Excess Growth and Apoptosis
Is Hypertension a Case of Accelerated Aging of Cardiovascular Cells?

Pavel Hamet, Nathalie Thorin-Trescases, Pierre Moreau, Pierre Dumas, Ben-Sung Tea, Denis deBlois, Vladimir Kren, Michal Pravenec, Jaroslav Kunes, Yulin Sun, Johanne Tremblay

Abstract—Several groups including ours have demonstrated cardiac hyperplasia in neonates from genetically hypertensive rat strains. We have shown that similar problems exist in the kidney as well. More recently, we found that excessive heart and kidney weight is neonatally related to inhibition of apoptosis. Using recombinant inbred strains derived from a reciprocal cross between Brown Norway and spontaneously hypertensive rat progenitor strains, we mapped the inhibition of neonatal apoptosis to 2 distinct loci on chromosomes 1 (Myl 2) and 18 (Abrb 2). Positional candidate genes at these loci are being explored. These studies have also demonstrated that the loci determining kidney and heart weights in neonates are distinct from those determining increased organ weight in adults. The impact of blood pressure per se is also divergent because adult kidney weight is negatively correlated whereas heart weight is positively correlated with it. Analyses by extremes of low and high percentiles from fetal life to adulthood identified a single locus determining heart weight at Acaa on chromosome 8 in newborn (P=0.0003) and adult (P=0.016) rats. The Acaa region contains a DNA mismatch repair gene (hMLH1). The kinetics of neonatal growth through adulthood by prelabeling DNA with [3H]thymidine in pregnant mares showed that although the growth process is complex and nonlinear in the kidney of hypertensive rats, there is an increased turnover of cells, that is, reduced half-life of DNA. This observation is supported by the presence of shorter telomere fragments in kidneys of spontaneously hypertensive rats. These studies suggest that cardiovascular cells from hypertensive animals are subject to accelerated turnover, potentially leading to their accelerated aging. (Hypertension. 2001;37[part 2]:760-766.)

Key Words: hypertension ■ genetics ■ telomeres ■ growth ■ apoptosis ■ aging

Hypertension, as a component of cardiovascular disease, as a risk factor as well as a complication, is clearly not a simple increase of blood pressure. Some time ago, Folkow1 and several other groups2-3 developed the notion that cardiovascular system proliferation may be an important component in the pathogenesis of hypertension. We proposed4 that a proliferative process may actually be primarily involved in hypertension development because it is present at birth and persists in cultured cells of genetically hypertensive rats.5-8 More recently, we have added the notion of imbalance of proliferation and apoptosis, which is apparently enhanced in later stages of hypertension and occurs in “time windows.”9-11 This phenomenon can be modulated by antihypertensive medication, with an initial increase in apoptosis leading to improvement of hyperplasia12 and resulting in long-term normalization of the balance between apoptosis and proliferation,13 findings that were recently confirmed by others.14 We have realized that apoptosis is actually suppressed in newborn offspring of hypertensives,15 at a time when the kidneys, heart, and aorta are already expressing hyperplasia,16 which suggests a potential mechanism of increased organ mass at that point in life. Follow-up of the genetic determinants from birth to adulthood provided evidence of a nonlinear, dichotomous process, leading us to the hypothesis that cardiovascular cells from hypertensive subjects are subjected to accelerated turnover, potentially culminating in accelerating aging.

Methods

Animals

This investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and all procedures were approved by the institutional Animal Care Committee of the Centre hospitalier de l’Université de Montréal (CHUM). The rats used to study neonatal apoptosis originated from 2 reciprocal crosses developed in the Biology Department of the Faculty of Medicine, Charles University (BN[Brown-Norway].Lx/Cub females×SHR[spontaneously hypertensive rat]/Ola males) (the BXH set) in the Institute of Physiology, Academy of Sciences, Prague, Czech Republic (SHR/Ola females×BN.Lx/Cub males) (the

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760
The samples were incubated for 60 minutes at 37°C. We loaded 0.2 μL of water. DNA content was measured by spectrophotometry, and DNA precipitated with 100% cold ethanol and resuspended in deionized water. After extraction with phenol and chloroform, DNA was electrophoresed at 90 V for 3.5 hours, transferred onto Hybond N membrane (Amersham), exposed to a phosphor-sensitive screen, and analyzed by PhosphorImager (Molecular Dynamics). One microgram of extracted DNA was labeled by enzymatic assay, with tdt. Aliquots of frozen tissue powder were weighed and incubated in buffer containing 20 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.0), 0.5% SDS, and 500 μg/mL proteinase K for 3 hours at 50°C, then treated with RNase A (final concentration, 250 μg/mL) for 1 hour at 37°C. After extraction with phenol and chloroform, DNA was precipitated with 100% cold ethanol and resuspended in deionized water. DNA content was measured by spectrophotometry, and DNA concentration per milligram of tissue powder was calculated.

One microgram of extracted DNA was labeled by enzymatic assay, with tdt. DNA was labeled in buffer containing 2 mmol/L CoCl₂, 0.5 mmol/L DTT, 100 mmol/L potassium cacodylate, 166 mmol/L [³²P]dCTP (3000 Ci/mmol, Amersham), and 20 U of tdt. The samples were incubated for 60 minutes at 37°C. We loaded 0.2 μL of labeled DNA on 1.5% agarose gel, which was then electrophoresed at 90 V for 3.5 hours, transferred onto Hybond N⁺ nylon membrane (Amersham), exposed to a phosphor-sensitive screen, and analyzed by PhosphorImager (Molecular Dynamics). One microgram of standard DNA molecular weight (λ DNA/HindIII fragments, Gibco BRL) underwent radiolabeling, electrophoresis, and transfer at the same time as the extracted DNA to control the variability of the procedure and to increase reproducibility. The optical density of each lane was calculated for the region between 180 and 1500 bp, then divided by the value obtained by the standard DNA molecular weight of the same bp length.

A total genome search for quantitative trait loci (QTL) of newborn cardiac apoptosis, DNA content, and organ/body weight was performed with 453 markers. Strain distribution patterns of these markers were obtained from the Ratmap World Wide Web site (http://ratmap.gen.gu.se). QTL were identified by means of Pearson’s product-moment correlation analysis, in which genetic marker information is correlated with the quantitative phenotype. Significant correlations suggest associations between markers and the quantitative trait and index the strength of the association. In previous RIS analysis, a statistical significance corresponded to P<0.0003. Instead of using a strict cutoff to define statistical significance, we identified genetic markers that yielded the lowest probability value in Pearson’s correlation analysis.

### In Utero DNA Labeling
Pregnant SHR (SHR/NrCrlBR, n=29) and Wistar-Kyoto rats (WKY) (WKY/NrCrlBR, n=17) (Charles River) were purchased at exactly 14 days of gestation and housed for 2 days before the initiation of labeling. They were then injected subcutaneously, in the neck, with [methyl-³²P]thymidine (0.5 mCi/kg; specific activity, 35 Ci/mmol, ICN Biomedical). One injection was given per day, always at the same time (10 AM), during the last 5 days of gestation (days 16 to 20). This resulted in near total fetal DNA labeling. The newborns (0 to 16 hours) and the 2-, 5-, 8-, 12-, and 20-week-old rats were then killed.

### TABLE 1. Newborn Body Weight, Cardiac Hypertrophy, DNA Fragmentation, and DNA Concentration in Progenitor Strains

<table>
<thead>
<tr>
<th>Trait</th>
<th>SHR</th>
<th>BN.Lx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>6.8±0.22</td>
<td>5.7±0.2</td>
</tr>
<tr>
<td>Heart wt/body wt, mg/g</td>
<td>5.3±0.2</td>
<td>6.0±0.2*</td>
</tr>
<tr>
<td>DNA fragmentation, au</td>
<td>1.2±0.4</td>
<td>0.5±0.2*</td>
</tr>
<tr>
<td>DNA concentration, μg DNA/mg heart tissue</td>
<td>1.5±0.2</td>
<td>2.1±0.1*</td>
</tr>
</tbody>
</table>

Data are shown as mean±SEM. *P<0.05 vs BN.Lx.

To evaluate cell death as a dynamic, DNA disappearance over time was measured by quantifying the decline of [³²P]thymidine–prelabeled DNA from birth to 20 weeks of age. Radioactivity from a given amount of extracted DNA (5 to 100 μg) was counted. Cumulative cell death was expressed as total [³²P]cpm/[^³²P]cpm/μg of DNA×total organ DNA content. A decline with time in total radioactivity of [³²P]prelabeled DNA suggested that some cells died that had been prelabeled in utero.

### Southern Blot Analysis of Telomere Restriction Fragment Length
For determination of telomeres, fresh isolated kidney tissue was obtained from SHR and BN progenitors of various ages, as indicated in Results.

Total genomic DNA was extracted from normotensive and hypertensive rat kidneys with phenol, precipitated with ethanol, and dissolved in Tris-EDTA buffer. For telomere restriction fragment (TRF) analysis, 10 μg of DNA was digested to completion with 50 U of HinI, electrophoresed for 90 hours on 0.3% agarose gel at 20 V, then transferred to a nylon membrane (Hybond N⁺, Amersham Pharmacia Biotech Ltd), according to the manufacturer’s protocol. To exclude a possible effect of DNA degradation, we also electrophoresed 10 μg of undigested DNA. Membranes were hybridized overnight with a [³²P]-labeled (TTAGGG), probe at 45°C, washed with 2× SSC, 1% SDS, followed by 0.1× SSC, 0.1% SDS, and exposed to a phosphor screen. The screen plate was scanned with a Molecular Dynamics Typhoon 8600 Variable Mode Imager, and TRF length was estimated by image analysis software (ImageQuant 5.1).

### Results

**QTL of Altered Balance Between Cell Replication and Apoptosis in Heart**

In progenitor strains, the neonatal heart weight–to–body weight ratio and DNA content were significantly higher in the hypertensive (SHR/Ola) than in the normotensive (BN.Lx/Cub) strain (by 12% and 29%, respectively) (Table 1). In contrast, no significant difference in newborn body weight was observed. On the other hand, the DNA fragmentation index indicative of apoptosis was lower by 64% in SHR hearts compared with BN.Lx. The strain distribution pattern of the DNA fragmentation index (Figure 1A) revealed that there was no significant difference in cardiac newborn apoptosis between BXH and HXB sets of RI strains, which suggests that the Y chromosome does not play an important role in the determination of this phenotype. Similar findings were obtained with the following phenotypes: body weight (not shown), heart weight–to–body weight ratio (Figure 1B), and DNA content (Figure 1C).

Progenitor strains were exceeded outward in a strain distribution pattern. To establish whether neonatal heart hyperplasia, as measured by DNA concentration per milli-
A gram of tissue, is related to the decreased apoptotic index, we estimated the correlation coefficient between the 2 phenotypes in RIS. Our results showed that newborn cardiac DNA concentration correlates negatively with apoptosis in the heart ($r = 0.64$, $P < 0.001$) (Figure 2). These data suggest that neonatal hyperplasia of the heart might be related (for 41% of its variance) to decreased apoptosis.

To identify the genetic determinants of newborn apoptosis and DNA concentration, total genome search was performed. Although no QTL were identified that would satisfy stringent statistical criteria of the genome search allowing a claim for linkage, several suggestive QTLs were demonstrated for the 2 phenotypes (Table 2). Interestingly, a marker of the $\beta$-adrenergic receptor (Adrb2) gene on chromosome 18 showed the strongest correlation with cardiac apoptosis ($r = 0.58$, $P < 0.005$). Within close proximity, a QTL was found to be significant for both cardiac apoptosis ($r = 0.50$, $P = 0.01$) and DNA concentration ($r = 0.50$, $P = 0.01$) at D18 Mit8. By synteny to humans, it is noteworthy that Adrb2 is located on 5q32-q34, where the cyclin G gene maps.

**QTL of Persistence of Neonatal Phenotypes Through Adulthood**

We studied RIS generated from crosses between SHR and BN.Lx that allow longitudinal investigation from birth to adulthood in phenotypes such as heart weight. Total genome scan was performed with nearly 500 markers. Although heart weight was significantly higher in newborn and adult SHR compared with BN.Lx rats, neither neonatal nor adult heart weight correlated with adult systolic or diastolic blood pressures in the RIS panel. Nevertheless, the heart weight/body weight (HW/BW) ratios correlated significantly ($r = 0.41$, $P = 0.03$) between newborns and adults. When we analyzed strains that kept the neonatal phenotype of highest and lowest terciles of HW/BW through adulthood, it correlated even more with adult phenotype ($r = 0.72$, $P = 0.0002$, $n = 16$), and HW/BW best correlated with locus PKATA (Acaa) on chromosome 8 ($P = 0.0003$ for newborn rats, $P = 0.0162$ for adult rats, $n = 16$) (Figure 3). The Acaa region of human chromosome 3 contains hMLH1, a DNA mismatch repair gene involved in apoptosis, CDDC2, a locus for dilated cardiomyopathy, and SCN5A, a voltage-gated sodium channel gene. It should, however, be kept in mind that with this

**TABLE 2. Total Genome Search: Suggestive Quantitative Trait Loci of Newborn Cardiac DNA Fragmentation Index and DNA Concentration**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Chromosome</th>
<th>Marker</th>
<th>$r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA fragmentation index</td>
<td>18</td>
<td>D18Mit3</td>
<td>−0.441*</td>
</tr>
<tr>
<td>DNA fragmentation index</td>
<td>18</td>
<td>GrI</td>
<td>−0.441*</td>
</tr>
<tr>
<td>DNA concentration, µg DNA/mg heart tissue</td>
<td>5</td>
<td>D5ucsf1</td>
<td>+0.484*</td>
</tr>
<tr>
<td>DNA concentration, µg DNA/mg heart tissue</td>
<td>18</td>
<td>D18Mit8</td>
<td>+0.484*</td>
</tr>
</tbody>
</table>

Results of Pearson’s correlation analysis are presented as correlation coefficients ($r$).

* $P < 0.05$, † $P < 0.01$.  

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**Figure 2.** Correlation between neonatal cardiac DNA fragmentation indicative of apoptosis and DNA concentration (µg/mg) in 20 recombinant inbred strains and 2 progenitor strains. There was negative correlation between newborn cardiac DNA concentration and apoptosis in heart ($r = -0.64$, $P < 0.001$). Data shown are mean values of 5 rats per strain.

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**Figure 1.** Strain distribution pattern in recombinant inbred strain panel showing A, DNA fragmentation index correlated from quantification of intensity of radioactivity incorporated into 150- to 1500-bp fragments by means of PhosphorImager as described in Methods. B, HW/BW ratio. C, DNA concentration measured as amount of micrograms of DNA per milligram of cardiac tissue. Black boxes indicate values of progenitor strains. Numbers indicate recombinant inbred strain originating from BN.Lx×SHR cross. A indicates strains originating from crosses from male BN.Lx and female SHR and C from male SHR with female BN.Lx.
relatively low number of RIS, the significance of association is limited. We conclude that although neonatal and adult heart weights (both high in SHR) are determined by different genes, progression from neonatal hyperplasia to adult heart hypertrophy may be mediated by common genes throughout life.

DNA Turnover Studies: Evolution of Kidney Weight

The renal mass index (kidney wt/body wt, mg/g) was already increased at birth in SHR as demonstrated previously by our group. As shown in Figure 4, the initial excess renal mass index diminished with time.

Decline of \([^{3}H]\)Thymidine-Prelabeled DNA With Time

Over the first 8 weeks of life, in utero–labeled DNA decayed progressively in both SHR and WKY kidney. Interpretation of the data were complicated by the fact that from birth to 2 weeks of age, the total radioactivity of \([^{3}H]\)-prelabeled DNA increased (Figure 5). One possibility is that the kidney is able to reuptake \([^{3}H]\)thymidine from the bloodstream. Nevertheless, when this increase was stabilized, there was a decline between 2 and 8 weeks of age in the level of total radioac-

Discussion

We and others have reported cardiac enlargement in newborn SHR when compared with WKY. Increased DNA synthesis and DNA concentration were documented, suggesting cellular hyperplasia in the SHR heart. Similar findings
were reported for newborn organs from stroke-prone SHR, GH (New Zealand), and Lyon hypertensive strains.16 Our results presented here confirm the increased cardiac mass and DNA concentration in newborn SHR compared with BN rats. Moreover, with the use of a set of RIS, we extended these observations by showing that 41% of increased cardiac DNA concentration might be explained by a reduction of apoptotic activity in the neonatal heart. In support of this view, induction of a window of apoptosis precedes the decline of DNA concentration in the heart of SHR treated with several antihypertensive agents.

Croses of hypertensive and normotensive strains have shown that blood pressure may not be the only determinant of heart size in SHR. The independence of heart weight and blood pressure has been well documented. Tanase et al27 reported that for different strains, there is no correlation between blood pressure and left ventricular mass. Then, Harris et al28 determined the genetic loci of growth hormone and guanylyl cyclase to be independently related to heart weight, and we found that a locus on Chr 12 containing HSP with its mutation in the 3′ UTR is a determinant of left ventricular weight in RIS as well as in an F2 cross.12 Pravenec et al29 demonstrated in the same RIS that there are possibly different genetic loci contributing to blood pressure and cardiac mass. Because of the early onset of hyperplasia associated with reduced cardiac apoptosis, it does not appear that these alterations are secondary to the development of hypertension but are rather genetically determined. Indeed, total genome search showed that significant QTL for both cardiac apoptosis (r = −0.50, P = 0.01) and DNA concentration (r = +0.50, P = 0.01) may be located at D18 Mit8. This may indicate the effects of a single gene in this region that influences cardiac hyperplasia and apoptosis. In fact, within close proximity, a marker of the Adrb2 gene on Chr 18 showed the strongest correlation with cardiac apoptosis (r = −0.58, P < 0.005). Although the strength of the correlation is relatively weak, the Adrb2 gene might play a significant role in cardiac apoptosis. In agreement to this view, we demonstrated selective induction of apoptosis in the heart of adult SHR during β-adrenergic blockade with propranolol.

The sympathetic nervous system is an important regulator of blood pressure through alterations in vascular responsiveness, renin release, renal sodium handling, and cardiac output.30 Interestingly, genetic variations in β-adrenergic receptors in an African Caribbean population, leading to attenuated vasodilation, have been associated with increasing total peripheral resistance and hence, blood pressure.31 Furthermore, association and linkage studies in humans have shown the contribution of β-adrenergic receptors to the inheritance of essential hypertension.32,33 Altered contractile function and decreased responsiveness to adrenergic stimulation are among the earliest reported changes with myocardial hypertrophy.34,35 Several abnormalities in the adenyl cyclase cascade have also been documented in SHR with stable hypertrophy.36 Moreover, we have already reported that cAMP production promotes apoptosis of vascular smooth muscle cells in culture.37 Thus, the new hemodynamic role brought by the transition from the fetal to the newborn state might implicate altered sympathetic dysfunction that is responsible for the reduced apoptotic activity seen in the SHR heart. In support of this view, the low ratio of right ventricular mass37 and elimination of arrhythmogenic areas of the cardiac conduction system38 involve postnatal apoptosis. Further investigations will be needed to confirm the association between abnormalities in β-adrenergic receptors and its related pathway (eg, adenyl cyclase activity, cAMP production) in neonatal SHR hearts and the decreased cardiac apoptotic response observed.

The search for syntenic regions in humans allows us to establish a positional candidate gene for neonatal cardiac apoptosis. Indeed, by synteny to human genes, Adrb2 is located on Chr 5 (5q32-q34), where the cyclin G gene is localized. Interestingly, cyclin G was previously found to have a distinct putative binding site for the p53 tumor suppressor gene product.39 p53 has been shown to induce apoptosis in the cardiovascular system of a number of models, including cardiomyocyte apoptosis, by activation of the renin-angiotensin system in cultured cells.40 Use of RIS permitted us to localize genetic determinants associated with several phenotypes, including that of the stress response in rats, on 2 autosomal chromosomes as well as on the Y chromosome.41 We have also mapped QTL for differential stress gene expression, which led us to uncover mutations of the transcription factor HSF1 on Chr 7 as a major determinant of several stress gene expressions.42 We have already proposed the use of newborn and adult RIS as a tool to search for genetic determinants of target organ damage in hypertension.43 This study has determined a locus distinctly related to kidney weight in newborns and in adults as well as loci that appear to be implicated at both ages. Although there is also hyperplasia of the kidney in SHR neonates, blood pressure in itself correlated negatively with adult kidney weight. Thus, for the kidney also, as demonstrated in the current study (Figure 4), the neonatal hyperplasia disappears with age.44 Distinct QTL were significant both in newborn and adult relative weight, such as D3 Mit9, whereas others such as R6 on Chr 1 were significant only in adult relative kidney size. Of interest is that this QTL was found in the
region of susceptibility to renal disease in Fawn-hooded rats.\textsuperscript{45}

In this study, we have demonstrated that kidney hyperplasia, although present in the newborn, disappears with time. Most importantly, however, our prelabeling studies have clearly shown that neonatal cells disappear in the first period of life and are replaced by new ones and that this phenomenon occurs more rapidly and more frequently in hypertension. Further and extensive studies\textsuperscript{44} have permitted us to conclude that the half-life of cells in the kidney but also in the heart and the aorta is actually decreased by $\approx 50\%$ in SHR compared with WKY. This strongly suggests increased cellular turnover. We have demonstrated shortening of the cell cycle by direct observation of cell numbers accumulated in differential phases of the cycle of aortic smooth muscle cells from SHR\textsuperscript{46} with a shortening of the S phase between C and R restriction points, as well as in the G2 M phase. It is of interest that this acceleration of the cell cycle, at least in vitro, can be not only genetically determined, as suggested by its presence in SHR and persistence in long-term culture, but it also can be induced in response to an infectious environment,\textsuperscript{47} actually a very powerful illustration of environmental impact on cell cycle and a good example of environment-gene interaction in hypertension.\textsuperscript{48} This accelerated proliferation may even be a basis of the somewhat increased susceptibility to cancer of certain hypertensives, particularly of the kidney.\textsuperscript{49,50} On the basis of these findings, we have proposed a hypothesis of accelerated senescence with increased cell turnover.\textsuperscript{50} This process would in itself lead to a shortening of telomeres, or mitotic clocks. Actually, Aviv and Aviv\textsuperscript{51} were the first to point out that the length of telomeres, their growth, and aging can all be related to essential hypertension. This led that group to evaluate pairs of twins from the Danish Twin Register and to recently demonstrate that telomere length is inversely correlated with pulse pressure\textsuperscript{52} even in normotensive subjects. Telomeres, TTAGGG tandem repeats that protect the ends of chromosomes, shorten with each cell division. In fetal life and in most cancer tissues, they are kept inactive in somatic cells in adulthood. The shortest telomeres described are those of the kidney.\textsuperscript{53} Shortening of telomeres was documented even in aortic vascular smooth muscle cells of humans with age.\textsuperscript{54}

We are presenting initial new data suggesting that telomeres of genetically hypertensive rats are significantly shorter than those of normotensives. It must be emphasized that rat telomeres are different from those of humans, which are much shorter in length (from 20 to 100 kb in the rat and 3 to 5 kb in humans of [TTAGGG]\textsubscript{4} repeat). However, all these telomeres contain nucleosome arrays.\textsuperscript{23} If our observation is confirmed by further investigation, it would be direct evidence of increased cell turnover and therefore of accelerated cell aging in hypertension. We propose a testable hypothesis that hypertension is a case of accelerated aging, and if proven correct, a novel approach to therapy should be contemplated.

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**References**


Workshop: Excess Growth and Apoptosis: Is Hypertension a Case of Accelerated Aging of Cardiovascular Cells?

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