Elastin and Collagen Accumulation in Rabbit Ascending Aorta and Pulmonary Trunk during Postnatal Growth

Correlation of Cellular Synthetic Response with Medial Tension

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SUMMARY Absolute and relative quantities of elastin, collagen, and DNA in anatomically defined segments of rabbit ascending aorta (AA) and pulmonary trunk (PT) were compared at intervals from birth to 2 months of age. Identical in size, weight, and composition at birth, the vessels maintained similar lengths and diameters at each age but diverged markedly in weight and scleroprotein content after 1 week. By 2 months, 3 times as much elastin and 1.7 times as much collagen had accumulated in the AA as compared to the PT. By contrast, the increase in total DNA content was the same for both segments. Differences in total fibrous protein accumulation, total elastin accumulation, and elastin content relative to DNA paralleled differences in estimated total medial tangential tension. Proportions of elastin and collagen relative to dry weight increased markedly only between 1 and 2 weeks of age and not thereafter despite continuing rapid growth, steadily increasing medial tension, and increasing total scleroprotein content. Thus, medial cells were capable of adapting their quantitative scleroprotein synthetic response to differences in medial tension throughout growth but established a fixed qualitative response within 2 weeks.

IN GENERAL, normal mammalian arterial vessels with large diameters, whose walls sustain relatively high total tangential tensions, have relatively thick medias containing abundant collagen and elastin fibers, whereas vessels of small diameter, bearing lower tensions, have correspondingly thinner walls containing less fibrous protein. A comparative study of the adult mammalian aorta revealed that the total number of medial fibroelastic layers (lamellar units) increases linearly with radius, so that the average tension per medial lamellar unit was nearly constant at about 2,000 dyn/cm regardless of species. Similar relationships prevail for other major homologous arteriologies. Hypertension in young rats and in monkeys results in increased absolute quantities of aortic collagen and elastin although relative proportions of these substances remain unchanged. Medial cross-sectional area increases approximately 0.1 mm² for each tension increment of 5,000 dyn/cm, but the number of medial lamellar units remains the same as in normotensive controls. These data suggest that both genetic and mechanical factors are operative in determining medial structure and composition.

Since any alterations of arterial architecture and composition that occur immediately after birth do so in the presence of rapidly changing pressure and flow, we reasoned that a quantitative investigation relating medial fibrous protein accumulation to medial tension during this period might provide insights into the nature of the medial synthetic response. We compared the ascending aorta (AA) with the pulmonary trunk (PT) because these vessels remain very nearly the same in length and diameter as they grow despite the development of marked differences in medial thickness, structure, and composition soon after birth as pressure rises in the systemic circulation and falls in the pulmonary circuit. That a comparative quantitative chemical study of these vessels could provide insights into vessel adaptation to changing tensions was suggested by earlier observations that persistence of elevated pulmonary pressures in both congenital heart disease and other states is associated with the persistence of "aortic" medial structure and composition in the PT.

In the present report we will show that in rabbits from birth to 2 months of age (1) differences in total fibrous protein accumulation for AA and PT segments correspond closely to developing differences in medial tension, and (2) despite the continuing rise in medial tension and scleroprotein content during this period, proportions of elastin and collagen relative to dry weight do not increase after 2 weeks. We also will present evidence that differences in accumulation of fibrous proteins in response to tension probably reflect differences in the elicited synthetic response of individual cells.

Methods

Ascending aortas and pulmonary artery trunks were studied in newborn, 1-week-old, 2-week-old, 1-month-old, and 2-month-old New Zealand white rabbits (Thompson Farms, Monee, Illinois). Newborn rabbits were killed within 24 hours after birth. The ages of the 1- and 2-week-old rabbits were accurate to within 24 hours, and the ages of 1- and 2-month-old rabbits were accurate to within 2...
days. For the chemical analyses, we removed the entire PT from the pulmonic valve ring to the division into main left and right pulmonary arteries, and the entire AA from the aortic valve ring to the first brachiocephalic branch. In other rabbits, vessels were fixed under controlled pressure perfusion to ensure a reasonably close approximation to dimensions in vivo and sampled at standard sites for morphometric study. The age span from birth to 2 months was selected because it represents the period during which systemic pressure increases steadily;18 beyond 2 months both systemic and pulmonic pressures remain at very nearly adult levels. It was also the period of most rapid growth as determined from data obtained by one of us (S.G.) and given below. Finally, it is the period during which consistent deviations in morphology between AA and PT become manifest. The 2-week observation point was chosen because it is the time at which the vasopressor response becomes established in the rabbit;13 the 1-week point was chosen to anticipate the rapid changes that are likely to accompany the usual perinatal pulmonary-systemic circulatory adjustments and the development of the vasopressor response. The 1-month time was then chosen to complete an array of time intervals.

PREPARATION OF TISSUES FOR CHEMICAL DETERMINATIONS

The rabbits were anesthetized with sodium pentobarbital, 50 mg/kg, injected intraperitoneally or intravenously. A longitudinal midline incision was made in the abdomen and exsanguination was carried out immediately by drawing blood from the abdominal aorta. The thoracic cage was opened by bilateral transection of the ribs. After measuring the length of the AA and of the PT from the pulmonic valve to its division into the main pulmonary arteries, the vessel segments were excised. Adventitia was removed by careful sharp dissection under a dissecting microscope.14 Small transmural fragments were taken for histological section from several rabbits in each group to ensure that the dissecting technique resulted in complete removal of adventitia. Tissues were minced and defatted by extraction with an ethanol-ether mixture (3:1) for 1 day, followed by extraction with anhydrous ether. After drying to constant weight in a vacuum oven at 50°C for 3 days, individual segments were pooled to provide sufficient material for determination of collagen, elastin, and DNA content. In all, 80 newborn, 80 1-week-old, 50 2-week-old, 30 1-month-old, and 20 2-month-old rabbits were used. All determinations were made on at least two sets of pooled tissues. The data were analyzed with a two-way analysis of variance. The standard deviations were estimated from the replicate measurements at each point. Values that were derived as products or quotients of the data were analyzed with Student’s t-test. Standard errors at each point were approximated by using a function of the variances of the original observations. Data are presented in Figure 1 as means ± 1 SD and in Figures 2–5 as means ± 1 SEM.

COLLAGEN AND ELASTIN DETERMINATION

Collagen and elastin were separated by a modification of the method of Lansing et al.18 Samples were suspended in 0.1 N NaOH and heated in boiling water for 50 minutes. The residue was washed twice with 0.1 N NaOH, and then with distilled water. The alkali extract and the subsequent washes were combined. The extract and the residue were each hydrolyzed in 6 N HCl at 105°C for 40 hours in sealed tubes. Measured samples of the hydrolysates were analyzed for nitrogen and hydroxyproline content and samples were also used for amino acid analysis on a Technicon analyzer. The amino acid profile of the residue fraction indicated a protein with the same amino acid pattern as elastin. Nitrogen was determined by the Kjeldahl procedure16 and hydroxyproline by a modification of the Neuman and Logan method.17 Hydroxyproline concentration of 14.4% for collagen was assumed for calculation of collagen content. Elastin was calculated from the nitrogen content of the residue fraction, assuming a nitrogen content of 14.8% for elastin. This percentage was computed from published reports of the amino acid analyses of elastin.18

DNA DETERMINATION

DNA was isolated either by (1) digesting tissues in 1 N NaOH overnight (16 hours) at 37°C, neutralizing, and then precipitating DNA by adjusting the solution to 5% trichloroacetic acid (TCA), or (2) precipitating a papain digest of these tissues in the cold with TCA. The papain digestion was carried out as described in standard procedures for the isolation of mucopolysaccharides.19 DNA was extracted from the TCA precipitate with 0.5 N perchloric acid at 70°C for 20 minutes and the quantity was determined in duplicate by the diphenylamine method of Burton.20

PREPARATION OF SPECIMENS FOR MORPHOMETRIC STUDIES

In a different series of experiments, the AA and PT were fixed while distended at physiological pressures. Five to seven rabbits were used at each age interval. The rabbits were selected so that their body weights fell within 1 SD of the means for their respective ages as determined from data obtained by one of us (S.G.) on approximately 500 rabbits in conjunction with other experiments. The selected weight ranges were as follows: newborn, 45–50 g; 1 week, 133–153 g; 2 weeks, 235–281 g; 1 month, 670–730 g; 2 months, 1,450 to 1,550 g. The rabbits were anesthetized as described above. The abdominal cavity was opened in the midline and catheters of suitable diameter were inserted and tied into the inferior vena cava and the abdominal aorta. Each catheter was connected to a separate controlled pressure perfusion circuit utilizing an apparatus similar to one described previously.21 For each circuit, physiologic saline at 37°C, phosphate-buffered 2.5% glutaraldehyde at 37°C, or 10% gelatin solution at 45°C could be introduced between the pressure system and the catheter. Pressure was maintained at the desired levels by a Nullmatic pressure-reducing valve, and monitored by a manometer at the catheter connection. The vena cava and the aortic cannulas were perfused simultaneously, first with saline for 10 minutes and then with the glutaraldehyde fixative for 1/2 hour. The values used for systemic pressure were those reported by Mott.15 The pulmonary artery distending pressures are based on reported determinations on rabbits and other animals of comparable size.22, 23 The pressure in the vena cava, and
MEASUREMENTS OF DIMENSIONS

Vessel diameters were determined directly from the transected vessels by means of a dissecting microscope equipped with a micrometer eyepiece. The histological sections were examined by means of a light microscope equipped with a micrometer eyepiece in order to ascertain the overall appearance and architecture of the media, measure medial thickness, and count the number of medial fibrocellular layers. Measurements were corrected for shrinkage during fixation and processing according to correction factors reported previously. Estimates of total tension were calculated from an approximation of the law of Laplace applicable to cylindrical vessels, i.e., \( T = \frac{P r}{R} \), where \( T \) is the tangential tension in dyn/cm, \( P \) is the pressure in dyn/cm², and \( r \) is the inner radius of the vessel in cm. In the present report, relationships between total tangential tension and total scleroprotein synthesis were considered.

Results

The medial lamellar structure of the two vessels was similar at birth. Medial layering in the AA became increasingly prominent as the width of each layer increased and individual aortic elastin plates became increasingly continuous. The medial lamellar architecture of the PT became somewhat less distinct with age as elastin lamellae became less prominent. Extracellular fibers were more prominent in the AA than in the PT by 2 weeks of age, but no differences in number, size, or staining properties of medial cells could be discerned by light microscopy.

DIMENSIONS AND WEIGHT OF VESSELS DURING GROWTH

Length, inner radius, and dry weight of the rabbit AA and PT are compared for each age in Figure 1. Values for each of these variables were identical at birth. Length and radius increased markedly with age, but the radius remained nearly identical for the two vessels. Length of the AA appeared to increase somewhat more rapidly than that of the PT, but the difference in length between the two segments was significant only at 2 months of age, when the aorta was approximately 20% longer than the pulmonary trunk. By contrast, dry weight increased much more rapidly in the AA than in the PT (\( P < 0.01 \)), and the significant difference between the segments was already well established at 2 weeks; by 2 months, the AA weighed twice as much as the PT.

Figure 1 Length, inner radius, and dry weight of ascending aorta (AA) and pulmonary trunk (PT) of the rabbit are plotted against age during growth from birth (NB) to 2 months. Increases in length, radius, and dry weight with time were significant for both vessels. The slight difference in length between the vessels was significant only at 2 months, whereas the more marked difference in dry weight were already established and significant at 1 week and remained significant thereafter.
TOTAL ELASTIN, COLLAGEN, AND DNA CONTENT OF EACH SEGMENT DURING GROWTH

Differences in dry weight for the two vessels at each age corresponded to differences in extracellular fibrous protein accumulation (Fig. 2), but total DNA content remained nearly the same for the two vessels at each age (Fig. 3). Although total elastin, collagen, and DNA were nearly identical for the two vessels at birth and at 1 week, significant differences in total elastin and collagen content were evident by 2 weeks and became increasingly evident thereafter. The increasing difference in total elastin content was more marked (Fig. 2). Each segment contained 0.18 ± 0.004 mg of elastin at birth, but by 2 months the AA contained 3.5 times more elastin than the PT. Each vessel contained 0.04 ± 0.002 mg of collagen at birth. The difference in collagen content between the vessels was significant at 2 weeks and remained so throughout, but the AA contained only 1.7 times more collagen than the PT at 2 months. Increases in total DNA, more or less the same for AA and PT (Fig. 3), paralleled the increases in segment length and radius rather than weight (Fig. 1). Each vessel contained 6.5 ± 0.37 μg of DNA at birth. Although the difference in mean total DNA content of about 8% at 2 weeks was significant (P < 0.05), this difference was small. At 2 months the AA contained 58 ± 2.38 μg of DNA and the PT contained 48 ± 4.35 μg, but this difference was not significant.

ELASTIN AND COLLAGEN ACCUMULATION RELATIVE TO DRY WEIGHT

Although the divergence in total elastin and collagen content for the two segments increased steadily, elastin and collagen content relative to dry weight changed and diverged markedly between the 1st and 2nd weeks of life (Fig. 4). At birth elastin concentrations were identical at 30% for both AA and PT. Between 1 and 2 weeks, elastin concentration rose to 48.3% (P < 0.01) in the AA and to only 37% (P < 0.05) in the PT. From 2 weeks to 2 months, elastin concentration of the AA remained constant; elastin concentration of the PT remained constant from 2 weeks to 1 month, but appeared to return to the newborn 30% level by 2 months. Collagen concentration, identical at about 8.5% for AA and PT at birth, rose subsequently in both arteries. As with elastin, the divergence in collagen concentration occurred between 1 and 2 weeks, but in contrast to elastin, collagen concentrations rose more rapidly in the PT. Between 1 and 2 weeks, collagen concentration rose significantly in the PT from 8.6% to 15.4% (P < 0.01) and from 8.4% to 11.3% in the AA (P < 0.025). After 2 weeks there was little change...
FIGURE 4  Concentrations of elastin (left) and of collagen (right) relative to dry weight are shown for the ascending aorta (AA) and the pulmonary trunk (PT) of the rabbit during growth from birth (NB) to 2 months. The change in AA elastin concentration was significant between 1 and 2 weeks but not thereafter. Elastin concentration in the PT increased less, but significantly, between 1 week and 2 weeks and fell to newborn levels by 2 months. Collagen concentration increased significantly in both vessels between 1 and 2 weeks, but the increase was greater in the PT. The relatively small increase in AA collagen concentration between 1 month and 2 months was significant.

in collagen concentration. Only the small increase from 11.2% to 13.8% between 1 month and 2 months in the AA was significant ($P < 0.01$).

ELASTIN AND COLLAGEN CONTENT RELATIVE TO DNA

Since the increasing differences in dry weight for the two vessels corresponded closely to the differences in total extracellular fibrous protein accumulation while total DNA content of the vessels remained the same at each age, changes in total elastin and collagen relative to total DNA from point to point may reflect differences in average synthetic activity per cell for the two vessels. Therefore elastin and collagen content are expressed in relation to DNA content in Figure 5. Elastin and collagen content relative to DNA content was the same at birth for AA and PT. Elastin accumulation relative to DNA content increased very rapidly in the AA, but remained nearly constant for the PT. Collagen accumulation relative to DNA also increased in the AA but markedly less than did elastin content. In the PT, however, collagen content relative to DNA increased significantly but did not exceed the rate of aortic collagen accumulation relative to DNA. Thus, although relative proportions of elastin and collagen synthesized in each vessel during growth became fixed by 2 weeks (Fig. 4), the net elaboration of scleroprotein per cell appeared to continue to increase. The increasing accumulation of scleroprotein per cell was attributable mainly to elastin synthesis and was more prominent in the aorta.

MEDIAL TENSION

Total medial tangential tension calculated from distending pressure and measured radius is plotted in Figure 6 for each age. The divergence in total medial tension between the two vessels paralleled the divergence in scleroprotein content. Tension rose by a factor of 10 in the aorta, from 2,500 dyn/cm at birth to 25,000 dyn/cm at 2 months; in the PT however, estimated tension rose to a mean value of only 4,200 dyn/cm at 2 months and this change was not significant. Total medial tension increased rapidly and continuously in the AA at about the same rate as the increases in dry weight (Fig. 1), total elastin content (Fig. 2), or elastin content relative to DNA (Fig. 5). The slight increase in medial tension in the PT was comparable to the smaller and more gradual increase in dry weight, total fibrous protein content, and accumulation of fibrous protein relative to DNA.

Discussion

Comparisons of the AA with the PT during the immediate postnatal period provided insights into some of the factors which may govern differentiation of the arterial media. Previous investigations by others have revealed increased fibrous protein and mucopolysaccharide production in aortas and pulmonary arteries subjected to hypertension, but it had not been shown that fibrous protein accumulation also correlates well with differences in increasing medial tension which occur normally in different arteries during growth. Nearly identical in size, weight, and composition at birth, the AA and PT remained similar
in length and diameter but diverged markedly in wall thickness and mass as differences in intraluminal pressure became manifest. By 2 months, 3 times more elastin and almost twice as much collagen had accumulated in the AA, as compared to the PT. At this time total medial tension, equal for the two vessels at birth, was about 6 times greater in the AA than in the PT. The data indicate that individual medial cells are capable of a remarkable range of biosynthetic response during growth, for total DNA content increased at about the same rate for each segment and therefore remained nearly the same for the two vessels. Since DNA content is an acceptable approximate measure of cell number, it is reasonable to conclude that cell proliferation in these arteries during normal growth is relatively independent of increasing medial tangential tension and is probably related instead to more general humoral factors which regulate overall growth and/or to hemodynamic factors other than medial tension.

Thus, cell populations of comparable size are called on to produce markedly different quantities of extracellular fibrous proteins, presumably in response to the different levels of medial tension. It also should be noted that, similarly to our initial observations in rabbits, quantitative stereological and chemical analyses of rat thoracic aorta during normal growth have revealed that increases in wall thickness and in the proportion of scleroproteins relative to cells correspond well with estimates of increasing medial tension. It is not clear from other studies whether the increased elaboration of fibrous protein associated with hypertension is due mainly to synthesis by an increased number of cells or to increased synthesis by each cell. The evidence suggests that in hypertension arterial cell mass increases initially. Evidence with regard to DNA production is contradictory; it is not significantly increased in the hypertensive monkey aorta, but is increased in the hypertensive rabbit. There also is evidence of increased thymidine uptake during the development of hypertension in rats.

In addition to postnatal differences in intraluminal blood pressure and medial mechanical stress, differences in oxygen tension between systemic and pulmonary circulations also supervene rapidly after birth. It is conceivable that the relative oxygen lack in the PT inhibits or limits protein synthesis. Such an explanation for the marked difference between the two vessels is, however, unlikely, for although oxygen tension is 25% higher in the systemic circulation than in the pulmonary, the aorta rapidly becomes much thicker than the pulmonary artery. Since
transmural diffusion of oxygen diminishes very rapidly across the aortic wall,29 most of the media of the AA would receive no more oxygen than the PT.

The continuing, steady increase of medial tension in the AA did not appear to influence the relative proportions of its fibrous proteins once the concentrations of 48% elastin and 11% collagen were established at 2 weeks. A similar constancy in the relative proportions of elastin and collagen persists when hypertension induces increased absolute fibrous protein accumulation in the thoracic aorta3-5 and other vessels.30 Actually, each anatomically defined artery31 or aortic segment32 appears to establish and maintain its own characteristic fixed ratio. Harkness et al.,32 studying canine aortas, found that proximal regions contained relatively more elastin and distal regions contained relatively more collagen. They felt that these differences were determined genetically and established at birth because they were present in young dogs. Information concerning breeds, ages, or sampling sites were not furnished by these authors and it may well be that a brief period of rapid change during the immediate postnatal period, such as we observed in rabbits, was overlooked.

McClosey and Cleary33 determined absolute and relative elastin and collagen content in descending thoracic and abdominal aortas of rabbits 1-130 weeks of age and found that the adult pattern of elastin and collagen distribution along the aorta was evident at 1 week and that large changes in relative quantities of these components occurred only during the early postnatal period. Although rates of elastin and collagen accumulation were different for the thoracic and abdominal aorta, relative proportions of these proteins remained fixed for each segment. In earlier work, Cleary34 had shown that each mammalian species had a characteristic adult gradient of elastin decrease and collagen increase with distance from the heart along the aorta while total scleroprotein content relative to dry weight remained quite constant. In the present study the wide range of elastin synthetic response per cell appeared to reflect the marked differences in medial tension, and the smaller range of the collagen synthetic response bore a closer relationship to DNA content or cell number.

Modifications in relative proportions of elastin and collagen were therefore due principally to modifications in elastin synthesis. The mechanisms by which medial tensile stresses may induce fibrous protein synthesis and accumulation are unknown, nor are there data that identify the factors that determine the early establishment of fixed biosynthetic ratios of elastin and collagen. Cyclic stretching or displacement of medial cells and fibers associated with the pulse pressure wave could facilitate synthesis by enhancing diffusion of metabolites to and from medial cell surfaces, by altering the configuration and the immediate electrochemical environment of cell membranes35 or by changing the configuration and mechanical tension of other cell structures. It has been shown that tensile forces play a role in determining the orientation of extracellular matrix components;36 modifications in relative orientation of extracellular macromolecules could promote interactions which favor cross-linking and stabilization of extracellular fibrils. Degradative processes also could be affected by changes in protein and enzyme concentration or by mechanical activation of proteases such as collagenase and elastase.

With regard to the elastin and collagen gradients characteristic for each location along the aorta, it is unlikely that this vessel is populated by overlapping clones of cells, each with a slightly different synthetic potential. It is more reasonable to presume that some graded mechanical stimulus is effective during a short initial induction period. The pulse pressure wave, for example, could be such a stimulus, for one would expect a gradient of attenuation with distance from the heart. Species differences in gradient could be due to differences in heart rate, cardiac output, aortic geometry, peripheral resistance, and pattern of arterial runoff. Similarly the differences in relative elastin and collagen content between the AA and PT which supervene after birth could be a function of differences in amplitude or form of the pulse pressure wave related to differential changes in pressure and flow. If such adjustments do indeed result in the induction of an acceptable adult biosynthetic differential for any given location, one wonders why the adaptive period for establishing it is operative only for a brief interval during the postnatal period. Our findings provide no obvious clues to help identify any functional advantage for the stabilization of collagen-elastin ratios in rabbit arterial trunks as early as 2 weeks of age, i.e., while growth, medial tension, and cell number are still increasing rapidly. It is probable that the enabling hormonal or metabolic milieu is optimal during the 2nd week of life and/or that the fetal phase of differentiation of the major trunks and other organs reaches completion only at the end of the 2nd week. Indeed, the rabbit becomes furred and opens its eyes at 2 weeks and its vasopressor response is not fully established until 2 weeks.13

The comparison of the PT with the AA described in this report and elsewhere37 suggests that mechanical tension plays an important role in the growth, differentiation, and composition of blood vessels. Similar comparisons among various arterial segments in animals of different adult size, different gestational periods, different postnatal growth rates, and different local hemodynamic conditions, both native and surgically induced, may be expected to provide insights into the effects of these variables on vessel wall components. Other experiments provide direct evidence that cyclic stretching of medial cells grown on extensible membranes results in stimulation of both collagen and mucopolysaccharide synthesis.38-39 Refinement of such experiments to vary rate and amplitude of stretch in the presence of different humoral factors and metabolites may help to illuminate the mechanisms by which mechanical stresses control smooth muscle cell proliferation, differentiation, and biosynthesis.

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