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Differences in binding of glucocorticoid receptor to DNA in chronic renal graft rejection

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Abstract Although chronic rejection is the most common reason for late allograft loss, its pathophysiology and etiology are unclear. Attempts to prevent chronic rejection are now focused on the modulation of transcriptional regulation. We evaluated the ability of glucocorticoid receptors (GR) to bind to the DNA binding site in peripheral blood mononuclear cells (PBMC) of five patients with chronic rejection and seven without it. Using an electrophoretic mobility shift assay, we measured the amount of nuclear glucocorticoid receptor capable of binding to its specific DNA recognition sequences, termed glucocorticoid response elements (GRE). GR binding was significantly greater in control patients than in those with chronic rejection ($P < 0.01$). The retarded band was almost undetectable in two patients with chronic rejection even though they were taking more prednisolone than the seven control patients, all of whom had clearly identifiable retarded bands. These results suggest a decreased ability of GR to bind to GRE in chronic rejection, resulting in a reduced ability to block key proinflammatory promoter sites. This reduced binding may be one molecular basis of chronic rejection.

Key words Activator protein-1 · Chronic rejection · Electrophoretic mobility shift assay · Glucocorticoid receptor · Kidney transplantation · Nuclear factor κB

Abbreviations *AP-1* Activator protein-1 · *CyA* Cyclosporine · *GR* Glucocorticoid receptors · *GRE* Glucocorticoid response elements · *NFκB* Nuclear factor κB · *PBMC* Peripheral blood mononuclear cells · *PCR* Polymerase chain reaction · *RT* Reverse transcription

Introduction

Despite increased knowledge of immunobiology and improved perioperative care, only 50% of cadaver kidney grafts continue to function at 10 years, the others failing as a result of chronic rejection. Neither the etiology nor the pathophysiology of this chronic rejection is yet understood [14]. Long-term maintenance immunosuppressive regimens including cyclosporine (CyA) and steroids are now commonly used world-wide and are used routinely in our hospital. These two drugs are believed to complement each other in preventing graft rejection, and their combined use thus permits maintenance therapy at low doses with the associated reduction of side effects [9]. While the occurrence of early graft rejection has been markedly reduced in recent years, the long-term attrition rate has not exhibited similar improvement. Some patients appear to have a higher risk of chronic rejection despite similarities to other patients in terms of age, gender, HLA match, time since transplantation, dose of prednisolone, and trough concentration of CyA. Such patients with chronic rejection require higher doses of steroids to maintain graft function than other recipients. To explore the re-

Table 1 Baseline characteristics of patients. Values are expressed as the mean ± SD (*NS* not significant, *CyA* cyclosporine)

	Chronic rejection group $n = 5$	Stable function group $n = 7$	Difference
Gender (female/male)	2/3	3/4	NS
Age (years)	47 ± 7	43 ± 9	NS
Donor age (years)	49 ± 18	53 ± 11	NS
HLA-A mismatch	1.2 ± 0.3	1.4 ± 0.4	NS
HLA-B mismatch	1.8 ± 0.5	1.8 ± 0.4	NS
HLA-DR mismatch	1.4 ± 0.5	1.3 ± 0.5	NS
Time since transplantation (years)	8 ± 2	9 ± 3	NS
Prednisolone dose (mg/day)	7.5 ± 2.5	4.6 ± 1.7	NS
CyA trough concentration (ng/ml)	49 ± 30	60 ± 33	NS
Serum creatinine (mg/dl)	4.0 ± 2.2	1.2 ± 0.3	$p = 0.0068$
Urinary protein (mg/dl)	146 ± 105	0	$p = 0.0038$

lationships between steroid therapy and chronic allograft rejection, we obtained mononuclear blood cells from patients with and without this form of rejection to compare the capacity of glucocorticoid receptors (GR) to bind to glucocorticoid response elements (GRE). We also studied the effect of steroids on the DNA binding of transcription factors likely to be activated in chronic inflammation, such as nuclear factor κB (NFκB) and activator protein-1 (AP-1), in cells from patients with or without chronic rejection. Our ultimate goal is to develop criteria for adjusting certain maintenance immunosuppressive protocols based on the results of these assays.

Patients and methods

Patient selection

The subjects were 12 living renal transplant patients including 5 with immunologically mediated chronic rejection and 7 control patients with stable renal function, no histologic evidence of chronic rejection, and no rejection episodes. Patients whose grafts were HLA-identical or ABO-incompatible were excluded from the study. Chronic rejection, defined as ongoing or recurring immune reaction to the graft, was carefully distinguished from nonimmune chronic changes in this study. Chronic rejection was identified from clinical data and from histopathologic findings for a biopsy specimen from the graft. The main clinical criterion for chronic rejection was a gradual rise in serum creatinine concentration. Histopathological criteria were fibrointimal cellular proliferation in arteries larger than interlobular arteries, diffuse interstitial fibrosis with inflammatory cell infiltration, and significant tubular atrophy [5]. All cases of chronic rejection were categorized according to the grading system for chronic allograft nephropathy in the Banff 97 working classification [10]. All patients gave their informed consent to the study, which followed the ethical standards of the 1964 Declaration of Helsinki.

No intergroup differences were noted in baseline demographic characteristics with regard to age, gender, HLA-mismatch, time since transplantation, prednisolone dose, or CyA trough concentration. Urinary protein and serum creatinine differed between the two groups (Table 1).

Isolation of mononuclear cells and nuclear protein preparation

Cell isolation and nuclear protein extraction followed procedures previously described [1]. Twenty ml heparinized peripheral blood was obtained from each patient. Peripheral blood mononuclear cells (PBMC) were purified by separation on Ficoll gradients. Cells were gently lysed in 400 µl of buffer including 10 mM HEPES-NaOH at pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM PMSF. After centrifugation, the nuclear pellet was lysed with 50 µl of chilled buffer including 20 mM HEPES-NaOH at pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM PMSF. The lysate was kept on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation at 10,000 × *g* for 2 min, and the supernatant fraction was stored at –80 °C.

RNA extraction and amplification

Total RNA was extracted from PBMC using an Isogen kit (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. The extract was dissolved in diethylpyrocarbonate-treated water and quantitated spectrometrically by measurement of absorbance at 260 nm. Reverse transcription (RT) of RNA followed by polymerase chain reaction (PCR) was used to detect GR gene expression. A cDNA was synthesized with 500 ng of total RNA in 10 µl of RT reaction mixture using a GeneAmp RNA PCR kit (Perkin-Elmer-Cetus, Norwalk, Conn.). The reaction mixture included 50 mM KCl, 10 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM dNTP, 1 U/µl RNase inhibitor, 2.5 U/l reverse transcriptase, and 2.5 µM oligo-d(T)16 as a primer. The mixture was incubated at 42 °C for 15 min, 95 °C for 5 min, and 5 °C for 5 min. The PCR reaction mixture contained 10 µl of RT product as a template, 1 × PCR buffer, 2 mM $MgCl_2$, 1.25 U/50 µl AmpliTaq DNA polymerase (Perkin-Elmer-Cetus), and primers at a concentration of 0.15 µM each. Sequences of the GR primers were designed according to the DNA binding site of the GR sequence as follows: sense, 5'TGCCTGGTGTGCTCTGATGAA-3'; and antisense, 5'TCCAGGTTCATTCCAGCCTGA-3' [8]. PCR conditions for GR were one cycle of 94 °C for 3 min and 30 cycles including 94 °C denaturation for 30 s, 50 °C for 1 min, and 72 °C extension for 1 min. PCR products were separated by electrophoresis on 2% agarose gels in the presence of ethidium bromide. We amplified the glyceraldehyde-3-phosphate dehydrogenase gene as an internal control as previously described [2].

Electrophoretic mobility shift assays

Probes were end-labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase. Binding reaction was performed in a total volume of 25 µl including 12 mM HEPES-NaOH at pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 12% glycerol, 1 µg poly (dI-dC), 50,000 cpm of probe, and 10 µg of nuclear extract. Samples were loaded on a 4% polyacrylamide gel and run at 10 V/cm in a running buffer after incubation at room temperature. The gels were dried and exposed to radiographic films. Activity in autoradiographs representing the protein-DNA complexes was measured by a Fuji bioimaging analyzer (BAS 2000; Fuji Film, Tokyo, Japan).

To confirm that the binding observed was specific, target DNAs containing point mutations were used as probes. Target and mutant oligonucleotide probes were GR (5'GAC CCT AGA GGA TC**T GTA CA**G GA**T GTT CT** A GAT-3') and GR mutant (5'GAC CCT AGA GGA TC**T** *CA* A CAG GA**T** *CA* T CT A GAT-3'); NF$\varkappa$B (5'AGT TGA **GGG GAC TTT CCC** AGG C-3') and NF$\varkappa$B mutant (5'AGT TGA **GG***C* GAC TTT CCC AGG C-3'); and AP-1 (5'CGC TTG A**TG ACT CA**G CCG GAA-3'), and AP-1 mutant (5'CGC TTG A**TG ACT** *TG*G CCG GAA-3'). Target and mutant oligonucleotides were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.).

Statistical analysis

Values are means ± SD. Differences among groups were examined by the Mann-Whitney U test. *P* values of less than 0.05 were considered significant.

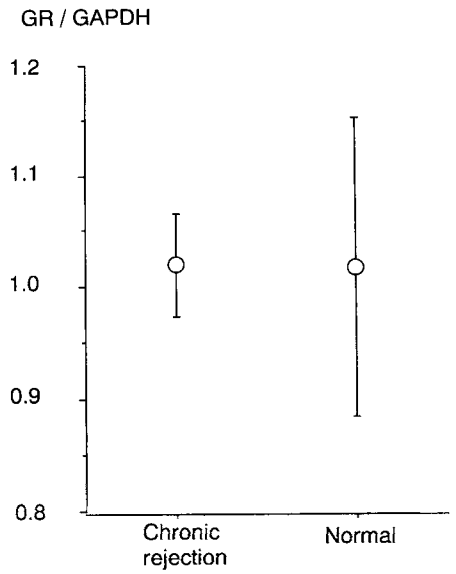

Fig. 1 Comparison of baseline expression of glucocorticoid receptor (*GR*) mRNA in transplant patients with chronic rejection with that in transplant patients without rejection. After reverse transcription-polymerase chain reaction, GR expression in the patient groups was compared as the ratio of GR to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). No significant difference was observed in the amount of GR mRNA between the two groups

Results

No difference found in the baseline level of GR between groups

To determine why some patients appear to be at a higher risk of developing chronic rejection despite background similarity in age, gender, HLA match, time since transplantation, prednisolone dose, and trough concentration of CyA (Table 1) to others without rejection, we first examined whether the level of GR expression was the same in both groups, since low expression of GR might influence the effect of glucocorticoid on immunosuppression. RT-PCR was used to measure basal levels of GR mRNA in PBMC from the patients in these groups. No significant difference in the amount of GR mRNA was found between the two groups (Fig. 1).

Differences in DNA binding level between patients with and without chronic rejection

The finding of no difference in baseline level of GR between the two groups led us to investigate whether the level of GR binding in these groups was the same. We used electrophoretic mobility shift assays to measure the amount of GR capable of binding to the specific DNA recognition sequence, GRE, in nuclear extracts from five patients with chronic rejection and seven patients with stable renal function. Interestingly, levels of GR binding were significantly higher in the patients with stable renal function than in the patients with chronic rejection (Fig. 2A). Since other transcription factors might influence the binding of GR, we further examined the ability of AP-1 and NF$\varkappa$B to bind to their DNA-binding sites in the same patients. Higher basal levels of AP-1 and NF$\varkappa$B DNA binding were observed in the nuclei from patients with chronic rejection than in those from patients without rejection (Figs. 2B and C).

DNA binding was specific

Although electrophoretic mobility shift assays with GR-, AP-1, and NF$\varkappa$B-specific sequence were used to search nuclear extracts prepared from PBMC for binding activity, binding specificity required confirmation. As shown in Fig. 2D, with control incubation with the radiolabeled oligonucleotide loaded in the absence of protein in lane "cold", no band was found, while nuclear proteins incubated with radiolabeled oligonucleotides containing specific sequences for GR, AP-1, or NF$\varkappa$B exhibited clear bands. Binding of protein to oligonucleotides containing point mutations was inhibited. We thus confirmed that target oligonucleotides for GR,

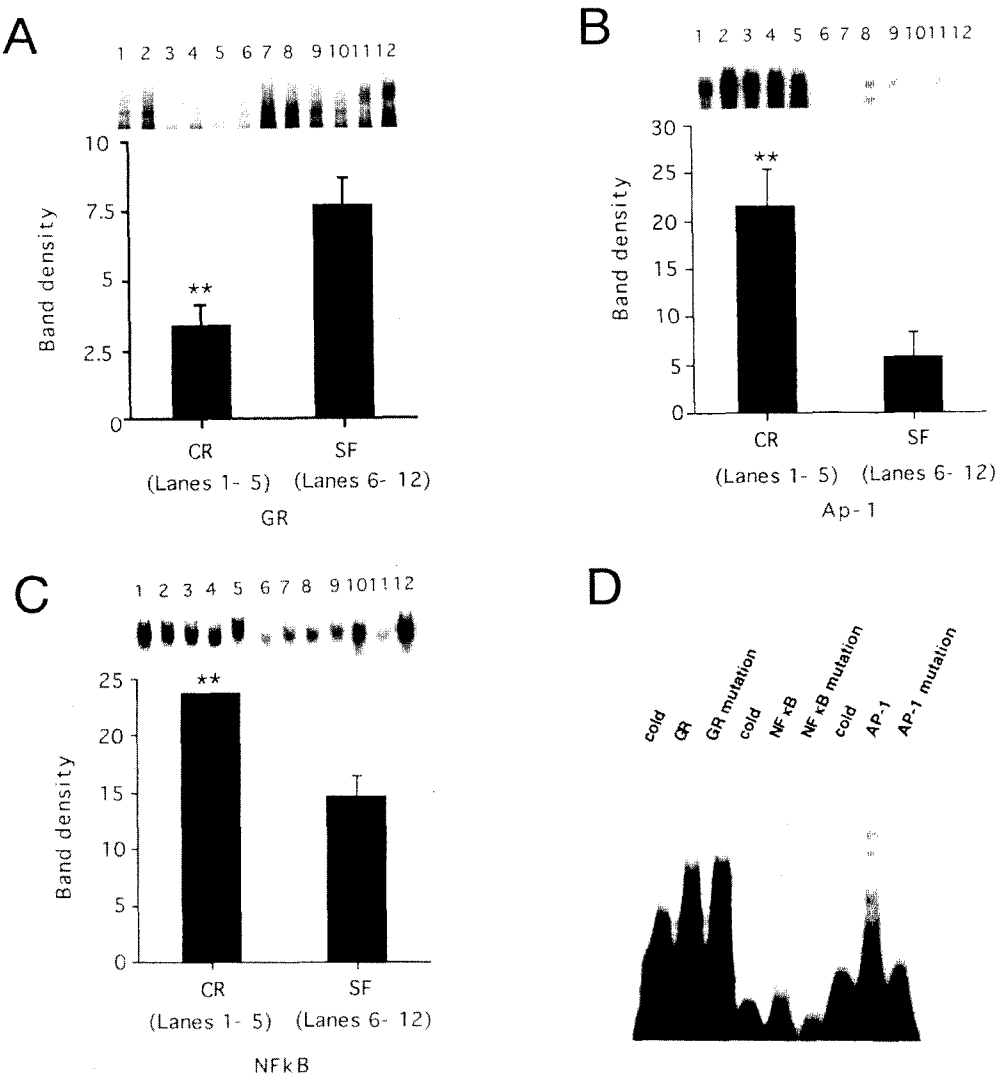

Fig. 2 Comparison of glucocorticoid receptor (*GR*) (**A**), activator protein-1 (*AP-1*) (**B**), and nuclear factor $\varkappa$B (*NF$\varkappa$B*) (**C**) DNA binding in nuclei from peripheral blood mononuclear cells of patients 1–5 with chronic rejection (*CR*) and patients 6–12 with stable renal function (*SF*). Band density is measured in arbitrary units. The mean values of band density are shown (** $P < 0.01$). Specificity of binding in mobility shift assays (**D**): target DNA was labeled with phosphorus-32. ^{32}P-labeled target DNA alone (*cold*) exhibits a position for "free" DNA. Specificity of binding was demonstrated using target DNA containing point mutations

AP-1, and NF$\varkappa$B all produced a specific band which was not found when the corresponding mutant oligonucleotides were used (Fig. 2D).

Discussion

In 1960, Goodwin and coworkers found that cortisone could reverse acute rejection of renal allografts, although the mechanism by which it did so was unclear [13]. The molecular basis of this effect is now being uncovered. Glucocorticoids enter the cell and bind to an inactive cytosolic GR, which translocates the hormonal signal to the nucleus and participates directly in gene regulation. In the absence of hormone, the receptor is found predominantly in the cytoplasm in complexes with other proteins, most notably hsp90. Upon binding of steroids, these complexes dissociate, permitting receptors to enter the nucleus, dimerize, and bind to specific DNA sequences designated GRE. This binding results in increased or decreased transcription of a number of genes involved in the inflammatory process [4]. Of particular importance for chronic rejection, cytokine gene transcription is repressed by GR. Direct interaction occurs between GR and other transcription factors activated in chronic inflammation [6]. An interaction has been demonstrated between the proinflammatory transcription factor AP-1, which is a heterodimer of Fos and Jun oncoproteins, and GR. GR/AP-1 interaction can affect the integrity of the DNA binding domain of GR, which appears to be critical for the repression activity of transcription [7]. NF$\varkappa$B also exhibits protein-protein interactions with GR that inhibit DNA binding by NF$\varkappa$B and thereby downregulate transcription of genes dependent on NF$\varkappa$B [12]. Moreover, glucocorticoids induce synthesis of the inhibitor protein I$\varkappa$B. In

unstimulated cells, NFκB is retained in the cytoplasm because it is bound to IκB. Thus, an increase in the synthesis of IκB will decrease the amount of NFκB in the nucleus [3, 11]. In our study, levels of GR binding were significantly higher in patients with stable renal function than in patients with chronic rejection.

To study the effects of reduced DNA binding in patients with chronic rejection, we examined the ability of AP-1 and NFκB to bind to their respective DNA binding sites in the same patients. Increased basal levels of AP-1 and NFκB DNA binding were detected in cell nuclei from patients with chronic rejection, indicating increased activation of these transcription factors. Use of a higher dose of CyA in these patients might have avoided increased activation of the proinflammatory transcription factors. CyA decreases transcription of certain cytokine genes via inhibition of a transcription factor complex that includes AP-1 and NFκB [13]. The anti-inflammatory effects of glucocorticoids are thought to be mediated predominantly through suppression of the activation of these transcription factors via direct interaction between GR and these factors, and, conversely, these effects of steroids can be overcome by the activation of AP-1 and NFκB: excessive amounts of AP-1 and NFκB reduce the number of activated GR within the nucleus available for binding to GRE, which can lead to a further increase in cytokine levels and growth factor expression. This sequence may be part of the molecular basis of chronic rejection in renal transplant recipients and might be interrupted by increasing the dose of CyA.

Important remaining questions are whether patients such as ours with chronic rejection have a GR mutation and whether these individuals have alterations in GR affinity or number. However, had these factors been responsible for low GR binding activity in these patients, they would have exhibited steroid resistance during induction of immunosuppression. Moreover, we found no differences in amount of GR mRNA between patients with and without chronic rejection.

In conclusion, our findings suggest that doses of steroid and CyA administered to transplant recipients can be adjusted based on measured binding activity of GR, AP-1, and NFκB. Further investigation is needed to determine whether adjustment of doses of steroid and CyA can alter the binding activity of these transcription factors in transplant patients.

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