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Short Communications

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TLR-4+ peripheral blood monocytes and cardiovascular events in patients with chronic kidney disease—a prospective follow-up study

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Abstract

Background. Atherosclerosis is an inflammatory process mediated by circulating immune cells, including monocytes. There is accumulating evidence for the involvement of Toll-like receptor 4 (TLR-4) as a mediator of atherogenesis.

Methods. We evaluated the association between CD14+/TLR-4+ monocytes in peripheral blood (flow cytometry) and future cardiovascular events (CVE), e.g. myocardial infarction, percutaneous transluminal coronary angioplasty (including stenting), aortocoronary bypass, stroke and angiographically verified stenosis of peripheral arteries and cardiovascular (CV) death, in 191 patients with chronic kidney disease Stage V receiving hemodialysis therapy.

Results. At baseline, CD14+/TLR-4+ monocytes correlated significantly with age ($r = 0.2$; $P = 0.007$), high-sensitivity C-reactive protein ($r = 0.2$; $P = 0.008$) and mean arterial pressure ($r = -0.2$; $P = 0.02$), but not with gender ($P = 0.5$), smoking ($P = 0.6$) and the presence of diabetes ($P = 0.5$). During a median follow-up period of 36 [1–54] months, 79 (41%) patients experienced a CVE. A total of 55 patients died during the follow-up period, 25 of those due to a confirmed CV cause. Log-rank test did not reveal statistical significance for TLR-4+ monocytes concerning incident CVE ($P = 0.3$), CV death ($P = 0.85$) and overall death ($P = 0.8$). In a multiple Cox-regression analysis, we identified age ($P = 0.003$) and smoking ($P = 0.001$) as the only independent variables associated with incident CVE.

Conclusions. Unexpectedly, we could not detect an association between CD14+/TLR-4+ monocytes and incident CVE as well as CV death in stable hemodialysis patients. Further studies have to clarify the potential role of this cell population for CV outcome in this population.

Introduction

Atherosclerosis is an inflammatory process that selectively affects arteries and that is present in a variety of clinical conditions such as hypertension and diabetes mellitus [1–5]. It has been shown that circulating immune cells, including monocytes, that attach to inflamed vessels by interacting with adhesion molecules and chemokines secreted by the activated endothelium play a pivotal role in atherogenesis [6]. Subsequently, secreted cytokines can activate residing macrophages, endothelial cells and vascular smooth muscle cells, which actively participate in cellular immunity and contribute to local inflammation [3,7].

A family of Toll-like receptors (TLRs) has recently been defined as key molecules of the ontogenetically highly preserved pathogen recognition receptors in mammals [8,9]. They are expressed by a variety of immune and nonimmune cells and recognize antigens of infectious organisms, so-called pathogen-associated molecular patterns. With these receptors, host cells can thus discriminate pathogens from self-tissues and, if necessary, activate their defense mechanisms. There is accumulating evidence for the involvement of TLR-4 as an important mediator of atherogenesis [10]. Its activation stimulates mononuclear phagocytosis to secrete chemokines that are involved in the recruitment of monocytes and T-lymphocytes to the arterial wall [11].

Accelerated atherosclerosis is a frequent complication of patients with end-stage renal disease. Since patients with chronic kidney disease (CKD) represent a high-risk population with regard to cardiovascular events (CVE), we set out to explore the correlation between circulating TLR-4+ monocytes and traditional CV risk factors in a cohort of 191 stable CKD Stage V patients on maintenance hemodialysis (HD). Moreover, we studied the association between TLR-4+ monocytes and incident CVE at initiation of the study, as well as survival during a prospective follow-up.

Materials and methods

Study design

The study protocol was approved by the Hannover Medical School Ethics Committee. Stable Caucasian patients with CKD Stage 5 from six HD centers were studied between October 2004 and January 2006. Ethylene-

Table 1. Clinical and laboratory data of HD patients with and without incident CVE during follow-up[a]

	All patients	Incident CVE	No CVE	P-value
n	191	79	112	
Age (years)	69 (61–76)	72 (64–77)	67.5 (58–76)	0.02*
Male/female	107/84	47/32	60/52	0.41
Body mass index (kg/m^2)	25.2 (23–28)	25.2 (23–28)	25.2 (23–28)	0.92
Dialysis vintage prior to inclusion (months)	40 (18–81)	34 (14–63)	43 (21–86)	0.72
Diabetes (*n*)	62	28	34	0.46
Patients on statins (*n*)	115	50	65	0.47
Patients on AT_1-antagonists (*n*)	41	17	24	0.49
Patients on erythropoietin (*n*)	164	68	96	0.94
Current smoker (*n*)	16	10	6	0.07
Mean arterial blood pressure (mmHg)	97 (89–103)	95 (87–103)	97 (90–103)	0.89
High-sensitivity C-reactive protein (mg/L)	3.6 (1.5–7.9)	3.6 (1.8–7.1)	3.8 (1.3–8.3)	0.67
Serum total cholesterol (mg/dL)	166 (145–194)	174 (151–209)	162 (143–188)	0.05*
Serum triglycerides (mg/dL)	164 (116–244)	180 (117–280)	154 (113–220)	0.24
Serum albumin (g/L)	39 (36–42)	40 (37–43)	39 (36–42)	0.56

[a]Data are displayed as median (interquartile range); *P < 0.05.

diaminetetraacetic acid (EDTA) blood samples for blood count, routine chemistry and fluorescence activated cell sorting (FACS analysis) were drawn from the dialysis cannulas immediately before the HD session was started. We carried out these investigations in all patients after a 2-day dialysis-free interval (long interval). All routine laboratory measurements including high-sensitivity C-reactive protein (hsCRP) were done using certified assay methods.

After the initial screening, all patients entered a prospective follow-up [median of 36 (1–54) months]. In this period, we assessed all CVE defined as myocardial infarction, percutaneous transluminal coronary angioplasty (including stenting), aortocoronary bypass, stroke and angiographically verified stenosis of peripheral arterial vessels (carotic, aortoiliac or femoral arteries). Furthermore, we also assessed overall mortality (including CV death).

Flow cytometry of circulating CD14+/TLR-4+ monocytic cells

We isolated peripheral blood mononuclear cells from 14 mL blood by Ficoll density gradient centrifugation. We analyzed the total number of circulating CD14+/TLR-4+ monocytes using a gating strategy flow cytometry (*Epics XL cytometer*; Coulter Beckman). In brief, we stained whole EDTA blood within 6 h after drawing the blood. Thereafter, we incubated a volume of 100 μL with an appropriate amount of fluorescein isothiocyanate-labeled monoclonal mouse anti-human-CD45 antibody (Coulter Beckman) for 20 min. For detection of CD14+/TLR-4+ monocytes, we added allophycocyanin-labeled monoclonal mouse anti-human-CD14 antibody and anti-phycoerythrin (PE)-labeled monoclonal mouse anti-human-TLR-4 antibody (both Coulter Beckman) for 30 min. As an isotype control, we used anti-phycoerythrin labeled mouse IgG1 antibody (Coulter Beckman) to a second anti-CD45-stained blood sample. Two blinded investigators independently assessed the number of CD14+/TLR-4+ cells.

Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) 15.0 for Windows and GraphPad Prism software (GraphPad Prism Software Inc., San Diego, CA). Two-sided P-values <0.05 were considered statistically significant for all statistical procedures. Follow-up time is displayed as median (minimum to maximum). Univariate comparisons of continuous variables between groups were performed using an unpaired *t*-test or the nonparametric Wilcoxon rank sum test in the case of nonnormally distributed variables. Dichotomized variables were compared using Pearson's χ^2 test. Univariate correlation analysis was performed by Spearman correlation analysis. In addition, parameters independently associated with incident CVE were identified by univariate and multivariate Cox proportional hazards models. Variables found to be statistically significant at a 10% level in the univariate analysis were included in the multivariate model using backward elimination. The distribution of the time-to-event variables was estimated using the Kaplan–Meier method with log-rank testing.

Results

We completed a total of 191 data sets from CKD patients on maintenance HD. Patient characteristics are shown in Table 1. In this baseline cohort, we found a significant correlation between CD14+/TLR-4+ monocytes and age ($r = 0.2$; P = 0.007), hsCRP ($r = 0.2$; P = 0.008) and mean arterial pressure ($r = -0.2$; P = 0.02), but not gender (P = 0.5), smoking status (P = 0.6) and the presence of diabetes (P = 0.5). A total of 164 patients received recombinant human erythropoietin (86%) and 115 patients were on statins (60%). We found no association between recombinant human erythropoietin (P = 0.2) and statin (P = 0.8) treatment and CD14+/TLR-4+ monocytes.

During a median follow-up period of 36 (1–54) months, 79 (41%) patients experienced a CVE. A total of 55 patients died during the follow-up period, 25 of those due to a confirmed CV cause. In a multiple Cox-regression analysis performed with CV risk factors listed in Table 2, we identified age (P = 0.003) and smoking status (P = 0.001) as the only independent variables associated with incident CVE. Figure 1 shows the Kaplan–Meier curve for incident CVE over a median follow-up period of 36 (1–54) months stratified to levels of CD14+/TLR-4+ monocytes above and below median. Log-rank test did not confirm statistical significance for CD14+/TLR-4+ monocytes concerning incident CVE (P = 0.3). Similarly, CD14+/TLR-4+ monocytes were not predictive for CV death (P = 0.85) and overall death (P = 0.8).

Discussion

Surprisingly, in the present study, we could not confirm a relationship between the number of CD14+/TLR-4+ monocytes and incident CVE as well as CV death in 191 patients at very high risk for CVE (i.e. Stage 5 CKD patients on maintenance HD). However, CD14+/TLR-4+ monocytes showed a positive correlation with age as well as hsCRP and a negative correlation with mean arterial pressure, while there was no correlation with other traditional CV risk factors (e.g. total serum cholesterol) and

Table 2. Simple Cox-regression analysis concerning incident CVE during follow-up[a]

	Univariate			Multivariate		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (years)	1.024	1.004–1.045	0.02*	1.032	1.011–1.054	0.003*
Serum cholesterol (mg/dL)	1.004	0.999–1.010	0.12			
Gender (male/female)	1.073	0.685–1.682	0.76			
Body mass index (kg/m^2)	0.994	0.952–1.037	0.77			
Dialysis vintage (months)	0.999	0.995–1.002	0.45			
Diabetes	1.280	0.807–2.031	0.29			
Current smoker	2.183	1.123–4.243	0.02*	3.210	1.587–6.491	0.001*
MAP (mmHg)	1.003	0.990–1.017	0.63			
hsCRP (mg/L)	1.005	0.993–1.017	0.39			
Triglycerides (mg/dL)	1.001	1.000–1.002	0.14			
Serum albumin (g/L)	1.006	0.960–1.005	0.79			
CD14+/TLR-4+ monocytes (per µl)	1.000	0.999–1.001	0.74			

[a]MAP, mean arterial blood pressure; HR, hazard ratio; CI, confidence interval. *P < 0.05.

with nontraditional CV risk factors such as nutritional status (body mass index and serum albumin level) detectable.

Our findings are in contrast to those reported from studies in patients with acute coronary syndrome [12] and animal models of advanced atherosclerosis, in which the expression level of TLR-4 in atherosclerotic plaques and on circulating monocytes correlates with severity of atherosclerotic disease [13]. We have to admit, however, that in patients with advanced CKD, numerous factors are thought to play a role in the pathophysiology of CV complications and that the extremely high CV mortality among these patients has been explained by more than the traditional CV risk factors [14–16]. This fact has been underscored by the results of the German Diabetes and Dialysis Study, in which treatment with atorvastatin had no significant effect on CV mortality in HD patients with type 2 diabetes [17]. Since this outcome is in sharp contrast to results of major statin intervention trials in nonrenal patients as well as subgroup analyses in patients with CKD [18,19], the authors concluded that the most plausible explanation for the absence of a significant effect on mortality in HD patients is the presence of additional pathogenetic pathways in CV disease.

The 'malnutrition, inflammation and atherosclerosis syndrome', which is associated with an exceptionally high mortality rate [20], describes metabolic alterations in CKD patients. The permanent (micro)inflammatory condition present in many patients on HD may influence the biology and possibly the number of this cell population, as we and others have shown for mature monocytes [21]. Moreover, the dialysis procedure itself—particularly the blood–membrane contact—might influence this cell population. We have studied CD14+/TLR-4+ monocytes in our patients after the long HD interval in order to minimize this potential confounding factor. The association with age in our cohort might be a nonspecific reflection of increasing atherosclerotic burden rather than a direct marker of specific CVE. Finally, in our follow-up period, we observed sufficient CVE in order to perform a proper multivariate analysis, but we cannot definitely exclude the possibility that the study was inadequately powered in order to detect very minor associations between CD14+/TLR-4+ monocytes and CVE.

In conclusion, we found no relationship between CD14+/TLR-4+ monocytes and incident CVE as well as CV death in stable CKD Stage V patients receiving HD therapy. Further experimental and clinical studies to clarify the role of TLR-4 for the progression of atherosclerotic disease in patients with CKD are highly desirable.

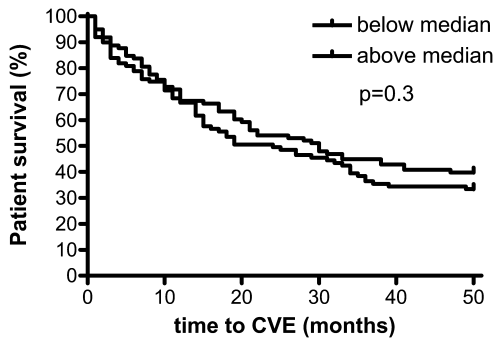

Fig. 1. Kaplan–Meier curve for incident CVE in 191 stable hemodialysis patients. Patients were grouped above and below median of the number of CD14+/TLR-4+ monocytes. In the follow-up period of 36 (1–54) months, 79 patients experienced a CVE. Log-rank testing did not confirm statistical significance for CD14+/TLR-4+ monocytes with respect to incident CVE (P = 0.3).

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Conflict of interest statement. None declared.

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Immunogenicity of an adjuvanted 2009 pandemic influenza A (H1N1) vaccine in haemodialysed patients

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Abstract

Background. The 2009 pandemic of influenza A (H1N1) prompted an urgent worldwide vaccination campaign, especially of high-risk subjects, such as maintenance haemodialysis (HD) patients. Still the immunogenicity of the pandemic A (H1N1) vaccine in HD patients is unknown.

Methods. We prospectively studied the immunogenicity of a monovalent adjuvanted influenza A/California/2009 (H1N1) vaccine (Pandemrix®, GSK Biologicals, Rixensart, Belgium) in HD patients and controls. Antibody level was measured using a seroneutralization assay before (D_0) and 30 days after (D_{30}) a single 3.75 μg vaccine dose. Specimens were tested in quadruplicates. Geometric mean (GM) antibody titers were determined in each subject at D_0 and D_{30}. Seroconversion was defined as an increase in GM titers by a factor 4 or more.

Results. Fifty-three adult HD patients [aged 71 ± 10, 58.5% males, on HD for a median of 38 (3 − 146) months] and 32 control subjects (aged 47.3 ± 14, 31.3% males) were analyzed. Baseline GM titers were similar in HD patients and controls [7.9 (6.6 − 9.6) vs 10 (6 − 17); p = 0.69]. Seroconversion was observed in 30 (93.8%) controls and 34 (64.2%) HD patients (p = 0.002). In addition, GM titers at D_{30} were significantly higher in controls than in HD patients [373 (217 − 640) vs 75.5 (42.5 − 134); p = 0.001]. HD patients were significantly older than controls