Title: Effects of Diabetes and Hypertension on Macrophage Infiltration and Matrix Expansion in the Rat Kidney

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Author's response to reviews: see over
Response to Editorial Decision Letter

TO: BMC Nephrology / Editorial Office

Dear Madam or Sir,

Please find enclosed the revised version of our manuscript, now entitled “Effects of Diabetes and Hypertension on Macrophage Infiltration and Matrix Expansion in the Rat Kidney”.

The manuscript was revised according to the reviewer’s suggestion which we found both helpful and informative. We have added several additional data, including real-time RT-PCR (Figure 3 and Table 2) and measurements of glomerular and interstitial fibrosis (Figure 7).

We have now cited and discussed the report by Kelly et al. (NDT 2002) in the last paragraph of the background section (page 3, 2nd paragraph) as well as in the second paragraph of the discussion section (page 11, 2nd paragraph). We believe that our report is an advance over that by Kelly et al. because we focused on another Chemokine, MCP-1, whereas Kelly et al. focused on osteopontin, and because we included normotensive, normoglycemic as well as normotensive, diabetic control animals which were omitted in the study by Kelly et al. Further, the results of our study are partly at variance with those obtained by Kelly et al. as discussed on page 11 (2nd paragraph).

The title of our manuscript was altered to reflect the fact that we obtained results on matrix expansion. The remaining changes made in the manuscript are detailed below in the point-by-point responses to the reviewer’s comments.

Sincerely

On behalf of the authors Karl F. Hilgers, MD, FASN
Response to Reviewer Prof. Enyu Imai

- We have now cited and discussed the report by Kelly et al. (NDT 2002) in the background section (page 3, 2nd paragraph) as well as in the discussion section (page 11, 2nd paragraph). We believe that our report extends the paper by Kelly et al. because we focused on another Chemokine, MCP-1, whereas Kelly et al. focused on osteopontin, and because we included normotensive control animals (normoglycemic as well as diabetic) which were omitted by Kelly et al. Further, the results of our study are partly at variance with those obtained by Kelly et al. who reported very little inflammation in hypertensive rats in the absence of diabetes whereas we observed a marked effect of hypertension on macrophage infiltration. These contrasting findings are discussed on page 11 (2nd paragraph).

- We have now cited the report by Chow et al. (Kidney Int 2004) in the background section (page 3, 1st paragraph, last line), and discussed it in the discussion section (page 11, 1st paragraph, last 3 lines).

- The gene expression of MCP-1 has been studied in a higher number of rats by real-time RT-PCR (Figure 3 which replaces the Northern blot). The methods are described on page 5 (2nd paragraph). Statistical analysis revealed a significant effect of hypertension but not of STZ diabetes on MCP-1 expression in kidney cortex.

- Immunohistochemistry (rather than isolated glomerular protein which would have required additional animals) was used to investigate MCP-1 protein in glomeruli (Figure 5). Semi-quantitative evaluation of immunostaining revealed an increase by STZ diabetes and by hypertension (Figure 6). The Western blot (Figure 4) is shown to demonstrate the specificity of the antiserum, not for quantification.

- To provide more information on glomerular and tubular lesions, we evaluated tubulointerstitial fibrosis by immunostaining for collagen I, and glomerulosclerosis by immunostaining for collagen IV (page 7, 1st paragraph, last 6 lines). The results are presented on the newly added Figure 7.

- The figures are now numbered in order of the citation in the results section.

- The written English style was judged to be acceptable by a native English speaker (Prof. Topley).
RE: MS 1103319792492640 – First Revision
Effects of Diabetes and Hypertension on Macrophage Infiltration and Matrix Expansion in the Rat Kidney

Response to Reviewer Prof. Nicholas Topley

- Following the reviewer’s suggestion, we tried to clarify throughout the manuscript that the effects of diabetes and hypertension combined on inflammation in the kidney are, for the most part, not even additive. This notion is now emphasized in the concluding statement of the abstract (page 2, last paragraph), in the discussion (page 11, 2nd paragraph), and in the conclusions (page 13, 1st paragraph). Moreover, the lack of an additive effect on inflammation contrasts sharply with the results on glomerular matrix expansion where an additive effect is clearly evident (see the newly added figure 7).

- The notion of a threshold level of macrophage infiltration is intriguing but we have no data to support or reject it. Therefore we hesitate to discuss this issue in the manuscript. We do not believe that the level of blood pressure is so high in our model as to exclude further macrophage influx, however. At least in glomeruli, we have previously observed a 3fold higher degree of macrophage influx in a different model of hypertension on a similar genetic background, using the same methods for staining and counting (Hartner et al. 2001, Am J Kid Dis 38:164).

- According to the reviewer’s suggestion, we have screened for the expression of MCP-1, osteopontin, VCAM-1, ICAM-1 and RANTES by real-time RT-PCR. The methods are described on page 5 (2nd paragraph), and the results are shown on figure 3 and table 2. The most prominent changes were seen for MCP-1 and osteopontin. We did not further investigate osteopontin because Kelly et al. (reference 13) had already focused on osteopontin in hypertensive TGR with STZ diabetes.

- Variability in RNA loading / GAPDH hybridization: The Northern blot has been replaced by real-time RT-PCR data (see above). These data were normalized to the individual levels of 18 S RNA as determined by real-time PCR. The results did not differ when GAPDH was used instead of 18 S as a housekeeping gene in the real-time RT-PCR (data not shown).

- The observation that the Western blot for MCP-1 shows more than one band is probably due to different glycosylation of the protein (Yoshimura & Leonard 1992, Cytokines. 4:131). A double band similar to that shown in figure 4 has been observed in Western blots for rat MCP-1, even with the use of other antisera (for example, Suda et al. 2001, Kidney Int. 60:1705). There is no loading control because the Western blot is shown to demonstrate the specificity of the antiserum, not for quantification. The immunostaining for MCP-1 protein was evaluated semi-quantitatively.
Response to Reviewer Prof. Dick de Zeeuw

- The reviewer is concerned that the aggravation of diabetic renal injury by TGR hypertension which was described by Kelly et al. (reference 14) may not be present in the rats studied by us. To test the model, we evaluated tubulointerstitial fibrosis by immunostaining for collagen I, and glomerular sclerosis by immunostaining for collagen IV (page 7, 1st paragraph, last 6 lines). The results are presented on the newly added Figure 7. Glomerular matrix expansion was clearly aggravated by the presence of hypertension and diabetes combined, compared to animals which were either diabetic or hypertensive.

- We investigated rats 5 weeks after induction of STZ diabetes whereas Kelly et al. (reference 13) used rats after 12 weeks of diabetes. We are currently investigating renal injury at later time points in this model. However, we selected the 5 weeks time point for this study because we observed little or no macrophage infiltration induced by STZ diabetes at later time points (for example, at 10 weeks), as discussed on page 11 (1st paragraph). The differences between our results and those of Kelly et al. are also discussed in detail on page 11 (2nd paragraph).

- In individual rats, the expression level of MCP-1 correlated with macrophage counts in the interstitial space (r2=0.47, p=0.002) but not in glomeruli (r2=0.002, p=0.857).

- In the discussion, the paragraphs relating to mechanisms of MCP-1 induction and the role of MCP-1 in Glomerulonephritis were deleted. Instead, we discussed the findings of Kelly et al. in more detail, and refer briefly to the potential role of other chemokines and adhesion molecules.

- The gene expression of MCP-1 has been studied in a higher number of rats by real-time RT-PCR (Figure 3 which replaces the Northern blot). The methods are described on page 5 (2nd paragraph). Statistical analysis revealed a significant effect of hypertension but not of STZ diabetes on MCP-1 expression in kidney cortex. The Western blot (Figure 4) is shown to demonstrate the specificity of the antiserum, not for quantification of MCP-1 protein. Semi-quantitative evaluation of immunostaining was used to investigate MCP-1 protein (Figures 5 and 6).

- The figures are now numbered in order of the citation in the results section.