Heat shock protein (HSP) expression and proliferation of tubular cells in end stage renal disease with and without haemodialysis

Amit K. Dinda¹, Meer Mathur¹, Sandeep Guleria², Sanjeev Saxena³, Suresh C. Tiwari³ and Satish C. Dash³

¹Department of Pathology, ²Department of Surgery and ³Department of Nephrology, All India Institute of Medical Sciences, New Delhi 29, India

Abstract

Background. Prolonged dialysis is associated with acquired cystic kidney disease (ACKD) and also higher incidence of renal cell carcinoma. Relationship among dialysis, tubular cell proliferation, development of cystic change and neoplastic transformation is not clearly known. Whether dialysis causes additional stress on tubular cells is also conjectural. Study of heat shock protein (HSP) expression which are rapidly synthesized in cells in response to a variety of stresses may be helpful in this regard.

Methods. To evaluate dialysis induced early changes in end stage renal disease (ESRD), kidneys from eight adult autopsied patients were examined (group I) who were on weekly maintenance haemodialysis for 3–12 months. The heat shock protein (HSP 72/73) expression of tubular epithelial cells and their proliferating cell nuclear antigen (PCNA) labelling index (LI) were studied by immunohistochemistry using monoclonal antibodies. For comparison similar study was carried out in 10 cases of ESRD (Group II) of similar age and sex distribution who were not dialysed. The atrophic tubules were subtyped morphologically into (1) classic, (2) thyroid, (3) endocrine and (4) super tubules.

Results. In the dialysed group (I) the percentage of hyperplastic super tubules (10.6±4.1%) was significantly higher than in the non-dialysed group (II) (5.2±1.3%) with a higher PCNA LI (6.8±2.04%) (group II 4.9±1.9%) (P<0.01 to <0.001). Though grossly not detected, but microscopic cysts and microadenoma like areas were seen in all the cases in group I with a mean diameter of 522.66±315.25 μm and 494.85±262.46 μm respectively. They were seen in one case of group II. PCNA LI of the cells in microadenoma (7.2±3.1%) and microcysts (6.6±2.6%) were similar to that of super tubules in group I. Quantitation of HSP expression by image analysis (optical density 2.309±0.155) showed a positive correlation (r=0.7555) (P<0.001) with PCNA LI in super tubules indicating a higher induction in the dialysed group.

Conclusions. This study suggests that haemodialysis may cause injury to tubular cells and aggravate stress on an already compromised situation of ESRD leading to increased cell proliferation and more hyperplastic supertubule formation which may be the forerunner of cyst formation as well as neoplastic transformation.

Key words: Dialysis; ESRD; HSP expression; PCNA labelling

Introduction

The relation of acquired cystic kidney disease (ACKD) with haemodialysis and peritoneal dialysis is well known [1,2]. A recent comprehensive review has shown up to 50-fold increased risk of renal cell carcinoma (RCC) in ACKD compared with the general population [3]. It is generally believed that development of ACKD and renal cell neoplasm is a continuous process with evolving phenotypic expression [3]. The pathogenetic mechanism which initiates the process is not clearly known. A large number of pathogenetic pathways have been proposed. However, their relative importance and consequences need further evaluation [3].

Dialysis may result in introduction of various chemicals into the circulation, such as nitrite, nitrosamine, formaldehyde and plasticizer (from dialysis tubing) which may induce sublethal injury to the tubular cells and induce their proliferation [4,5]. Recent studies have documented that the kidney in end stage renal disease (ESRD) is not a resting organ. It shows high proliferative activity of the tubular epithelial cells compared with normal kidney [6].

In response to stress due to various aetiologies, heat shock proteins (HSPs) are rapidly synthesized and expressed in the cells [7]. These HSPs are usually classified according to their size, HSP20 to 30 kDa, HSP60, HSP70, HSP90 [8]. These are highly conserved...
proteins throughout evolution. Although many of their functions are still speculative, several properties have been clearly defined. They are involved in protein folding and unfolding which may resist denaturation of proteins in response to various stresses such as heat [7,8], exposure to heavy metal [9] and hypoxia [10]. HSP may have an active role in transfer of antigenic peptides during antigen presentation [11]. In normal rat and human kidney localization of HSP73 has been studied [12,13]. In rat kidney HSP73 was localized in glomerular and Bowman's epithelia as well as epithelia of renal tubules from proximal to collecting duct in both nuclei and cytoplasm of the cells [12]. In normal human kidney, HSP72/73 showed a uniform fine granular cytoplasmatic staining of visceral glomerular epithelial cells and epithelia of distal convoluted tubules and collecting ducts without localization in proximal tubules [13]. In cases of tubular damage such as acute interstitial nephritis there was a significant increase in expression of HSP in tubular epithelium [13]. It was suggested that an increase in synthesis and cellular expression of HSP might indicate increased cellular stress [14]. The present study was undertaken on HSP expression and the proliferative activity of tubular cells in kidneys of ESRD with and without haemodialysis. Our aim was to investigate whether haemodialysis might cause increased expression of HSP in tubular epithelial cells as well as induce higher proliferative activity. Considering the heterogeneity of the morphological types of atrophic tubules in ESRD [6], we studied HSP and PCNA expression in each morphological subtype of tubules separately.

Subjects and methods

From the autopsy record of the past 15 years, 18 cases were included in this study who were suffering from ESRD. Eight patients were managed with weekly maintenance haemodialysis for periods of 3–12 months (group I). Ten patients did not receive any dialysis (group II). In all these cases autopsies were performed within 2 h of death. In five cases (two in group I and three in group II), kidney tissues were also obtained by blind needle biopsy immediately after death and fixed in 10% buffered formalin. Ten kidney biopsies of adults not receive any dialysis (group II). In all these cases autopsies were performed within 2 h of death. In five cases (two in group I and three in group II), kidney tissues were also obtained by blind needle biopsy immediately after death and fixed in 10% buffered formalin. Ten kidney biopsies of adults were taken from surgical pathology record as controls which were reported as normal.

Histology

Following gross examination and weighing of the kidneys, sections were taken from the upper pole, lower pole and mid-part of each kidney, fixed in 10% buffered formalin and routinely processed for histology. Sections were taken from these three regions in all the kidneys to avoid any sampling bias. Paraffin sections (5 μm thick) were stained with haematoxylin and eosin (H&E), periodic acid Schiff (PAS) and silver methanamine (SM) stains for routine histological evaluation. The atrophic tubules were classified into four morphological types as described earlier [6]: (1) 'classic' atrophic tubules with simple flat epithelium and thick wrinkled basement membrane, (2) 'thyroidized' tubules with uniform round shape and casts, (3) 'endocrine' tubules with clear epithelial cells, narrow lumen and thin basement membrane, and (4) 'super' tubules which were large dilated tubules with hyperplastic epithelial cells. In the majority of cases, a minimum of three paraffin blocks were examined from three different regions of each kidney as mentioned earlier. In all, 1000–2000 atrophic tubules were evaluated randomly in each case to determine the relative distribution of the four morphological types. A minimum of 20 serial sections were examined under light microscope from each block of tissue to identify the focus of cystic change or microadenoma formation.

Immunohistochemistry

Serial 5 μm thick paraffin sections were taken on poly-L-lysine (Sigma Chemical Co, St Louis, MO, USA) coated slides and numbered. Two consecutive slides were used for immunostaining with monoclonal antibody against HSP 72/73 (clone W27) and proliferating cell nuclear antigen (PCNA) (clone PC10). Both antibodies were obtained from Boehringer Mannheim Biochemica (Mannheim, Germany). Immunostaining was carried out using the avidin-biotin conjugate (ABC) immunoperoxidase technique using diaminobenzidine tetrahydrochloride (DAB) as chromogen [15]. Antigen retrieval was performed on deparaffinized sections by microwaving in citric acid buffer twice for 5 min [10,16]. Both the primary antibodies were used in 1:100 dilution with overnight incubation at 4°C. The ABC kit was procured from Vector Laboratories (Burlingame, CA, USA). Each batch of immunostaining was accompanied by a positive control, negative control and a control with substitution of primary with a non-related antibody of similar immunoglobulin type.

PCNA labelling index (LI)

PCNA LI was determined by calculating the percentage of positively stained cell nuclei out of the total number of cell nuclei [15]. This index was determined separately for the four atrophic tubule subtypes as well as in proximal and distal tubules of normal control cases. A minimum of 200 cell nuclei were examined for estimation of LI.

HSP expression and image analysis

HSP expression was studied in consecutive serial sections in the same group of tubules to compare with PCNA positivity. The degree of HSP expression was assessed by measuring the optical density (OD) of cytoplasmic brown staining following reaction with DAB. It was measured with the help of image analysis using a standard method [17]. The image analysis was performed using a Xilix microimager (Xilix Technologies Corporation, Richmond, Canada), F-64/oculus image grabber card (Coreco Inc, Quebec, Canada) based on a pentium IBM personal computer (Celebris XL, Digital Corporation, NY, USA) and Optimas 5.2 image analysis software (Optimas Corporation, Washington, USA). Briefly, a representative image was captured from an HSP-immunostained slide. The mean OD of the cytoplasmic area of the tubular cells was determined. A minimum of 200 cells of each tubular type were analysed. A negative control of the consecutive serial section with antibody substitution was similarly analysed to assess non-specific DAB staining. The essential prerequisites for a correct OD measurement such as identical thickness of paraffin
sections, identical performance of immunohistochemistry and avoidance of any counter staining were carried out [17].

Statistical analysis

The statistical analysis was performed using Microstat software package (CA, USA) [18]. Values within the same group were compared using the paired t-test and between groups I and II by unpaired t-test. The Wilcoxon rank sum and signed rank test were applied when required. Correlation regression analysis was done with determination of r value to evaluate the significance of a correlation between PCNA index and OD of HSP expression in each type of tubule; P < 0.05 was considered to be significant.

Results

All the cases included in this study were adult (Table 1). The age of eight patients in the dialysed group (I) varied from 40 to 56 (48 ± 8.4) years with a male:female ratio of 3:1. The duration of illness varied from 3 to 10 (6.8 ± 2.2) years. Previous biopsy records were available in five cases. Three cases presented with nephrotic syndrome diagnosed as membranous glomerulonephritis. Two cases presented with nephritic syndrome showed membranoproliferative and idiopathic crescentic glomerulonephritis. The remaining three cases presented initially with features of chronic renal failure. These patients were on maintenance haemodialysis of 4 h, twice a week for periods of 3–12 months.

The age range of the 10 patients in non-dialysed group (II) was 42 to 62 (50 ± 9.2) years with a male female ratio of 4:1 (Table I). The duration of illness varied from 4 to 12 (7.1 ± 3.2) years. Previous biopsy records were available in four cases. Three cases presented with nephrotic syndrome and were diagnosed as membranous glomerulonephritis (n = 2) and membranoproliferative glomerulonephritis (n = 1). One case presented as nephritic syndrome and was diagnosed as having membranoproliferative glomerulonephritis. The other six cases presented initially with features of chronic renal failure. None of these cases in these two groups had any clinical or serological evidence of collagen vascular disease or diabetes.

Gross examination revealed typical small granular contracted kidneys in all cases. There was no evidence of hydronephrosis. On gross serial sectioning, no cystic change or any abnormal space occupying lesion was detected. The mean weight of kidneys in group I was 62 ± 30 g (52–105) and in group II 68 ± 25 g (48–110).

Histology

On light microscopic examination, all four types of atrophic tubules were seen in both the groups. The distribution of classic, thyroid and endocrine type of tubules was similar in the two groups with no significant difference (Table 2). Super tubules were significantly more frequent in group I (10.6 ± 4.1%) than in group II (5.2 ± 1.326) (P < 0.001) (Table 2). These were tubules with a larger diameter lined by proliferating cuboidal to columnar cells. In focal areas they showed double or multilayering of lining cells. In some cases, these hyperplastic epithelial cells formed papillary luminal projections (Figure 1); such changes were noted more frequently in group I.

The microadenoma and microcystic areas were noted in all cases of group I and in one case of group II. The microadenoma areas varied from 120 to 922 μm in diameter with a mean of 494.85 ± 262.46 μm. The proliferating cells showed solid nests, aggregates, as well as tubular structures with focal areas of dilatation (Figure 2). Mitotic figures were noted in these cells. These lesions were randomly distributed in renal parenchyma with a pushing margin. The microcystic areas varied in diameter from 145 to 1125 μm with a mean of 522.66 ± 315.25 μm. These were lined by single layer of the columnar to cuboidal cells. These cysts were noted separately as well as in close relation to the microadenoma-like areas (Figure 2). Both microadenomas and microcysts were noted in close proximity to the super tubules. They were more frequent in areas with a high concentration of super tubules.

PCNA LI

The PCNA LI in normal kidneys showed lower proliferation rate in proximal tubular cells (0.24 ± 0.14%) than distal tubules (0.36 ± 0.16%) (P < 0.05). In ESRD, the proliferative potential of cells in all types of atrophic tubules in both the groups were significantly higher than normal (P < 0.001) (Table 2). Super tubules had the highest PCNA LI. It was (6.8 ± 2.04%) significantly higher than all other types in the dialysed group (I) (P < 0.05 to < 0.001) (Table 2 and Figure 3). In the non-dialysed group (II) the PCNA LI was 4.9 ± 1.96% higher than in the thyroid and endocrine types (P < 0.001) whereas it was not significantly different from the classic atrophic tubules (3.05 ± 2.40%) (P > 0.08). Comparison of PCNA LI of different types of tubules between the two groups showed a significantly high LI of only super tubules in group I (P < 0.05), whereas the difference was not significant for other types (Table 2). The PCNA LI of epithelial cells of microadenoma and microcystic areas were 7.2 ± 3.12% and 6.6 ± 2.64% respectively (P < 0.08), which were similar to those of super tubules in group I.

<p>| Table 1. Age, sex distribution and duration of illness of ESRD patients with haemodialysis (group I) and without dialysis (Group II). Values are given as mean ± SD with range shown in parentheses. |
|-----------------|----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age (years)</th>
<th>M:F</th>
<th>Duration of illness (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>8</td>
<td>48 ± 8.4</td>
<td>3:1</td>
<td>6.8 ± 2.2</td>
</tr>
<tr>
<td>(40–56)</td>
<td>3:1</td>
<td>(3–10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td>50 ± 9.2</td>
<td>4:1</td>
<td>7.1 ± 3.2</td>
</tr>
<tr>
<td>(42–62)</td>
<td>4:1</td>
<td>(4–12)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. The distribution of morphological types of atrophic tubules, their PCNA LI and HSP expression in dialysed (group I) and non-dialysed (group II) cases

<table>
<thead>
<tr>
<th>Tubules</th>
<th>Classic</th>
<th>Thyroid</th>
<th>Endocrine</th>
<th>Super</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distribution (%)</td>
<td>I</td>
<td>60.2±14.6</td>
<td>21.3±5.5</td>
<td>7.9±2.8</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>65.6±20.5</td>
<td>20.8±4.6</td>
<td>9.8±3.9</td>
</tr>
<tr>
<td>PCN LI (%)</td>
<td>I</td>
<td>3.45±2.25</td>
<td>1.58±0.92</td>
<td>1.62±0.81</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>3.05±2.40</td>
<td>1.65±1.02</td>
<td>1.48±0.92</td>
</tr>
<tr>
<td>HSP expression (optical density)</td>
<td>I</td>
<td>2.086±0.035&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2.072±0.016&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2.063±0.015&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>2.032±0.22</td>
<td>2.026±0.014</td>
<td>2.020±0.016</td>
</tr>
</tbody>
</table>

Significance of difference between groups I and II; <sup>*</sup><i>P</i>&lt;0.05, <sup>b</sup><i>P</i>&lt;0.01 and <sup>c</sup><i>P</i>&lt;0.001.

Discussion

A large number of studies are available in the literature regarding the association of dialysis with the development of ACKD and renal cell carcinoma, although the pathogenetic mechanism remains to be clearly defined [1–5,19]. Considering various theories and hypotheses,
it appears to be a multifactorial mechanism [3].

Relationships among tubular epithelial cell proliferation, development of cysts as well as adenoma or carcinoma in patients undergoing dialysis treatment needs further evaluation. The present post-mortem study was devised to include eight patients (group I) under haemodialysis treatment for a period ranging from 3 months to 1 year, to detect dialysis induced early renal changes. In these cases, no gross cyst or tumour was identified. For comparison, 10 non-dialysed cases of ESRD were taken (group II) having a similar age and sex distribution as well as similar duration of illness (Table 1). In ESRD the tubulo-interstitial compartment of the kidney undergoes extensive damage with tubular atrophy and loss as well as development of atubular glomeruli [20]. However, the morphological appearance of these atrophic tubules is heterogeneous and they have been classified into three subtypes; (1) ‘classic’ atrophic tubules, (2) tubules showing thyroidization and (3) endocrine tubules [6]. Along with these atrophic tubules, a fourth subtype of large tubules with hypertrophic or hyperplastic epithelial cells has been noted which is designated as ‘super’ tubules [6]. These tubules are thought to develop as a consequence of a compensatory growth mechanism and obstruction [21]. A previous study on ESRD using a panel of nephron-segment specific epithelial markers showed that the classic atrophic type of tubules were mostly derived from the proximal tubules [6]. The thyroid and endocrine types of tubules were mostly positive for distal tubular epithelial markers. The super tubules showed varying degrees of expression of both proximal and distal tubular markers [6].

Although no segment specific epithelial cell markers were used in the present study, the tubules were morphologically characterized into the four groups using histochemical stains. The relative distribution of all these different types of atrophic tubules was similar in dialysed (I) and non-dialysed (II) groups apart from the super tubules (Table 2). In group I, the percentage of super tubules (10.6±4.1%) was significantly higher (P<0.001) than in group II (5.2±1.3%). Morphologically, these tubules showed hyperplasia of the lining epithelial cells with focal areas of double or multiple layering and occasional mitosis. Some of them showed papillary projection in the lumen specially in dialysed (I) and non-dialysed (II) groups apart from the super tubules (Table 2). In group I, the percentage of super tubules (10.6±4.1%) was significantly higher (P<0.001) than in group II (5.2±1.3%). This finding was different from the earlier report [6] which showed classic atrophic tubules to have highest PCNA LI. This difference may be due to the extensive sampling done in the present study. The super tubules of group I showed significantly higher LI (6.8±2.04%) than those of group II (4.9±1.96%) (P<0.01) indicating a higher proliferation rate associated with dialysis. The PCNA LI of the cells lining the microcystic areas (6.6±2.64%) and in microadenomas (7.2±3.12%) were similar to the super tubules of dialysed group. It is possible that the morphological transformation to super tubules is associated with a higher proliferation rate which in turn may be associated with transepithelial secretion of fluid resulting in cyst formation [19,22]. We observed some super tubules with cystic dilatation as well as papillary projection of proliferating epithelial cells into the lumen (Figure 1). Close proximity of super tubules, microcysts and microadenoma was noted in the dialysed group (Figure 2). Cyst fluid from ACKD cases has been shown to have mitogenic effect on proximal tubular cells and renal carcinoma cells [23]. It also contains a significantly higher concentrations of epidermal growth factor than that of simple cysts [24]. All the cases in the dialysed group (I) showed microcyst as well as adenoma formation, whereas only one out of 10 cases in the non-dialysed group (II) had these changes.

To investigate whether haemodialysis causes additional stress to tubular epithelial cells in ESRD, we studied HSP expression using an anti-HSP 72/73 monoclonal antibody. HSPs are a group of highly conserved proteins developed during the evolution of species which are expressed or induced by stress [7–14]. They promote protein assembly and folding, elimination of malfolded proteins and stabilization of newly synthesized proteins in various cellular compartments [8,25]. Among the HSP families, the HSP70 series has been reported as the most abundant, participating in various cellular functions, both under normal and stress-related conditions [26]. A recent immunohistochemical study of HSP 72/73 in normal human kidney and kidney biopsies from cases of minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS), membranous glomerulonephritis (MGN) and chronic interstitial nephritis (CIN) showed 2+ to 3+ positivity of cells in distal convoluted tubules, cortical and medullary collecting tubules, portion of the loop of Henle and glomerular epithelium [13]. Increased staining of these tubules was noted in acute interstitial nephritis (AIN) and diffuse proliferative glomerulonephritis associated with active interstitial inflammation [13]. In an earlier study in 28 cases of human renal disease Dodd et al. [27] showed positive staining in proximal tubules in ischaemic transplant nephropathy and microPAN (poly-antieritis nodosa). In the present study we mainly observed cytoplasmic positivity in distal tubular epithelium of the normal kidney in line with Venkatasheshan et al. [13] and quantified the cytoplasmic expression of HSP by measuring the OD with the help of image analysis system.

Recently, image analysis or image cytometry has been used by several workers for selective quantitative analysis of the intensity of immunohistochemical reactions (optical densitometry) which appears to be proportional to the amount of antigen present at the reaction site [28–30]. Unlike the Western blot, this technique does not give a direct quantification of the antigen [31], but it is very helpful for studies with reference to morphology [29,30] like the present one,
where the different subtypes of tubules are intermingled with each other in the cortex.

In ESRD without dialysis (group II) all the different types of tubules showed significantly higher OD than the distal tubules of normal kidney (Table 2), (P < 0.05 to < 0.01). Among the different types of tubules the super tubules showed highest OD (2.106 ± 0.016) which was significantly higher than other types (P < 0.01 to < 0.001) (Table 2). The complex multifactorial pathogenesis of ESRD [20] had been proposed to be associated with marked alteration of tubulointerstitial microenvironment with ischaemic [32] and metabolic [33] stress which might increase HSP expression in all tubular cells in comparison with the normal kidney [13]. The cause of increased HSP expression in super tubules was not clear in our study. It is possible that they represent the remaining hypertrophic, hyperfunctioning nephrons which bear additional functional stress in an altered microenvironment [20, 21].

In the dialysed group (I) all the types of tubules had significantly higher HSP expression than the non-dialysed group (II) (P < 0.05 to < 0.001), super tubules showing the highest (2.309 ± 0.155) (Table 2). The increased expression of HSP with haemodialysis might be secondary to stress caused by dialysis in addition to alteration of ESRD. Dialysis may cause introduction of several potentially toxic exogenous chemicals into the circulation such as plasticizers, formaldehyde, nitrite and nitrosamine which may cause tubular injury as well as inducing tubular cell proliferation [3–5]. We observed a significant positive correlation between HSP expression and the proliferation of tubular cells (PCNA L1) in super tubules in both dialysed and non-dialysed groups as well as in cells of microcysts and microadenomas (P < 0.001). The present study did not reveal the mechanism of induction of HSP secondary to haemodialysis, although it showed a definite increase in HSP expression in all types of atrophic tubules (Table 2). It is also well known that cells with a higher rate of proliferation are more susceptible to an oncogenic hit leading to neoplastic transformation [34]. This may be one of the pathogenetic mechanisms responsible for the higher incidence of renal cell neoplasms associated with dialysis.

Thus it may be suggested from the present study that haemodialysis causes more stress or injury to the tubular cells superimposed on an already compromised situation of ESRD, leading to a higher rate of tubular cell proliferation associated with more hyperplastic super tubule formation which may be the forerunner of cyst formation as well as neoplastic transformation.

Acknowledgements. The financial support for the work was provided by Indian Council of Medical Research. The authors are grateful to Mr Charan Singh, Mohan Kumar, Kishore and Shikander for technical assistance as well as Mr K. K. Arora for secretarial help.

References

HSP expression and proliferative activity in ESRD with and without dialysis


Received for publication: 14.10.96
Accepted in revised form: 5.9.97