Concentrations of endogenous cortisol were examined in 34 kidney-transplant recipients by improved "high-performance" liquid chromatography. Ten recipients were treated with prednisolone and azathioprine, the others with prednisolone and cyclosporine. Peripheral serum samples were collected just before transplantation, daily for two weeks after the transplant, weekly until discharge for about two months, and then monthly or occasionally. Mean (±SD) cortisol concentrations, initially 145 ± 87 μg/L, decreased immediately to 5.93 ± 5.11 μg/L after transplant, remained at almost these same values for two months, and then swiftly increased to 51 ± 59 μg/L by 1000 days. Cortisol concentrations within the period characterized by a cumulative dose of prednisolone at 300–700 mg were correlated significantly with the presence or absence of acute allograft rejection; patients with cortisol >4 μg/L had a higher risk of rejection. The majority of stable patients showed cortisol concentrations between 1 and 4 μg/L throughout the cumulative prednisolone period characterized above. Concentrations <1 μg/L after high-dose administration of methylprednisolone were accompanied by severe lung infection. We conclude that suppressed concentrations of endogenous cortisol, as assessed by highly specific HPLC, might provide a basis for predicting the therapeutic efficacy and adverse effects of prednisolone.

Additional Keyphrases: hormones · variation, source of · immunosuppression · chromatography, liquid · rapid-flow fractionation

Renal transplantation has become the most effective therapeutic procedure for treating end-stage kidney disease (1). Improvements in surgical techniques have shifted attention to the immunosuppressive care of patients after transplantation. Prednisolone (Pred) was previously considered the most important drug for this until cyclosporine (Cya) was found to give better results for graft survival (2, 3); however, Pred continues to be used as a major immunosuppressant (4).

The greatest challenge still facing clinicians is how to identify the patients at highest risk for developing graft rejection. Another problem is tapering the Pred dosage after a patient's condition has become stable. The pharmacodynamics of Pred have been studied in regard to (a) Pred-induced lymphocytopenia (5–9), (b) the effects of Pred on mixed lymphocyte cultures (9–14), (c) the effects of Pred on mitogen-activated lymphocytes (15–17), and (d) the drug's effects on the hypothalamus–pituitary–adrenal axis, as assessed by the peripheral plasma concentration of cortisol (18–20). Pre-operative mitogen-activated lymphocyte testing has recently been shown to successfully predict the pharmacodynamic effects of Pred on graft outcome (15).

We have described in preliminary reports (21, 22) that concentrations of endogenous cortisol after transplantation are strongly correlated to rejection. This result is consistent with the results obtained from pre-operative mitogen-activated lymphocyte testing. Here we present technical details of those studies with cortisol, conducted with "high-performance" liquid chromatography (HPLC).

Materials and Methods

Materials

Before the HPLC determination of endogenous cortisol, we pretreated samples with rapid-flow fractionation (RFF), having purchased the components of the RFF system from Kusano Scientific (Tokyo, Japan). The standardized apparatus has been described elsewhere (23). In brief, the system consisted of two glass columns packed with diatomaceous earth granules (particle size 50–100 μm). The inner volume of the first column, 4 mL, retained 0.5 mL of sample; the second column, 0.8 mL inner volume, retained 50 μL of 50 g/L sodium hydroxide solution to eliminate acidic components in the extract. Extraction solvent, introduced in a reservoir glass tube, was delivered through the columns by nitrogen gas pressure at 1–2 kg/cm². Using this system, we obtained an extract containing only neutral components in the plasma or serum specimen. Our HPLC system, a U-880 series obtained from Jasco (Tokyo, Japan), consisted of a reciprocal pump, an ultraviolet absorbance detector, and a chromatogram recorder. We also used a Model 7125 syringe-loading sample injector (Rhodyne, Cotati, CA). A conventional Hiber column, 4 mm (i.d.) × 250 mm, purchased from Merck (Darmstadt, F.R.G.), was packed with 5-μm particles of LiChrosorb Si-60 prewashed with dilute (10 mL/L) sulfuric acid to maintain the surface pH below 7.0. The chemicals were obtained from Wako Chemical Co. (Osaka, Japan), except for steroid standards from Sigma Chemical Co., (St. Louis, MO).

Procedures

Extraction procedure. We added 5 μL of a standard ethanol solution containing 5 or 50 ng of dexamethasone to 0.5 mL of plasma or serum. The larger amount of internal standard was used for determining cortisol before transplant; the smaller, after transplant. To extract glucocorticoids by RFF, we used the procedure described previously (24), with modifications in the extraction solvent mixture. When we used diethyl ether for the extraction, the glucocorticoids decomposed during postextraction storage. Sometimes the very low concentrations of cortisol in the transplant patients were completely lost. These chemical reactions seemed to proceed under oxidative conditions caused by peroxide(s) in the diethyl ether, because when freshly distilled solvent or a solvent treated with reductive reagent such as sodium borohydride was used, no decomposition.
occurred and the glucocorticoids could be determined. For practical reasons, however, we used a mixture of ethanol/dichloromethane (1/99 by vol) in the present study, the solvent polarity of this mixture being essentially that of diethyl ether. No other alteration was required.

Chromatography. After evaporating the solvent, we redissolved the extract in 20 μL of a mixture of methanol/dichloromethane (2/98 by vol) for HPLC. The mobile-phase solvent was chosen on the following basis: solvent A consisted of distilled water/methanol/dichloromethane/n-hexane (0.1/6/30/63.9 by vol); solvent B had the same components, 0.1/4/30/65.9 (by vol). Differences in the volume fractions of these solvents depended on the clinical conditions of the patients, especially the doses of Pred given after transplantation. Solvent A was used for assays of samples from pre-operative patients, who were taking no Pred, and from postoperative patients with functioning grafts and receiving standard immunosuppressive medication. Solvent B was used in assays for patients with graft failure, who were taking high doses of methylprednisolone for acute allograft rejection.

When the separation of cortisol from methylprednisolone was not satisfactory, we rechromatographed the samples as follows. The eluent fraction containing dexamethasone and cortisol was collected in a 10-mL glass tube, evaporated, reconstituted in 20 μL of methanol/dichloromethane (2/98 by vol), and injected for rechromatography with solvent A.

In any case, the flow rate was 1.0 mL/min for solvent A and 2.0 mL/min for solvent B. The detector was set at 245 nm with an analytical sensitivity of maximum 0.001 A full-scale. The cortisol concentration was determined by comparison with a calibration curve made from the ratio of the peak-heights for cortisol standards (0.5–100 ng) to that for 5 ng or 50 ng of dexamethasone as the internal standard. Other chromatographic conditions were the same as described previously (23, 24).

Patients

Medication. The study included 34 kidney-transplant recipients, ages 19 to 53 years (mean ± SD: 33.3 ± 9.2), who underwent transplantation by conventional surgery. For immunosuppressive therapy, 10 patients were given prednisolone/azathioprine (Pred/Aza); the others, prednisolone/cyclosporine (Pred/Cya). The initial dose of Pred was 120 mg/day for Pred/Aza, 60 mg/day for Pred/Cya. The dose of Pred was gradually tapered over three months after transplant to 10–20 mg/day and then even more gradually thereafter. The Cya dose was varied according to its concentrations in plasma, the optimal considered to be between 50 and 150 μg/L at trough (25). The Aza dose was determined by the usual therapeutic drug monitoring.

Diagnosis of acute rejection or rejection episode was based on standard criteria. Serum creatinine increasing by 2.0 mg/L per day, oliguria, fever, graft swelling, and graft tenderness were considered indications of rejection. In some patients, graft rejection was verified by biopsy. In patients with oliguria or with serum creatinine >30 μg/L in the Pred/Cya group, the daily dose of Cya was decreased, to avoid Cya nephrotoxicity; patients in whom no improvement of graft function was noted were suspected of allograft rejection. Treatment in such cases was a two- to five-day course of intravenous methylprednisolone (250–1500 mg/day). All patients were followed up for four to 30 months post-transplant.

Sampling. Blood samples were collected early in the morning, between 0630 and 0800 h, before drug administration. Pre-operative samples were collected after hospitalization but before surgery. When possible, samples were obtained each day throughout the two-week period following transplant, weekly until discharge for about two months, and then monthly or only occasionally thereafter. Plasma or serum specimens were stored at −40 °C until assayed.

Statistical analysis. Student's t-test, paired t-test, and Fischer's exact probability test were carried out for the data analysis. A value of P < 0.05 was considered statistically significant.

Results

Chromatography

Cortisol was satisfactorily recovered from patients' sera, as has also been described in previous papers (23, 24). Typical chromatograms before and after transplant (Figure 1) show the immediate suppression of cortisol by 98.76% within four days after transplant. The capacity ratios (k' value) of dexamethasone and cortisol were 8.07 and 8.91, respectively, indicating that they were completely separated from each other. The base-line separation between cortisol and Pred was also satisfactory, with the k' value of the latter being 10.35. Calibration of cortisol concentration was carried out by using the regression equation, y = 6.86426x + 0.0087 (r = 0.9999, P < 0.0001), where y is cortisol concentration (μg/L) and x the peak-height ratio of cortisol to 50 ng of dexamethasone. Because complete linearity between peak height and injected amount of dexamethasone and cortisol was noted in each case, we used the coefficient of 6.8643 in the above equation, with the amount of internal standard for HPLC being 5 ng. The

![Fig. 1. Chromatograms of serum cortisol before (A) and four days after (B) transplantation](image-url)
quantification limit of cortisol was as low as 1 μg/L and the detection limit, at a signal-to-noise ratio of 3 ± 1, was 0.5 μg/L. The coefficient of variance (CV) for the quantification limit of cortisol at 1 μg/L was 10%. At higher concentrations, e.g., 50 μg/L, the CV was <3%.

The rechromatography of cortisol in the presence of a large amount of methylprednisolone is shown in Figure 2. The patient in this case had had 1.0 g of methylprednisolone administered intravenously for acute allograft rejection on the previous day. On the first chromatogram, obtained with solvent B, a cortisol peak overlapped that of methylprednisolone. The eluent corresponding to the peak area of cortisol was recovered along with that of dexamethasone as the internal standard. Rechromatography conducted with solvent A led to a satisfactory determination of cortisol. The patients who had been treated repeatedly with high doses of intravenous methylprednisolone within three months after transplant showed cortisol concentrations equal to or below the quantification limit. A total of 14 patients occasionally showed cortisol concentrations <1 μg/L after high-dose steroid therapy.

Differences in the cortisol concentrations in serum and plasma were not significant. In most cases, we used serum samples rather than plasma because RFF during repeated analytical runs took longer. Within-run and day-to-day CV in our method was within <2.5%.

We studied interference of cortisol determination by co-administered drugs. Pred and methylprednisolone did not interfere with the peak for cortisol, as mentioned above. Cya, though extracted quantitatively (25), showed no ultraviolet absorption at 245 nm and thus caused no interference. Aza could be only slightly extracted and showed a minor peak at 65 min when chromatographed with solvent A. Mizoribine was not extracted with our solvent system, owing to having greater polarity than the steroids. Acidic and basic antihypertensives such as furosemide, propranolol, and captopril were eliminated from the extract during the pH-dependent fractionation of RFF. Thus, the most troublesome interference was that of methylprednisolone, which was eliminated by the above rechromatography.

Endogenous Cortisol in Transplantation

The average serum concentration of cortisol in 34 recipients before transplantation was 145 (SD 87) μg/L, which is in the normal range for healthy subjects. After transplantation, the concentration decreased to 5.93 (SD 5.11) μg/L within four days. The concentration–time curve for cortisol (Figure 3) indicated that this low concentration of cortisol lasted for at least three months after transplantation. The concentration subsequently began to increase slowly to the original concentration during long-term administration of Pred for 1000 days. At this clinical stage, there were considerable individual differences in the rates at which cortisol concentrations returned to normal. However, the minimal concentration and recovery rate in patients medicated with Pred/Aza did not differ significantly from those in patients medicated with Pred/Cya.

Cortisol concentrations before and after transplant in patients with and without acute allograft rejection are shown in Table 1. Differences between rejection and no-rejection groups were statistically significant in the post-

![Fig. 2. Determination of cortisol after high-dose administration of methylprednisolone](image)

Chromatogram A was obtained with solvent B and 5 ng of dexamethasone, flow rate 2 mL/min. The eluent fraction identified by the shaded area was collected for subsequent injection to obtain chromatogram B (at flow rate 1 mL/min). Cortisol concentration (I) was defined to be 1.49 μg/L. A and B were recorded at 0.002 and 0.001 A full-scale, respectively. I and III as in Fig. 1; III, methylprednisolone; IV, prednisolone

![Fig. 3. Change in cortisol concentration after transplant, in response to prednisolone administration](image)

Trough value of around 1–10 μg/L lasted at least three months after transplant. The rate at which the cortisol concentration returned to pre-surgical values varied greatly according to the patients. Bars indicate 1 SD
Table 1. Data on Renal Transplant Patients with and without Rejection during the First Three Months after Transplantation

<table>
<thead>
<tr>
<th></th>
<th>Rejection</th>
<th>No rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>14</td>
<td>20</td>
</tr>
<tr>
<td>Age, years (mean ± SD)</td>
<td>32.1 ± 7.4</td>
<td>34.1 ± 9.9</td>
</tr>
<tr>
<td>Related/cadaveric grafts, no.</td>
<td>6/8</td>
<td>10/10</td>
</tr>
<tr>
<td>Pre-transplant blood transfusion, mean units received</td>
<td>10.8</td>
<td>7.8</td>
</tr>
<tr>
<td>HLA-AB mismatches, mean no.</td>
<td>0.89</td>
<td>0.95</td>
</tr>
<tr>
<td>HLA-DR mismatches, mean no.</td>
<td>0.93</td>
<td>0.90</td>
</tr>
<tr>
<td>Cortisol conc, µg/L (mean ± SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-transplant</td>
<td>130 ± 59</td>
<td>157 ± 99</td>
</tr>
<tr>
<td>Post-transplant</td>
<td>9.15 ± 6.08</td>
<td>3.68 ± 2.29*</td>
</tr>
<tr>
<td>Post/Pre conc ratio, %</td>
<td>7.39 ± 4.59</td>
<td>2.87 ± 2.13*</td>
</tr>
</tbody>
</table>

*Significantly different from rejection patients (P < 0.01).


transplant cortisol concentrations and in their post/pre-transplant concentration ratios. Other items such as type of graft, blood transfusion history, and HLA histocompatibility did not affect graft outcomes.

Critical cortisol concentrations, as correlated with early acute allograft rejection or rejection episode, were examined within three months after transplantation. Individual cortisol concentrations within the period beginning from the time of a cumulative Pred dose of 300 mg and ending at 700 mg proved useful for predicting rejection. Data between the third and 12th day after transplant were also useful (data not shown).

We used the cortisol concentration of 4 µg/L as our criterion for rejection. Of our 34 transplant recipients, 16 (47%) showed individual average cortisol concentrations >4 µg/L during the test period. Among these, 11 recipients (69%) had allograft rejection within three months after transplantation, whereas only 3 (17%) of the 18 recipients whose cortisol concentrations were <4 µg/L underwent allograft rejection. The data for all test samples in the period are given in Figure 4; in Figure 5 the average values for individual patients are compared with their graft outcomes. Cortisol concentrations exceeding the critical value were clearly correlated with the incidence of acute rejection and (or) rejection episode under both Pred/Aza (P < 0.01) and Pred/Cya (P < 0.01) treatment regimens.

Discussion

Because no significant relationship had been demonstrated between the plasma concentration of Pred and its therapeutic effects (26), Frey et al. (18, 19) studied Pred pharmacodynamics by inhibition of the hypothalamus–pituitary–adrenal axis. They compared suppressed endogenous cortisol concentrations in kidney-transplant recipients with their cushingoid habitus. The results, re-examined later by Öst et al. (20), were found to be at variance with those of previous reports. These respective studies, carried out independently, with use of their own HPLC methods (27, 28), showed patients with cushingoid habitus to apparently have lower cortisol concentrations than non-cushingoid recipients. Still, in most studies to date, no significant relationship has been shown between the plasma concentration of cortisol and adverse effects of Pred or graft outcome. This inconsistency may possibly arise from differences in either the particular patients examined, or the HPLC methodology, or both.

The detection limit of the previous HPLC (27, 28) was 5–10 µg/L, which is inadequate for determining accurately cortisol concentrations that have been greatly suppressed. The present HPLC, with a detection limit of 0.5 µg/L, appears to be adequate for the in vivo pharmacodynamic assessment of Pred in terms of its effect on the hypothalamus–pituitary–adrenal axis.

One possible mechanism for the suppressive effect of Pred on endogenous cortisol is that cortisol is replaced by Pred on glucocorticoid-binding proteins (20, 29, 30), thereby facilitating the elimination of cortisol (increased clearance) from plasma. The well-established negative feedback mechanism (31), with suppression of the hypothalamus–pituitary–adrenal axis during long-term or high-dose single administrations of Pred, may also be involved.

The most important finding of this study is that the suppression of the adrenal cortex (as determined from cortisol concentrations) is reversible for at least three weeks after transplant. Even though strongly suppressed, the adrenal cortex continues to function. Cortisol concentrations within this period were significantly correlated with later rejection episodes. Differences in cortisol concentrations between rejection and no-rejection recipients were statistically significant in each case (P < 0.01 for a week, P < 0.05 for two and three weeks), but none of any subsequently observed differences was significant. Thus, irreversible organ failure of the hypothalamus–pituitary–adrenal axis may occur as soon as a month after transplan-
tation. Great interindividual differences in the rate of recovery of cortisol concentration support the above possibility.

Because acute rejection occurs generally within three months, and especially within one month, after transplant, it is desirable to predict graft outcome at the earliest possible time. We found that there were statistically significant differences in cortisol concentrations between rejection and no-rejection recipients within a period in which the cumulative Pred dosage was 300–700 mg and daily dosage exceeded 30 mg/day. This period corresponds roughly to from three to 12 days after transplant in both immunosuppressive regimens. Using the data obtained within this period, we defined the critical concentration of cortisol as 4 µg/L. Patients with higher cortisol concentrations may have greater risk of rejection, so individualized optimization of steroid therapy is recommended.

Patients administered Pred/Cya had received a half-daily dose of Pred in contrast to patients given Pred/Aza. The average concentration of suppressed cortisol was a little higher in patients co-administered Cya instead of Aza, but not statistically significantly so. Nevertheless, the difference seemed correlated with changes in serum creatinine concentrations, possibly because of Cya nephrotoxicity. Failure of renal function in Cya-treated patients may lead to increased concentrations of cortisol in serum, owing to a pharmacokinetic decrease in the plasma clearance of cortisol. However, the adrenocortical toxicity of Cya (32) in humans also should be studied. Data on dose-dependent pharmacokinetics (29, 30) and on in vitro hyperbolic Pred effects in mixed lymphocyte cultures (10) indicate that the extent of increase in immunosuppression may decrease with an increase in the area under the concentration curve for Pred. This would also explain why the cortisol concentrations in both immunosuppressive regimens were essentially the same. According to Frey et al. (10), doses of Pred exceeding 50 mg/day may little enhance its therapeutic value.

Another significant finding of this study is that a cortisol concentration <1 µg/L was elsewhere correlated with severe lung infections (21). The pharmacokinetics of Pred have been studied in relation to its adverse effects including virus infection (19, 20, 26). However, Øst et al. (20) stated that the predictive value of Pred kinetics is probably small in routine clinical work because a significant relationship between plasma concentration and effect has yet to be demonstrated. In the present study, cortisol concentrations on some occasions were strongly suppressed to less than the critical value after repeated administrations of high doses of methylprednisolone (33). The therapeutic efficacy of Pred may thus be reflected in its alternative effect on endogenous cortisol concentrations through the hypothalamus–pituitary–adrenal axis.

As discussed above, we saw considerable interindividual differences in endogenous cortisol concentrations among patients whose clinical conditions differed. We could thus predict rejection and lung infection at the earliest possible time following transplant and high-dose steroid therapy for rejection episode, respectively. If the sensitivity of mitogen-activated lymphocyte to Pred (15, 16) is known pre-operatively, postoperative measurement of cortisol may serve to predict with great accuracy the transplant outcome for an individual patient.

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