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Polyploid nuclei in human artery wall smooth muscle cells

(atherosclerotic plaque/tetraploidy/arterial media)

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ABSTRACT **Although polyploid nuclei have long been known to be present in many adult human tissues, the ploidy of smooth muscle cells in human artery wall has never been determined. We measured DNA content in individual smooth muscle cell nuclei of artery wall specimens by two means: Feulgen microdensitometry and flow microfluorimetry. A significant percentage of nuclei were polyploid; most of these were tetraploid, although higher levels were also found. The frequency of polyploidy varied with age from less than 1% at birth to a mean of 7% in adult aortic, carotid, and iliac vessels. Atherosclerotic plaques had a lower tetraploid content than the underlying media, whereas normal intima was similar to the corresponding media. The increase in frequency of hyperploid smooth muscle cell nuclei correlates with the normal growth, development, and aging of human artery wall. We suggest that the regular existence of a subset of polyploid smooth muscle cells may indicate an important functional role for this phenotype.**

Polyploid nuclei have long been known to exist in a wide range of human and animal tissues including uterus, salivary gland, epidermis, urinary bladder, brain, liver, and trachea (1–7). Adult human myocardium has been observed to contain 50–60% tetraploid nuclei (8), but the ploidy of smooth muscle cell nuclei in the human artery wall has never been reported. Rat aorta has been observed to contain a significant number of polyploid smooth muscle cells: normotensive rats had 10% tetraploid nuclei while hypertensive rats developed 27% tetraploid nuclei (9). It was concluded that the appearance of polyploid nuclei is a regular occurrence in the normal growth and development of the rat artery wall and that artery wall hypertrophy in hypertension could be accounted for solely on the basis of cellular hypertrophy (without hyperplasia); thus the development of a chronic hypertensive state may be related to increases in smooth muscle cell nuclear ploidy.

The findings in the rat aorta indicated that the nuclear ploidy of human artery wall deserved study. We used two techniques, Feulgen microdensitometry on paraffin-embedded sections and flow microfluorimetry on isolated nuclei, to determine whether human arterial smooth muscle cells undergo polyploidization. We report here the existence of a subpopulation of smooth muscle cells in elastic and muscular arteries containing tetraploid and rare higher ploidy nuclei.

MATERIALS AND METHODS

Specimen Collection. Aortic, iliac, and carotid arterial specimens were obtained from autopsies with a postmortem interval of 4–14 hr. Specimens for Feulgen microdensitometry were immediately fixed in 4% phosphate-buffered paraformaldehyde. Nuclear isolation was either done on fresh tissue within 2 hr of collection or specimens were frozen in liquid nitrogen and held at −70°C until further processing.

Feulgen Microdensitometry. Arterial sections were fixed in 4% paraformaldehyde, embedded in paraffin, cut cross sectionally 8 μm thick, deposited on glass slides, and Feulgen stained as described by Fand (10). Individual nuclear DNA content was determined with a scanning integrating microdensitometer (Vickers M85; Vickers Instruments, York, England). Two hundred measurements were made per specimen, traversing the full thickness of the media and measuring each intact nucleus; chicken erythrocytes were used as a standard.

Nuclear Isolation and Flow Microfluorimetry. Isolated nuclei were prepared from arterial tissue obtained at autopsy by a modification of the procedure of Thornwaite *et al.* (11). The adventitia was removed; 200-mg specimens containing both media and intact intima were assayed to determine ploidy of the full artery wall; plaque, intima, and inner and outer media were separated and assayed individually to determine ploidy of these different layers. Specimens were minced in 1 ml of nuclear isolation medium (0.6% Nonidet P-40/Tris-buffered isotonic saline, pH 7.0/1 mM $CaCl_2$/21 mM $MgCl_2$/0.2% bovine serum albumin containing diamidinophenylindole at 10 μg/ml), allowed to sit at room temperature for 10 min, passed through an 80-μm filter, frozen in 10% dimethyl sulfoxide, and then thawed and syringed three times through a 26-gauge needle before doing flow microfluorimetry. Nuclear DNA content was assayed on an ICP-22 flow cytometer (Ortho Instruments, Westwood, MA), 5,000–10,000 nuclei per specimen, interfaced with a PDP-11/03 computer (Digital Equipment, Maynard, MA) for curve fitting and subtraction of an exponential noise background (12).

Statistical Analysis. The relationships between age at death and proportion of tetraploid nuclei in aortic, iliac, and carotid artery smooth muscle cells were examined with a multivariate linear regression model (13). The proportions were transformed to a logit scale, $\log(p/1-p)$, to obtain approximately normal homoscedastic residuals from fitted quadratic regressions on age. Because data points were missing (at random) for one or two of the arteries in 11 of the 34 cases, the parameters of the regression model were estimated by the "EM" algorithm for maximum likelihood estimation from incomplete data under the assumption that the trivariate vector of logits followed a normal distribution given age (14). The significance of the quadratic equations and comparisons of the equations for the three arteries were assessed with approximate multivariate tests. These were computed by substituting the EM algorithm estimates of the matrix of regression coefficients and the covariance matrix of the trivariate residual vectors into the usual likelihood ratio statistics assuming complete data (13). The test statistics, using Bartlett's approximation, were referred to χ^2 distributions with appropriate degrees of freedom.

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Analysis of percent tetraploid nuclei in portions of individual arterial specimens separated by dissection was done after transformation to a logarithmic scale to assess the significance of proportional changes rather than absolute differences. Mean differences of logarithms were determined and compared by Student's *t* test.

RESULTS

Feulgen Microdensitometry. Feulgen microdensitometry on nine normal iliac arteries showed the existence of a subpopulation of smooth muscle cell nuclei with tetraploid DNA content and rare (<1%) octaploid, 16-ploid, and 32-ploid forms. Tetraploids were rare in three infants (<1%) but increased with age (Fig. 1) to a mean of 6% in adults. By inspection, these nuclei appeared to be scattered throughout the media, with little tendency to cluster and no obvious predilection for inner or outer bands of medial smooth muscle. Unfortunately, this technique suffers from a possible selection bias, because only a portion of the nuclei in a given part of a tissue section can be measured and only a limited number of measurements can be done per specimen; therefore, we used flow microfluorimetry to make an alternative determination of smooth muscle cell ploidy (see below).

Nuclear Yield. The percentage of nuclei extracted from vessel wall specimens was estimated by comparing numbers of nuclei isolated with total DNA content, as assayed by a modification of the method of Fujimoto *et al.* (15). Three different specimens of normal iliac media were found to have 0.585 ± 0.005 μg of DNA per mg of tissue or 8.6×10^4 diploid nuclei per mg [using a value of 6.8 pg of DNA per nucleus (16)]. Between 5.5 and 9.1×10^3 nuclei per mg were isolated from these specimens as counted by hemocytometer; this gives estimated yields of 6–11% (calculated as diploid nuclei).

Flow Microfluorimetry. Aortic, iliac, and carotid vessels were collected from 34 autopsies. Flow microfluorimetry assays confirmed our Feulgen microdensitometry results and permitted more extensive analysis. Patients ranged from 14 to 96 years old; death was due to accident or suicide for approximately half of the cases and to natural causes for the remainder.

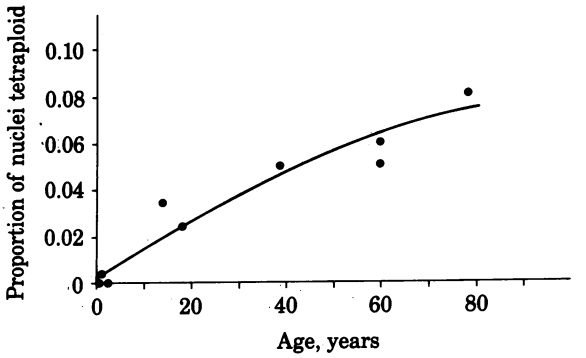

FIG. 1. Frequency of tetraploid nuclei in iliac artery as determined by Feulgen microdensitometry. Two hundred nuclei were measured for each point. Quadratic regression of frequency of tetraploid nuclei on age is shown ($n = 9$, $r^2 = 0.93$, $P < 0.001$).

Regressions of the frequency of tetraploid nuclei (transformed to a logit scale) on age were determined and compared for the three vessels. Each was fit best by a quadratic, rather than a linear, regression; plots are shown in Fig. 2. The general relationship of an increasing proportion of tetraploid nuclei with age was clearly significant for each of the three arteries (parameters and standard errors are given in Table 1). The three curves showed little divergence from one another up to 60 years of age, suggesting that the age-dependent polyploidization of arterial smooth muscle cells for these three vessels is similar. The only significant contrast was between the aorta and carotid equations ($P \cong 0.03$, 3 df). Overall evidence for different true regressions was rather weak, however, since a test of the equality of all three regression equations indicated less than significant discrepancy ($\chi^2 = 10.58$, $P \cong 0.10$, 6 df). The tendency for the curves to

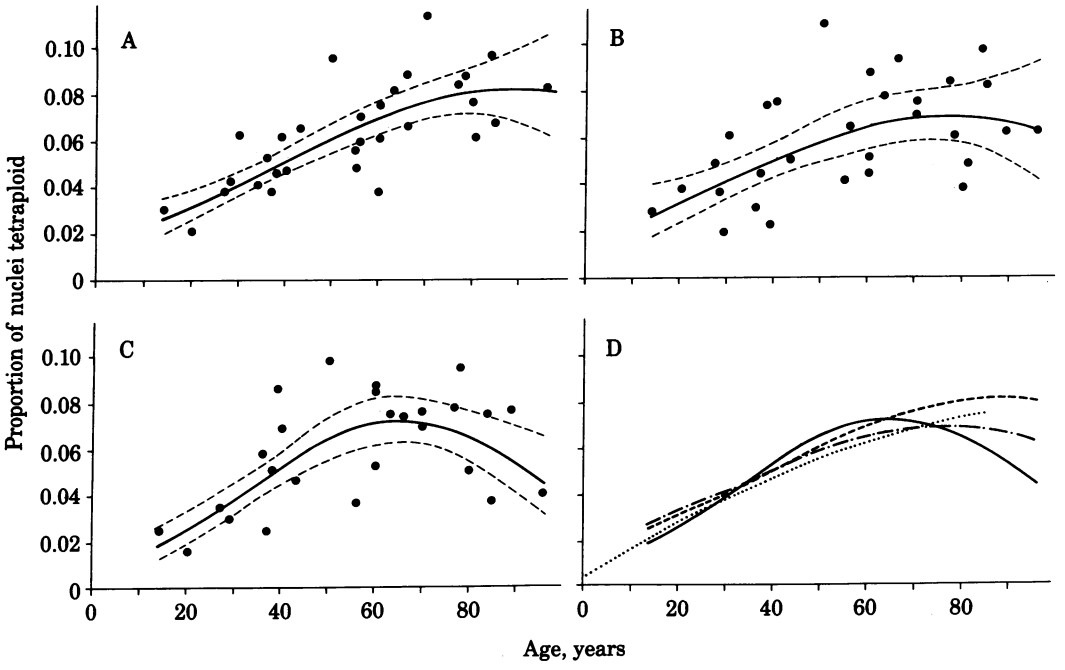

FIG. 2. Plots of quadratic regressions of flow microfluorimetry data on aorta (*A*) and iliac (*B*) and carotid (*C*) arteries. Proportions of tetraploid nuclei determined by flow microfluorimetry were transformed to a logit scale and examined with a multivariate linear regression model. ——, Fitted curves transformed back to the proportion scale; – – –, ± 2 SE. (*D*) Super position of curves for aorta (– – –), iliac artery (–·–), and carotid artery (——) together with the regression for the Feulgen data (·····) (Fig. 1).

Table 1. Summary statistics for regression analysis of proportion tetraploid nuclei, as determined by flow microfluorimetry, on age

Coefficient	Artery		
	Aorta	Iliac	Carotid
Constant	−4.107 (0.271)	−4.086 (0.410)	−4.817 (0.384)
Linear	0.0379 (0.0106)	0.0388 (0.0161)	0.0691 (0.0150)
Quadratic	−0.000214 (0.000095)	−0.000253 (0.000143)	−0.000527 (0.000134)
Standard error of regression	0.243	0.359	0.323
Residual correlation matrix			
Iliac	0.280		
Carotid	0.416	0.111	

Parameter estimates and standard errors are given for multivariate quadratic regression of $\log(p/1-p)$ on age, where p = proportion of tetraploid nuclei in aorta, iliac, and carotid arteries, as determined by flow microfluorimetry. Values in parentheses are standard errors of the coefficients and were computed from formulas for the matrix of second derivatives of the logarithm likelihood (or information matrix) for the incomplete data as given by Dempster *et al.* (14).

plateau beyond the age of 60 (as reflected by the negative quadratic coefficients) was significant ($P \cong 0.003$, 3 df). The divergence at extreme ages and the negative slope of the carotid artery curve have no obvious physiologic meaning; although they could reflect the actual living population at increased ages, they may well be due merely to sampling error, because we studied a relatively small number of cases from a heterogeneous population. Comparison of the Feulgen data for iliac vessels with the flow microfluorimetry results (Fig. 2*D*) showed close agreement between the two methods. Thus, statistical analysis of our flow microfluorimetry data substantiated the impression from the curves that nuclear polyploidization of the three large arteries studied progressed similarly with age and tended to plateau beyond 60 years.

Individual results on pairs of vessels from the same case gave positive but relatively small correlation coefficients (Table 1). It remains to be determined whether this variability is real or merely due to our sampling technique and whether such variability may exist along the length of a single artery. Graphical comparisons of male vs. female and of accidental deaths vs. natural deaths showed no distinctions between these different groups.

To see whether atherosclerotic lesions have different tetraploid content from normal media, six large fibrous plaques were dissected from the underlying media and assayed independently. In these paired samples (Table 2), fibrous atherosclerotic plaques were found to have an average of 35% fewer tetraploid nuclei than the corresponding media ($P < 0.001$). Similar comparisons of intima vs. media and inner media vs. outer media showed no consistent difference between corresponding members of these pairs.

DISCUSSION

In determining the ploidy of individual smooth muscle cell nuclei by flow microfluorimetry, we were concerned that the technique of nuclear isolation might introduce three major potential sources of error: (*i*) selection of a subset of nuclei (e.g., decreased isolation of tetraploid nuclei, which, being larger, might be more difficult to extract from tissue), (*ii*) failure of nuclei from binucleated cells to separate (which would therefore appear as polyploids), and (*iii*) clumping of normal diploids during flow microfluorimetry. These potential sources of error appear to be negligible for a number of reasons. Less than 2% clumping was observed by fluorescence microscopy of the nuclear preparations. More compelling is the close similarity of the Feulgen age–regression data when compared with the flow microfluorimetry age–regression data (Fig. 2*D*). Finally, a comparison was done on three specimens: in these, the percent tetraploidy was estimated by flow microfluorimetry and on the same nuclear isolate by measuring DNA in 200 nuclei with a Zeiss microfluorimeter. Agreement between the results obtained with the two techniques was within one standard error, indicating that any flow microfluorimetry error introduced by clumping or by the presence of two nuclei attached to each other was negligible.

Assuming the reliability of the estimates, we conclude that there is a subpopulation of smooth muscle cells in human artery wall that contain nuclei of polyploid DNA content–mostly tetraploid but occasional higher forms. The emergence of polyploidization in arterial tissue seems to correlate with normal growth and development as shown by the statistically significant regression of tetraploidy on age. Tetraploids, rare at birth, increase gradually to a mean of 6% or 7% at age 60. The process proceeds

Table 2. Comparisons of paired flow microfluorimetry data

	Mean (arithmetic) ± SEM							
	First component	Second component	Difference ± SEM	Estimated median ratio	95% confidence interval	t	P	n
Media vs. plaque	6.6 ± 1.5	4.3 ± 1.1	2.4 ± 0.9	0.64	0.57–0.72	8.6	<0.001	9
Media vs. intima	7.1 ± 2.5	6.7 ± 4.2	0.5 ± 3.4	0.85	0.59–1.20		NS	9
Inner media vs. outer media	8.4 ± 3.0	7.1 ± 1.6	1.4 ± 2.6	0.88	0.69–1.11		NS	16

Frequency of tetraploid nuclei was assayed by flow microfluorimetry for paired and separated portions of artery wall. Arithmetic means and mean differences for each of the two components of these pairs are shown. Median ratios (second component/first component) and 95% confidence intervals were estimated from logarithms of percentage tetraploid nuclei [e.g., estimated median ratio = $\exp(\log X_2 - \log X_1)$]. NS, not significant.

in a roughly parallel manner in the aorta and iliac and carotid arteries.

The age-related polyploidization of human arterial smooth muscle cell nuclei is probably one example of a phenomenon common to the normal growth and development of many organs, as evidenced by the fact that polyploidization has been found in most tissues examined (6). Of particular interest, human left ventricular myocardial cells, which have <6% polyploid nuclei at birth, are reported to develop 40–70% tetraploid nuclei by adulthood (8), with the most rapid increase occurring between 5 and 10 years of age, correlating with increasing heart weight. In arteries, polyploidization is a progressive process up to 40–60 years of age and the percent polyploid cells reached is much lower than in the heart. Although caution is needed in extrapolating a developmental process for an individual from fixed observations on a heterogeneous population, we conclude that nuclear polyploidization occurs regularly in normal growth and development of the artery wall. The amount and the time course of polyploidization seem to be somewhat different in the artery wall and in the heart.

A recent study of hypertension in rats found 10% tetraploid nuclei in aortic smooth muscle cells of 5- and 11-month-old normotensive Wistar–Kyoto male rats compared with 33% tetraploid nuclei in spontaneously hypertensive rats (9). A significant correlation between blood pressure and percent tetraploidy was found for these animals, when systolic pressures increased more than 60%. Furthermore, in rat aorta, cellular DNA content was found to be proportional to cell mass and the aortic vessel thickening accompanying hypertension was accounted for solely by cellular enlargement (with no change in cell number). It was proposed that "the nuclear response to hypertension may thus represent a fixed change related to the establishment of a chronic hypertensive state." Similarly, data on the human heart (8) were interpreted to indicate that polyploidization of myocardial cell nuclei in adults correlated with cardiac hypertrophy (whether of the left or the right chambers) rather than with age. Conceivably, some of the variation in tetraploidy we found among adults could correlate with differences in blood pressure. Similarly, the tendency noted for ploidy to increase during the adult years might not be a process due strictly to aging but could be associated with progressively increasing blood pressure. We cannot assess these possibilities with our data, although in future studies it will be important to do so.

The point at which arterial smooth muscle cell polyploid nuclei lie in the cell cycle remains to be determined. We do not believe these nuclei simply represent the G_2 and mitotic compartments of the classic cell cycle for the following reasons. Mitotic figures are extremely rare in the normal media of rat and man, suggesting a very low level of smooth muscle cell proliferation. Similarly, [^{3}H]thymidine labeling of swine (17), rabbit (18), and rat (9) vessel wall cells is very low, indicating that only a small number of medial smooth muscle cells are in the S phase. Two possibilities remain. First, some of these cells with tetraploid nuclei could be in the G_0/G_1 phases of the cell cycle and capable of DNA replication prior to division. The presence of octaploid and higher forms supports this possibility. Second, cells with tetraploid nuclei could be arrested in the G_2 phase of the cell cycle and be capable of dividing without DNA synthesis. There is no reported evidence for this in the vessel wall. However, subpopulations of cells arrested in the G_2 phase have been proposed to exist in a number of animal tissues (3, 7, 19, 20).

Increases in nuclear ploidy occur in many tissues (6) and in different circumstances. The role of these ploidy increases is not clear in relationship to possible functional differences. Concepts of atherosclerosis now focus on cell proliferation as an early and primary event in lesion formation. The atherosclerotic plaques examined here show both diploid and tetraploid populations with a small but significant decrease in the population of tetraploid nuclei compared with cell populations of media and adjacent intima. The presence of tetraploid cells in human artery walls represents an inhomogeneity in the smooth muscle cell population of artery walls whose ability to proliferate is worthy of study.

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