Renal failure induces telomere shortening in the rat heart

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Background. Renal failure aggravates pathological cardiac remodelling induced by myocardial infarction (MI). Cardiac remodelling is associated with telomere shortening, a marker for biological ageing. We investigated whether mild and severe renal failure shorten cardiac telomeres and excessively shorten telomeres after MI.

Methods. Rats were subjected to sham, unilateral (UNX) or 5/6th nephrectomy (5/6NX) to induce none, mild or severe renal failure. MI was induced by left coronary artery ligation. Renal function parameters and blood pressure were measured. DNA was isolated from non-infarcted cardiac tissue. Telomere length was assessed by quantitative polymerase chain reaction (PCR).

Results. Proteinuria was unchanged in UNX and MI compared with control, but strongly increased in 5/6NX, UNX+MI and 5/6NX+MI. Serum creatinine levels were increased fourfold in 5/6NX and tenfold in 5/6NX+MI. 5/6NX and groups with both renal failure and MI showed an approximate 20% reduction of telomere length, similar to the MI group. No excess telomere shortening was observed in hearts from rats with renal ablation after MI.

Conclusion. Severe renal failure, but not mild renal failure, leads to shortening of cardiac telomeres to a similar extent as found after MI. Renal failure did not induce excessive telomere shortening after MI. (*Neth Heart J* 2009;17:190-4.)

Keywords: telomere, kidney failure, myocardial infarction

Cardiovascular complications frequently occur in patients with renal failure¹ and renal failure aggravates unbeneficial cardiac remodelling.² These complications may be the consequence of systemic changes induced by renal failure, such as hypertension, anaemia, electrolyte and metabolic disturbances or (coronary) atherosclerosis. Biological ageing, indicated by short telomere length, might also be a contributor to the process of cardiac remodelling, since cardiac remodelling shows similarities to features of cardiac ageing. Telomeres form the end of chromosomes and consist of specific base-pair repeats. Functional telomeres protect the end of chromosomes, thereby preventing loss of genetic information and maintaining cellular stability. Damage to telomeres or excessive shortening of telomeres leads to genomic instability or activation of cellular damage responses, such as cellular senescence and apoptosis.³⁻⁵

Telomere length in the heart has been associated with cardiac disease. In patients with heart failure, the mean telomere length was decreased compared with healthy, age-matched controls.⁶ Another study showed that genetically modified mice with extremely short telomeres developed left ventricular failure and unbeneficial morphological changes of the heart, such as left ventricular dilatation.⁷

To date, it is not known whether effects of renal failure on cardiac condition are associated with telomere shortening. In this study, we investigated whether experimental renal failure is associated with telomere shortening in the heart and, if so, whether excess telomere shortening occurs in the already failing heart. To determine this, we assessed telomere

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length by means of quantitative polymerase chain reaction (PCR) on DNA from non-infarcted cardiac tissue of rats with mild or severe renal failure following renal ablation, with or without myocardial infarction.

Subjects and Methods

Experimental animals

Male Wistar rats (250–275 g) were housed under standard conditions. During the study, rats received a normal salt diet (0.3% NaCl) and had free access to food and water. Animal experiments were approved by the institutional animal ethical committee.

Surgical procedures

To induce mild renal failure, rats were subjected to unilateral nephrectomy under anaesthesia with 2.0% isoflurane in O₂/N₂O (1:2). Laparotomy was performed, with subsequent removal of the right kidney after careful separation of the kidney from adrenal gland and surrounding tissue.

For severe renal failure, 5/6th nephrectomy was performed in the rats under anaesthesia with 2.0% isoflurane in O₂/N₂O (1:2). The right kidney was removed as described above, with additional interruption of two-thirds of the blood supply of the left kidney, achieved by ligation of the proximal branch of the renal artery. If, after visual inspection, less than two-thirds of the renal perfusion appeared to be cut off, additional smaller branches of the renal artery were ligated or coagulated.

To induce heart failure, rats were subjected to myocardial infarction (MI). Briefly, thoracotomy was performed under anaesthesia with 2.0% isoflurane in O₂. MI was induced by ligating the left ascending coronary artery. In groups with mild or severe renal failure, additional MI was performed two weeks after renal ablation. Sham operations for renal ablation and MI were performed on the control group.

Animals with severe renal failure were sacrificed under 2.5% isoflurane anaesthesia in O₂/N₂O (1:2) 12 weeks after renal ablation with measurement of haemodynamic parameters and collection of the hearts. Animals with mild or no renal failure were sacrificed 16 weeks after renal ablation or sham operation, following the same procedure as in animals with severe renal failure.

Measurement of systolic blood pressure

Systolic blood pressure was measured by tail cuff plethysmography (IITC Life Science, Woodland Hills, CA) in awake and restrained animals. Rat tails were heated by placing the tails under a lamp (250 W) for five to ten minutes. Elevated temperature was maintained by a 150 W lamp. The tail cuff was inflated to a pressure of 200 mmHg and subsequently slowly deflated, while measuring the systolic blood pressure of the rats.

Measurement of kidney function parameters

Proteinuria was measured in samples of 24-hour urine collected with metabolic cages. For determination of total urinary protein the trichloroacetic acid (TCA) method (Nephelometer Analyser II, Dade Behring, Marburg, Germany) was used. Plasma creatinine levels were measured using the photometric determination according to the Jaffe method (Ecoline Mega, DiaSys Diagnostic Systems GmbH, Holzheim, Germany) described earlier.⁸

Kidneys were fixed in formalin for 48 hours and subsequently embedded in paraffin. For analysis of focal glomerulosclerosis (FGS), sections of 3 µm were stained with periodic acid Schiff (PAS). The degree of FGS was assessed in 50 glomeruli by scoring semi-quantitatively on a scale of 0 to 4, a method originally described by Raij et al.⁹ Overall FGS score is expressed as arbitrary units (AU) with a maximum of 200. An examiner blinded for the groups evaluated all sections.

DNA isolation and quantitative PCR

Genomic DNA was isolated from frozen cardiac tissue according to the manufacturer's instructions (QIAamp DNA Mini Kit, Qiagen). In groups that underwent MI, tissue was taken from the non-infarcted region and corresponding areas were used for the other groups. In brief, cardiac tissue was cut into small pieces and incubated overnight at 56 °C in a mixture of lysis buffer and proteinase. Buffer for precipitation was added and this mixture was incubated at 70 °C for ten minutes, followed by centrifugation. Subsequently, 96% ethanol was added to the supernatant and the sample was pipetted on a column and washed twice with washing buffers. Distilled water was used for elution of the genomic DNA. DNA concentration was measured with a spectrophotometer. Quantitative PCR on telomeres was performed using Cawthon's method¹⁰ in which mismatches in primers exclude the possibility of primer-dimer formation without affecting the hybridisation of the primers to the DNA.

Quantitative PCR was performed according to the thermal profile of incubation at 94 °C for five minutes, followed by 40 cycles of 94 °C for 15 seconds and 54 °C for two minutes. Each reaction consisted of a mixture of quantitative PCR reagents with a total volume of 50 µl, containing 10 µl of DNA dissolved in distilled water. Primers used for amplification of telomeres were Tel-1: 5'-ggt ttt tga ggg tga ggg tga ggg tga ggg tga ggg t-3' and Tel-2: 5'-tcc cga cta tcc cta tcc cta tcc cta tcc cta tcc cta-3'.

Quantities of telomeric DNA were normalised to the quantities of the genomic AT₁ receptor, also assessed by quantitative PCR. The thermal profile of this quantitative PCR consisted of incubation at 94 °C for five minutes, followed by 40 cycles of 94 °C for 15 seconds and 60 °C for one minute. Primers were: AT1rat-f: 5'-acg tgt tct cag cat cga ccg cta cc-3' and AT1rat-r: 5'-aga atg ata agg aaa ggg aac aag aag ccc-3' (Biogen, Malden, the Netherlands).

Mean cardiac telomere length is expressed as the ratio of the results of the quantitative PCR on telomeres and the single gene copy AT_1 receptor, referred to as T/S ratio.

Statistical analysis

All data are presented as mean $\pm$ SEM. Statistical analysis was performed by one-way ANOVA, followed by a least significant difference (LSD) post hoc test to identify the groups that were different from each other. P values <0.05 were considered statistically significant.

Results

General characteristics

No mortality was observed in sham or renal ablation groups. MI and UNX+MI resulted in an acute mortality of 20 and 25%, respectively. In 5/6NX+MI a 47% overall mortality was observed. Body weight was lower in 5/6NX+MI compared with UNX, 5/6NX and UNX+MI.

Cardiac characteristics

Left ventricular infarct size was not significantly different in the three groups that underwent MI and no significant differences in heart rate were observed between the groups (table 1). Heart weight was increased in UNX+MI, 5/6NX and 5/6NX+MI compared with control (table 1). Systolic blood pressure was increased in the 5/6NX and 5/6NX+MI group compared with all other groups (table 1).

Renal characteristics

To evaluate renal impairment after UNX and 5/6NX, we measured plasma creatinine, proteinuria, and focal glomerulosclerosis (FGS), as shown in table 1. Proteinuria and FGS were significantly higher in UNX+MI, 5/6NX and 5/6NX+MI compared with control. Plasma creatinine was increased in 5/6NX+MI compared with all other groups.

Telomere length

Mean telomere length was decreased by approximately 20% in rats that underwent MI compared with control (figure 1). Mean telomere length was decreased in 5/6NX to a similar extent as observed in MI. In contrast, telomere length was not affected in the UNX group. In groups with combined renal ablation and MI, telomere length was reduced to a similar extent as found in groups with either 5/6NX or MI (figure 1).

No statistical relationship between renal parameters or blood pressure and telomere length was found in any study group, as tested by Pearson's linear regression analysis (data not shown).

Discussion

In this study, we found that severe renal failure, but not mild renal failure, in rats led to a shortening of cardiac telomeres and an increase in heart weight. The changes observed in severe renal impairment were similar in degree to those observed in rats that underwent myocardial infarction. Furthermore, the combination of severe renal failure and myocardial infarction led to an excess increase in heart weight, but not to an excess shortening of cardiac telomeres.

Renal failure is known to cause alterations in heart function and morphology. It has been shown that severe renal failure leads to left ventricle hypertrophy, increased interstitial fibrosis and a decreased capillary density.¹¹ On the functional level, severe renal failure decreased cardiac output¹² and decreased ischaemia tolerance.¹³ In our study, we observed that severe renal failure causes a more distinct deterioration of the cardiac condition than mild renal failure.

Although a causal relationship remains to be established, previous studies have revealed a role of telomeres in heart failure. As mentioned before, heart failure patients showed a decreased cardiac telomere length compared with age-matched controls.⁶ This finding was confirmed in a large cohort of 620 patients with heart failure compared with 183 gender- and

Table 1. General, cardiac and renal characteristics.

	Control	UNX	5/6NX	MI	UNX+MI	5/6NX+MI
N	9	9	7	7	8	8
Body weight (g)	465 $\pm$ 11	485 $\pm$ 16	488 $\pm$ 9	472 $\pm$ 12	524 $\pm$ 18*	423 $\pm$ 26 ^{††¶}
SBP (mmHg)	132 $\pm$ 3	123 $\pm$ 3	174 $\pm$ 7* ^{†§¶}	118 $\pm$ 6	137 $\pm$ 4	160 $\pm$ 7* ^{†§¶}
Infarct size (% of LV)	-	-	-	17 $\pm$ 2	19 $\pm$ 3	30 $\pm$ 6
Heart rate (beats/min)	326 $\pm$ 12	336 $\pm$ 5	352 $\pm$ 16	352 $\pm$ 34	329 $\pm$ 7	371 $\pm$ 17
Heart weight (g)	1.31 $\pm$ 0.05	1.36 $\pm$ 0.04	1.57 $\pm$ 0.03*	1.48 $\pm$ 0.06	1.57 $\pm$ 0.05 *	1.85 $\pm$ 0.12* ^{†§}
Proteinuria (mg/24 h)	19 $\pm$ 3	22 $\pm$ 6	256 $\pm$ 36*	15 $\pm$ 5	154 $\pm$ 58*	215 $\pm$ 45*
FGS (%)	4 $\pm$ 1	5 $\pm$ 1	36 $\pm$ 4*	3 $\pm$ 1	19 $\pm$ 5*	43 $\pm$ 3*
Plasma creatinine (μ mol/l)	18 $\pm$ 2	20 $\pm$ 2	88 $\pm$ 13	29 $\pm$ 2	24 $\pm$ 1	193 $\pm$ 78* [¶]

Data expressed as mean $\pm$ SEM. UNX=unilateral nephrectomy, 5/6NX=5/6th nephrectomy, MI=myocardial infarction, SBP=systolic blood pressure, FGS=focal glomerulosclerosis. * p <0.05 vs. control, [†] vs. UNX, [‡] vs. 5/6NX, [§] vs. MI, [¶] vs. UNX+MI, [¶] vs. all other groups.

Figure 1. Mean cardiac telomere length of experimental groups expressed as T/S ratio (percentage of control). UNX=unilateral nephrectomy, 5/6NX=5/6th nephrectomy, MI=myocardial infarction. * $p < 0.05$ vs. control.

age-matched controls.¹⁴ In addition, telomere length inversely correlated with the severity of heart failure.¹⁴ Interestingly, in patients with heart failure, shorter telomere length was associated with decreased renal function.¹⁵

There are strong indications that oxidative stress plays an essential role in the process of telomere shortening. Cultured human fibroblasts exposed to mild oxidative stress clearly showed accelerated telomere shortening, while treatment with an antioxidant minimised telomere shortening under oxidative stress.¹⁶ Furthermore, overexpression of extracellular superoxide dismutase, an antioxidant gene, inhibited telomere shortening in cultured human fibroblasts under both normal cell culture conditions and oxidative stress.¹⁷

Several studies reported increased oxidative stress in animals or human with chronic renal failure.^{18,19} As mentioned before, oxidative stress causes telomere shortening.²⁰ In the severe renal failure groups, increased oxidative stress is a likely candidate to convey the cardiac telomere shortening. Another likely candidate to cause telomere shortening in the severe renal failure groups may be systolic hypertension. Yet, in another study increased blood pressure did not lead to cardiac telomere shortening in spontaneously hypertensive rats.²¹ Moreover, no relationship was found between systolic blood pressure and telomere length in this study. In addition, in this study telomere shortening was also observed in the non-hypertensive groups. Therefore, it is not very likely that the shortened telomere length in the severe renal failure groups is merely the result of increased systolic blood pressure. Increased oxidative stress is also observed in the heart after myocardial infarction, and is a possible

cause of telomere shortening in the heart. Increased oxidative stress has been reported in post-MI failing hearts due to mitochondrial dysfunction.²² Furthermore, increased levels of oxidative stress have shown to develop after an MI in rats.²³ To summarise, oxidative stress is likely to play an essential role in the process of telomere shortening both after MI and in severe renal failure.

Our study encounters some limitations. First, we determined telomere length from non-infarcted heart tissue. Thus, the determined telomere length does not only reflect mean telomere length of myocytes, but of all cells in heart tissue, including fibroblasts, endothelial cells, and inflammatory cells. However, previous studies suggest that the number of non-myocyte cells in non-infarcted areas of the post-MI heart is substantially less compared to myocytes. Studies using animal models with rats have demonstrated substantially less collagen accumulation in non-infarcted regions of the post-MI hearts as compared with infarcted areas, accounting for approximately 2 to 7% of the cardiac tissue.²⁴⁻²⁶ Also, the inflammatory cell reaction in non-infarcted areas was sparsely seen in non-infarcted areas.^{24,25} In conclusion, we cannot exclude the possibility of presence of non-myocyte DNA in the DNA we isolated from non-infarcted cardiac tissue. However, based on the above-mentioned findings,^{24,25} we conclude that our assessment of telomere length of cardiac tissue in the MI groups predominantly reflects telomere length of myocytes. This is also true for composition of cardiac tissue in renal failure. Assessment of cardiac fibrosis in renal failure showed that fibrotic tissue accounts for less than 3% of the left ventricle.^{27,28} Because assessment of fibrosis was performed with tissue area measurements, and not fibroblast cell count, it is not certain what the absolute number of fibroblasts in the ventricle was. However, 3% of fibrotic tissue is very little, and we reason that the absolute number of fibroblasts would still be considerably less than the number of myocytes. Thus, we postulated that the determined telomere length predominantly reflects mean telomere length of myocytes.

Secondly, the severe renal failure groups were sacrificed at week 12 because of the deteriorating clinical condition of the rats, whereas the other groups were sacrificed four weeks later. However, assuming telomere length would have further decreased in time, we may have merely underestimated the effects of severe renal failure on telomere length compared with mild or no renal failure. Therefore, neither of these practical features undermine the conclusions drawn from this study.

The traditional technique to determine telomere length is telomere restriction fragment (TRF) analysis by Southern blotting. However, TRF analysis has some limitations, as it also measures non-telomeric DNA restricted by the enzyme employed and it is sensitive to possible restriction site polymorphisms.¹⁰ TRF analysis furthermore requires relatively large amounts of DNA compared with quantitative PCR. In this

study, we used the relatively new technique of quantitative PCR to determine telomere length. Reliability of quantitative PCR for detection of telomere length was substantiated in an experiment with endothelial cells with known mean telomere length.²⁹ Furthermore, in experiments in which TRF analysis and quantitative PCR were compared, mean telomere lengths obtained by both techniques strongly corresponded with each other.^{10,29,30,31} Therefore, we concluded that the technique of quantitative PCR was suited for detecting differences in mean telomere length in our experiments.

Although evidence of an association between telomere length and cardiovascular disease is accumulating, the nature of this relationship – causal or merely associative – has not been determined. If telomeres are causally involved in the development and progression of cardiovascular disease, the possibility of telomere length as a modifiable factor is very interesting. This provides us with a possible new therapeutic target in the treatment of cardiovascular disease. Naturally, thorough research will be indispensable before practical implementation.

In conclusion, our main observation is that severe renal failure, but not mild renal failure, leads to hypertrophy and telomere shortening in the heart to a similar extent to that found after MI. Since shortening of telomeres is implied in cardiomyocyte dysfunction and cardiac failure, this mechanism may contribute to the increased cardiovascular morbidity and mortality in patients with renal failure.

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