Clearance of increased serum advanced glycosylation end products in patients with end stage renal disease by hemodialysis¹

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KEY WORDS  advanced glycosylation end-products  kidney diseases  flow injection analysis  enzyme-linked immunosorbent assay  fluorescence spectrometry  hemodialysis

AIM To study the effect of removal of hemodialysis using acetate membrane on serum advanced glycosylation end products (AGEP) in 36 patients 59.1 ± 1.6 with end stage renal disease (ESRD). METHODS Serum AGEP levels were determined with quantitative fluorescence spectrometry flow injection analysis (FIA) and competitive enzyme-linked immunosorbent assay (ELISA) using a polyclonal antibody directed against AGEP. RESULTS The serum AGEP levels in patients with ESRD quantified by fluorescence spectrometry-FIA and ELISA were higher than those in controls 25 ± 5 vs 7.5 ± 1.5 6.1 ± 1.8 vs 1.4 ± 0.5 and 37 ± 20 vs 9 ± 10 kU/L respectively and markedly reduced t(22 ± 6) 4.2 ± 1.4 and t(19 ± 14) kU/L respectively after hemodialysis. CONCLUSION Increased serum AGEP levels in the circulation of patients with ESRD were reduced effectively by hemodialysis.

INTRODUCTION

Advanced glycosylation end products (AGEP) are generated by the non-enzymatic reaction of reducing sugars with the free amino group of proteins or amino acids. They exhibit characteristics of brownness, fluorescence, intramolecular and intermolecular covalent crosslinks. AGEP in the circulation may be a heterogeneous unrecognized class of potentially toxic substances. A large number of studies suggest that the various chronic complications of diabetes and end stage renal disease (ESRD) result from the accumulation of tissue macromolecules that have been progressively modified by AGEP. The elevation of AGEP level in sera of patients with diabetes and ESRD is well confirmed serum AGEP levels in patients with ESRD is found to be higher than in patients with diabetes. Removal of AGEP from serum delays the onset and slows the progression of complications in patients with diabetes and ESRD. There is a controversy whether hemodialysis can reduce the increased serum AGEP level. In the present study we detected the serum AGEP level in patients with ESRD before and after hemodialysis by quantitative fluorescence spectrometry flow injection analysis (FIA) and enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Reagents  Bovine serum albumin  BSA  human serum albumin  HSA  gelatin  O-phenylenediamine  OPD  and proteinase K were purchased from Sigma. Biotinylated anti-rabbit IgG antibody and horseradish peroxidase-HRP linked streptavidin were obtained from Vector. Other chemicals were of AR.

Serum samples  Serum samples were obtained from 54 control subjects age 59.1 ± 1.6 a and 36 patients with ESRD pre- and post-hemodialysis age 65 ± 4 a at 6.9 ± 2.0 a of dialysis periods. The intervals between dialysis sessions were 48 – 72 h. The serum samples of post-hemodialysis were collected within 30 min after hemodialysis. Serum samples were stored at –20 ℃ till analysed.

Clinical data  The primary causes of ESRD in this study group were chronic glomerulonephritis  hypertension  chronic interstitial nephritis  polycystic kidney disease and unknown causes. Patients with diabetic nephropathy were excluded. All the patients accepted the conventional bicarbonate dialysis with blood flow 250 – 300 mL/min dialysate flow 500 mL/min. Each dialysis session took 4.5 – 5 h using acetate membrane 1.3 m². The
average serum creatinine pre-HD was 459 ± 95 μmol/L.

Preparation of AGEP-BSA and AGE-P-HSA
BSA or HSA 50 g/L were incubated with D-glucose 0.5 mmol/L in the phosphate buffer saline PBS pH 7.4 0.2 mmol/L at 37 °C for 90 d. The control samples of BSA or HSA were also incubated under same conditions but without glucose. After incubation dialysis against PBS was carried out to remove unbound glucose. Fluorescence spectra were recorded using a 650-60 fluorospectrometer Hitachi Japan. AGE-P-BSA and AGE-P-HSA were purified by Sephadex G-200. The method of Bradford was used for quantification of proteins.

Quantitative fluorescence spectrometry
Serum sample 50 μL was diluted with PBS upto 5.0 mL. After the samples were filtrated through 0.22 μm filters the fluorescence intensity was measured with a 650 – 60 fluorospectrometer at an excitation wavelength of 370 nm and an emission wavelength of 440 nm. Various dilutions of purified AGE-P-BSA were used as calibrator and the sample AGE levels were calculated according to the standard curve. The AGE value was defined as U/mL 1.0 U/mL equals to AGE-P-BSA 1.0 mg/L.

Competitive ELISA
The polyclonal rabbit antibody was raised against purified AGE-P-BSA as previously reported. The competitive ELISA was performed in triplicate with some modification. Briefly 96-well microtitre plates Nunc Denmark were coated with AGE-P-HSA 100 μL/well 5 mg/L overnight and blocked with 200 μL/well of 0.5 % gelatin for 1 h. After washing six times with PBS containing 0.05 % Tween-20 each well was added 50 μL of serum sample diluted 1:4 to be tested or calibrator purified AGE-P-BSA diluted as 0.625 2.5 10 50 100 mg/L and 50 μL anti-AGEP antibody 1:2000. After incubation for 1 h and washing 100 μL of biotinylated anti-rabbit IgG antibody 1:4000 was added. Following incubation for 1 h and washing again the plates were reacted with horseradish peroxidase HRP linked streptavidin. Color was developed with OPD and determined at absorbance 490 nm. The AGE values of sample could be calculated automatically by microplate reader Bio-rad 550 USA according to its calibration curve. One AGE unit was defined as the amount of antibody reactive material that was equivalent to AGE-P-BSA standard 1.0 mg/L.

Flow injection analysis. The 20 μL of serum samples were mixed with trichloroacetic acid 0.15 mol/L 480 μL and chloroform 100 μL in microcentrifuge tubes. The tubes were centrifuged 10 min 13 000 × g to complete the precipitation of proteins and were shaken vigorously to extract lipids into the organic phase. The 20 μL of the aqueous layer was injected to sample injector loop 20 μL of high performance liquid chromatography. Water flow rate was at 0.5 mL/min and spectrofluorometric detector was set with emission wavelength at 440 nm and excitation wavelength at 370 nm for detection of AGE-peptide. The samples were analyzed in triplicate and peak height mode was used for signal measurement. Standard AGE-peptide obtained by hydrolysis of AGE-P-BSA with proteinase K were diluted as 0.1 0.5 1 5 10 50 100 mg/L and used for preparing calibration curve and calculating sample AGE-peptide as described above. The value was also defined as kU/L 1.0 kU/L equals to standard AGE-peptide obtained from hydrolysis of AGE-P-BSA 1.0 mg/L.

Statistical analysis
Data were expressed as x ± s and compared by paired or unpaired t-test and ANOVA.

RESULTS

AGEP determined by ELISA E-AGEP
The serum E-AGEP levels in patients with ESRD were markedly increased as compared with those in controls 37 ± 20 vs 9 ± 10 kU/L P < 0.01. The serum E-AGEP levels in patients with ESRD were markedly decreased by 48.5 % after hemodialysis 19 ± 14 kU/L Tab 1.

Tab 1. AGEP level in patients with ESRD pre- and post-hemodialysis determined by three independent assays.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>F-AGE</th>
<th>E-AGE</th>
<th>AGE-peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>54</td>
<td>7.5 ± 1.5</td>
<td>9 ± 10</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>ESRD</td>
<td>36</td>
<td>25 ± 5</td>
<td>37 ± 20</td>
<td>6.1 ± 1.8</td>
</tr>
<tr>
<td>ESRD + Hemodialysis</td>
<td>36</td>
<td>22 ± 6</td>
<td>19 ± 14</td>
<td>4.2 ± 1.4</td>
</tr>
</tbody>
</table>

AGEP determined by fluorescence spectrometry

F-AGEP The serum F-AGEP levels in patients with ESRD were markedly elevated compared with those in control 25 ± 5 vs 7.5 ± 1.5 kU/L P < 0.01. The serum F-AGEP levels in patients with ESRD significantly decreased by 12.1 % after hemodialysis 22 ± 6 kU/L Tab 1.
AGEP determined by FIA□ AGE-peptide□ The levels of serum AGE-peptide in patients with ESRD were markedly higher than those in control subjects□$6.1 \pm 1.8\%$ vs $1.4 \pm 0.5\%$ kU/L□ $P < 0.01$□. The levels of serum AGE-peptide in patients with ESRD were decreased by $30.9\%$ after hemodialysis□$4.2 \pm 1.4\%$ kU/L□ Tab 1□. Almost all patients showed a markedly decreased AGE-peptide after hemodialysis□Fig 1□.

Fig 1. Pre- and post-hemodialysis levels of AGE-peptide in individual patients with ESRD.

Comparison of three analytical approaches
The AGE-peptide determined by FIA showed the most excellent precision with lowest inter-assay coefficient of variation□ within-day $1.21\%$ □ between-day $6.35\%$ □ and best recovery rate for assay□ $94.88\% - 101.89\%$ □. Also□ it was less time and money consuming. The AGE level determined by ELISA□ E-AGE□ showed the highest inter-assay coefficient of variation□ within-day $9.51\%$ □ between-day $15.18\%$ □ worst recovery rate□ $90.92\% - 121.43\%$ □ for three analytical approaches. The precision and accuracy of F-AGE□ within-day $3.14\%$ □ between-day $7.73\%$ □ recovery rate $95.8\% - 115.13\%$ □ was superior to those of E-AGE□ Tab 2□.

DISCUSSION

Determination of circulating AGEP is of great value to assess impending cardiovascular risks□ to monitor the efficacy of AGEP removal methods□ and to test novel pharmacological approaches for inhibition of AGEP formation. However□ application of AGEP measurements in clinical practice is still limited by lack of specific□ simple□ and rapid analytical procedures. Because AGEP produced in vitro and prepared in vitro by incubation of diverse proteins with diverse reducing sugars contain a common immunological epitop□ it is reasonable and possible to measure the total AGEP in the circulation by this antibody. F-AGE determined by fluorescence spectrometry only represents the AGEP that can produce fluorescence□ not including imidazolone□ CML□ pyrroline□. The AGE-peptide just represented the low

Tab 2. Comparison of three analytical approaches for AGEP assay.

<table>
<thead>
<tr>
<th></th>
<th>F-AGE</th>
<th>E-AGE</th>
<th>AGE-peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coefficient of variance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within-day assay</td>
<td>$3.14%$</td>
<td>$9.51%$</td>
<td>$1.21%$</td>
</tr>
<tr>
<td>Between-day assay</td>
<td>$7.73%$</td>
<td>$15.18%$</td>
<td>$6.35%$</td>
</tr>
<tr>
<td>Recovery</td>
<td>$95.8% - 115.13%$</td>
<td>$90.92% - 121.43%$</td>
<td>$94.88% - 101.89%$</td>
</tr>
<tr>
<td>Sample size</td>
<td>$50 \mu$L</td>
<td>$50 \mu$L</td>
<td>$20 \mu$L</td>
</tr>
<tr>
<td>Analysis time</td>
<td>$&lt; 10$ min</td>
<td>$&gt; 6$ h</td>
<td>$&lt; 20$ min</td>
</tr>
<tr>
<td>Expenditure□ R. M. B□</td>
<td>$&lt; 1$ yuan</td>
<td>$&gt; 5$ yuan</td>
<td>$&lt; 1$ yuan</td>
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<td>ESRD/Controls</td>
<td>$3.28$</td>
<td>$4.28$</td>
<td>$4.4$</td>
</tr>
<tr>
<td>The number of overlap</td>
<td>0</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>between ESRD and Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>The number of decrease case in ESRD post-hemodialysis</td>
<td>24</td>
<td>32</td>
<td>35</td>
</tr>
</tbody>
</table>
molecular weight of AGEP. It is respected that AGEP
peptide is a novel and specific marker for monitoring the
removal efficacy of AGEP by some procedures.
Each assay for AGEP level showed significant
differences between the controls and patients with ESRD or
between the pre- and post-hemodialysis. Especially the
AGE-peptide level of all patients with ESRD was
significantly decreased after hemodialysis. These results
suggest that the clearance of AGEP was mainly mediated by
kidney and was markedly impaired in the patients with
ESRD which might play an important role in the
development of chronic complications of ESRD. The
serum AGEP level determined by three independent assays
in patients with ESRD were significantly decreased after
hemodialysis it was valuable to find out that the different
parameters of AGEP level for each patient showed different
removal effect and the decreased extent of each patient was
not similar. These results suggest that the difference for
reduced AGEP level could be explained by the fact that the
three AGEP parameters reflect heterogeneous classes of
AGEP. Although AGEP level could not return to the
normal level after hemodialysis it can be regarded that
hemodialysis is a useful and practical therapy for clearing
the serum AGEP in patients with ESRD. It is necessary to
further investigate the factors that influence the removal
efficacy of AGEP or specific portions of AGEP including
different modalities of dialysis the dialysis session the
primary causes of ESRD the remaining renal function the
age of patients and so on. It was reported that high flux
hemodialysis was more effective 35 % – 40 % AGEP
clearance however the clinical application of this
technology is still limited. Renal transplantation is the
best therapeutic modality that can normalize the serum and
tissue levels of AGEP. Using a lysozyme-linked
dialyzer as an adjunctive therapy might be an effective way
to eliminate toxic AGEP from the sera of patients with
ESRD.

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reactive advanced glycosylation end products from diabetic
血液透析清除终末期肾病患者增高的血清高级糖基化终产物

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关键词 高级糖基化终产物; 肾疾病; 流动注射分析; 酶联免疫吸附测定; 荧光光谱法; 血液透析

目的: 研究血液透析对例终末期肾病患者血中高级糖基化终产物的清除效果

方法: 分别用荧光分光光谱、流动注射分析法和酶联免疫吸附法测定终末期肾病患者血中高级糖基化终产物的水平及比较血液透析前后的变化

结果: 荧光分光光谱、流动注射法和酶联免疫吸附法测定的终末期肾病患者血中高级糖基化终产物的水平显著高于对照组，分别为(1) (2)和(3)。

透析后上述水平分别降到(4)、(5)和(6)。

结论: 血液透析能有效地清除终末期肾病患者血中增加的高级糖基化终产物