Serum Neopterin Levels Among Hepatitis C-Positive Living-Donor Renal Transplant Recipients

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ABSTRACT

BACKGROUND: The role of neopterin as a marker of cell-mediated immunity for immunological monitoring after transplantation is of great potential interest. Neopterin levels among hepatitis C virus (HCV)-positive recipients of living-donor renal transplantation (LDRT) have not been previously described.

METHODS: Twenty-two HCV-positive (group I) and 10 HCV-negative (group II) recipients of LDRT were serially monitored for serum neopterin levels by enzyme-linked immunosorbent assay (ELISA). Group I patients were monitored thrice, ie, before transplantation, day 10, and 6 months post transplantation, while group II patients were monitored twice (day 10 and 6 months post transplantation). Peripheral blood T-lymphocyte subsets (CD3, CD4, CD8, CD4+CD25+, CD16+) and Th1/Th2 cytokines were monitored concomitantly by flow cytometry.

RESULTS: Ten days post transplantation, there was a significant increase in neopterin and neopterin/creatinine levels among group I patients. There was a positive correlation between activated T-lymphocyte (CD4+CD25+) and neopterin early post transplantation (day 10). Th2 cytokines IL-10 and IL-5 showed a positive correlation with neopterin levels on day 10 and 6 months post transplantation, respectively. Neopterin levels did not show association with either HCV viral load or allograft rejection among our study cohort.

CONCLUSION: Increased monocyte/macrophage activation with elevated serum neopterin was detected among group I patients on day 10 post transplantation, but it could not predict rejection. It appears that IL-10 either from a regulatory or nonregulatory source helps in the maintenance of stable graft early post transplantation. Further, it would be of interest to assess the role of neopterin in chronic allograft nephropathy and long-term graft outcome.

KEYWORDS: hepatitis C, living-donor renal transplantation, neopterin, rejection, viral load

Introduction

Cell-mediated immunity is an integral component seen in autoimmune diseases, malignant tumors, and allograft transplantation. Neopterin, a pyrazinopirimidine compound, is a marker of cellular immune response in various diseases. The compound was termed “neopterin” to denote that it might start a new (Greek, neo) epoch in pteridine research. Neopterin belongs to a group of compounds known as pteridines and is a pyrazinopirimidine derivative, namely, 2-amino-4-hydroxy-(1′,2′,3′-trihydroxypropyl)pteridine, and its biosynthesis begins with guanosine triphosphate. Increased amounts of neopterin are produced by human monocytes/macrophages upon stimulation with the cytokine interferon-γ (IFN-γ). Other interferons are less active, and stimulation with other cytokines does not induce neopterin in vitro.

Increased concentration of neopterin has been reported in conditions causing stimulation of cellular immunity, such as viral and other infectious diseases, autoimmune diseases, heart and kidney failure, coronary artery disease, and allograft rejection. Monitoring of solid allograft (kidney, heart, liver, and pancreas) recipients by neopterin measurements in morning urine or serum/plasma has become a useful tool in surveillance after transplantation. The bulk of evidence regarding the diagnostic value of neopterin in transplantation stems from the field of kidney transplantation. Increased neopterin has been shown to be significantly associated with impaired graft function and rejection in many studies.

Renal transplantation is currently the treatment of choice for patients with end-stage renal disease. However, despite continuous advances in immunosuppressive therapy and prophylaxis of infectious complications, acute rejection still remains a problem following kidney transplantation. As neopterin is formed during the course of cell-mediated immune response, its monitoring allows us to determine the effect of therapeutic interventions that are assigned to interfere with the degree of immune activation. Serum neopterin levels are a good marker...
of Th1 activation, and hence may provide a potential noninvasive adjunctive tool to monitor acute rejection.8

Renal transplant patients represent an important group with high prevalence of hepatitis C virus (HCV) infection acquired during hemodialysis. Elevated neopterin levels have been reported during viral infection including hepatitis, Epstein–Barr virus, and cytomegalovirus infection.9 Neopterin levels among HCV-positive recipients of renal transplantation have not been examined to date, and it would be of interest to assess this inflammatory marker among these patients.

The aim of this study was to assess the dynamics of the serum neopterin as well as neopterin/creatinine (Neo/CR) levels during the first 6 months post transplantation among HCV-positive (group I) and negative (group II) recipients of living-donor renal transplantation (LDRT). Furthermore, the possible relationship between the serum neopterin levels and viral load, peripheral blood T-lymphocyte subsets, Th1/Th2 cytokines levels, and allograft rejection were also investigated. To the best of our knowledge, this is the first study monitoring serum neopterin levels among HCV-positive recipients of LDRT at different time periods, ie, before transplantation, and day 10 and 6 months post transplantation.

Materials and Methods

Study subjects. 22 HCV-positive, HIV and HBV negative (group I) recipients of LDRT were recruited in this study. Control group included 10 age-matched HCV, HIV, and HBV negative recipients of LDRT (group II). All the patients in group I and II were antiviral treatment naïve and underwent transplantation in the Department of Renal Transplant Surgery, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, between September 2005 and October 2008. Serum samples were collected at three time points, ie, before transplantation, as well as day 10 and 6 months post transplantation, from group I patients, while group II were monitored post transplantation at two time points (day 10 and 6 months post transplantation). Serum creatinine levels of patients were measured at all time points. Patients with rejection received pulse methylprednisolone (500 mg intravenous daily for 3 days), and none of the patient received steroid taper therapy. The study protocol was approved by the ethics committee of the Institute, and informed consent was obtained from all the participants prior to inclusion in the study. The demographic and clinical features of the patients included in this study are detailed in Table 1. Our research complied with principles of the Declaration of Helsinki.

DNA extraction and HBV PCR. Blood samples of all the patients were tested for HBV DNA by polymerase chain reaction (PCR) to exclude this infection in our study groups. DNA was extracted using the commercially available AxyPrep viral DNA extraction kit (Axygen Biosciences), and PCR was carried out using primers specific for the surface HBV genome, sense primer 5′-YCCTGCTGGTGGCAGTTC and antisense primer 5′-AAGCCANACARTGGGGAAAGC.10 PCR products were then analyzed by electrophoresis in 2% agarose gel stained with ethidium bromide, and visualized under ultraviolet light. All the samples were negative for HBV.

Quantitative detection of HCV RNA by real-time PCR. RNA was extracted from serum using the QIAamp Mini Virus spin kit (Qiagen) and was reverse-transcribed to cDNA using the first-strand cDNA synthesis kit (MBI Fermentas). Quantitative real-time reverse-transcriptase PCR was performed (Roche LightCycler® 480) using HCV standard, which was commercially synthesized (Tib Mol). The primer specific for the 5′UTR region of HCV

| VARIABLE | GROUP I (N = 22) | GROUP II (N = 10) | P-VALUE |
|----------|-----------------|------------------|---------|
| Recipient age | 32.27 ± 10.01 | 34.60 ± 9.90 | NS |
| Sex (M:F) | 20:2 | 10:0 | NS |
| No. of previous Tx | 0(21), 1(1) | 0(10) | NS |
| Pre-Tx cross match | All negative | All negative | |
| HLA matching* | Haplo-match-12 | Haplo-match-10 | |
| Minimum follow-up | 6 months | 6 months | |
| Potential risk factors | | | |
| No. of dialysis | 74.54 ± 58 | 48.40 ± 12 | 0.005 |
| No. of blood transfusion | 2.59 ± 4.45 | 2.1 ± 2.13 | NS |
| No. of rejection episodes | 5 | 4 | NS |
| Immunosuppression | | | |
| CycI/MMF/Wys | 6 | 2 | |
| Tacro/MMF/Wys | 14 | 8 | |

Notes: Values represented as mean ± SD. *Spouses were not tested.
Abbreviations: Tx, transplant; NS, not significant; CycI, Cyclosporine; MMF, Mycophenolate mofetil; Tacro, Tacrolimus; Wys, Wysolone.
genome was used to carry out single-round real-time PCR assay using the SYBR green dye format, forward primer 5'-AGCGTCTAGGCTGCGT-3, and reverse primer 5'-GGTGTAATCTACCGGTTCCG-3.11,12

**Analysis of phenotypic markers and TH1/Th2 cytokine estimation.** T-cell subsets (CD3, CD4, CD8, and C4+CD25+) and natural killer (NK) (CD56+CD16+) cells were enumerated by surface staining with fluorochrome-conjugated antibodies: simultest CD4- FITC/CD8-PE, simultest CD3-FITC/CD16+CD5-PE, and CD25-PE from BD Biosciences. The cells were acquired using a FACSCAN flow cytometer, and CeliQuest software was used for analysis. Frozen supernatants from phytohemagglutinin (PHA)-stimulated cultures were thawed and tested for cytokines IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ using the Cytometric Bead Array Human Th1/Th2 cytokine kit (BD Biosciences). Serum TGF-β was quantified using a commercially available ELISA kit (Bender Med Systems).

**Measurement of serum neopterin.** Serum neopterin levels were measured using a commercially available ELISA kit (Immuno Biological Laboratories).

**Diagnosis of rejection.** The histological changes occurring within the first 6 months after renal transplantation were evaluated using the Banff classification system.13

**Statistical analysis.** All statistical analyses were carried out using SPSS software (version 13, SPSS, Inc.) and differences were considered significant if the P-value was less than 0.05 (P < 0.05). Statistical differences among different groups were evaluated using an independent t-test and within the groups using a paired t-test. Spearman's rho correlation was used to correlate different variables.

**Results**

**Serum neopterin.** The mean serum neopterin levels among group I patients at three time points, ie, before transplantation as well as day 10 and 6 months post transplantation, were 40.11 ± 32, 92.28 ± 46.5 and 83.94 ± 44.41 nmol/L, respectively. There was a significant increase in serum neopterin on day 10 as well as 6 months post transplantation in comparison to pre-transplantation levels (P = 0.001 and 0.02, respectively). Among group II patients, serum neopterin levels on day 10 and 6 months post transplantation were 94.11 ± 57.7 and 55.11 ± 55.09 nmol/L (Fig. 1A). There was no significant difference in serum neopterin levels between group I and II on day 10 as well as 6 months post transplantation (P > 0.05).

**Neopterin/creatinine (Neo/CR).** Neopterin is removed from the circulation by renal excretion, and therefore it is necessary to calculate the measured neopterin concentrations in relation to the renal function. To exclude the effect of renal function on neopterin levels, we calculated the Neo/CR levels by dividing the neopterin values by the serum creatinine (in nmol/mg). Neo/CR levels among group I patients at three time points, ie, before transplantation as well as day 10 and 6 months post transplantation, were 5.71 ± 7, 80.33 ± 48, and 72.09 ± 54 nmol/mg, respectively. There was a significant upregulation of Neo/CR levels on day 10 as well as 6 months post transplantation in comparison to pre-transplantation levels (P = 0.001 and 0.001) among group I patients (Fig. 1B). The Neo/CR levels of group II patients on day 10 and 6 months post transplantation were 48.68 ± 45 and 37.82 ± 41 nmol/mg. There was no significant difference in the mean Neo/CR levels between group I and II on day 10 as well as 6 months post transplantation (P > 0.05).

**Immunosuppressive regimens and serum neopterin levels.** Out of 22 group I patients, 14 were administered Tacro/MMF, 6 CsA/MMF, 1 CsA/Aza, and 1 CsA/Certican. In the Tacro/MMF group (N = 14), the mean neopterin levels were 36.44 ± 14.98, 92.08 ± 21.98, and 91.78 ± 23.98 nmol/L at the three time points, respectively. Six patients of group I were administered CsA/MMF, and the mean neopterin levels were 56.38 ± 14.98 nmol/L (before transplant), 88.48 ± 31.87 nmol/L (day 10 post transplant), and 62.15 ± 19.78 nmol/L (6 months post transplant). Within group I, day 10 neopterin levels were significantly increased as compared to pre-transplant levels in the Tacro/MMF group (P = 0.001). In group II, eight patients who were given Tacro/MMF showed a mean

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**Figure 1.** (A) Scatter diagram showing serum neopterin levels among group I patients at three different time points (ie, before transplantation and day 10 and 6 months post transplantation). Dotted line indicates the mean value. (B) Bar diagram showing sequential changes in neopterin/creatinine (Neo/CR) levels among group I patients at three different time points (ie, before transplantation and day 10 and 6 months post-transplantation).
neopterin level of 85.06 ± 19 nmol/L on day 10 post transplant and 61.36 ± 17 nmol/L at 6 months post transplant. Two patients in group II were administered CsA/MMF, and the mean neopterin levels were 130.28 ± 12 nmol/L on day 10 and 30.12 ± 17.54 nmol/L at 6 months post transplant. There was no significant difference in Tacro/MMF vs CsA/MMF neopterin levels for both group I and II.

Serum neopterin levels among rejectors and non-rejectors. No difference in the neopterin levels of group I patients was observed between patients with (N = 5) or without rejection episodes (N = 17) at all three time points (P > 0.05). Within group II, rejection episodes were observed in 4 out of 10 patients, and there was no difference in the neopterin levels between rejectors and non-rejectors at both day 10 and 6 months post transplantation (Table 2).

HCV viral load and neopterin levels among group I patients. There was no association between serum viral load with neopterin levels among group I patients at all three time points during the study (P > 0.05) (Table 3). Further, there was no significant difference in viral load between rejectors and non-rejectors among group I patients (P > 0.05).

Serum neopterin levels and T-lymphocyte subsets. There was a significant positive correlation between serum neopterin levels and activated T-lymphocyte (CD4+CD25+) subsets among group I patients on day 10 post transplantation (r = 0.410, P = 0.05) (Fig. 2). Group I patients, however, did not show any correlation between serum neopterin and CD4+CD25+ cells 6 months post transplantation. Interestingly, though not significant, there was a decrease in CD4+CD25+ subsets 6 months post transplantation as compared to day 10 post transplantation. No correlation was found between serum neopterin levels and CD4+CD25+ cells among group II patients (P > 0.05).

Serum neopterin levels and NK cells. There was no association between serum neopterin levels and NK (CD16+56) cells among group I and group II patients at different time points during the study (P > 0.05).

Serum neopterin and Th1/Th2 cytokines. There was a significant positive correlation between serum neopterin and IL-10 levels among group I patients on day 10 post transplantation (r = 0.422, P = 0.05) (Fig. 3A). A positive correlation was also observed between neopterin and IL-5 levels 6 months post transplantation (r = 0.456, P = 0.033) (Fig. 3B).

Group II patients did not show any correlation with Th1/Th2 cytokine levels either on day 10 or 6 months post transplantation.

Discussion
Immunological monitoring of renal transplant recipients is of great potential interest, as it may identify patients at risk of allograft rejection and thereby enable recipient-tailored immunosuppressive therapy, resulting in prolonged graft survival. Investigating the cytokine network of the immune system is complicated by the fact that most of these mediators are biologically labile and bind to target cells, and, moreover, interaction between cytokines can modify, potentiate, or even diminish a specific effect. In contrast to cytokine levels, the IFN-γ-inducible macrophage product neopterin is biochem-

| VARIABLE | GROUP I (N = 22) | GROUP II (N = 10) |
|----------|------------------|-------------------|
|          | PRE TX           | DAY 10 POST TX    | 6 MONTHS POST TX | 6 MONTHS POST TX | P-VALUE |
| Neopterin (nmol/L) | 40.11 ± 45.7 | 92.28 ± 46.5 | 83.9 ± 54.4 | 40.11 ± 45.7 | 92.28 ± 46.5 | 83.9 ± 54.4 | NS |
| Viral load (log_{10} copies/mL) | 7.03 ± 0.914 | 7.13 ± 1.19 | 5.8 ± 0.641 |

Note: NS, not significant.

Figure 2. Scatter diagram showing positive correlation between day 10 post-transplantation neopterin and activated T-lymphocyte subsets CD4+CD25+ among group I patients.

Table 3. Serum neopterin and viral load among group I patients.
 Serum neopterin level is a good marker of Th1 activation, and hence may provide a useful noninvasive adjunct tool to monitor acute rejection. In a retrospective analysis of 172 renal transplant recipients, plasma neopterin was found to be useful in the early detection of acute rejection, with an overall sensitivity of around 90%. Reibnegger et al evaluated 294 consecutive recipients of renal allografts and showed that measurement of urine neopterin can be of help as an additional marker in early diagnosis of renal allograft rejection and that high neopterin values during the initial posttransplant period are associated with poorer long-term graft survival. Recent studies have also demonstrated significant increases in post-transplant neopterin levels in those with allograft rejection. A positive correlation between serum neopterin levels and the severity of chronic kidney disease has also been reported recently.

In our study, serum neopterin levels did not differ between patients with or without rejection episodes at all three time points among group I patients. However, unlike other studies, we did not measure the neopterin levels at the time of rejection, which might explain this discrepancy. Put together, in the present study, because of the relatively small sample size, we found no association between serum neopterin at early time point and rejection episodes. Further studies with a larger number of patients and longer follow-up period are needed to confirm whether neopterin levels at early time point can be used as predictive biomarker for rejection episodes.

It is well accepted that there exists a cross-regulation between Th1- and Th2-type immune response, by which Th1 (IFN-γ and IL-2) and Th2 (IL-4, IL-5, IL-10) cytokines negatively regulate each other. IL-12 is the major regulatory stimulus for Th1-type immunity, triggering IFN-γ production by Th1 and NK cells. IL-10 is produced by both innate and adaptive immune cells, including, in addition to Th2 cells, monocytes, macrophages, dendritic cells B cells, regulatory T cells, Th1 cells, and, most recently, Th17 cells. Irrespective of the cellular source, the principal role of IL-10 appears to be containment and suppression of inflammatory responses. It has been shown that IL-10 downmodulates a variety of inflammatory conditions including autoimmune diseases and transplant.

**Figure 3.** (A) Scatter diagram showing positive correlation between day 10 post-transplantation neopterin and IL-10 among group I patients. (B) Scatter diagram showing positive correlation between 6 months post-transplantation neopterin and IL-5 among group I patients.
rejection. The neopterin levels of group I patients showed a significant correlation with cytokines IL-10 and IL-5 on day 10 and 6 months post transplantation, respectively. A significant positive correlation between plasma neopterin and IL-10 has been observed earlier in other clinical conditions. The anti-inflammatory role of cytokine IL-10, probably from a non-T-regulatory source, in maintaining stable graft function in early post transplant period among patients on calcineurin inhibitors (CNI) has been reported earlier. Although we have not investigated the cellular source of the IL-10, our data reinforce the role of IL-10 in the maintenance of graft stability in the early post-transplant period. For ethical reasons, patients in our study groups did not receive the same immunosuppressive regimen, which potentially could have confounded the results. It would be pertinent to design a case–control study in future with a similar immunosuppressive regimen.

Conclusion

In summary, our data demonstrate pronounced immune activation in HCV-positive recipients of LDRT with increased activated T-lymphocyte subset CD4^+CD25^+ and serum neopterin levels in the early period post transplantation (day 10). This opens up the possibility to further analyze the peripheral blood of a large number of patients to potentially identify acute rejection. Such a procedure would not necessitate invasive diagnostic procedures such as protocol biopsy. The obvious limitation of this study is the relatively small sample size and short follow-up period. Future studies are warranted to investigate neopterin levels in a larger number of patient populations, either in a single or multicentric study to confirm our conclusions.

Author Contributions

Designed the study, collected samples, performed experiments, analyzed the data and wrote the manuscript: SJ. Conceived and designed the study, helped with data analysis, and edited the manuscript: RWM. Helped in execution of work and performed some experiments: SA. Drafted and approved the final manuscript: MM. All authors reviewed and approved of the final manuscript.

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