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ORIGINAL ARTICLE

Role of bone marrow-derived stem cells, renal progenitor cells and stem cell factor in chronic renal allograft nephropathy

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Abbreviations: CAN, chronic allograft nephropathy; HSCs, Hematopoeitic stem cells; MSCs, Mesenchymal stem cells; SCF, stem cells factor; AKI, acute kidney injury; ARF, acute renal failure; CKD, chronic kidney disease; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor; ASMA, alpha smooth muscle actin; IF/TA, interstitial fibrosis/tubular atrophy; CMV, cytomegalo virus; HIV, human immunodeficiency virus; CRP, C-reactive protein; UAE, urinary albumin excretion; UALP, urinary alkaline phosphatase; RI, resistivity index; PI, pulsitility index; RBF, renal blood flow; MoAbs, monoclonal antibodies; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

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KEYWORDS

Chronic allograft nephropathy;
Hematopoietic stem cells;
Mesenchymal stem cells;
Stem cell factor;
Renal regeneration

Abstract *Introduction:* Chronic allograft nephropathy (CAN) is a poorly understood clinico-pathological entity associated with chronic allograft loss due to immunologic and non-immunologic causes. It remains the leading cause of late allograft loss. Bone marrow derived stem cells are undifferentiated cells typically characterized by their capacity for self renewal, ability to give rise to multiple differentiated cellular population, including hematopoietic (HSCs) and mesenchymal stem cells (MSCs). Characterization of HSCs includes their multipotency, expression of typical surface markers such as CD34 and CD45, while characterization of MSC includes their multipotency, expression of typical surface markers such as CD90 and CD105, and the absence of hemopoietic lineage markers.
Aim & methods: The aim of the present work was to study the role of bone marrow-derived HSCs and MSCs, renal progenitor cells and SCF in chronic renal allograft nephropathy in relation to renal hemodynamics and histopathological changes. We studied 30 patients with kidney transplantation for more than 6 months, divided into 15 patients with stable serum creatinine and 15 patients who developed CAN. Detection of HSCs and MSCs in the peripheral blood using flow cytometry via detection of CD34, CD45, CD117 and CD106, as well as immunohistochemical detection of CD34, CD133, VEGF and αSMA in transplanted kidney biopsies of patients with CAN were done.
Results: There was a significant increase in the levels of SCF, number of peripheral blood HSCs and MSCs in both transplanted patient groups than the controls and they were higher in patients of group Ia than patients of group Ib, ($F = 39.73$, $P < 0.001$), ($F = 13.28$, $P < 0.001$), ($F = 11.94$, $P < 0.001$), respectively and this was accompanied by evident expression of markers of renal repair.
Conclusion: Stem cells might have a role in renal regeneration in CAN and this may pave the way toward the use of stem cells in correction of CAN.

1. Introduction

Chronic allograft dysfunction (CAD) is a clinico-pathological entity associated with chronic allograft loss caused by immunologic and non-immunologic causes.[1]

Chronic allograft injury is the leading cause of late graft loss after kidney transplantation[2] characterized by progressive interstitial fibrosis and tubular atrophy (IF/TA) as well as microvascular and glomerular damage accompanied by declining graft function months to years after transplantation.[3]

In spite that the incidence of acute rejection and early graft failure had declined dramatically as a result of development in immunosuppressive medications and protocols, and the one year graft survival is now close to 90% in most transplant centers, yet, late allograft failure remains the problem to overcome.[4]

Significant attention has been directed to study the potentiality of stem cells (SCs) in the treatment of a number of acute and chronic diseases.[5,6]

The bone marrow (BM) derived SCs are undifferentiated cells typically characterized by its capacity for self renewal, ability to give rise to multiple differentiated cellular populations (often termed cellular plasticity)[7] and the ability to generate many if not all of the differentiated cell types that are contained in an organ,[8] so that in the presence of damage, these cells can replace the injured ones.[9] The BM harbors two distinct stem cell populations: hematopoietic stem cells (HSCs)[10] and mesenchymal stem cells (MSCs), which provide stromal support for HSCs.[11]

The bone marrow derived HSCs, are pluripotent undifferentiated cells that give rise to all blood cells (erythrocytes, thrombocytes, and leukocytes) and move between the bone marrow and the peripheral blood. The CD34 antigen is highly expressed in pluripotent cells and its expression gradually reduces as the level of maturation of hematopoietic cell lineages increases, to the point of becoming completely absent in fully mature cells. HSCs can be mobilized into the circulation in response to multiple cytokines, chemokines and adhesion molecules.[12,13]

Stem cell factor (SCF), also known as Steel factor or c-kit ligand, is a cytokine produced by stromal cells and is important for mobilization, proliferation and differentiation of HSCs, specifically myeloerythroid lineages. SCF functions by binding to CD117/c-Kit, a tyrosine kinase receptor, highly expressed on HSCs.[14] The SCF/c-Kit signaling pathway promotes cell survival by inhibition of apoptosis in multiple cell types, including HSCs. Interestingly; HPCs express the HSC marker CD34 and SCF and its receptor c-kit.[15]

The other BM derived SCs are the MSCs. MSCs are pluripotent stromal cells which are defined by their plastic adherence, surface marker expression of CD73, CD90, CD105 and CD106 (vascular cell adhesion molecule [VCAM]-1) combined with a lack in expression of hematopoietic markers CD34, CD45, CD14 and HLADR, and the capacity to differentiate into cells of mesodermal lineage including adipocytes, osteocytes, chondrocytes and myocytes.[16]

The MSCs during tissue injury, can be released from their niche in the BM into circulation and recruited to sites of inflammation by migrating toward inflammatory chemokines and cytokines where they differentiate into specialized cells and promote local tissue repair by preventing apoptosis and/or control of inflammation in situ through secretion of growth factors and cytokines and activation of endogenous progenitor cells.[17] MSCs are potent immunomodulators of both the innate and adaptive immune systems.[18] MSCs have been shown to exert a profound inhibitory effect on T cell proliferation and function. MSCs can regulate an innate immune response by signaling dendritic cells to direct an anti-inflammatory T-cell

response and by directly suppressing natural killer cell functions. MSCs also affect an adaptive immune response by exerting their immunoregulative effects through direct interaction with T-cells. Also, MSCs can inhibit B lymphocytes' proliferation.[19]

Moreover, MSCs have the ability to differentiate into vascular cell types and release proangiogenic factors such as vascular endothelial growth factor, which can promote the recruitment of endothelial cells for angiogenesis.[20]

The immune-privileged properties attributed to MSCs make them a powerful tool that could be used in many inflammatory and immune-mediated diseases.[21]

The identification of SCs both inside the kidney pave the way toward the future regeneration of the damaged kidney.[22] There is evidence of engraftment and differentiation of stem cells during normal renal cellular turnover,[23] acute kidney injury (AKI), chronic kidney disease (CKD) and in different forms of acute and chronic glomerular diseases.[24–26]

So, the aim of this study was undertaken to investigate the role of bone marrow-derived HSCs and MSCs, renal progenitor cells and SCF in chronic renal allograft dysfunction in relation to renal hemodynamics and histopathological changes.

1.1. Subjects

The present study included 45 subjects who are divided into the following groups:

Group I 30 Patients with renal transplantation who were transplanted at the Nephrology and Transplantation Unit, Main Alexandria University Hospital. They were subdivided into two subgroups.

Group Ia 15 Patients with renal transplantation and with biopsy proven chronic allograft nephropathy (CAN). Their ages ranged between 17 and 54 years with a mean 33.33 ± 11.02 years.

Group Ib 15 Patients with renal transplantation and with stable allograft function. Their ages ranged between 22 and 50 years with a mean 29.07 ± 7.85 years.

Group II A control group of 15 healthy subjects with matched age and sex. Their ages ranged between 18 and 41 years with a mean 28.67 ± 6.58 years.

The study included patients transplanted for more than six months and seronegative for hepatitis B, C, cytomegalovirus (CMV) and human immunodeficiency (HIV) virus. Patients with chronic diseases such as diabetes mellitus, connective tissue diseases or other autoimmune diseases, infections or any kind of malignancy, cardiac and respiratory diseases were excluded. None of the patients had history of previous renal transplantation.

The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and an informed consent was obtained from each patient and control in the study.

2. Methods

All patients and controls were subjected to thorough history taking which included previous diseases, original renal disease, complications after transplantation, e.g. infection or rejection including duration of rejection and number of attacks. Also, a complete physical examination was done with special observation for signs of rejection including tender graft, change in blood pressure and urine volume.

Laboratory investigations included hemoglobin concentration, total white blood count, lymphocyte count,[27] renal function tests (blood urea, serum creatinine, and creatinine clearance),[28] and complete urine analysis with measurement of 24-h urinary albumin excretion (UAE).[28] Serum C-reactive protein (CRP) measurement was done by turbidimetry.[29,30] Estimation of urinary alkaline phosphatase (U.ALP), as a marker of tubular function, was done by the spectrophotometric method.[31]

Radiological study for the assessment of the renal allograft was done by ultrasound examination as regards the graft size, cortical echogenicity, parenchymal thickness, corticomedullary differentiation, and the presence of hydronephrosis or lymphocele. Renal hemodynamic study of the graft was measured by duplex Doppler ultrasonography, with calculation of the resistive and pulsatility indices (RI, PI).[32,33]

2.2. SCF assay:[34]

Detection of serum level of SCF was done using enzyme linked immunosorbant assay (ELISA) kit as described by Galli.[34]

2.3. Enumeration of hemopoeitic and mesenchymal stem cells in the peripheral blood by flow cytometry[35–37]

The HSCs (CD34 + 117 + 45+) and MSCs (CD106 + 34−) in the peripheral blood are detected using 3-color flow cytometric assay.[35] The detailed characterization of hematopoietic stem cells was obtained by analyzing the expression of a given set of antigens in a cell population.

Monoclonal Antibodies (MoAbs) used are: Fluorescein isothiocyanate (FITC)-conjugated anti-CD45, Phycoerythrin (PE)-conjugated anti-CD34 and Phycoerythrin (PE)-conjugated anti-CD117 (c-Kit) mAbs for identification of HSCs, (eBiosciences. Inc),[36] and PE-conjugated anti-CD34 and FITC− conjugated anti-CD106 mAbs for the identification of MSCs (eBiosciences Inc).[37] The flow cytometer used was Becton Dickinson, FACS caliber flow cytometer equipped with Cell Quest software. The procedure was done as shown by Gajkowska, et al.[35]

2.4. Histopathological examination

An ultrasound guided renal biopsy was done to all patients who were clinically suspected to have CAN (Group Ia). Biopsy was subjected to:

2.4.1. Light microscopic examination:[38]

A routine light microscopic examination using variant stains (H&E, Masson Trichrome, PAS, Silver methanamine stains) was performed. The pathological report did describe the occurrence and staging of CAN according to Banff classification 2009.[39]

2.4.2. Immunohistochemical Staining:[40–43]

All sections were mounted on glass slides and subjected to monoclonal antibody staining for the following antibodies:

Table 1 Mean ± SD and statistical comparison of age, hemoglobin concentration, blood urea, serum creatinine, creatinine clearance and urinary proteins, serum C-reactive protein, urinary alkaline phosphatase in the studied groups.

Parameters	Group Ia	Group Ib	Group II	*F*	*P*	L S D		
						GIa/GIb	GIa/GII	GIb/ GII
Age (years)	33.33 ± 11.02	29.07 ± 7.85	28.67 ± 6.58	1.33	0.28	NS	NS	NS
Bld. Hb (g/dl)	10.4 ± 1.29	12 ± 93	13.42 ± 1.15	26.55	< 0.001	*	*	*
Bld. Urea (mg/dl)	92.67 ± 47.98	30.93 ± 6.79	27.27 ± 5.05	25.60	< 0.001	*	*	NS
S. Cr (mg/dl)	2.67 ± 1.12	1.07 ± 0.17	0.97 ± 0.17	31.32	< 0.001	*	*	NS
Cr. Cl (ml/min)	44.73 ± 9.74	68.00 ± 4.24	113.00 ± 15.06	159.52	< 0.001	*	*	*
U. Pr (mg/24hs)	1348.00 ± 999.19	194.00 ± 82.10	19.22 ± 6.84	23.34	< 0.001	*	*	NS
S. CRP (mg/l)	17.40 ± 11.34	6.73 ± 3.01	3.40 ± 1.29	17.260	< 0.001	*	*	NS
U. ALP (umol/min)	0.06 ± 0.03	0.09 ± 0.02	0.10 ± 0.06	6.230	0.004	*	*	NS

Group Ia = Renal transplant patients with chronic allograft dysfunction, Group Ib = renal transplant patients with stable allograft function, Group II = control subjects Bld. Hb = blood hemoglobin, S. Cr = serum creatinine, Cr. Cl = creatinine clearance, U. Pr = urinary protein, S. CRP = serum C-reactive protein, U. ALP = urinary alkaline Phosphatase.
* = Significant *P* value at 5% level, NS = insignificant difference between the 2 groups.

CD133 as a marker for renal progenitor cells, CD34 as stem cell marker, vascular endothelial growth factor (VEGF) as vascular markers, alpha smooth muscle actin (ASMA) as fibrotic marker. The four antibodies were provided by lab vision incorporation (Neo Markers, Fremont, USA). Expression of all antibodies used was visualized using the streptovodin-biotin-immunoenzymatic antigen detection system which was performed according to manufacturers' protocol provided by labvision incorporation (Neo Markers, Fremont, USA). Using standard Immunohistochemical techniques as briefly described by Ramani et al.[40] for CD34, and Yin et al.[41] for CD133, Tyrley, et al.[42] for VEGF and Skalli, et al.[43] for αSMA

2.5. Statistical analysis

All calculations were performed on a personal computer with SPSS software (version 11.0) for Windows (SPSS Inc., Chicago, IL, USA). Qualitative data were presented as numbers (n) and percentages (%). Quantitative data were presented as means and standard deviation (SD). Comparison between the means of quantitative variables was performed using the one-way ANOVA (*F*-test) for comparison between three means. The correlations between different variables were evaluated by Pearson or Spearman correlation coefficients according to the distribution of variables (continuous or discontinuous quantitative variables respectively). A value $\leqslant 0.05$ was accepted as statistically significant.

3. Results

3.1. Clinical data

In the present study, in group Ia, 3 patients suffered from acute rejection episodes, 1 with urinary tract infection, cyclosporine toxicity, ATN, lymphocyle and urinary leak. In group Ib, 4 patients suffered from acute rejection episodes, 2 with urinary tract infection, 1 with cyclosporine toxicity, 1 with CMV disease, 1 with ATN, 2 with lymphocyle and 1 with urinary leak.

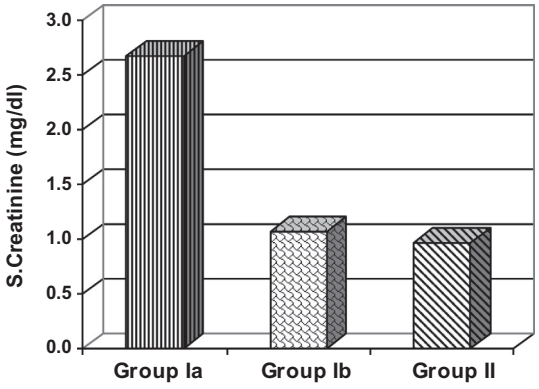

Figure 1A Serum creatinine (mg/dl) in patients in renal transplant recipients with chronic allograft dysfunction (Group Ia), stable allograft function (Group Ib) and in controls (Group II).

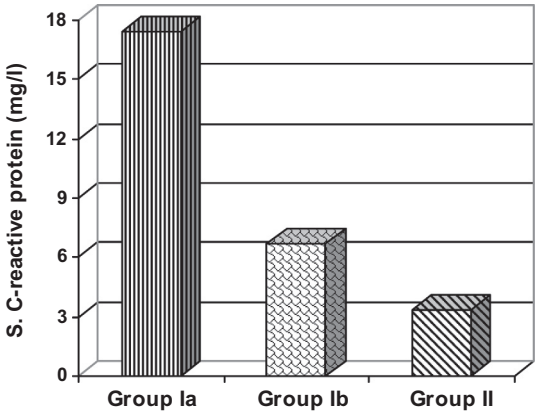

Figure 1B C-reactive protein (mg/l) in patients in renal transplant recipients with chronic allograft dysfunction (Group Ia), stable allograft function (Group Ib) and in controls (Group II).

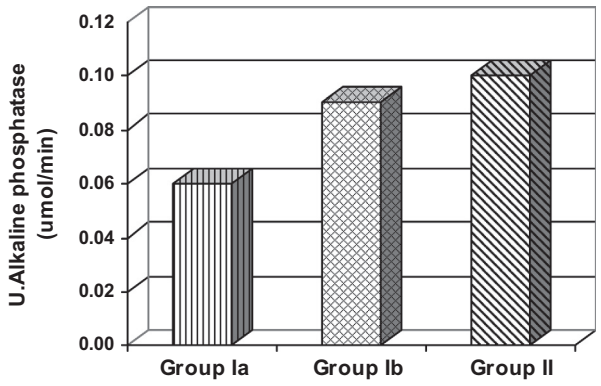

Figure 1C U. Alkaline phosphatase (μmol/min) in patients in renal transplant recipients with chronic allograft dysfunction (Group Ia), stable allograft function (Group Ib) and in controls (Group II).

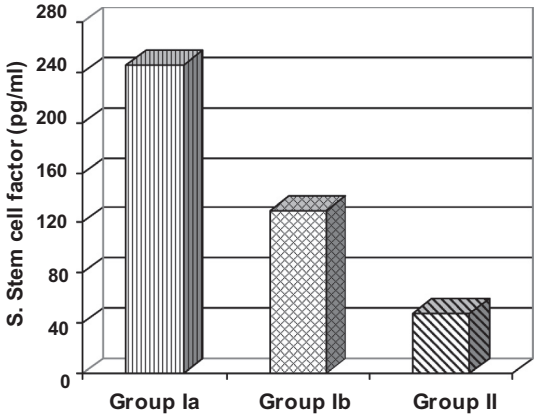

Figure 1D S. Stem cell factor (pg/ml) in patients in renal transplant recipients with chronic allograft dysfunction (Group Ia), stable allograft function (Group Ib) and in controls (Group II).

3.2. Laboratory results

Table 1 shows that hemoglobin level was significantly lower in both groups of patients than the controls and in group Ia than group Ib ($F = 26.55$, $P < 0.001$).

The blood urea, serum creatinine, CRP, and urinary protein excretion were significantly higher in group 1a patients (patients with CAN) than group 1b (patients with stable allograft function) and controls. While there was no statistically significant difference between both group 1b and controls ($F = 25.6$, $P < 0.001$), ($F = 31.32$, $P < 0.001$) ($F = 23.34$, $P < 0.001$) ($F = 17.260$, $P < 0.001$), respectively. Creatinine clearance and U.ALP were significantly lower in patients than controls and in patients of group Ia than patients of group Ib ($F = 159.52$, $P < 0.001$), ($F = 6.230$, $P = 0.004$), respectively. (Figs. 1A–1C)

Table 2 shows that there was a significant increase in the levels of SCF, number of peripheral blood HSCs and MSCs in both transplanted patient groups than the controls and they were higher in patients of group Ia than patients of group Ib, ($F = 39.73$, $P < 0.001$), ($F = 13.28$, $P < 0.001$), ($F = 11.94$, $P < 0.001$), respectively (Figs. 1D and 1E). Renal hemodynamic study revealed a significant increase in RI and PI with a significant decrease in renal artery cross sectional area and RBF in patients of group Ia than group Ib and controls, insignificant difference was found between group Ib and controls (Fig. 1F).

3.3. Histopathology results

All biopsies showed picture of CAN. Nine biopsies showed CAN1a, and 6 biopsies showed CAN1b (according to Banff classification 2011) (See Figs. 2A–2F)

The glomeruli in seven biopsies showed double contour of the peripheral capillary loops in 25–50% (cg2). Six biopsies showed an increase in mesangial matrix in 0–25%. 0–25% of the cortical tubules was infiltrated by mononuclear inflammatory cells in seven biopsies (t1) and mild to moderate degree of tubular atrophy (ct1, ct2) was found. Eight biopsies showed 0–25% interstitial infiltration by mononuclear inflammatory cells (i1), mild to moderate degree of interstitial fibrosis (ci1, ci2), was found in eleven and four biopsies respectively. This was obviously shown with masson trichrome stain. None of the biopsies showed evidence of intimal arteritis (v0), while twelve

Table 2 Mean ± SD and statistical comparison of serum stem cell factor, Peripheral blood count of hematopoietic stem cells, mesenchymal stem cells and HSCs/MSCs ratio, resistive index, pulsatility index, cross sectional area of the renal allograft artery and renal blood flow in the studied groups.

Parameters	Group Ia	Group Ib	Group II	F	P	L S D		
						GIa/GIb	GIa/GII	GIb/GII
SCF (pg/ml)	246.47 ± 72.51	129.67 ± 67.04	47.40 ± 17.71	39.73	< 0.001	*	*	*
HSCs (cell/μL)	7.20 ± 3.53	4.87 ± 1.96	2.80 ± 0.86	13.28	< 0.001	*	*	*
MSCs (cell/ μL)	12.27 ± 7.14	8.07 ± 2.31	4.40 ± 1.45	11.94	< 0.001	*	*	*
HSCs/MSCs ratio	0.64 ± 0.19	0.62 ± 0.23	0.69 ± 0.28	0.384	0.683	NS	NS	NS
RI	0.73 ± 0.07	0.60 ± 0.05	0.59 ± 0.05	29.52	< 0.001	*	*	NS
PI	1.28 ± 0.20	1.08 ± 0.10	1.04 ± 0.09	13.46	< 0.001	*	*	NS
Diameter (mm^2)	21.44 ± 4.64	34.89 ± 3.79	37.70 ± 3.92	66.29	< 0.001	*	*	NS
RBF (ml/min)	475.33 ± 108.75	790.00 ± 112.25	862.33 ± 85.33	60.08	< 0.001	*	*	NS

SCF = Stem cell factor, HSCs = hematopoeitic stem cells, MSCs = mesenchymal stem cells, RI = resistive index, PI = pulsatility index, Diameter = renal artery cross sectional area, RBF = Renal blood flow.
Abbreviations as in Table 1.

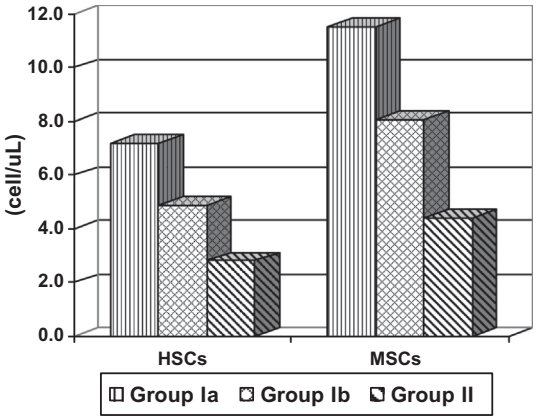

Figure 1E Hematopoeitic stem cells (HSCs) and Mesenchymal stem cells (MSCs) (cell/μl) in patients in renal transplant recipients with chronic allograft dysfunction (Group Ia), stable allograft function (Group Ib) and in controls (Group II).

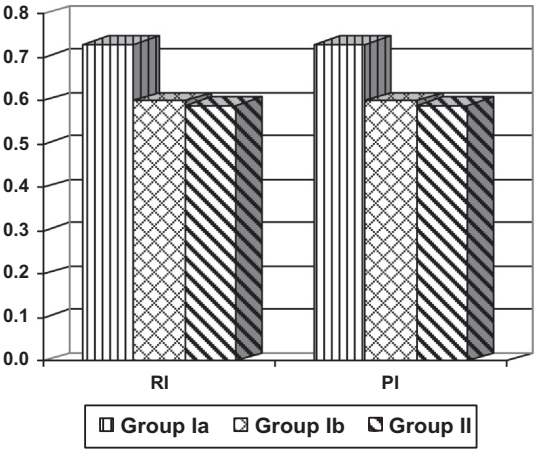

Figure 1F Resistive index (RI) and Pulsatility index (PI) in patients in renal transplant recipients with chronic allograft dysfunction (Group Ia), stable allograft function (Group Ib) and in controls (Group II).

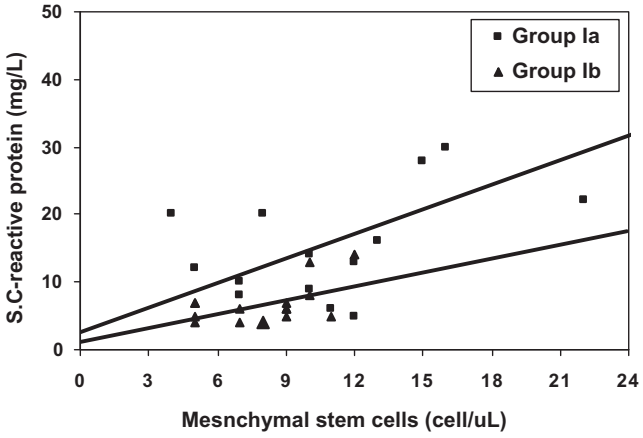

Figure 2A Correlation between mesenchymal stem cells (cell/uL) and C-reactive protein (mg/L) in renal transplant patients with chronic allograft nephropathy (group Ia) and with stable allograt function (Group Ib).

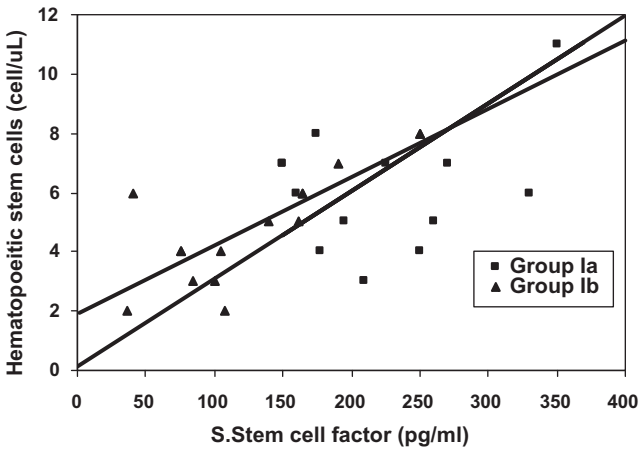

Figure 2B Correlation between stem cell factor (pg/ml) and hematopoietic stem cell (cell/uL) in renal transplant patients with chronic allograft nephropathy (group Ia) and with stable allograt function (Group Ib).

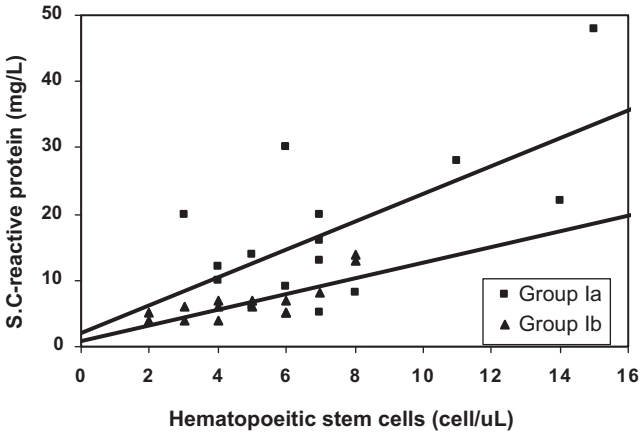

Figure 2C Correlation between hematopoietic stem cells (cell/uL) and S. C-reactive protein (mg/L) in renal transplant patients with chronic allograft nephropathy (group Ia) and with stable allograt function (Group Ib).

of them displayed fibrous intimal thickening and narrowing of the included arteries up to 25% (cv1). Three biopsies displayed fibrous intimal thickening and narrowing of the included arteries 25–50% (cv2). Mild arteriolar hyalinosis was detected in eight biopsies (ah1). (Fig. 3 {A, B})

Table 3 shows the immunohistochemical results of biopsies of patients with CAN using CD34, CD133, VEGF and ASMA antibodies. It ranged from minimal to moderate in distribution and showed the intensity and distribution of these markers (glomerular, mesangial, or tubular) (Fig. 3 {C–F}).

3.4. Statistical correlations

The correlations between the different studied parameters in both groups of patients are present in Tables 4 and 5, and Fig. 2.

In patients with group Ia there was a positive correlation between SCF and HSCs ($r = 0.643$, $P = 0.010$), also, HSCs were

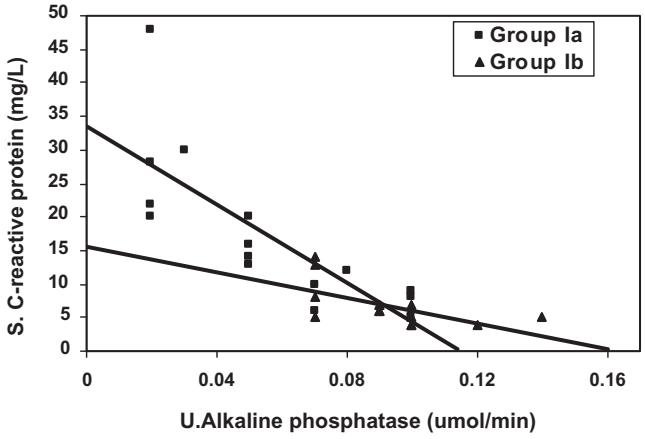

Figure 2D Correlation between urinary alkaline phosphatase (umol/uL) and C-reactive protein (mg/L) in renal transplant patients with chronic allograft nephropathy (group Ia) and with stable allograt function (Group Ib).

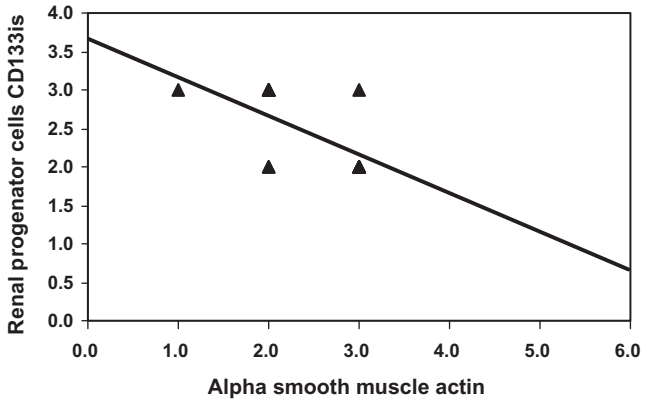

Figure 2E Correlation between renal alpha smooth muscle actin and renal progenitor cells CD133 in renal transplant patients with chronic allograft nephropathy (group Ia).

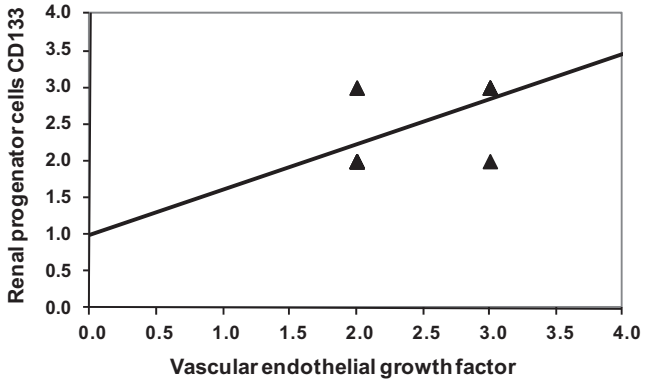

Figure 2F Correlation between renal vascular endothelial growth factor and renal progenitor cells CD133 in renal transplant patients with chronic allograft nephropathy (group Ia).

positively correlated with MSCs, S.CRP and creatinine ($r = 0.877$, $P < 0.001$), ($r = 0.651$, $P = 0.009$), ($r = 0.668$, $P = 0.006$) respectively. U.ALP was negatively correlated with S. creatinine, S.CRP and RI ($r = -0.652$, $P = 0.008$), ($r = -0.782$, $P = 0.001$), ($r = -0.751$, $P < 0.001$) respectively. The renal progenitor cells CD133 were positively correlated with CD34 ($r = 0.873$, $P < 0.001$) and both were positively correlated with VEGF ($r = 0.600$, $P = 0.018$). ($r = 0.722$, $P = 0.002$) and negatively correlated with ASMA ($r = -0.612$, $P = 0.015$), ($r = -0.757$, $P = 0.001$) respectively. In group Ib there was a positive correlation between SCF and HSCs ($r = 0.790$, $P < 0.001$), also, HSCs were positively correlated with MSCs, S.CRP and. creatinine ($r = 0.617$, $P = 0.014$), ($r = 0.767$, $P = 0.001$) ($r = 0.799$, $P < 0.001$) respectively. U.ALP was negatively correlated with S. creatinine, S.CRP and RI ($r = -0.879$, $P < 0.001$), ($r = -0.609$, $P < 0.001$) ($r = -0.756$, $P = 0.001$) respectively.

4. Discussion

In the present work, blood urea and creatinine levels were significantly higher in group Ia than Ib and the difference was insignificant between group Ib and control groups, but creatinine clearance was significantly lower in group Ia than Ib and in Ib than control group. This goes with earlier studies which reported that successful kidney transplantation at the best transfers patients from chronic kidney disease stage 5 (CKD5) to CKD2 but never normalized GFR.[44,45] As expected CAN group had a significant lower GFR than stable transplanted patients but moreover, the difference between the two groups beside being statistically significant, was of critical clinical value as the patients were transferred from CKD2 to CKD3b.

Also hemoglobin level was lower in patient group than control and lower in patients with CAN than patients without CAN, this finding may be attributed to reduction in GFR in CAN group and the use of immunosuppressant drugs with or without RAS blockers in transplanted cohort, this was also shown and explained by Winkelmayer et al.[46] this anemia in patients with CAN was described by Choukroun et al.,[47] who also described a good relation between correction of anemia and retarding the progression of CAN, also Winkelmayer et al.[48] described a definite relation between anemia and chronic allograft loss.

Urinary protein level was significantly higher in group Ia than group Ib, also it was significantly higher in group Ia than control subjects, while the difference was insignificant between group Ib and control group. Proteinuria was correlated negatively with GFR. This result was matched with what was shown by Fernandez-Fresnedo et al. as they showed in their work a significant increase of proteinuria in transplanted patients that was also correlated with renal allograft dysfunction.[49] Many other investigators showed the same results repeatedly over the years.[50–55]

CRP was significantly higher in the CAN group than transplanted group with stable renal functions and there was no significant difference between the latter group and control subjects. In concordance to our findings, Sezer et al.[56] and Fink et al.[57] found similar data with elevation of CRP level in transplant population. Not only in transplant patients but also in non diabetic CKD there was an increase in CRP as seen in the study done by Stuveling et al.[58] in the absence of any evidence of infection goes with the assumption that there is a

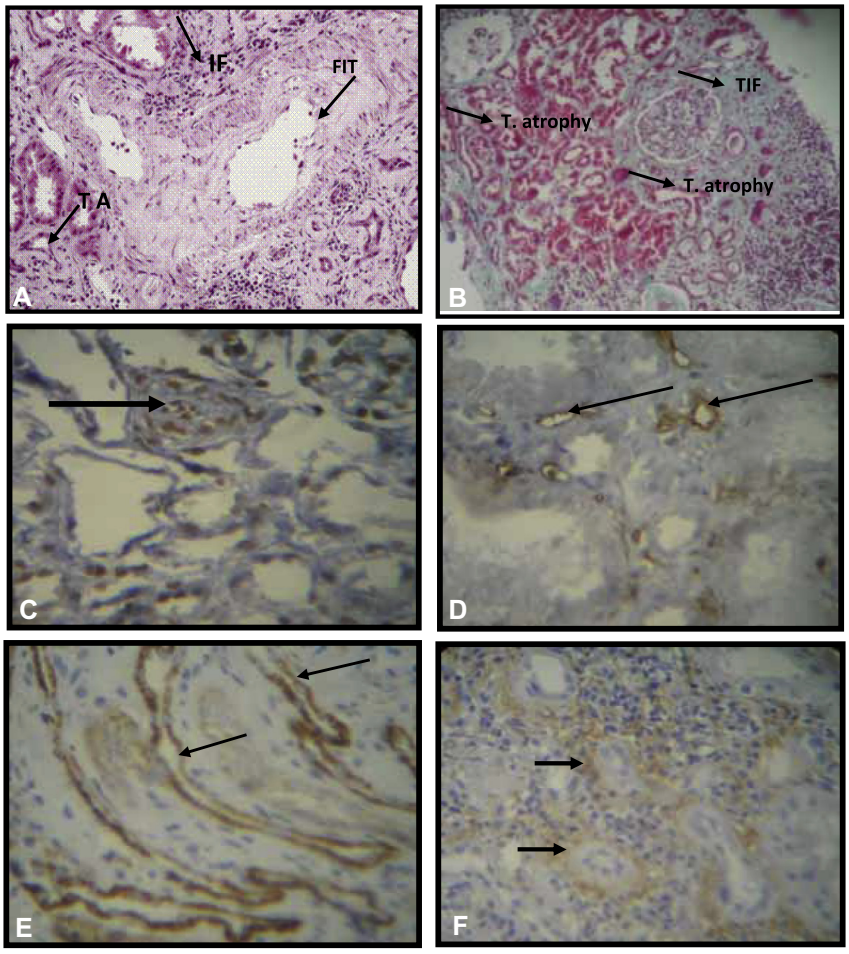

Figure 3 Light microscopy of CANIa (A) showing tubular atrophy with interstitial fibrosis (Masson trichrome) (arrows). (B) Fibrous intimal thickening (FIT), tubular atrophy (TA), and interstitial fibrosis (IF). Immunostaining (C) with anti CD 133 displayed nuclear deposits within the mesangium (arrow) (D) with anti CD34 demonstration positive membranous staining of hemopoeitic stem cell (HSC) around blood vessels (E) with anti vascular endothelial growth factor (VEGF) showing positive endothelial cytoplasmic staining (arrow) (F) with anti alpha smooth muscle actin (ASMA) demonstrating positive membranous staining within the interstitium with peri-tubular and peri-vascular distribution (arrow).

persistent state of micro inflammation in patients who develop CAN.

Urinary alkaline phosphatase was significantly less in patients who developed CAN than patients without CAN, and control subjects, this could be explained by significant degree of tubular atrophy in patients who developed CAN producing less amount of alkaline phosphatase in response to injury due to less tubular cell mass in a way that resemble liver enzymes in cirrhosis. Although the data of urinary alkaline phosphatase in kidney transplant are scarce, yet in another kidney disease model viz diabetic nephropathy, it was shown that urinary alkaline phosphatase can mark early proximal tubular injury even preceding the appearance of micro albuminuria.[59]

In the present work the levels of SCF in the peripheral blood of the patient group are significantly higher than control and regarding the subgroups of renal transplantation, its level in the patients with CAN was significantly higher than patients without CAN. A decade ago, a putative role for SCF in the pathogenesis of various forms of kidney disease progression was observed in several studies.[60,61] On the other hand, other studies suggested a regenerative role of SCF in renal diseases.[62] In concordance with the finding of previously mentioned studies, in the present study SCF was positively correlated with each of HSC markers (CD34, CD117 and CD45), MSC markers (CD106) in serum of both transplanted groups.

In the present work, stem cell markers were upregulated in transplanted patients and even more in patients who developed CAN in the absence of any other inflammatory condition that explains this finding. This may support the assumption that stem cells and SCF upregulation in patients with CAN may point out their potential role in the process of repair of a failing graft. On the contrary to this view, it may represent a mechanism by which ongoing fibrosis of the graft causes progressive deterioration of its function. Many studies have supported the former concept both in experimental and human models, in both kidney transplantation and other forms of kidney diseases. On the other hand other studies advocate for the latter view.

Imasawa et al.[63] clarified the role of BM derived stem cells in mesangial cell regeneration in a rat model. Similarly Ito et al.[64] proved the upregulation and migration of CD45 + ve BM-derived stem cells for glomerular repair in a rat model after anti Thy-1 glomerulonephritis and they concluded that the bone marrow can give rise to mesangial cells in vivo.

Table 3 Frequencies of immunohistochemical expression of $CD133^+$, $CD34^+$ cells, vascular endothelial factor, alpha-smooth muscle actin and renal fibrosis in renal biopsies of patients with chronic allograft dysfunction.

Immunohistochemical	Number of patients	Percentage of patients
$CD133^+$		
Minimal	0	0
Mild	7	53.3%
Moderate	8	46.7%
$CD34^+$		
Minimal	0	0
Mild	11	73.3%
Moderate	4	26.7%
VEGF		
Minimal	0	0
Mild	10	66.7%
Moderate	5	33.33%
ASMA		
Minimal	1	6.7%
Mild	11	73.3%
Moderate	3	20%
Fibrosis		
Minimal	0	0%
Mild	8	53.3%
Moderate	7	46.7%

VEGF = Vascular endothelial growth factor.
ASMA = Alpha smooth muscle actin.

Table 4 Statistical correlation between some of the studied parameter in renal transplant recipient with chronic allograft dysfunction.

Variable		S. Cr	Cr Cl	U. Pr	CRP	U. ALP	S. SCF	HSCs	MSCs
S. Cr	*r*								
	P								
Cr Cl	*r*	−0.955*							
	P	< 0.001							
U. Pr	*r*	0.521*	−0.554*						
	P	0.046	0.032						
S. CRP	*r*	0.910*	−0.829*	0.528*					
	P	< 0.001	< 0.001	0.043					
U. ALP	*r*	−0.652*	0.658*	−0.265	−0.782*				
	P	0.008	0.008	0.340	0.001				
S. SCF	*r*	0.838*	−0.773*	0.457	0.854*	−0.764*			
	P	0.000	0.001	0.087	< 0.001	0.001			
HSCs	*r*	0.668*	−0.630*	0.625*	0.651*	−0.432	0.643*		
	P	0.006	0.012	0.013	0.009	0.108	0.010		
MSCs	*r*	0.816*	−0.832*	0.714	0.763*	−0.507	0.669*	0.877*	
	P	< 0.001	< 0.001	0.003	0.001	0.053	0.006	< 0.001	
RI	*r*	0.577*	−0.534*	0.549*	0.768*	−0.751*	0.724*	0.546*	0.530*
	P	0.024	0.040	0.034	0.001	< 0.001	0.002	0.035	0.042

Abbreviations as in Tables 1 and 2.

Sugimoto et al.[65] reported a significant clinical and histological improvement in cases of Alport syndrome who were treated with stem cells and stated that their data confirm that BM derived stem cells could have a positive role in renal regeneration.

Semedo et al.[66] studied the effect of MSCs on renal inflammation and fibrosis in a rat model of chronic renal failure and their results suggested that MSC therapy can indeed modulate the inflammatory response that follows the initial phase of chronic renal injury. The immunosuppressive and remodeling properties of MSCs may be involved in the decreased fibrosis in the kidney. Marina Morigi et al.[67] studied the effect of MSCs derived from male mice in treating cisplatin induced ARF in female mice and their results offered a strong case for exploring the possibility that mesenchymal stem cells by virtue of their renotropic property and tubular regenerative potential may have a role in the treatment of acute renal failure in humans.

Table 5 Statistical correlation between some of the studied parameter in renal transplant recipient with stable allograft function.

Variable	S. Cr	Cr Cl	U. Pr	CRP	U. ALP	S. SCF	HSCs	MSCs	
S. Cr	*r*								
	P								
Cr Cl	*r*	−0.935*							
	P	< 0.001							
U. Pr	*r*	0.848*	−0.874*						
	P	< 0.001	< 0.001						
S. CRP	*r*	0.762*	−0.822*	0.750*					
	P	< 0.001	< 0.001	< 0.001					
U. ALP	*r*	−0.879*	0.892*	−0.714*	−0.609*				
	P	< 0.001	< 0.001	0.003	< 0.001				
S.SCF	*r*	0.618*	−0.778*	0.678*	0.735*	−0.726*			
	P	0.014	< 0.001	0.005	0.002	0.002			
HSCs	*r*	0.799*	-0.834*	0.736*	0.767*	−0.834*	0.790*		
	P	< 0.001	< 0.001	0.002	0.001	< 0.001	< 0.001		
MSCs	*r*	0.546*	−0.728*	0.653*	0.526*	−0.687*	0.895*	0.617*	
	P	0.035	0.002	0.008	0.044	0.005	< 0.001	0.014	
RI	*r*	0.893*	−0.779*	0.787*	0.668*	−0.756*	0.530*	0.598*	0.506
	P	< 0.001	< 0.001	< 0.001	0.007	0.001	0.042	0.019	0.054

Abbreviations as in Tables 1 and 2.

Kunter et al.[68] studied the reparative role of MSCs in a rat model of glomerulonephritis through infusion of MSCs in the rat renal artery after induction of GN, then detection of these intraglomerular cells and correlation with glomerular healing. Acute renal failure was ameliorated by MSC injection into the left renal artery on day 2 after disease induction. Again, MSC led to more rapid recovery from mesangiolysis, increased glomerular cell proliferation, and reduction of proteinuria by 28%.

Chen et al.[69] studied the ability of kidney derived MSCs to produce endothelial and smooth muscle like cells under the influence of angiogenic factors as VEGF both in vivo and *in vitro* and stated that kidney mesenchymal stem cells are capable of differentiation toward endothelial and smooth muscle cell lineages *in vitro* and in vivo, support new blood vessel formation in favorable conditions and promote functional recovery of an ischemic kidney.

Togel et al.[70] studied the positive effect of MSC infusion in the setting of AKI and concluded that MSC and endothelial cells interact and that these interactions are likely responsible, at least in part, for the kidney-protective effects of MSC in AKI, mediated by complex paracrine actions that are able to significantly protect and regenerate the damaged vasculature in AKI.

Wide spread distribution of CD133 positivity was detected in nearly all histopathology domain of the, interstitial and tubular) might point out to the potential reparative role of renal progenitor cells in CAN. This goes with the finding of Ronconi E et al. in a different model of kidney disease.[71]

CD34 was detected obviously around blood vessels and within the interstitium, this exceeds the limits of renal endothelial cells and presents the upregulation of HSCs and their homing in the transplanted kidney in patients with chronic allograft injury and may be a part of the setup of renal repair. Di Marco et al.[72] detected an increased level of endothelial progenitor cells which are CD34 + ve, and VEGFR2 + ve in renal transplant patients who had endothelial dysfunction than matched controls and assumed that it has a reparative role. BM derived stem cells have been reported by Rookmaaker et al.[73] to contribute to glomerular endothelial repair following thrombotic microangiopathy.

VEGF is a known endothelial marker that upregulates in situations where there is neoangeogenesis. Rate of VEGF in different clinical situations is controversial whereas it might be considered a harmful player in cases of cancer metastasis,[74] and proliferative diabetic retinopathy,[75] to the extent that specific anti-VEGF mono clonal antibodies,[76] are now in clinical utility for such condition, Its role in situation like kidney diseases is to be more clarified.

In this work VEGF was located by immunohistochemistry in endothelial cytoplasmic distribution both in peritubular capillaries as well as blood vessels whether this represents a normal finding or an up regulation of the level of its expression due to CAN is still unclear and is an interesting point to be further studied

The positivity of ASMA in this group of patients is an expected finding as the latter is known to be highly associated with fibrosing situations in different models of kidney diseases.[77]

Both RI and PI were significantly higher in group Ia than group Ib, also they were significantly higher in group Ia than control subjects, while the difference was insignificant between group Ib and control group. Elster et al.[78] suggested that elevated RI is an early predictor of histologically relevant CAN, possibly a result of undergoing vasculopathy, and stated that early evidence of CAN may allow for a targeted change in therapy before clinically significant injury. Ultrasonography should become a routine part of a transplantation clinic evaluation.

Radermacher et al.[79] studied 122 kidney allograft recipients regarding Doppler parameters and suggested that a Doppler ultrasonographic study performed three or more months after transplantation can predict long-term allograft outcomes. Their data also suggest that longitudinal Doppler studies may be useful in monitoring interventions such as different immunosuppressive protocols or in comparing the capability of various antihypertensive drugs to improve allograft outcomes. Such studies may reduce the need for sequential renal biopsies, with their associated risks. However, an increased

resistance index could mean acute vascular rejection with endarteritis, chronic allograft nephropathy, or both. Only a renal biopsy can distinguish among these conditions.

In this study RI and PI were correlated negatively with creatinine clearance, the same was shown by Nezami et al.[80] who studied the correlation of Doppler parameters to renal allograft dysfunction after kidney transplantation in 273 kidney allograft recipients and found that there is a strong correlation between the PI and PI that allow physicians to use each of them instead of the other in patients with a kidney allograft undergoing Doppler ultrasound., and stated that there is a significant correlation between these Doppler ultrasound indexes and serum creatinine level. Doppler ultrasound can be used as a tool to predict kidney function in association with serum creatinine, but its applicability depends heavily on the operator's skill.

In conclusion, upregulation of stem cell markers in transplanted patients and even more in patients who developed CAN in the absence of any other inflammatory condition that explain this finding may support the assumption that stem cells and SCF upregulation in patients with CAN might point out their potential role in the process of repair of a failing graft.

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