Vascular Smooth Muscle Cell Hypertrophy and Hyperploidy in the Goldblatt Hypertensive Rat

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SUMMARY. Our major objective in this study was to examine the hypothesis that the aortic smooth muscle cell hypertrophy and hyperploidy observed in previous studies of spontaneously hypertensive rats is not peculiar to that model, but also occurs in Sprague-Dawley rats made hypertensive by a Goldblatt procedure (two-kidney, one-clip model). Flow microfluorometric and microdensitometric analysis of smooth muscle cell DNA content showed a significant increase in the frequency of tetraploid smooth muscle cells from 5.6 ± 0.9% in controls to 14.6 ± 1.9% in hypertensives 1 month after Goldblatt surgery. Neither differences in ploidy nor elevation in blood pressure were apparent 2 weeks after surgery. The frequency of polyploid smooth muscle cells increased with age, duration of hypertension, and level of blood pressure. Analysis of the interrelationship between smooth muscle cell ploidy and hypertrophy in 5-month post-surgery Goldblatts by cytospectrophotometric measurements of the protein and DNA content of individual smooth muscle cells showed that tetraploid and octaploid cells from Goldblatt rats had 64% and 129% greater protein mass, respectively, than diploid cells. In addition, the mean protein mass of smooth muscle cells from Goldblatts was approximately 100% greater than that of normotensive controls, with each of the ploidy classes in Goldblatts having a higher frequency and mass than the corresponding cells in controls. Estimates of cell number per centimeter aortic length, based on measurements of average DNA/cell and total aortic medial DNA, showed no difference between hypertensives and controls. Furthermore, the rate of accumulation of polyploid cells could account for the increased frequency of cells undergoing DNA synthesis as measured by \[^{3}H\text{thymidine}\] autoradiography. Thus, smooth muscle cell hypertrophy, not hyperplasia, was responsible for the increased mass of smooth muscle in aortas of Goldblatt hypertensive rats compared with normotensive controls, and this smooth muscle cell hypertrophy was accompanied by an increase in DNA ploidy. (Circ Res 53: 491-501, 1983)

ARTERIES from hypertensive patients and animals have a greater smooth muscle mass than those of their normotensive counterparts (Furuyami, 1962; Wolinsky, 1970; Jurukova et al., 1976; Wiener et al., 1977; Warshaw et al., 1979; Olivetti et al., 1980; Arner and Uvelius, 1982). Folkow and others (Folkow et al., 1973; Lundgren et al., 1974; Berecek and Bohr, 1977) have suggested, based on hemodynamic evidence, that an increase in medial wall thickness, wall:lumen ratio, and mass of smooth muscle in resistance vessels of hypertensive animals confers both a functional and a structural advantage, so that, at any given level of smooth muscle activation, vascular resistance is greater in hypertensives than controls. Whereas the role of structural decreases in vessel luminal diameter as a factor in increased peripheral resistance in hypertension remains a highly debated issue, there is good evidence supporting the idea that vessel hypertrophy confers a functional advantage. Mivlaney, Halpern and co-workers (Mulvany et al., 1978; Warshaw et al., 1979) found, in studies of isolated mesenteric arteries (150-200 \(\mu\text{m}\) in diameter), that vessels from spontaneously hypertensive (SH) rats developed greater maximal contractile force than those of normotensive Wistar-Kyoto (WKy) controls. The increased force was due to an increase in the mass of smooth muscle in SH rats rather than to a change in the force development per unit of contractile mass. Similarly, Arner and Uvelius (1982) demonstrated that, whereas maximum tension development of abdominal aortic-segments was greater in SH rats than in WKy controls, no differences were found in either force development per unit cell area or in maximum shortening velocity. Data thus suggest that the characteristics of actomyosin interaction in SH and WKy rats are similar, and that differences in force-generating capacity are due to differences in total contractile mass.

The studies above point out the potential significance of changes in wall mass in hypertension and emphasize the importance of understanding the cellular basis of medial hypertrophy. In recent studies (Owens et al., 1981; Owens and Schwartz, 1982) we demonstrated that the increased smooth muscle mass in aortas of SH rats relative to WKy controls was due solely to smooth muscle cell hypertrophy without detectable hyperplasia. Our findings were recently confirmed in morphometric studies by Olivetti et al. (1982), who found that smooth muscle...
cell number did not differ, between aortas of SH and WKy rats, and that differences in mass of smooth muscle were due to cellular hypertrophy. An additional finding in our studies was that smooth muscle cell hypertrophy was accompanied by an increase in DNA ploidy with up to 30% of the smooth muscle cell population in SH rats being polyploid. Thus, the increase in DNA synthesis and content in an aortic coarctation model of hypertension in rabbits (Bevan, 1976; Bevan et al., 1976) in the absence of any increase in cell number in this same model in rats (Olivetti et al., 1980) could be accounted for by changes in smooth muscle cell ploidy. A critical remaining issue, however, is whether changes in smooth muscle cell ploidy are unique to a genetic model of hypertension such as the SH rat. Thus, in the present study, we have examined whether smooth muscle cell hypertrophy and hyperploidy also occur in normotensive rats made hypertensive by a Goldblatt procedure (Goldblatt et al., 1934). In addition we have studied the time-course of changes in ploidy and hypertrophy relative to changes in blood pressure, examined interrelationships between smooth muscle cell hypertrophy, hyperploidy, and blood pressure, and assessed the relative roles of smooth muscle cell hypertrophy vs. hyperplasia in aortic medial thickening in Goldblatt hypertension.

Methods

Animals

Male, Sprague-Dawley rats, obtained from Tyler Laboratories, were used throughout these experiments. Rats were maintained on normal sodium (110 mEq/µg) chow and both food and water were administered ad libitum.

Blood pressures were measured via a tail-cuff method, using a piezoelectric pressure transducer (Buffington Clinical Systems). Animals were lightly anesthetized with ether, and the animal’s temperature was maintained with an electric heating pad. Although pressures measured in this manner are slightly lower than measurements in conscious animals, the variance between measurements is less (unpublished observations). All animals were identified by number, and the blood pressure technician was not aware of experimental groupings. Heart weight:body weight ratios were determined at sacrifice, as previously described (Owens and Schwartz, 1982).

Surgical Procedure: Induction of Goldblatt Hypertension

Surgery was performed on rats that were 4–6 weeks of age. Two blood pressure measurements were taken during the week before surgery. Animals were divided randomly into control and Goldblatt groups, and were anesthetized with sodium pentobarbital (24 mg/kg, intraperitoneal). The left renal artery was exposed through a midline incision and was dissected free. A 0.23-mm silver clip was closed with 000 monofilament nylon. A midline incision

or biweekly thereafter. Operated animals were considered hypertensive when their systolic blood pressure exceeded 140 mm Hg for three consecutive readings. The only exception to this was with the 2-week post-surgery group in which all operated animals, other than those showing left kidney necrosis on necropsy, were grouped as ‘hypertensive’ despite the fact that their pressures were not yet elevated (Fig. 1).

At either 2 weeks, 1 month, 3 months, or 5 months after surgery, rats were killed by cervical dislocation following ether anesthesia. The thoracic aorta and heart were excised, perfused with Hanks’ solution (Gibco) (pH 7.4) and placed in fresh Hanks’. Hearts were cleaned of loose connective tissue, dried for 48 hours, and weighed. An intima-medial preparation of the aorta was made by carefully dissecting away the adventitia and intercostal arteries (Wolinsky and Daly, 1970). The endothelium then was scraped off with a piece of Teflon. Resultant medial preparations were used for subsequent isolation of smooth muscle cells and for flow cytometric ploidy determinations described below.

Evaluation of Aortic Hypertrophy

A quantitative assessment of the difference in mass of smooth muscle between thoracic aortas of hypertensive and normotenotive rats was obtained by the method of Anversa et al. (1980). This involved multiplying the wet weight (of aortic medial preparations) per vessel length by the fraction of aortic media occupied by smooth muscle (i.e., volume density). Several vessel segments for transmission electron microscopy were taken from the midpoint of the thoracic aorta, fixed in 1% paraformaldehyde, 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0), placed in 1% osmium tetroxide for 2 hours, dehydrated, en bloc stained with 3% uranyl acetate, and embedded in Epon. Sections were cut and post-stained with uranyl acetate and Reynolds’ lead citrate. Volume densities were determined on electron micrographs by standard point-counting techniques (Weibel and Bolender, 1973). A minimum of 15 electron micrographs (approximately 1325 µm²/ micrograph) were analyzed from each animal. By this sampling method, the variability between electron micrographs from individual animals (expressed as the standard deviation) was 13%.

FIGURE 1. Time-course of systolic blood pressure changes in two-kidney, one-clip Goldblatt rats and control non-clipped rats. Blood pressures were measured via a tail-cuff method under light ether anesthesia. Each data point represents the mean value (± SE) at each respective time point for animals utilized in this study (i.e., 34 Goldblatts and 28 controls).
Smooth muscle cell nuclei were prepared for flow cytometric evaluation of DNA ploidy as previously described (Owens et al., 1981). In brief, medial thoracic aortic preparations were incubated (37°C, 5% CO₂, 95% air) for 1.5 to 2 hours in a collagenase and elastase solution. Fetal calf serum (20% vol/vol) was added and the tissue centrifuged. This cell-tissue pellet then was suspended in nuclear isolation medium containing Tris-buffered isotonic saline (pH 7.0), 10 μg/ml diamidino-phenylindole (DAPI), 0.6% Nonidet P-40 (vol/vol) 1.0 mM CaCl₂, 21 mM MgCl₂, and 0.2% bovine serum albumin (wt/vol). Samples were vortexed gently and cooled on ice. Suspensions were filtered through 50 μm plastic mesh to remove tissue debris. Nuclei then were syringed three times through a 26-gauge needle to ensure single nuclei. DAPI-stained nuclei were observed in a Leitz fluorescent microscope to determine the percentage of nuclei aggregates. If greater than 2%, nuclei were resyringed and examined again. Nuclear yields were about 25%, as determined by DNA assay. As reported previously (Owens and Schwartz, 1982), the results obtained with this assay agree closely with those obtained by Feulgen-DNA microdensitometric analysis in tissue sections. Thus, the flow cytometric assay provides a valid measure of the frequency of polyploid smooth muscle cells in the intact aorta. Measurement, acquisition, and analysis of DNA content was done on an ICP-22 flow cytometer (Ortho Diagnostic Systems, Inc.). Cell cycle compartments were estimated by an adaptation of the method of Dean and Jett (1974), including fitting and subtraction of an exponential noise background. Non-linear least squares fitting was by the method of Marquardt (1963).

Aortic smooth muscle cells for microdensitometric evaluation of cellular hypertrophy (protein) and hyperplordy (DNA), and for autoradiography were enzymatically dissociated into single cells using methods previously described (Owens et al., 1981; Owens and Schwartz, 1982). Isolated cells were fixed overnight in 10% neutral-buffered formalin. Cells were then washed with distilled water, and cell smears made on glass slides and air-dried.

### Protein: DNA Microdensitometry

To examine the relationship between smooth muscle cell hypertrophy and hyperplordy, and to provide a quantitative measure of the degree of cellular hypertrophy, we made simultaneous microdensitometric determinations of the protein and DNA content of individual smooth muscle cells, as previously described (Owens and Schwartz, 1982). In brief, smooth muscle cell smears, prepared as described above, were stained for Fuaggén DNA reaction product and naphthol-yellow S-protein product were sufficiently separated to permit simultaneous measurements of DNA and protein in the same cell (Owens and Schwartz, 1982). Protein measurements were made at the naphthol-yellow S absorption maximum of 445 nm, whereas DNA measurements were made at 565 nm. Standards of chick red blood cells (2.5 pg DNA) and cultured rat aortic smooth muscle cells were processed simultaneously. Cellular protein content of standards was determined by the method of Lowry et al. (1951). By comparison to standards, the absolute quantity of DNA and protein for individual smooth muscle cells was calculated.

### [3H]Thymidine Autoradiography

The frequency of cells undergoing DNA synthesis was determined by [3H]thymidine autoradiography on smooth muscle cell dispersions, as previously described (Owens and Schwartz, 1982). Animals for autoradiography were given a single intraperitoneal dose of [3H]thymidine (50 μCi/100 g body weight; 6.7 Ci/mM; New England Nuclear) 1 hour before sacrifice. This essentially gives an instantaneous estimate of the frequency of cells in S phase of the cell cycle. Autoradiography was done on smooth muscle cell dispersions prepared as described above. This method has the advantage over conventional autoradiography on tissue sections of permitting analysis of large numbers of cells—a necessity when dealing with very low labeling frequencies. Slides containing cell dispersions were coated with autoradiographic emulsion (Kodak, NTB-2) exposed for 2 weeks (4°C), developed by standard procedures, and stained with hematoxylin. The frequency of labeled cells was determined using counting techniques for determining the frequency of a rare event, as described by Schwartz and Benditt (1973). This involves counting the total number of labeled cells (Nₖ) per slide. An estimate of the total number of cells per slide (Nₖ) was obtained by counting the number of cells per microscopic field in 40 randomly selected fields. This then was multiplied by the number of fields per slide to give Nₖ. Thymidine index was estimated as Nₖ/Nₖ. An average of 50,000 cells were counted per animal.

### Determination of Smooth Muscle Cell Number

To assess whether smooth muscle cell hyperplasia occurred in aortas of Goldblatt hypertensive rats, an estimate of smooth muscle cell number per unit aortic length was obtained as previously described (Owens et al., 1981). In brief, total DNA content of aortic tissue was determined on medial preparations of thoracic aortas by the diphenylamine method of Burton (1955). DNA content was expressed per unit in situ vessel length. Smooth muscle cell number then was calculated by dividing the DNA content per aortic length by the average DNA per smooth muscle cell determined by flow cytometry.

### Results

#### Blood Pressure

Figure 1 illustrates the time course of blood pressure changes after partial constriction of the renal artery. Blood pressures were typically elevated between 16 and 20 days following surgery. Thirty-four percent of the animals which were clipped did not show a blood pressure increase within 30 days (i.e., non-responders), and were discarded. It should be noted, however, that selection of hypertensive animals was not possible with the 2-week postsurgery group, because their pressures were not yet
TABLE 1
Blood Pressure, Heart Weight, and Heart Weight:Body Weight Ratio of Goldblatt Hypertensive (GB) and Control Sprague-Dawley Rats

<table>
<thead>
<tr>
<th>Time post-clipping</th>
<th>Blood pressure† (mm Hg)</th>
<th>Average dry heart wt (g)</th>
<th>Heart wt ( \times 10^{-4} ) body wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-week, GB ( n = 8 )</td>
<td>128.4 ± 7.6</td>
<td>0.279 ± 0.010</td>
<td>7.88 ± 0.24</td>
</tr>
<tr>
<td>2-week, control ( n = 6 )</td>
<td>120.3 ± 7.7</td>
<td>0.269 ± 0.014</td>
<td>7.62 ± 0.30</td>
</tr>
<tr>
<td>1-month, GB ( n = 7 )</td>
<td>154.0 ± 1.9‡</td>
<td>0.412 ± 0.017§</td>
<td>10.06 ± 0.74‡</td>
</tr>
<tr>
<td>1-month, control ( n = 5 )</td>
<td>112.2 ± 3.9</td>
<td>0.268 ± 0.004</td>
<td>7.34 ± 0.19</td>
</tr>
<tr>
<td>3-month, GB ( n = 6 )</td>
<td>163.3 ± 6.6‡</td>
<td>0.470 ± 0.019‡§</td>
<td>10.18 ± 0.61‡</td>
</tr>
<tr>
<td>3-month, control ( n = 6 )</td>
<td>116.7 ± 5.7</td>
<td>0.375 ± 0.029</td>
<td></td>
</tr>
<tr>
<td>5-month, GB ( n = 6 )</td>
<td>163.7 ± 3.1‡</td>
<td>0.477 ± 0.030‡</td>
<td>8.83 ± 0.56‡</td>
</tr>
<tr>
<td>5-month, control ( n = 6 )</td>
<td>114.7 ± 2.2</td>
<td>0.366 ± 0.015</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM.
† Time following surgical clipping of the renal artery of 4- to 6-week-old rats. Controls were age-matched.
‡ Significantly greater than age-matched control (\( P < 0.025 \), analysis of variance).
§ Significantly greater than 1-month Goldblatt (\( P < 0.05 \), analysis of variance).
|| Significantly different from the 2-week and 1-month control groups (\( P < 0.025 \), analysis of variance).

Smooth Muscle Cell Polyploidy and Rates of DNA Synthesis

Results of flow cytometric evaluations of the frequency of polyploid cells (Figs. 2 and 3) showed that substantial numbers of tetraploid (4C) aortic smooth muscle cells were present in both Goldblatt hypertensive and normotensive rats. A marked increase in the frequency of polyploid smooth muscle cells was apparent by 1 month following renal-artery clipping. Whereas no significant differences were seen at 2 weeks post-surgery, it should be noted that none of these animals were as yet hypertensive, and that some animals included in analysis probably would never have developed hypertension. As observed previously in our studies of SH rats (Owens and Schwartz, 1982), the frequency of polyploid smooth muscle cells increased with age, although, for controls, ploidy did not increase significantly (analysis of variance) beyond 3 months following sham operation—i.e., after animals were more than 4–5 months old. Linear regression analysis showed a significant correlation between ploidy and animal age in both Goldblatt hypertensive (\( r = 0.83, P < 0.001 \)) and control groups (\( r = 0.85, P < 0.001 \)). The fractional increase in polyploid smooth muscle cells with age (i.e., the slope of the linear regression line) was more than four times greater in hypertensives (slope = 8.27%/month) than in controls (1.96%/month). A significant linear relationship was also found between the frequency of polyploid smooth muscle cells and systolic blood pressure (Fig. 4) for combined Goldblatt and control animals (\( r = 0.63, P < 0.001 \)), for Goldblatt animals alone (\( r = 0.56, P < 0.005 \)), but not for controls alone (\( r = 0.02, \) not significant). When the relationship between ploidy and blood pressure was analyzed in animals of the same age, significant relationships were observed for the 1-month (\( r = 0.80, P < 0.01 \)), 3-month (\( r = 0.80, P < 0.01 \)), and 5-month (\( r = 0.83, P < 0.001 \)) groups.
FIGURE 2. Representative DNA determinations of individual smooth muscle cell (SMC) nuclei by flow microfluorimetry for a 5-month post-clip Goldblatt hypertensive rat (upper) and a normotensive age-matched control. Nuclei were stained for DNA with dianiadphene-indole as described by Owens et al. (1981). Distinct populations of diploid (7 pg DNA) and tetraploid (14 pg DNA) smooth muscle cells are apparent.

0.88, P < 0.01), and 5-month (r = 0.83, P < 0.02) post-clip groups but not for the 2-week group (r = 0.40, not significant). Despite this apparent relationship between polyploidy and blood pressure, it is interesting that, although ploidy increased by more than 200% (P < 0.001, analysis of variance) in Goldblatt rats between 1 and 5 months post-clipping (Fig. 3), there was not a corresponding change in blood pressure during this interval (Fig. 1). This emphasizes the importance of an age-pressure component and shows that blood pressure appears to peak much sooner than does the polyploid response.

To determine the possible contribution of proliferating smooth muscle cells to the tetraploid population measured by flow cytometry, the incidence of cells in S phase of the cell cycle was measured by [3H]thymidine autoradiography. This was done in the 1-month post-clip group, only since it would be expected to be highest at this early time point (Bevan, 1976). The frequency of cells in S phase (mean ± SEM) was 3.50 ± 0.67 (× 10^-4) and 1.26 ± 0.27 (× 10^-4) for Goldblatt hypertensive and normotensive controls, respectively. This difference was significant (P < 0.025, analysis of variance). The fact that labeling frequencies were on the order of 10^-4 demonstrated that the high frequency of tetraploid cells observed by flow cytometry did not represent the G2 component of a rapidly proliferating population of cells. Likewise, an extremely low S phase population was evident by flow cytometry (Fig. 2) in all age groups studied, in that cells with intermediate DNA content between diploid and tetraploid were not observed.

FIGURE 3. Frequency of tetraploid aortic smooth muscle cells in Goldblatt hypertensive and in normotensive controls as determined by flow microfluorimetry. Goldblatts were significantly greater than controls (P < 0.001, analysis of variance) at 1, 3, and 5 months post-clipping. In addition, there was a significant increase in tetraploidy with age at all time points examined for Goldblatt rats but only between 1 and 3 months post-clipping (i.e., from 3 to 5 months of age) for controls (P < 0.025, analysis of variance).

FIGURE 4. Linear regression of the frequency of tetraploid aortic smooth muscle cells to systolic blood pressure for both Goldblatt and control rats. Blood pressures utilized represented the mean value of at least two determinations taken during the week before sacrifice for the 2-week post-clip group, during the 2 weeks before sacrifice for the 1-month post-clip group, and during the month before sacrifice from the 3- and 5-month post-clip groups.
It is interesting to note that, if the rate of DNA synthesis measured at 1 month were to continue until 3 months post-clipping, and assuming an S phase duration of approximately 8 hours (Ross et al., 1978), the calculated change in the fraction of tetraploid smooth muscle cells in Goldblatts \((3.50 \times 10^{-4} \times 24 \text{ hr/8 hr } \times 60 \text{ days})\) and controls \((1.26 \times 10^{-4} \times 24 \text{ hr/8 hr day } \times 60 \text{ days})\) would be 6.3% and 2.3%, respectively. The observed change in the fraction of tetraploids during this interval (Fig. 3) was 9.1% for Goldblatts and 3.8% for controls. Thus, the change in the fraction of polyploids could account for the number of cells expected to undergo DNA synthesis during this interval. In other words, data suggest that the increased smooth muscle cell DNA synthesis observed was associated with generation of polyploid cells rather than more cells. This idea is supported by results of cell number determinations discussed elsewhere.

Interrelationship between Smooth Muscle Hypertrophy and Hyperploidy: Simultaneous Protein and DNA Measurements

To examine the interrelationship between cellular hypertrophy and hyperploidy, and to provide a quantitative measure of the degree of smooth muscle cell hypertrophy, protein and DNA content of freshly isolated smooth muscle cells were determined by scanning microdensitometry (Figs. 5 and 6). These measurements are extremely time-consuming and were performed only on cells from the 5-month post-clip group. Although there was considerable overlap in protein content between ploidy classes (Fig. 5), polyploid cells from both hypertensive and normotensive rats had a greater average protein mass than diploid cells (Fig. 6). In addition, diploid and tetraploid cells from Goldblatt hypertensive rats had 76% and 164% greater protein mass respectively than the corresponding cells in normotensive controls. Thus, there was also an increase in cellular mass which was independent of a change in cellular ploidy. Data for octaploid cells showed a high variance, but sample size was small (8 in a total of 380 Goldblatt cells measured, and 0 in 487 for control cells).

Based on the frequency and respective cellular mass of each ploidy class, the average mass for smooth muscle cells from Goldblatt and normotensive rats was calculated (shaded bars figure 6). As shown, the average protein mass of smooth muscle cells from 5-month post-clip Goldblatt rats was more than twice that in age-matched normotensive controls.

Role of Smooth Muscle Cell Hypertrophy vs. Hyperplasia in Vessel Hypertrophy

The relative role of smooth muscle cell hypertrophy vs. hyperplasia to aortic wall hypertrophy was evaluated in 3-month post-clip Goldblatt hypertensive rats compared to their normotensive controls. A quantitative estimate of the degree of vessel hypertrophy was obtained by the method of Anversa et al. (1980), in which aortic medial wet weight (mg)
is multiplied by the fraction of the media occupied by smooth muscle. Results (Table 2) showed a 32.2% and 39.7% increase ($P < 0.01$, analysis of variance) in aortic mass and smooth muscle mass, respectively, in Goldblatt hypertensive rats, compared with controls. Results are in agreement with those of numerous other investigators (Wolinsky, 1970; Wiener et al., 1977) who have demonstrated an increase in aortic smooth muscle mass in Goldblatt hypertensive rats.

To assess the relative roles of smooth muscle cell hypertrophy vs. hyperplasia to this increase in total mass of smooth muscle, we made DNA and cell number determinations on aortic medial preparations from these same animals. Whereas the DNA content per unit in situ length was increased by 20% ($P < 0.05$, analysis of variance) in Goldblatts, compared with controls, the smooth muscle cell number per unit vessel length (calculated by dividing total medial DNA by the average DNA/cell determined from flow cytometric measurements of ploidy) was not different. Average DNA/cell was 8.7 pg for Goldblatts, and 7.75 pg for controls. Results thus showed that the increased mass of smooth muscle in aortas of Goldblatt hypertensive rats compared with controls occurred without a detectable increase in cell number.

**Discussion**

As observed previously in our studies of the SH rat (Owens et al., 1981; Owens and Schwartz, 1982), smooth muscle cell hypertrophy and hyperplody also occurred in Goldblatt hypertension. The rate of increase in polyplody cells in Goldblatt hypertensive rats was very similar to that in SH rats, suggesting that there is not a difference in inherent propensity for development of smooth muscle cell ploidy between these models. This is important, since it shows that changes in smooth muscle cell ploidy in conjunction with cellular hypertrophy are not a genetic anomaly unique to the SH rat. Although Olivetti et al. (1980) did not measure DNA contents, they did observe an increase in nuclear volume in rats made hypertensive by aortic coarctation, suggesting that changes in ploidy occur in this hypertensive model, as well.

In previous studies we demonstrated that differences in mass of aortic smooth muscle between SH rats and WKy controls were due solely to cellular hypertrophy without detectable hyperplasia (Owens et al., 1981; Owens and Schwartz, 1982). Recent morphometric studies (Olivetti et al., 1982) likewise showed that, whereas smooth muscle cell hyperplasia occurred in both SH and WKy rats between 21 and 45 days of age, differences in mass between SH and WKy rats were due to cellular hypertrophy without detectable differences in cell number. Similarly, this same group also reported (Olivetti et al., 1980) that smooth muscle cell hypertrophy, not hyperplasia, was responsible for aortic medial thickening in rats following subdiaphragmatic aortic stenosis. Three lines of evidence are presented in this study to support the idea that changes in medial smooth muscle mass in Goldblatt hypertension result from cellular hypertrophy, not hyperplasia. First, based on biochemical measurements of total vessel DNA and flow cytometric measurements of cellular DNA, we were unable to detect an increase in smooth muscle cell number in hypertrophied aortas from Goldblatt hypertensive rats compared with normotensive controls. Second, evaluation of smooth muscle cell hypertrophy by cytospectrophotometric measurements of the protein content of individual cells showed a 100% increase in average cellular mass in Goldblatts compared with controls. Third, the rate of accumulation of polyplody cells in Goldblatt rats could account for the increased incidence of cells undergoing DNA synthesis in these animals. These data thus provide very strong evidence that differences in mass of aortic smooth muscle between Goldblatt hypertensive and normotensive controls are also due to cellular hypertrophy rather than hyperplasia. Whereas the studies
above are in apparent contrast with a number of studies that have implicated smooth muscle cell hyperplasia in vessel thickening, the results of this study demonstrate how the increased DNA synthesis and/or content observed by Bevan and others (Crane and Dutta, 1963; Bevan et al., 1976; Bevan, 1976; Seidel, 1979; Rorive et al., 1980) could be accounted for by increases in smooth muscle cell ploidy. Additional studies implicating hyperplasia are based on rather imprecise measurements of cell cross-sectional areas or on assumptions that nuclear size, shape, and orientation are the same in SH and WKy rats (Mulvany et al., 1979; Warshaw et al., 1979; Arner and Uvelius, 1982).

Our observation that the growth response of vascular smooth muscle cells in hypertension is limited to cellular hypertrophy rather than hyperplasia has important implications with regard to smooth muscle cell growth control. Cells hypertrophy in hypertension, whereas, in lipid and traumatic models of atherogenesis, cells undergo proliferation (Imai et al., 1970; Spaet et al., 1975; Thomas et al., 1976). Chamley-Campbell et al. (1979, 1981) have presented evidence showing that smooth muscle cells do not replicate in cell culture until they have lost much of their contractile apparatus. Based on this and other data, the Campbells have suggested that smooth muscle cells normally exist in a nonproliferative, contractile state, and that the cells must first dedifferentiate before proliferating. The evidence presented in this study, as well as data of Olivetti et al. (1982), indicate that cellular hypertrophy is the principal mode of smooth muscle growth in both normal and hypertensive adult rats. It is thus interesting to speculate that the hypertrophic growth response of smooth muscle cells may represent a mechanism whereby smooth muscle mass can increase without loss of differentiated function. There is precedent for this idea in both cardiac and skeletal myocytes, which respond to 'hypertrophic' stimuli by increasing cell mass without loss of differentiation (Carlson, 1973; Baserga, 1976; Rumyantsev, 1977). Furthermore, it is interesting that, like smooth muscle cells, significant numbers of normal adult cardiac myocytes are polyploid and that the incidence and degree of polyploidy increases in myocardial hypertrophy (Sandritter and Scomazzoni, 1964; Grove et al., 1969). Whereas our speculation remains to be tested, it is clear from our observations that the problem of initiation of growth of the smooth muscle cell must be examined in a new light.

We should also point out that the cell cycle status of the polyploid smooth muscle cell has not yet been clearly established. We do know that tetraploid cells do not represent the G2 plus mitotic component of a rapidly proliferating cell population, since the frequency of smooth muscle cells undergoing DNA synthesis was of the order of $10^{-4}$. However, there are at least two other possibilities. First, they could represent cells arrested in the G2 phase of the cell cycle. Arrest of cells in G2 has been shown to occur in a number of other cells and tissues (Gelfant, 1962; Gordon and Lane, 1980). A G2-arrested cell would contain 4C DNA but 2N chromosomes (where C = haploid DNA content, N = haploid number of chromosomes) and, upon appropriate stimulation, would be capable of undergoing mitosis without prior DNA synthesis. The increased protein content which we observed in polyploid cells would be quite consistent with the increase in cell mass known to occur as cells progress through the cell cycle (Darzynkiewicz et al., 1979). A second possibility is that our polyploid cells are true tetraploids. A true tetraploid is a cell which, in the resting phase of the cell cycle (Go), contains double the number of chromosomes (4N) and, upon appropriate mitogenic stimulation, would initiate DNA synthesis rather than mitosis. The fact that we observed a small number of octaploid cells by microspectrophotometry suggests that at least some 4C cells are true "tetraploids" and can reenter S phase without undergoing mitosis.

An obvious question that needs to be addressed deals with the mechanism responsible for initiating the smooth muscle growth response. It has been suggested that the medial hypertrophy that occurs in vessels of hypertensive patients and animals represents an adaptive response to normalize vessel wall tension. Whereas the demonstration in this study of a significant linear relationship between the frequency of tetraploid-hypertrophied vascular smooth muscle cells and blood pressure would be consistent with this idea, our data also suggest that polyploidy is more than just a simple response to increased pressure. For example, we observed a significant increase in the frequency of polyploid smooth muscle cells in both hypertensive and normotensive rats between 1 and 5 months post-surgery (Fig. 3), despite the fact that blood pressures did not change during this same interval (Fig. 1). Thus, if cellular hypertrophy is an attempt to normalize tension, then it appears to be a very slow adaptive response. Our demonstration of a higher correlation between polyploidy and animal age than between polyploidy and blood pressure emphasizes the importance of a time-pressure component. Finally, our previous observations (Owens and Schwartz, 1982) of polyploid cells in veins, although at equal frequencies in SH and WKy rats, also suggest that hypertrophy and hyperplpoy represent a response to factors other than just increased pressure.

Whereas the studies reported here have focused on a large vessel, there is reason to suspect that similar changes occur in resistance vessels. There is good evidence that increases in wall thickness and smooth muscle mass occur in microvessels of hypertensive animals (Ichijima, 1969; Mulvany et al., 1978; Warshaw et al., 1979) and man (Furuyama, 1962). Mulvany, Halpern and co-workers (Mulvany et al., 1978; Warshaw et al., 1979, 1980) have consistently shown an increase, although not always significant, in cross-sectional area of individual smooth muscle cells in mesenteric arteries of SHR.
suggesting that cellular hypertrophy is present. Bevan (1976) examined rates of DNA synthesis by \[^{3}H\]thymidine autoradiography in a variety of vessels from rabbits made hypertensive by aortic stenosis. Although she did not collect quantitative data on DNA replication in microvessels, she did suggest, based on inspection of autoradiograms, that increases in DNA synthesis occur in small vessels as well. Whether this reflected DNA endoreplication or cellular hyperplasia remains to be determined. In preliminary studies (unpublished observations), we have observed that polyploid smooth muscle cells are present in mesenteric arteries (approximately 150 \(\mu m\) in diameter) of 3-month Goldblatt hypertensive rats. However, we do not have quantitative data on their frequency at this time.

Assuming that medial hypertrophy occurs in pressure-regulating vessels of hypertensive animals, what role might it play in the hypertensive process? In this study we were not able to detect a significant increase in the frequency of polyploid cells prior to onset of hypertension. However, it is likely that nearly half of the 2-week Goldblatt rats analyzed would not have developed hypertension. Thus it is not entirely clear whether changes in ploidy preclude, parallel, or follow development of hypertension. As noted previously, an increase in mass of smooth muscle would confer a mechanical advantage so that, for a given level of smooth muscle cell activation, total force development would be greater in hypertensives than controls (Folkow et al., 1973; Berecek and Bohr, 1977; Mulvany et al., 1978; Warshaw et al., 1979). Whether this would in itself result in an increase in vascular resistance as suggested by Folkow et al. (1973), or whether it is simply an adaptive response to normalize wall stress, is controversial. In either case, it would be an extremely important component of a chronic elevation in vascular resistance.

Finally, we should comment on the significance of a change in smooth muscle mass due to cellular hypertrophy and hyperplasty as opposed to cellular hyperplasia. Whereas an increase in cell number would not be expected to result in a change in individual cell function, there is reason to suspect that cellular enlargement or hypertrophy may be associated with functional and/or structural alterations. Hypertrophy of cardiac myocytes, for example, is associated with profound alterations in myosin isozymes and contractile properties (Alpert and Mulieri, 1982, Litten et al., 1982). Data of Berner et al. (1981) suggest that smooth muscle cell hypertrophy may also be associated with major changes in cellular protein composition. Using morphometric techniques, they found a decrease in the relative number of myosin filaments along with a 250% increase in intermediate filaments in hypertrophic venous smooth muscle of the ligated portal vein. Consistent with this, Johansson (1976) and others (Uvelius et al., 1981) have reported a decreased force-generating capacity, a reduced frequency of spontaneous contractions, and a lower sensitivity to exogenous norepinephrine in this same model. Using microchemical techniques, Brayden et al. (1983) found decreased actin and myosin content and decreased stress-generating capabilities in cerebral (170 \(\mu m\) internal diameter) but not mesenteric arteries of SH rats, compared with WKy controls. Our data (Owens et al., 1981) and those of Seidel (1979) showed that actin and myosin content increased proportionately with other cellular proteins in hypertrophic aortic smooth muscle cells from SH rats. A decrease in the surface area:volume ratio as cells enlarge (Gabella, 1979), could influence cell function through a variety of mechanisms, including alterations in membrane receptor number and/or density. It is interesting to speculate that development of smooth muscle cell hypertrophy and hyperplasty might be related to some of the functional alterations reported for vascular smooth muscle in hypertensive animals (Holloway and Bohr, 1973; Jones, 1973; Hansen and Bohr, 1975; Lais and Brody, 1975). The change in ploidy would be particularly important, since changes in DNA, unlike changes in other macromolecules, are irreversible except by cell death. Although we have not as yet determined whether hypertrophy and hyperplasty are reversible by antihypertensive therapy, Van der Heijden and James (1975) have shown that the polyploidy which occurs in uterine smooth muscle during pregnancy does not reverse postpartum. The polyploidy change observed in this and previous studies (Owens et al., 1981; Owens and Schwartz, 1982) may relate to observations that treatment of animals with chronic hypertension (Wolinsky, 1971; Hamilton, 1975; Lundgren and Weiss, 1979) is not totally effective in reversing structural or functional vascular alterations. Thus, the development of smooth muscle cell hyperplasy may represent a fixed change related to the establishment of a chronic hypertensive state.

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