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BRIEF REPORT

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Increased expression of p21 (WAF1/CIP1) cyclin-dependent kinase (CDK) inhibitor gene in chronic allograft nephropathy correlates with the number of acute rejection episodes

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Abstract The p21 (WAF1/CIP1) cyclin-dependent kinase (CDK) inhibitor gene is considered to be the senescence marker in some recent publications. Expression of the gene was evaluated in 14 normal human kidney tissues of different ages and in nine chronically rejected renal allografts. All normal kidneys were negative for p21 expression. Glomerular, tubular and interstitial expression of the marker was detected in 88.9% ($P < 0.0001$) and vascular expression in 66.7% of chronically rejected grafts ($P < 0.001$). No correlation was found between the intensity of p21 expression and recipient age, donor age or number of human leukocyte antigen (HLA) mismatches. The marker was expressed more in grade 3 of chronic allograft nephropathy (CAN) than in grade 2 ($P = 0.059$ for glomerular score). Tubular expression of p21 was correlated with the number of acute rejections: $P < 0.05$ for three vs one and two, and $P = 0.0046$ for three vs no previous acute rejection episodes.

Keywords Kidney transplantation · p21 · Chronic allograft nephropathy · Kidney senescence · Cyclin-dependent kinase inhibitors

Introduction

Chronic allograft nephropathy (CAN) is the most frequent cause of late kidney-graft loss. Although extensively studied, the pathogenesis and the mechanisms of disease development are not completely understood [1]. A new theory has recently been proposed, according to which all transplantation (Tx)-associated stresses cause acceleration of graft senescence [2]. This has not been proved in clinical studies due to the absence of reliable markers.

The p21 (WAF1/CIP1) cyclin-dependent kinase (CDK) inhibitor gene is considered to be one of the senescence markers in recent publications [3, 4]. The studies on p21 expression in human kidneys do not analyse the age dependency of marker expression, proving its potential as a senescence marker [5, 6]. It is not clear from existing data whether p21 expression is stimulated by cell senescence, by DNA damage, or by a combination of both. Marker expression in CAN has not been investigated, to date, either.

In this study we evaluated the expression of p21 (WAF1/CIP1) CDK inhibitor gene in normal human kidneys of patients of different ages and in chronically rejected kidney grafts. To our knowledge, it is the first report on the value of the gene as a potential kidney senescence marker and on its up-regulation in CAN.

Patients and methods

Patient groups

Expression of p21 (WAF1/CIP1) CDK inhibitor was studied immunohistochemically in:

- Group I. Fourteen normal human kidney tissue samples resected from patients of various ages for renal cell carcinoma (71.4%) and kidney trauma (28.6%)
- Group II. Nine chronically rejected kidney grafts

The mean age of the patients in group I was 54 ± 16 years (range 21–79 years); 71% were male and 29% were female. Characteristics of transplant patients and their donors are shown in Table 1. The mean recipient age was 42.6 ± 13.7 years (range 19–60 years); 55.6% were male and 44.4% were female. All patients received their first kidney transplant from cadaveric donors aged between 14 and 60 years (mean age 47.4 ± 20.7 years). All patients were on haemodialysis before undergoing transplantation. Mean human leukocyte antigen (HLA) mismatch number was 3.8 ± 1.2 (range 2–5), and the mean cold ischaemia time was 16.8 ± 2.4 h (range 13–20 h). Three donors were cytomegalovirus (CMV) positive; no other donor co-morbid factors were detected. Recipient co-morbid conditions at the time of transplantation were hypertension (66.7%), hepatitis C (22.2%) and CMV (11%) infections. Immediate graft function was achieved in all nine cases. Initial immunosuppression was a cyclosporin A (CsA)-based triple-drug regimen (CsA + azathioprine/mycophenolate mofetil + prednisolone) in eight recipients (88.9%) and azathioprine with prednisolone in one early transplant patient (11.1%). Five out of nine patients (56%) experienced at least one episode (maximum: three) of acute cellular rejection. Other post-transplantation complications were CMV (33.3%) and sepsis (22.2%), and urinary tract infection, haemolytic-uremic syndrome and urinary fistula (each 11.1%, i.e. each in one patient). Immunosuppression was discontinued due to graft failure in all patients, and they were switched to haemodialysis. In all cases, clinical signs of graft intolerance (fever, hypertension etc.) were indications for transplantectomy. CAN, diagnosed by graft needle biopsy, was the cause of graft failure at an average of 6 years post-Tx (range 1–24 years) in all patients.

Tissue sampling and immunohistochemistry

All resected kidney specimens were evaluated macroscopically. After being sampled according to the main disease, 1.5×2-cm tissue samples containing cortex and medulla were taken from two separate, macroscopically normal kidney regions. The samples were fixed in 4% formaldehyde and embedded in paraffin; 4 to 5-μm-thick serial sections were stained with haematoxylin and eosin (HE) according to conventional techniques. The sections from the patients in group I were analysed by the pathologist to exclude oncological or other pathological changes. Only kidneys that were considered to be normal and with acceptable age-associated morphological changes (mild interstitial infiltration and/or tubular atrophy) were immunohistochemically evaluated further and included in the study ($n = 14$). The sections from the patients of group II were fixed, stained and evaluated according to Banff 97 criteria [7]. Repeated pathological assessment confirmed the initial diagnosis of CAN in all nine cases.

For p21 immunohistochemistry, 4-μm-thick sequential tissue sections were used. They were pre-treated by microwave antigen retrieval procedures for 20 min in 10 mmol/l citrate buffer solution (pH 6.0). Tissue sections were then incubated overnight at 4 °C with primary antibody (p21 WAF1/CIP1, clone F-5, Santa Cruz Biotechnology, code: SC-6246), diluted (1:25) in phosphate-buffered saline (PBS) plus 1% of bovine serum albumin (BSA; Sigma Co., 20K7607). The tissue sections were revealed by Envision monoclonal system (Envision+, Dako Co., code: K-4001) and developed with a liquid diaminobenzidine DAB+ substrate-chromogen system (Dako, code: 3468). Tissues from ovarian cancer were used as a positive control.

Sample evaluation and statistical analysis

Slides were evaluated blind under 20–40× magnification objectives. Only nuclear staining for p21 was considered positive and was counted. Glomerular, tubular, interstitial and vascular expression of the marker was analysed separately by calculation of respective scores. All calculations were done for the whole tissue area of the same size (1.5×2 cm). The total number of positive glomeruli, tubules and crosscut blood vessels were counted in glomerular (GS), tubular (TS) and vascular (VS) scores, respectively. The total number of positive interstitial nuclei was counted for an interstitial score (IS).

Statistical analysis was performed with computer-assisted software (Stat View 5.0, SAS Institute, 1998). Marker expression in group I and group II was compared by the Wilcoxon rank sum test for non-parametric variables. Normality of the data distribution was examined by the Shapiro–Wilk test. Correlation of marker expression with the age, degree of CAN, number of HLA mismatches and the number of previous acute rejections was analysed with a one-way ANOVA.

Table 1 Characteristics of transplant patients. *ESRD* end-stage renal disease, *CGN* chronic glomerulonephritis, *Aza* azathioprine, *Pred* prednisolone, *CsA triple* CsA + Aza/mycophenolate mofetil + Pred, *ND* not defined

Patient number	Age	Gender	Donor age	Cause of ESRD	Number of mismatches	Cold ischaemia time	Induction immuno suppression	Number of acute rejections	Graft survival	Grade of CAN
1	29	M	20	CGN	2	14	CsA triple	1	4	3
2	57	M	68	CGN	3	16	CsA triple	0	3	2
3	46	F	47	CGN	ND	16	Aza + Pred	3	24	3
4	36	F	44	CGN	4	18	CsA triple	1	1	3
5	60	M	68	Idiopathic nephrosclerosis	3	19	CsA triple	0	3	3
6	45	M	58	CGN	3	18	CsA triple	1	8	3
7	55	F	60	CGN	5	20	CsA triple	0	7	3
8	19	M	14	CGN	5	13	CsA triple	0	3	3
9	36	F	45	CGN	5	18	CsA triple	2	1	2

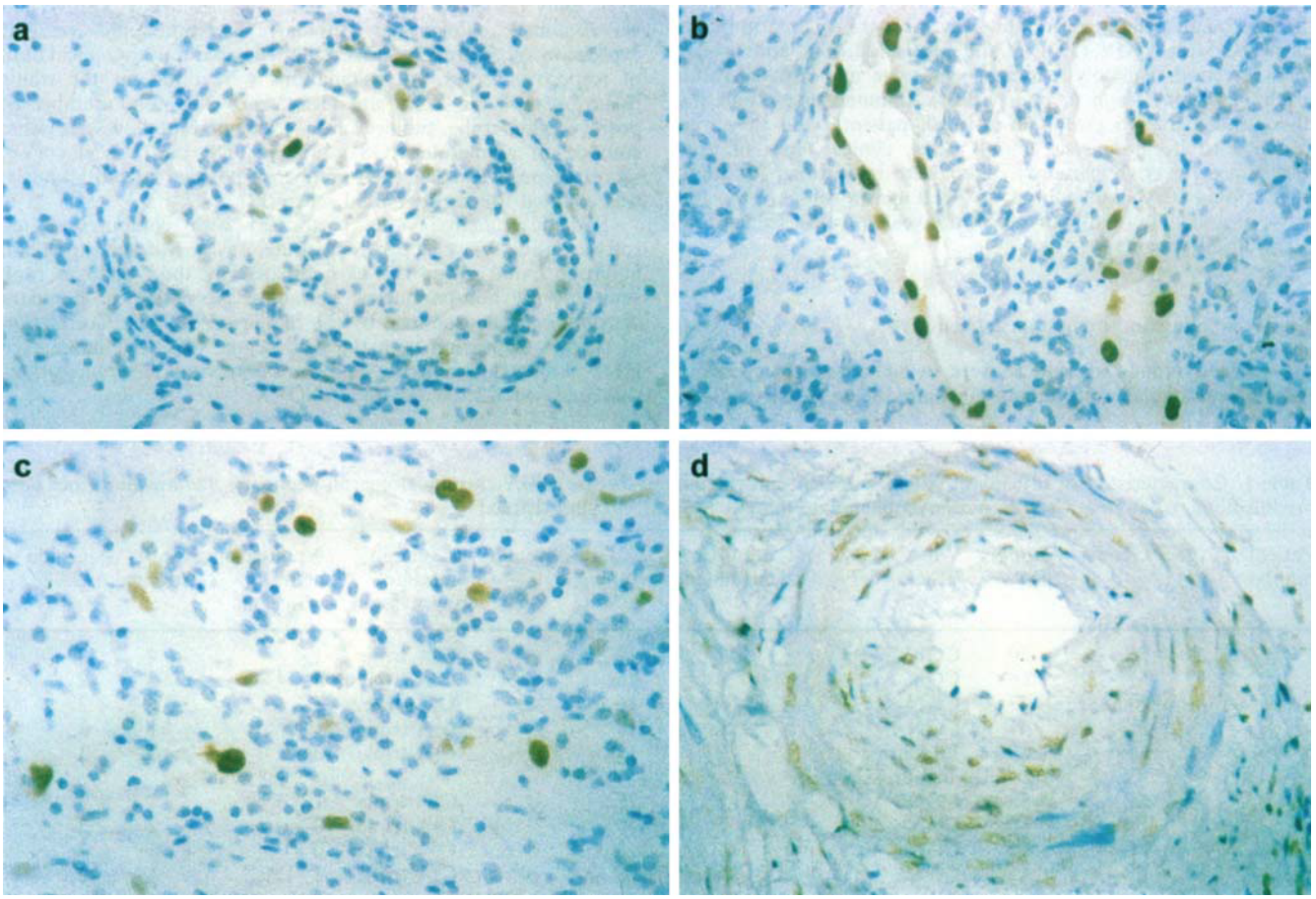
Results

All kidneys from group I were negative for marker expression, independent of patient age, glomerular, tubular and interstitial expression of p21 was detected in 88.9% ($P < 0.0001$) and vascular expression in 66.7% of chronically rejected kidney grafts ($P < 0.001$) (Fig. 1).

Evaluation of chronically rejected kidneys revealed pathological changes characteristic of CAN: glomerulopathy, cortical interstitial infiltration and fibrosis, atrophy of tubular epithelium with loss of brush borders, proliferation of myofibroblasts and fibrous intimal thickening of arteries with narrowing of lumen. Gene expression was detected in the following cells: podocytes and mesangial glomerular cells (Fig. 1a), tubular epithelial (Fig. 1b), interstitial (Fig. 1c), vascular myofibroblasts and endothelial cells (Fig. 1d).

In the chronically rejected kidneys (group II) the marker expression did not correlate with recipient age, donor age and the number of HLA mismatches. All four scores were higher in grade 3 of CAN than in grade 2, with a trend to significance for the glomerular score ($P = 0.059$). All scores were higher in patients who had had three previous acute rejection episodes. No significant correlations were found for the glomerular, interstitial and vascular scores. The tubular score was found to be significantly correlated with the number of acute rejections, in that (a) three acute rejections were associated with a higher marker expression than one and two rejections ($P < 0.05$), and (b) patients with no history of acute rejections had a significantly lower tubular score than those with three previous rejection episodes ($P = 0.0046$; Fig. 2).

Fig. 1a–d Expression of p21 in CAN. **a** Glomerular, **b** tubular, **c** interstitial, **d** vascular. Note the tubular atrophy and fibrous intimal thickening of the artery – characteristic pathological features of CAN. Magnification ×40



Discussion

P21 (WAF1/CIP1) is a CDK inhibitor gene, preventing the cell cycle from proceeding to mitosis. The importance of the marker in kidney damage is supported by up-regulation of the gene in cisplatin-induced acute renal failure [8], cyclosporine toxicity [9] and experimental diabetic nephropathy [10]. The existing studies demonstrate two potential mechanisms of p21 expression—tissue senescence and DNA damage. However,

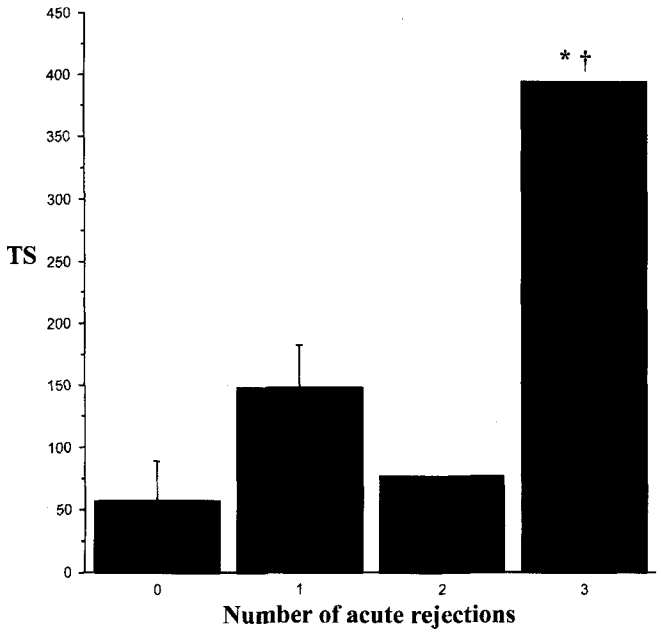


Fig. 2 Correlation of tubular score with acute rejection. $*P < 0.05$ for three vs one and two rejections, † $P = 0.0046$ for three vs no rejections

the evidence is not sufficient to answer the question of whether p21 (WAF1/CIP1) is a marker of cell senescence, DNA damage, or both. No studies have been conducted on its value in CAN.

We found no expression of the marker in normal kidney tissue samples independent of age, while the characteristic pathological features of CAN were associated with a significantly higher marker expression by all tissue structures. These data are in contrast to the results of studies favouring p21 as a marker of kidney senescence [3] and support the opinion that p21 is upregulated as a result of tissue damage caused by various stress influences [8, 11]. Intensity of the marker expression in CAN was not related to donor or recipient age, supporting the above statement. A positive trend in the relationship between gene levels with the grade of CAN ($P = 0.059$ for glomerular score) indicates a possible correlation of p21 expression with the degree of kidney tissue damage. More cases must be evaluated if this is to be proven.

An important finding, from our point of view, is the relationship between the gene expression and the number of previous acute rejections. The significance of acute rejection in the development of CAN is still widely debated. Numerous results in favour as well as against this view have been reported [12, 13]. Our results support the opinion that acute rejection is, indeed, an important factor in subsequent CAN development. Parenchymal infiltration caused by previous episodes of acute rejection and/or inflammation was found in almost all kidney grafts. The vast majority of positive cells were the infiltrating cells. We assume that tissue damage due to acute rejection/inflammation episodes could cause the gene over-expression. A correlation between tubular expression of the marker and the number of acute rejection episodes supports this interpretation. In patients without any history of rejection, inflammation caused by various Tx-associated stresses (explantation, preservation, ischaemia/reperfusion) and post-Tx complications (subclinical or unrecognised rejections, immunosuppressant toxicity, infectious diseases, etc.) could cause over-expression of the gene.

The primary results of this study indicate the direction that future research on this marker should take. Its value in the prediction and early detection of CAN should be evaluated next. The impact of injuries associated with organ procurement (explantation and preservation) on the gene expression profile has not been defined. The influence of various post-Tx complications on marker expression is also the subject of further research.

In conclusion, the current study shows that renal expression of p21 (WAF1/CIP1) CDK inhibitor is not age-dependent in normal or chronically rejected kidneys and cannot be considered as a marker of human kidney senescence. Chronic allograft nephropathy is associated with increased expression of p21 in all kidney tissue structures due to previous acute rejection and inflammation episodes.

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References

1. Paul LC (1999) Chronic allograft nephropathy: an update. Kidney Int 56:783
2. Halloran PF, Melk A, Barth C (1999) Rethinking chronic allograft nephropathy: the concept of accelerated senescence. J Am Soc Nephrol 10:167
3. Ding G, Franki N, Kapasi A, Reddy K, Gibbons N, Singhal P (2001) Tubular cell senescence and expression of TGF-β1 and p21 WAF1/CIP1 in tubulointerstitial fibrosis of aging rats. Exp Mol Pathol 70:43
4. Munro J, Steeghs K, Morrison V, Ireland H, Parkinson EK (2001) Human fibroblast replicative senescence can occur in the absence of extensive cell division and short telomeres. Oncogene 20:3541

5. Mateo MS, Saez AI, Sanchez-Beato M, et al. (1997) Expression of p21 WAF1/CIP1 in fetal and adult tissues: simultaneous analysis with Ki67 and p53. J Clin Pathol 50:645
6. Shakland SJ, Eitner F, Hudkins K, Goodpaster T, D'Agati V, Alpers CE (2000) Differential expression of cyclin-dependent kinase inhibitors in human glomerular disease: role in podocyte proliferation and maturation. Kidney Int 58:674
7. Racusen LC, Solez K, Colvin RB, et al. (1999) The Banff 97 classification of renal allograft pathology. Kidney Int 55:713
8. Miyaji T, Kato A, Yasuda H, Fujigaki Y, Hshida A (2001) Role of the increase in p21 in cisplatin-induced acute renal failure in rats. J Am Soc Nephrol 12:900
9. Khanna AK, Hosenpud JD (1999) Cyclosporine induces the expression of the cyclin inhibitor p21. Transplantation 67:1262
10. Kuan CJ, al-Douahji M, Shakland SJ (1998) The cyclin kinase inhibitor p21WAF1, CIP1 is increased in experimental diabetic nephropathy: potential role in glomerular hypertrophy. J Am Soc Nephrol 9:986
11. Vogt BL, Rossman RG (2001) Effects of arsenite on p53, p21 and cyclin D expression in normal human fibroblasts—a possible mechanism for arsenite's comutagenicity. Mutat Res 478:159
12. Cecka JM (1997) The UNOS Scientific Renal Transplant Registry. In: Cecka JM, Terasaki PI (eds) Clinical transplants 1996. UCLA Tissue Typing Laboratory, Los Angeles, USA, p 1
13. Isoniemi HM, Krogerus L, Von Willebrand E, Taskinen E, Ahonen J, Hayry P (1992) Histopathological findings in well functioning, long-term renal allografts. Kidney Int 41:155