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ORIGINAL ARTICLE

Up-regulation of cell cycle regulatory genes after renal ischemia/reperfusion: differential expression of $p16^{(INK4a)}$, $p21^{(WAF1/CIP1)}$ and $p27^{(Kip1)}$ cyclin-dependent kinase inhibitor genes depending on reperfusion time

Archil B. Chkhotua,[1] Dietmar Abendroth,[2] Gebhard Froeba[3] and Hubert Schelzig[4]

1 National Centre of Urology, Tbilisi, Georgia
2 Transplantation Centre, University Clinic Ulm, Ulm, Germany
3 Department of Anesthesiology, University Clinic Ulm, Ulm, Germany
4 Thoracic and Vascular Surgery, University Clinic Ulm, Ulm, Germany

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Correspondence
Archil B. Chkhotua, National Centre of Urology, Tsinandali st. 9, 380044 Tbilisi, Georgia. Tel.: +995 99 210021; fax: +1 484 2883641; e-mail: achkhotua@gmail.com

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Summary

The aim of this study was to evaluate the influence of renal ischemia, cold preservation and reperfusion on the degree of renal kidney senescence. An experimental model of *ex vivo* renal hemoperfusion was used. Expression of $p16^{(INK4a)}$, $p21^{(WAF1/CIP1)}$ and $p27^{(Kip1)}$ cyclin-dependent kinase inhibitor genes (CDKIGs) was studied immunohistochemically in kidney biopsy samples at baseline and different time points after reperfusion. All three markers were up-regulated in kidney tissue after the reperfusion; however, their activation in different renal cells varied according to the reperfusion time. Expression of p16 was significantly increased in tubular cells at 180 min of reperfusion when compared with the baseline. Activation of p27 was detected in glomerular cells at 15 min and was significantly higher at 60, 120 and 180 min of reperfusion. The marker started increasing in tubular cells at 15 min and was elevated at every time point afterwards. p21 was significantly over-expressed in all renal cells after the reperfusion. It has been shown by the results of the current study that renal ischemia/reperfusion is associated with over-expression of CDKIGs indicating on substantial DNA damage and/or accelerated tissue senescence. For the first time it has been shown that tissue expression of CDKIGs is positively related with the reperfusion time.

Introduction

Renal ischemia/reperfusion (I/R) activates a cascade of cellular events with subsequent tissue damage and substantial clinical morbidity [1]. It has been shown that delayed graft function caused by renal I/R considerably influences graft survival [2]. There are numerous studies conducted on the pathophysiology of I/R injury in recent years; however, its exact mechanisms are still controversial [3]. A better understanding of the mechanisms of I/R injury will help in protecting the graft from damage and developing the therapeutic strategies against it.

Numerous enzyme systems like proteases, nitric oxide syntases, phospholipases etc. are activated after I/R causing cell membrane damage, cytoskeleton disruption and DNA degradation with eventual cell death [4]. It also activates complement, cytokines and adhesion molecules, which are cytotoxic themselves and attract leukocytes into the ischemic area causing further tissue damage [5]. The role of reactive oxygen species (ROS)-mediated immune system activation, Toll-like receptors, apoptosis markers and proinflammatory cytokines have also been evaluated in the most recent studies [6–8].

The senescence theory can give new insight into this problem. The role of senescence markers in I/R induced tissue damage is actively evaluated in the last years. Up-regulation of $p27^{(Kip1)}$ cyclin-dependent kinase inhibitor gene (CDKIG) has been shown after I/R [9]. p53 was

significantly activated in rat kidney tissue following I/R [10]. The telomere shortening, $p21^{(WAF1)}$ and $p16^{(INK4a)}$ CDKIG over-expression has been detected in experimental kidney transplantation (Tx) model [11]. Senescence-associated β-galactosidase was also found over-expressed in kidney tissue after I/R [12].

Despite its popularity in cell biology and molecular biology, importance of the senescence markers in organ Tx is not sufficiently evaluated. There are no studies on the influence of renal I/R on the intensity of different senescence markers. The impact of injuries associated with organ procurement (harvesting and preservation) on the markers' level has not yet been studied. Influence of reperfusion time on the degree of tissue damage is also the subject of further research.

In our previous study, we have shown that renal I/R was associated with kidney telomere shortening and over-expression of cell cycle regulatory genes indicating on substantial DNA damage and/or accelerated tissue senescence [13]. The influence of other transplant-related stresses on renal senescence has not been investigated. As a continuation, in the current experimental study, we evaluated the influence of renal ischemia followed by cold preservation and reperfusion on intensity of the senescence markers' expression. The impact of reperfusion time on the degree of renal tissue senescence was also studied.

Materials and methods

Experimental protocol

An experimental model of *ex vivo* hemoperfusion of pig kidneys was used as described earlier [14]. On the whole 12 pigs (German Landrace) were used in the study. The animals were kept under the conditions meeting the international principles of laboratory animal care. The study was carried out according to the German Law on the Protection of Animals and the protocol was approved by the local ethical committee.

Prior to the experiment the animals were fastened for 24 h with unlimited access to water. After premedication with intramuscular atropine (0.05 mg/kg Atropinsulfat®; B. Braun, Melsungen, Germany) and azaperone (4 mg/kg, Stresnil®; Janssen-Cilag, Neuss, Germany) an ear vein was secured. Following preoxygenation, general anesthesia was introduced with intravenous atropine (0.5 mg), Na-pentobarbital (6–10 mg/kg, Narkodorm-n®; Alvetra, Neumünster, Germany) and ketamine (2 mg/kg Ketavet®; Pharmacia & Upjohn, Erlangen, Germany) under pulsoxymetric monitoring with a probe placed on the tail. The pigs were orotracheally intubated and mechanically ventilated [Servo 900B®; Siemens, Erlangen, Germany; fraction of inspired oxygen (FiO_2), 0.35; respiratory rate, 10–12/min; positive end-expiratory pressure, 4 cm H_2O] in the supine position. Ventilation was adjusted during the surgical preparation to maintain arterial PCO_2 between 35 and 40 mmHg and hemoglobin oxygen saturation >90% (transcutaneous pulsoxymetry, (Datex Capnomac Ultima®; ULT-S-3301, Datex Instrumentarium Corp, Helsinki, Finland).

From a left anterior thoracotomy the descending thoracic aorta was exposed and a polyester surgical retraction tape (Mersilene®; Johnson & Johnson Intl., Brussels, Belgium) was placed around the aorta immediately below the origin of the left subclavian artery. The retraction tape was passed through plastic tubes out of the thoracic cavity as well as a drain (28F) to prevent tension pneumothorax. The thoracic cavity was then closed.

After the surgical preparation 120 min of recovery time was allowed. After baseline data collection the retraction tape around the aorta was fastened for 30 min with checking the disappearance of the blood pressure curve in the femoral artery. After that a 4-h reperfusion time was allowed. The kidneys were harvested, flushed *ex vivo* with cold Euro Collins solution and kept in ice for 12 h. At the end of the experiment the animal were sacrificed under deep anesthesia with additional dose of pentobarbital and intravenously administered 20 mmol bolus of KCl.

After storage, the renal artery was canulated and reperfused for 3 h with autologous blood using the specially designed perfusion system. The blood was heparinized (10 IU/ml), diluted in a hematocrit to 25% and adjusted to a pH of 7.4 within the perfusion circuit using bicarbonate titration and CO_2 insufflation into the oxygenator.

Perfusion system

An *ex vivo* hemoperfusion of kidneys was performed using the perfusion system described earlier [14]. Briefly, the system consists of a perfusion circuit through a closed chamber with a parallel separate membrane oxygenator circuit. Via an arterial canulation, pressure controlled perfusion was achieved by a computer-driven roller pump with on-line recording of flow, pressure and resistance. A second independent pump controlled the oxygenator circulation. The venous effluate was collected and continuously re-entered into the circuit.

The tissue biopsies were taken from the kidney before (0 min) and 15, 60, 120 and 180 min after the reperfusion. The tissue samples were than fixed in 4% formaldehyde and embedded in paraffin.

Immunohistochemistry

Sequential tissue sections consisting of cortex and medulla of 4-μm thickness were used for immunohistochemistry. Endogenous peroxidase was blocked by incubation in 1%

hydrogen peroxide. Sections were then incubated for 1 h at room temperature with either anti-$p16^{(INK4a)}$ (Santa Cruz Biotechnology Inc., Clone F-12, Code: SC-1661. Santa Cruz, CA, USA), anti-$p21^{(WAF1/CIP1)}$ (Santa Cruz Biotechnology Inc., Clone F-5, Code: SC-6246.) or anti-$p27^{(Kip1)}$ (Transductions Laboratories, Clone 57, Code: K25020. Heidelberg, Germany) antibodies diluted (1:100, 1:20 and 1:200 respectively) in PBS plus 1% of bovine serum albumin (Sigma Co. 20K7607. Munich, Germany). After PBS washing, tissue sections were revealed by Envision monoclonal system (Envision + TM; Dako Co. Code: K-4001. Carpinteria, CA, USA). Samples were developed with liquid diaminobensidine DAB+ substrate-chromogen system (Dako Co., Code: 3468. Glostrup, Denmark) and counterstained with hematoxylin. Tissues from ovarian cancer were used as a positive control and nonimmune mouse serum as a negative control.

Sample evaluation and scoring

Slides were evaluated with a microscope under 20–40× magnification objectives. Glomerular, tubular, interstitial and vascular expression of the markers was analyzed separately by calculating respective scores. The total number of glomeruli, tubules, interstitial nuclei and crosscut blood vessels, which were positive for p21 were counted in glomerular (GS), tubular (TS) interstitial (IS) and vascular (VS) scores, respectively. The total number of positive glomeruli and blood vessels were counted for p16 and p27. Tubular and interstitial expression of the above-mentioned markers was assessed semi-quantitatively with the following scoring system: no expression, 0; mild, 1; moderate, 2; and strong expression, 3.

Statistical analysis

Statistical analysis was performed with a computer-assisted software (StatView 5.0, SAS Institute Inc. 1998). Normality of the data distribution was examined with the Shapiro–Wilk test. Dependence of the intensity of the markers' expression on the reperfusion time was analyzed with one-way ANOVA and Kruskal–Wallis tests. In case of significant difference the means of the data were compared with Bonferroni test for homogeneity of means and nonparametric comparison of ranks.

Results

The genes were expressed to various degrees by all structures of the kidney tissue: tubular epithelial, interstitial and vascular endothelial cells, as well as parietal and visceral epithelial glomerular cells. The statistical analysis of variation of different gene scores with reperfusion time is shown in Table 1. Expression of p16 in glomerular cells did not change after the reperfusion. There was a trend towards its increase in vascular cells; however, without statistical significance ($P = 0.0747$). Expression of p16 in tubular and interstitial renal cells significantly increased after the reperfusion (Table 1). To determine when exactly the gene was significantly over-expressed, the mean data of the scores were compared by the test for homogeneity of the means. It was found that the marker was significantly up-regulated in tubular cells at 180 min of reperfusion when compared with the baseline values. Difference of the gene expression in interstitial cells was not significant, which probably can be explained by insufficient number of the cases (Fig. 1).

Expression of p27 changed in glomerular, tubular and interstitial renal cells (Table 1). Activation of the gene expression in glomerular cells started at 15 min and was significantly higher at 60, 120 and 180 min when compared with 0 and 15 min of reperfusion. More complex correlation was found between the gene expression levels in the tubular cells: it started increasing at 15 min and was significantly higher at every time point afterwards (Fig. 2).

p21 was significantly over-expressed in all renal cells after the reperfusion (Table 1). The gene expression in glomerular and tubular cells started increasing immediately after the reperfusion and was significantly higher at each time point afterwards when compared with the baseline values. The difference was especially prominent in interstitial cells. Significantly more marker was expressed in vascular cells at 180 min when compared with 0 and 15 min of reperfusion (Figs 3 and 4).

Discussion

Ischemia/reperfusion is a major transplantation-related stress substantially determining the treatment outcome. Numerous pathological events have been attributed to the I/R injury; however, its exact mechanisms and the ways of protection are not completely understood [6]. The various therapies have been suggested to diminish the I/R injury, yet the complexity of the pathological process makes their effectiveness doubtful [15].

The role of apoptosis, adhesion molecules, proinflammatory cytokines and toll-like receptors has been evaluated in I/R injury. Up-regulation of the several adhesion molecules: LFA-1, ICAM-1 and VCAM-1 were detected in renal grafts after Tx [16]. Renal I/R caused activation of proinflammatory cytokines [17]. Importance of the toll-like receptors in this process is a topic of ongoing investigation [18].

The fact that the senescence process may have a potential application in I/R is supported by several recent

Table 1. Correlation of the genes' scores with the reperfusion time.

	P16		P27		P21	
Score	Mean ± SD	*P*-value	Mean ± SD	*P*-value	Mean ± SD	*P*-value
Glomerular						
0′	7.1 ± 1.5	0.3420†	5.7 ± 1.1	0.0001†	3.6 ± 1.4	0.0001
15′	8.9 ± 2.4		7.3 ± 2.1		7.4 ± 2.1	
60′	9.4 ± 1.8		9.7 ± 2.8		10.6 ± 2.1	
120′	8.8 ± 1.9		10.1 ± 2.3		11.1 ± 4.0	
180′	9.3 ± 2.7		10.1 ± 2.5		11.3 ± 2.3	
Tubular						
0′	1.6 ± 0.7	0.0062*	1.4 ± 0.5	0.0003*	2.9 ± 1.2	0.0001†
15′	1.9 ± 0.8		2.4 ± 0.8		7.7 ± 3.3	
60′	2.3 ± 0.8		3.0 ± 0.7		19.6 ± 5.8	
120′	2.6 ± 0.7		3.0 ± 1.0		31.6 ± 13.3	
180′	3.3 ± 1.0		3.3 ± 0.8		46.3 ± 21.0	
Interstitial						
0′	1.1 ± 0.3	0.0210*	1.1 ± 0.3	0.0115*	10.8 ± 5.6	0.0001†
15′	1.4 ± 0.5		1.5 ± 0.6		47.4 ± 21.6	
60′	1.5 ± 0.5		1.9 ± 0.6		96.5 ± 26.9	
120′	1.8 ± 0.4		1.8 ± 0.7		114.0 ± 13.3	
180′	2.1 ± 0.7		2.0 ± 0.7		121.4 ± 19.4	
Vascular						
0′	0.7 ± 0.7	0.0747*	1.1 ± 0.9	0.1602*	0.2 ± 0.4	0.0005*
15′	1.7 ± 0.4		1.4 ± 1.3		0.2 ± 0.4	
60′	1.6 ± 0.5		2.0 ± 1.4		0.6 ± 0.5	
120′	1.3 ± 0.4		2.0 ± 0.9		1.0 ± 0.6	
180′	1.7 ± 0.8		1.9 ± 1.0		1.2 ± 0.4	

*Kruskal–Wallis test, †ANOVA test.

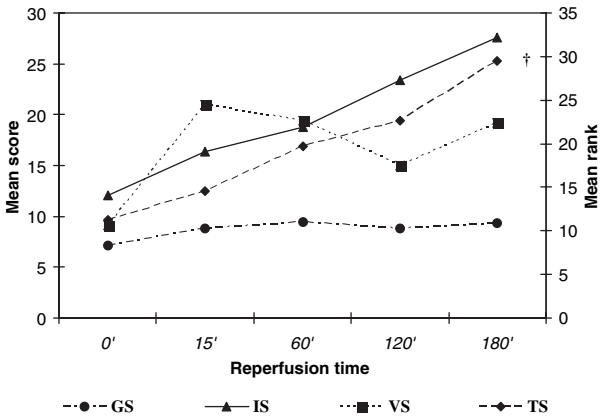

Figure 1 Expression of p16 in different renal cells after the reperfusion. †$P < 0.05$ for 180′ vs. 0′.

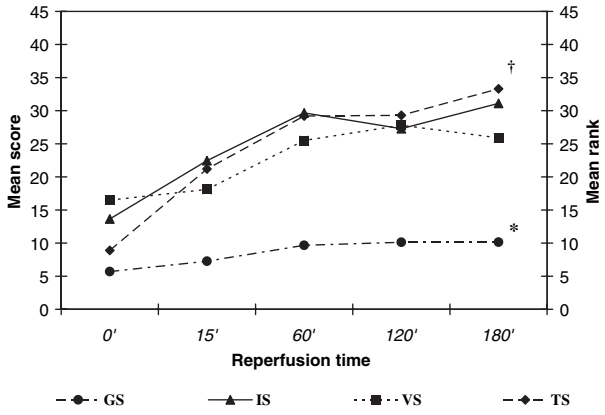

Figure 2 Expression of p27 in different renal cells after the reperfusion. *$P < 0.005$ for 60′, 120′ and 180′ vs. 0′ and 15′.†$P < 0.005$ for 180′ vs. 0′ and $P < 0.05$ for 60′ and 180′ vs. 0′ and 15′.

studies. It was found that skeletal muscle I/R caused induction of p27 expression [9]. Renal I/R was followed by p53 protein activation [10] and over-expression of senescence-associated β-galactosidase [12]. Increased p16 expression was detected in renal allograft biopsies [19].

In our previous study we have shown that I/R is causing the telomere shortening and activation of some of the cell cycle regulatory genes. The influence of other transplant-related stresses on renal senescence was not evaluated [13]. As a continuation of the previous study, in the current experiment the organ was subjected to the same stresses as in clinical transplant situation: ischemia followed by cold preservation, and reperfusion. Thus, we think that the model itself and the study results could be applicable to the clinical situation of cadaver kidney Tx.

It was found that renal ischemia with subsequent cold preservation and reperfusion causes activation of CDKIGs in almost all tissue structures. This indicates on a substantial DNA damage caused by the stress. It also can be interpreted as acceleration of the senescence process in accordance with the concept of stress-induced premature senescence [20].

Another important aspect of the present study is the influence of reperfusion time on tissue damage. The reperfusion itself has been defined as an additional stress to the organ. Reactive oxygen species (ROS) produced after I/R can contribute to diverse molecular changes in tissue including immune system regulation through toll-like receptors and nuclear factor kappa B [21]. The production of toxic hydroxyl radicals in the venous blood of cold-stored and reperfused human renal allografts has been detected [6].

There are very few studies on the influence of reperfusion time on tissue damage after ischemia. It was found that intestinal ischemia followed by continuous reperfusion caused various changes in the expression of apoptosis markers: number of apoptotic cells, Fas, FasL and caspase-3 was markedly increased, and Bcl-2 production was decreased according to the reperfusion time [22]. In an experimental study of rat kidney I/R vascular endothelial growth factor and interleukin-1beta expression was increased at 2 and 24 h of reperfusion [23]. p21 was up-regulated at 12 and 24 h of reperfusion in liver tissue [24]. There are no studies on the influence of renal postischemic reperfusion time on tissue expression of the

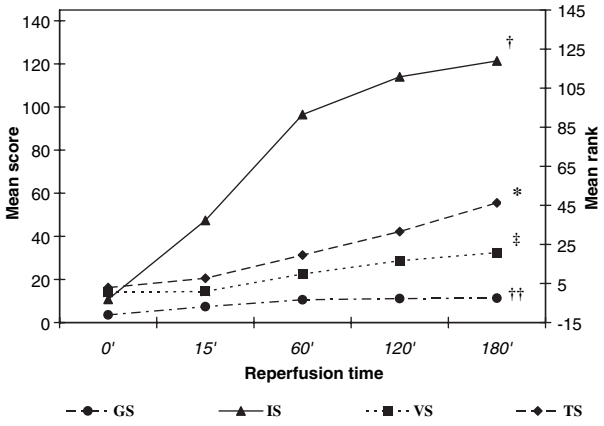

Figure 3 Expression of p21 in different renal cells after the reperfusion. †$P < 0.001$ for 60′ and 120′ vs. 0′. *$P < 0.001$ for 180′ vs. 0′, 15′ and 60′‡$P < 0.05$ for 180′ vs 0′ and 15′.†† $P < 0.05$ for 180′ vs. 0′ and 15′.

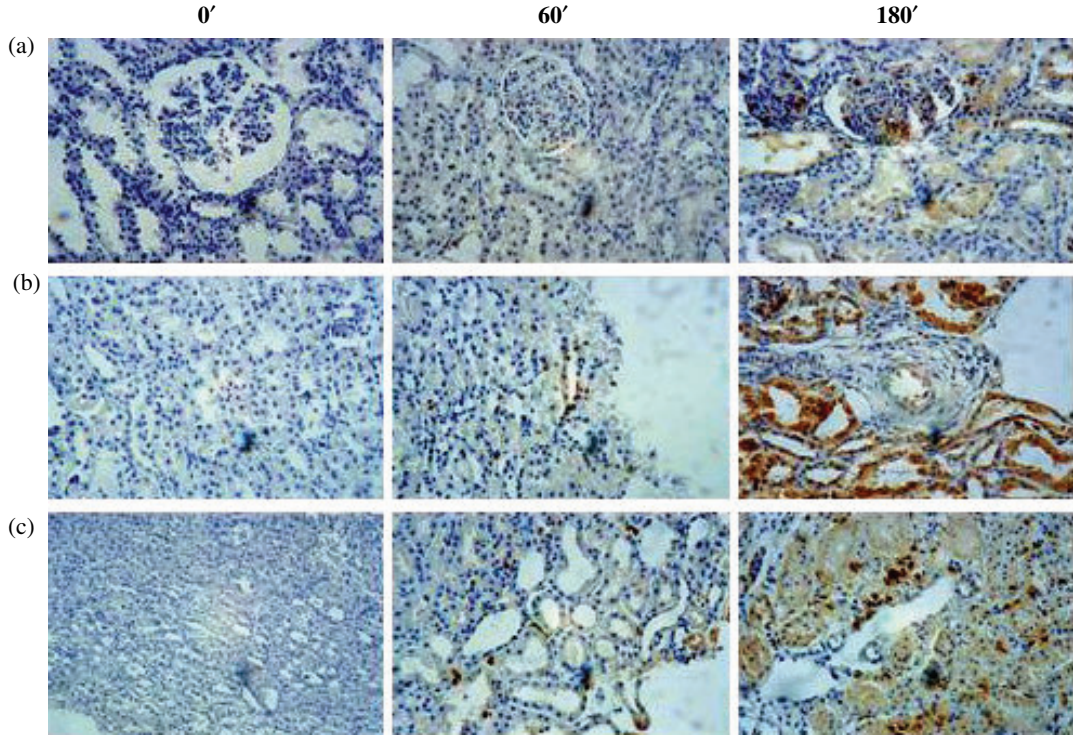

Figure 4 Expression of p21 in glomerular (a), tubular (b) and interstitial (c) renal cells at baseline and after the reperfusion. Counterstained with hematoxylin, ×40 magnifications.

senescence markers. In the current study we have found that reperfusion time is an additional stress for renal tissue causing activation of CDKIGs. With increasing time the genes' expression is increasing in different tissue structures. This finding can be interpreted as an acceleration of renal senescence with reperfusion. It also can be a protective mechanism against replication of the DNA damaged cells in the process of poststress tissue regeneration. Both these concepts should be further evaluated in order to assess the importance of above findings.

In conclusion, it has been shown by the results of the current study that renal ischemia followed by cold preservation and reperfusion is associated with over-expression of CDKIGs. For the first time it has been shown that reperfusion time is an additional stress for the renal tissue and causes the time-dependent activation of the above-mentioned genes. These findings indicate on a substantial DNA damage and/or accelerated tissue senescence caused by some of the transplantation associated stresses.

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