaction in multiple spindle cells (arrows) among the lining of CT (renal medulla; CD105, $\times 1,000$).

Fig. 12. +ve immunostaining in multiple CT (arrows) in subgroup S1 ($\times 200$) (renal medulla; CD105, $\times 200$).

Fig. 14. +ve immunostaining in some CT (arrows) in subgroup S2 (renal medulla; CD105, $\times 200$).

tic potential of doxorubicin was related to the formation of doxorubicin-DNA adducts (20).

In subgroup A1, occasional desquamated nuclei were detected in the lumen of CT. While in subgroup A2, detached epithelial lining and desquamated nuclei were found in the lumen of some CT. In subgroup A3, multiple CT revealed desquamated dark nuclei. Morphometric results were concomitant, indicating significant increase in mean count of CT containing desquamated nuclei in subgroups A2 and A3. Medullary tubular changes can be correlated to tubular injury developing in nephritis as confirmed by Stambe et al. (21), Ayla et al. (22) ADR-induced tubular atrophy can be related to formation of reactive metabolites that attack cell membranes and result in perox-

idation of polyunsaturated fatty acids. Damaged cell membranes resulted in detachment of tubular epithelium that ended in desquamation of the nuclei into the lumen (22).

In the present study, mononuclear cellular infiltration including fibroblasts was noticed between the CT in subgroup A1. It was stated that in ADR-induced nephrosis, cell infiltration is a hallmark that precedes the development of tubular cell atrophy (23).

Medullary peritubular capillary congestion was observable in subgroup A3. It was recorded that early alterations in peritubular capillary blood flow during reperfusion have been documented and associated with loss of function (24).

The mean value of serum creatinine was significantly increased in ADR group which may denote impairment

Fig. 15. +ve immunostaining in few CT (arrows) in subgroup S3 (renal medulla; CD105, ×200).

Fig. 16. Mean count of collecting tubules with desquamated nuclei.

Fig. 17. Mean area% of Prussian blue positive cells in medulla.

Fig. 18. Mean area% of CD105 positive cells in medulla.

Table 1. Mean ± standard deviation (SD) of the count of collecting tubules with desquamated nuclei, area% of Prussian blue +ve cells, area% of CD105 +ve cells and serum creatinine in control and experimental groups

Groups and subgroups	Count of CT with desquamated nuclei	Area% of Pb +ve cells	Area% of CD105 +ve cells	Serum creatinine
Control group	—	—	—	0.4100
Subgroup A1	1.6 ± 0.46	—	—	1.0300 ^d
Subgroup A2	6.2 ± 0.84 ^a	—	—	1.1360 ^d
Subgroup A3	8.4 ± 0.89 ^a	—	—	1.6420 ^d
Subgroup S1	—	6.56 ± 0.28 ^b	7.77 ± 0.63 ^b	0.8140
Subgroup S2	1.4 ± 0.25	2.95 ± 0.26 ^c	4.67 ± 0.26 ^c	0.7960
Subgroup S3	2.2 ± 0.39	1.92 ± 0.47	2.14 ± 0.19	0.7600

^asignificant compared to group 1 and subgroups A1, S1, S2 and S3, ^bsignificant compared to subgroups S2 and S3, ^csignificant compared to subgroup S3, ^dsignificant compared to group 1, subgroups S1, S2 and S3.

of renal function. It was proved that oxidative stress augments urea and creatinine levels in serum and albumin excretion in urine (25). The extent of ADR nephrosis was

not modified by medical treatment in rat (26). This point is controversial.

In S1 subgroup, mononuclear infiltrating cells were no-

Fig. 19. Mean value of serum creatinine.

ticed in the medulla, in addition to congestion of some peritubular capillaries. It could be commented that these reflex vascular changes help attraction and migration of injected therapeutic SCs to the site of injury. Verma et al. (27) considered and evaluated the high angiogenic efficiency and anti-inflammatory effect of endothelial progenitor cells (EPCs).

Vacuolated cytoplasm and dark nuclei of the cells lining few CT, less detachment and desquamation of the tubular lining were seen in subgroups S1, S2 and S3 in comparison to subgroups A1, A2 and A3 respectively. It was stated that experimental progressive renal disease is characterized by the development of tubulointerstitial damage. Intrarenal injection of SCs stimulated regenerating ability of the damaged tubules (28). It was added that acute tubular necrosis (ATN) in mouse is characterized by acute tubular cell injury and renal dysfunction. Human amniotic fluid SCs contributed to kidney repair either through paracrine effects and/or integration into damaged structures (29). Chen et al. (30) commented on effectiveness and safety of adipose derived MSC treatment in preserving renal parenchymal integrity from acute ischemic injury. They improved kidney functions in tubular structures.

In S1 subgroup, multiple spindle and few cuboidal Pb +ve cells were found in some peritubular capillaries and among the epithelial lining of the CT. The +ve cells were fewer in subgroup S2 and fewest in subgroup S3. The previous findings were confirmed by a significant increase in the mean area % of +ve cells in subgroup S1.

In subgroup S1, multiple spindle CD105+ve immunostained cells were evident among the lining epithelium of multiple cortical tubules. Recently, nephron regeneration and repair of tubular atrophy and interstitial

nephritis were reviewed by adipose-derived mesenchymal stem cells (31). Umbilical cord blood (UCB) is a reasonable option for the treatment of patients with hereditary BM failure syndromes. In patients given an unrelated UCB transplantation, only cord blood units containing a high number of cells should be considered to improve the results (32).

In the present study, a significant decrease in the level of serum creatinine was noted in SC therapy subgroups compared to ADR subgroups. Recently, transplantation of pluripotent SCs appears to improve serum indices of liver function and survival rate in mice after CCl4-induced hepatic damage (33).

In conclusion

ADR induced renal damage, represented by progressive degeneration of collecting tubules which was denoted by nuclear and cytoplasmic changes of the lining cells that ended into desquamation. The morphological findings were confirmed by morphometric assessment and correlated to progressive deterioration of the renal function manifested as progressive elevation of serum creatinine. Cord blood HMSC therapy proved definite amelioration of the tubulointerstitial and serological changes. A reciprocal relation was recorded between the extent of renal regeneration and the distribution of undifferentiated mesenchymal stem cells.

Potential conflict of interest

The authors have no conflicting financial interest.

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