Comparative Rates of Decline in the Primary Cloning Efficiencies of Smooth Muscle Cells From the Aging Thoracic Aorta of Two Murine Species of Contrasting Maximum Life Span Potentials

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Primary cloning assays of thoracic aortic smooth muscle cells from an F_1 hybrid strain of *Mus musculus* demonstrated linear regressions of replicative potentials as functions of donor age (6–30 months), with regression coefficients, in two independent cohorts, of -1.69 ± 0.27 (SE) and -1.88 ± 0.19 (SE) clones per milligram wet weight of intima-media per month and correlation coefficients of -0.83 and -0.92 ($P < 0.0001$). Secondary cloning (dilute plating from first passages) also demonstrated a high negative correlation ($r = -0.90$) between donor age and cloning efficiency, thus implicating intrinsic differences in cell populations. Comparable primary cloning assays on the aortas of aging cohorts of *Peromyscus leucopus*, a murine species with a maximum life span potential approximately twice that of *Mus musculus*, yielded about twice the number of clonable smooth muscle cells per unit weight; the rate of decline with age was slightly but significantly greater ($P < 0.01$). Electron-microscopic studies revealed cellular alterations confined to the first subintimal layer of aortas from mice (*Mus musculus*) 18 months and older. (Am J Pathol 1983, 110:236–245)

THERE IS BY NOW a large body of evidence that indicates that cultured fibroblastlike cells from the dermis of human subjects have limited replicative life spans *in vitro* and, moreover, that their life spans are inversely related to the age of the donor.[1–4] In comparative studies with dermal biopsies from a series of mammals, Rhome[5] recently presented evidence that the cumulative population doubling achieved by such cultures is directly proportional to the maximum life span potential of the donor species. There have been comparatively less intensive investigations of the limited replicative life spans of other somatic cell types, but the bulk of the evidence supports the conclusion that all or most somatic cell types have limited replicative life spans *in vitro*.[1,2,6–11]

Of considerable interest is the evidence that the replicative life spans of somatic cells vary according to cell type and, even when apparently identical cell types are cultured, there are significant differences related to the precise sites of biopsy.[4,7]

An important implication of these various studies is the possibility that species-specific and age-related differences in the replicative potentials of somatic cells may play important roles in the determination of tissue-specific age-related disorders and of the maximum life span potentials (MLSP) of mammals. We therefore believe that it is important to extend comparative studies to cell types other than skin fibroblastlike cells. It would also be desirable to employ alternative assays of replicative potential, particularly those less removed from *in vivo* conditions. In preliminary experiments with intact aortas from aging cohorts of a noninbred strain of *Mus musculus*, our laboratory reported results with three such alternative assays: primary cloning, secondary cloning, and autoradiographic analysis of ^{3}H-thymidine-

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labeled organoid cultures.[12] We now report more extensive and statistically analyzed cloning studies of intimal-medial aortic segments from aging cohorts of a genetically defined colony of *M musculus*. These studies document and quantitate an age-related decline in replicative potential of medial smooth muscle cells. Moreover, we have compared these results with those obtained using intimal-medial aortic segments from a comparatively long-lived murine species, *Peromyscus leucopus*,[13,14] and have carried out preliminary electron-microscopic assessments of the aortic wall in the aging *M musculus*.

Materials and Methods

Animals

(BALB/cNNia × C57Bl/6NNia) hybrid male mice ($CB6F_1$) were obtained from the specific-pathogen-free (SPF) aging colony maintained by Charles River Breeding Laboratories, Boston, Mass. under contract from the National Institute on Aging. They were maintained in filter-topped cages and fed acidified (pH 2.5) autoclaved water and Wayne sterilizable chow (Lab-Blox) (Continental Grains, Chicago, IL) ad libitum for the several days (not for more than 1 week) from receipt of each of the two shipments of 6–30-month-old cohorts to their sacrifice by cervical translocation. Male and female *Peromyscus leucopus* (white-footed deer mice) were from a line of randomly bred animals maintained by the late George A. Sacher at the Argonne National Laboratories, Chicago, Illinois. They were maintained in our vivarium under the conditions described above.

(A/HeTrWo × C57Bl/6JTrWo)F_1 hybrid male mice ($AB6F_1$), which provided aortas for DNA and Factor VIII antigen assays, were from an SPF aging colony maintained in our laboratories (Norman Wolf, DVM, PhD) and having aging parameters comparable to the $CB6F_1$ hybrids; a contemporary sample size of 200 gave a mean life span of 29.5 months and a maximum life span of 1266 days.

Primary and Secondary Cloning Assays

For *M musculus*, the following protocol was established. Immediately after cervical translocation, the entire thoracic aorta (from the arch of the aorta to the diaphragm) was aseptically removed and transferred to a 35-mm Petri dish with 2 ml of minimal essential Eagle's medium (MEM) with a reduced bicarbonate concentration (9 mM) and supplemented with 16% fetal bovine serum (heat-inactivated), 33 mM Hepes buffer (pH 7.4 at 37 C), and antibiotics (200 units/ml penicillin G and 200 μg/ml streptomycin (Hepes-MEM + serum). After measurement of the length of the aorta, it was transferred to a Petri dish with fresh media and completely stripped of its adventitial layer with watchmaker's forceps and microdissecting scissors (No. C-1225, Clay Adams, New York, NY). Light-microscopic examination of 5-μ sections of randomly selected blocks of stripped aorta did not reveal any residual adventitia. Specimens were then pooled in preweighed 35-mm Petri dishes with fresh media, and the dishes were reweighed to give average wet weights per stripped aorta. After midthoracic segments were obtained for light- and electron-microscopic study (see below), the specimens were again transferred to fresh medium and reweighed. They were then cut into approximately 1-mm segments with No. 10 scalpel blades, rinsed twice with phosphate-buffered saline (PBS) (0.1 M, pH 7.4 at 37 C) minus calcium and minus magnesium, transferred to a 50-ml round-bottom tube with a magnetic stirring bar and 1–2 ml of an elastase solution (100 units/ml PBS, pH 7.4), and stirred with a magnetic stirrer in a 37 C water bath. Lyophilized Type II elastase (90–110 units/mg) was purchased from Sigma Chemical Company (St. Louis, Mo). The total tissue substrate for the incubation was derived from pools of 10 aortas per age group, equivalent to 46–74 mg wet weight of intima-medi per milliliter of elastase solution. Tissue digestion was allowed to proceed for 30 minutes (in two 15-minute sequential treatments). After the first treatment, the intact segments of tissue were allowed to settle by gravity, and the supernatant cell suspension was transferred to a 15-ml test tube containing 5 ml Hepes-MEM plus serum. After the second enzymatic treatment of the residual tissue, 5 ml Hepes-MEM + serum was added, the residual intact segments of tissue were again allowed to settle by gravity, and the cell suspension was added to the 15-ml test tube containing the initial cell suspension. This was centrifuged at 1000*g* and resuspended in 5 ml of Hepes-MEM + serum. Aliquots of the cell suspensions were plated in sets of Petri dishes at concentrations of 1.0, 0.5, 0.25, 0.1, and 0.05 ml/60-mm dish in 4 ml of a growth medium consisting of bicarbonate-buffered (27 mM) MEM supplemented with 16% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. The same batch of fetal calf serum was used for all cloning experiments reported in this paper. Low-density dishes (those with 0.1 or 0.05 ml inocula) were left undisturbed for 6 days in a water-jacketed humidified incubator in 5% CO_2 in air at 37 C and then fixed and stained with 0.5% gentian violet in 20% ethanol. Colonies greater than 10

cells were counted with a dissecting microscope (×100 magnification). High-density dishes (those with 1.0, 0.5, and 0.25 ml inocula) were grown as above, except that they underwent a complete media change after 4 days and were harvested with trypsin versene[15] at 7 days. After cell concentrations were determined with a hemocytometer, these harvested cell suspensions were dilute-plated at various concentrations for the determination of "secondary" cloning efficiencies; after 10 days in growth medium, and plates were stained and counted as above.

The procedure for *P leucopus* was exactly as above, except that, because of the limitations on the availability of aging animals, the pooled specimens consisted of only two aortas per age group, and there was a single digestion period of 30 minutes in order to minimize loss of material. The concentration of tissue substrate per milliliter elastase solution (100 units/ml) was 20–33 mg wet weight intima-media. Experiments with thoracic aortas from young (3-month-old) *M musculus* did not reveal systematic differences in the yield of primary clones within the range of tissue concentrations and enzymatic treatment protocols used in the experiments with the two species of mice.

Immunofluorescence Assay for Factor VIII Antigen

The indirect immunofluorescence method employed for the detection of the Factor VIII antigen in the aortic clones was that of Gopspodarowicz et al,[16] with the exception that rabbit anti-human Factor VIII (Calbiochem-Behring, La Jolla, Calif) was diluted 1:20 instead of 1:100, goat anti-rabbit Ig (Calbiochem-Behring) was diluted 1:10 instead of 1:50, and the preparations were mounted for microscopy in phosphate-buffered glycerol. Positive controls consisted of cultures of bovine aortic endothelium (kindly provided by Dr. S. Schwartz), frozen sections of mouse aorta, and heterogeneous early outgrowths from collagenase digests[17] of mouse aorta. Negative controls included established cultures of human diploid skin fibroblastlike cells and nonendothelial components of the above positive controls.

Light and Electron Microsopy

Segments of aorta were fixed in neutral buffered formalin for light-microscopic examination, embedded in paraffin, sectioned at 5 μ, and stained with hematoxylin and eosin. Other aortic segments were fixed for electron-microscopic examination in 3% glutaraldehyde buffered with 0.1 M sodium cacodylate buffer containing 7.5% sucrose (pH 7.4) at 4 C for 3 hours. The tissue was subsequently rinsed overnight in 0.1 M cacodylate, postfixed in 1% osmium tetroxide, stained *en bloc* with 2% aqueous uranyl acetate, dehydrated in graded alcohols, and embedded in Epon 812.[18] Thin sections were cut with diamond knives on an LKB ultratome III (LKB Instruments Inc., Rockville, Md), double-stained with uranyl acetate and lead citrate,[19] and examined at 60 kv with an AEI 801 electron microscope.

DNA Assay

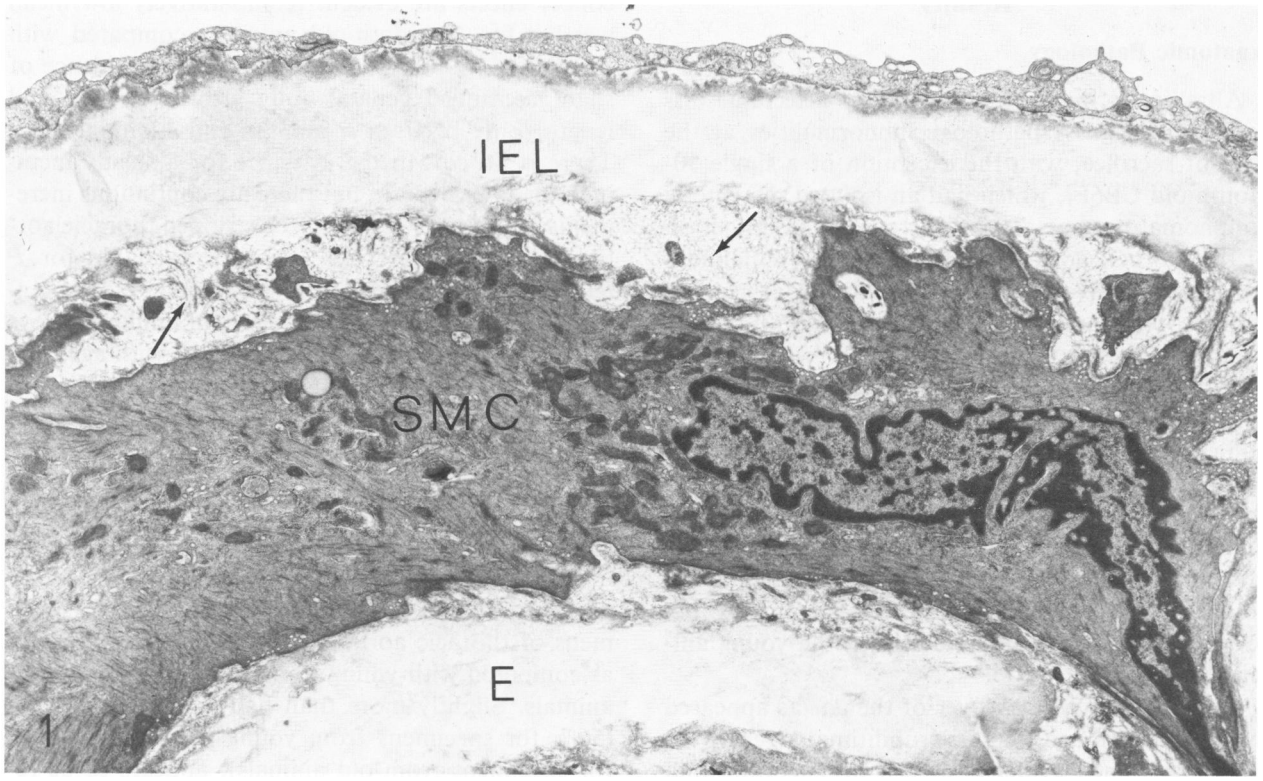
The fluorescent dye, Hoechst 33258 (Calbiochem-Behring) was employed to determine the DNA concentration of intimal-medial segments of aorta in tissue homogenates and in cell suspensions prepared by elastase treatment as described for primary cloning assays. Multiple independent extracts from 10–20 mg of wet tissue or from cell suspensions derived from comparable amounts of tissue were prepared. Samples were digested 1 hour at 55 C in 0.024 M EDTA, 0.075 M NaCl, and 0.1 μg/ml proteinase K (Boehringer-Mannheim, Indianapolis, Ind). A second 1-hour digestion (55 C) was carried out with fresh proteinase K and 0.1 μg/ml ribonuclease (Worthington, Freehold, NJ), followed by gentle homogenization with a Dounce homogenizer. This homogenate was extracted twice with phenol, twice with chloroform/isoamyl alcohol, and then ethanol-precipitated.

Samples were assayed in 2 M LiCl and 50 mM Tris buffer at pH 7.5 with 0.4 μg/ml and Hoechst 33258 dye with the use of a Perkin-Elmer (Norwalk, Conn) fluorimeter (excitation wavelength, 458 nm).[20] Calf thymus DNA (Sigma) was used for the preparation of standard curves.

Statistical Analysis

Linear regression analysis was carried out by the method of least squares.[21] Both parametric[22] and nonparametric[23] methods were employed as tests of the parallelism of the linear regressions obtained for the two different murine species.

Figure 1 – Electron micrograph from a cross-section of the thoracic aorta of a 6-month-old CB6F$_1$ male mouse. A differentiated smooth muscle cell is shown within the first layer of media (*SMC*). This layer is limited by the internal elastic lamina (*IEL*) and a layer of elastic tissue (*E*). The intercellular matrix contains sparse collagen fibrils and amorphous granular material (*arrows*). (×5000) **Figure 2** – Electron micrograph from a cross-section of the thoracic aorta of an 18-month-old CB6F$_1$ male mouse demonstrating a cell undergoing complete dissolution. Part of a necrotic nucleus can be seen in the lower lefthand portion of the figure (*N*). This first layer of the media is frequently enlarged when compared with the same layer in younger animals. *IEL*, internal elastic lamina. (×8000)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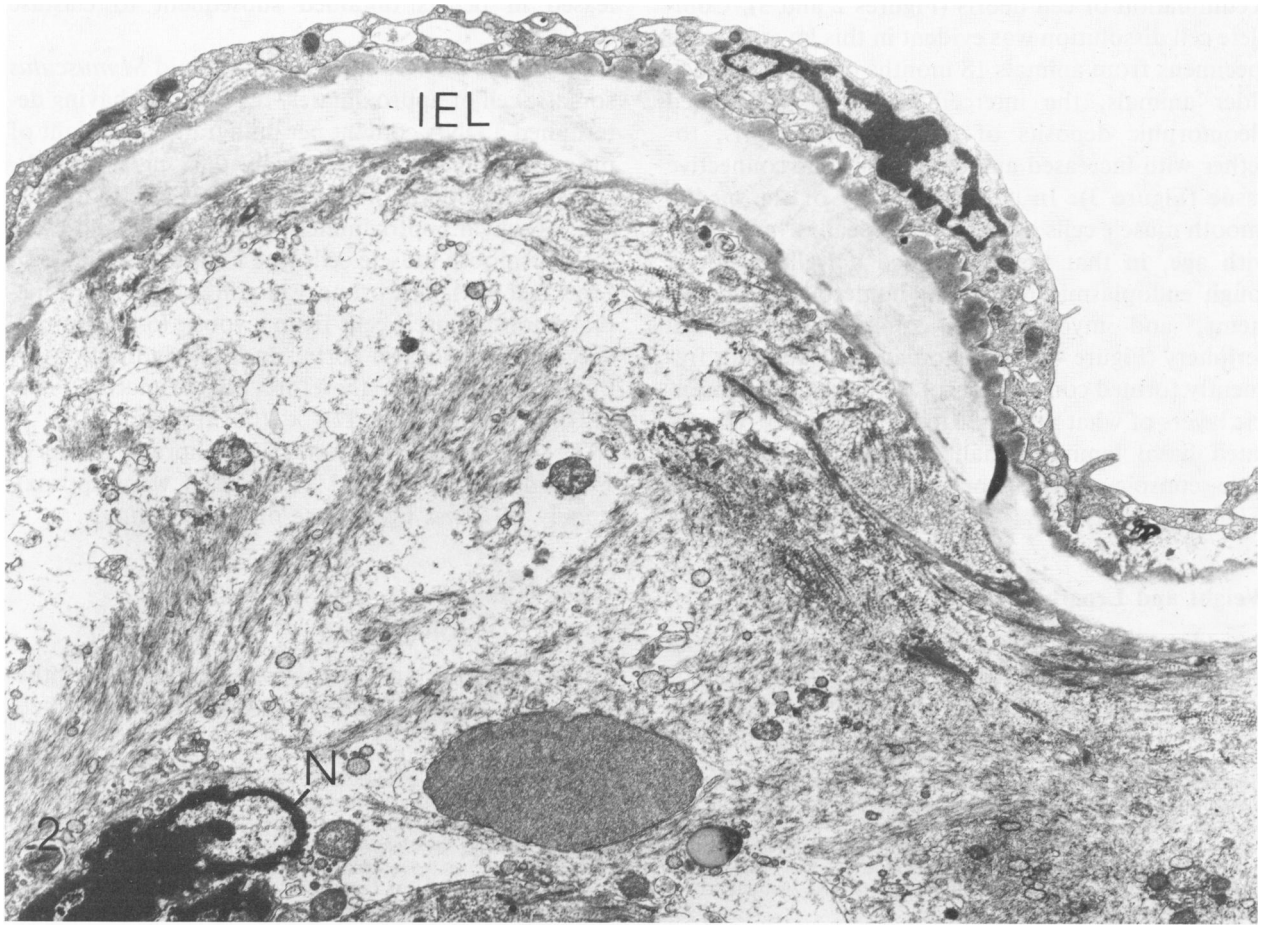
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Results

Anatomic Pathology

All animals were free of clinically detectable disease and of gross pathologic abnormalities at the time of sacrifice, with the exception of a single 30-month-old $CB6F_1$, which had an isolated mesenteric lymphomatous mass. Light-microscopic examination of the thoracic aorta failed to reveal any inflammatory, degenerative, or proliferative disease. Electron-microscopic examination of young (6-month-old) *M musculus* specimens ($CB6F_1$) revealed "well-differentiated" medial smooth muscle cells containing large complements of myofilaments and associated dense bodies (Figure 1); other organelles, such as rough endoplasmic reticulum, Golgi, and mitochondria, were sparse and usually confined to the perinuclear region of the cell. Sparse collagen fibrils, fine filamentous material, and occasional small membrane-bound vesicles characterized the intercellular matrix between medial smooth muscle cells in the young animals (Figure 1).

Although the intimal layer of the aortas appeared unaffected with age, the first subintimal layer of the media contained evidence of cell degeneration and accumulation of cell debris (Figures 2 and 3). Complete cell dissolution was evident in this layer in some specimens from animals 18 months or older. In such older animals, the intercellular matrix contained pleomorphic deposits of membranous debris, together with increased amounts of fibrous connective tissue (Figure 3). In addition, many of the medial smooth muscle cells of the layer appeared "modified" with age, in that they contained a well-developed rough endoplasmic reticulum, numerous Golgi elements, and myofilaments confined to the cell periphery (Figure 4). The cytoplasm of such cells frequently formed convoluted folds outlined by concentric layers of what appeared to be extensively reduplicated basal lamina. Small electron-dense granules were conspicuously associated with these lamina (Figure 4, inset).

Weight and Length Measurements

Tables 1 and 2 give several relevant weight and length measurements. The following points can be made with respect to *M musculus*: 1) Although cohort effects are evident (comparatively low mean weights for 18-month-old animals, compared with 12- and 24-month-old animals), there is evidence of slight continued general body growth (weight and length) only between 6 months and 12 months. 2) There is no convincing evidence for a steady increment in aortic length, but there are continuous increments in the wet weights of the stripped thoracic aortas. Although there are less data available for *P leucopus*, the wet weights of the intimal–medial segments of the thoracic aortas are quite similar to those of *M musculus*, and they also increase with age. The body weights of *P leucopus* are relatively stable with age from 8 months to 69 months and are somewhat less than those from the F_1 hybrid *M musculus*.

DNA Assays

Table 3 shows no significant difference in the total amount of DNA per milligram wet weight of specimens of thoracic aorta from old (27–28-month-old) as compared with young (6-month-old) *M musculus* animals. Slightly more than half of the total DNA (56% for specimens from young animals and 60% for specimens from old animals), appears to be released in pellets obtained subsequent to elastase digestion.

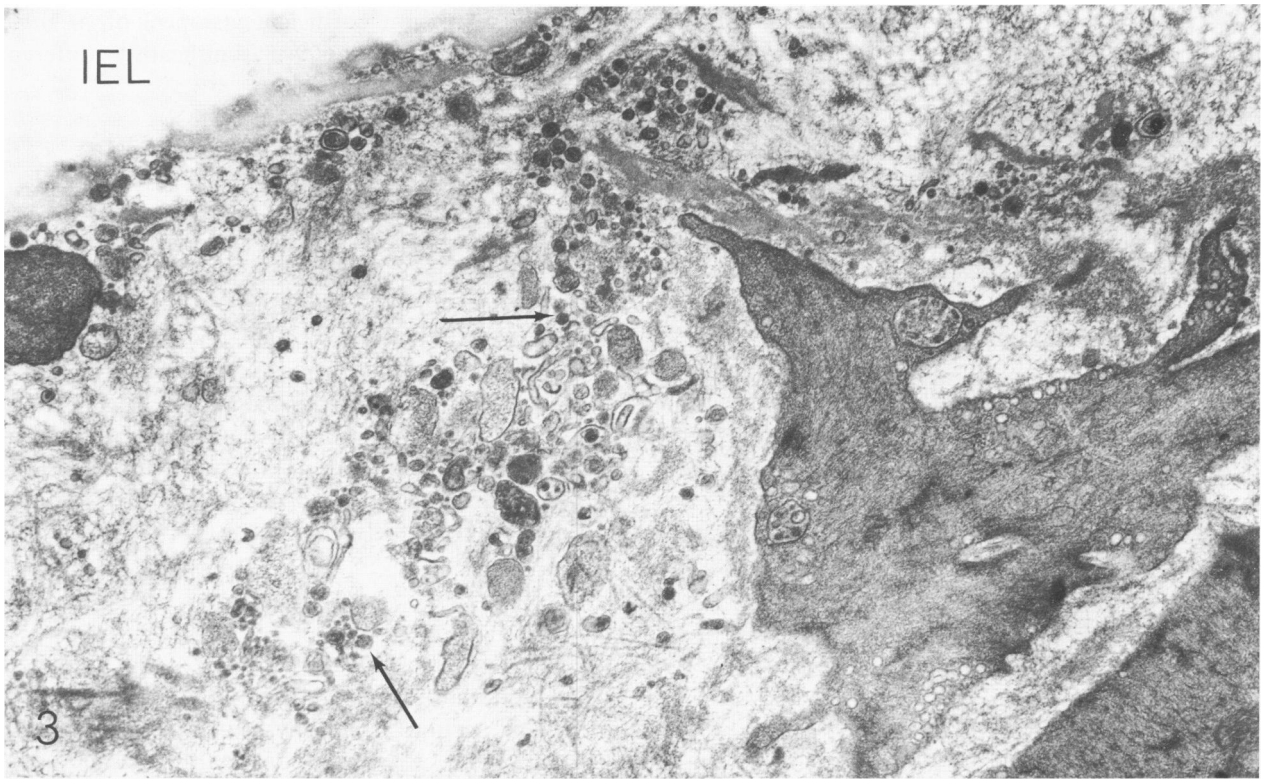
Assuming a DNA content per euploid *M musculus* somatic cell of approximately 6.3 pg[24] and having determined a DNA content per milligram wet weight of intima–media of approximately 0.32 μg (Table 3), there should be approximately 50,794 cells/mg tissue, of which approximately 72% (35,571) represent medial smooth muscle cells (see Figure 12A and B of Martin et al[12]). The primary cloning efficiency of medial smooth muscle cells from a young (6-month-old) specimen of thoracic aorta, under the conditions of our experiments, is therefore approximately 0.16–0.27% ($\sim$ 60–100/36,571 $\times$ 100) (Figure 5).

The DNA concentrations of the thoracic aortas of *P leucopus* (ages 6 and 24 months) were approximately 17% less than those of *M musculus*.

Immunofluorescent Assay for Factor VIII Antigen

While concurrent controls with bona fide endothelial cell cultures and histologic sections of endotheli-

Figure 3—Electron micrograph from a portion of the first medial layer of the thoracic aorta of a 30-month-old $CB6F_1$ male mouse. Note the accumulation of membranous cell debris (*arrows*) within the intracellular matrix. *IEL*, internal elastic lamina. (×9000) **Figure 4**—Electron micrograph showing a portion of a modified smooth muscle cell in the first layer of the thoracic aorta media of a 24-month-old $CB6F_1$ male mouse. The cell contains abundant rough endoplasmic reticulum (*rer*) and numerous Golgi complexes (*G*). Numerous cytoplasmic projections give the cell a villiform appearance. Concentric layers of basal lamina (*bl*) fill the space between the cytoplasmic processes. (×6800) **Inset**—A higher magnification of the redundant basal lamina, demonstrating the presence of numerous small electron-dense granules resembling proteoglycans (*arrow*) (×75,000)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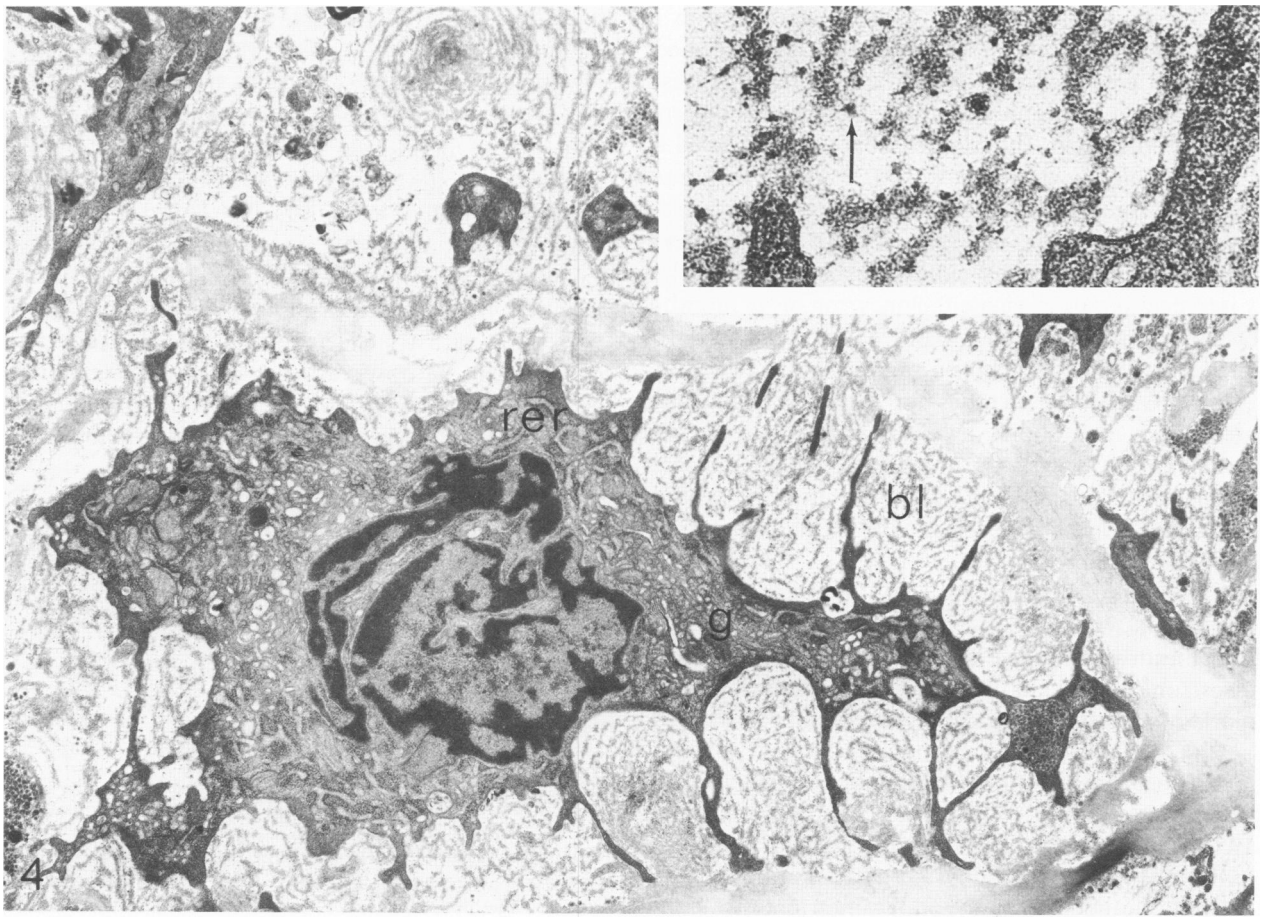
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Table 1—Total Body Weights, Body Lengths (Nose Tip to Tail Base), Thoracic Aortic Length (after Excision) and Wet Weights of Thoracic Aortas after Adventitial Stripping—*Mus musculus* (CB6F1 Males, Group 1), Ages 6 through 30 Months

Age (mo)	Body weight (g)	Body length (cm)	Aortic length (cm)	Aortic weight (mg)
6	30.6 ± 2.4	9.5 ± 0.3	1.4 ± 0.12	10.5
12	42.2 ± 5.6	10.0 ± 0.8	1.2 ± 0.25	11.1
18	36.5 ± 3.1	10.0 ± 0.7	1.4 ± 0.16	13.3
24	42.6 ± 2.5	10.5 ± 0.4	1.5 ± 0.15	13.8
30	37.0 ± 3.3	10.5 ± 0.4	1.6 ± 0.17	15.5

An average weight for stripped thoracic aorta (intimal-medial segment) was calculated by dividing by 10 the wet weight of a pool of 10 specimens for each age group. Other data are given as means ± standard deviations.

um were positive, 96 randomly selected clones from 8 separate culures of primary clones from elastase digests of intimal-medial strips of thoracic aorta of *M musculus* were all negative, as were cultures of human skin fibroblastlike cells.

Primary Cloning

Primary cloning experiments were carried out with two independent cohorts of $CB6F_1$ males. Group I animals arrived in the laboratory and were processed 4 months before Group II animals; a linear regression analysis of primary clones from elastase digests of their aortas as a function of donor age revealed a regression coefficient of -1.69 ± 0.27 (SE) clones/mg wet weight intima-media per month and a correlation coefficient of -0.83 ($P < 0.0001$); the y intercept would indicate 74.1 clones/mg at birth, and the x intercept would indicate 0 clones/mg at 43 months of age (Figure 5, Group I, $CB6F_1$). Experiments with Group II $CB6F_1$ mice gave a regression coefficient of -1.88 ± 0.19 (SE) clones/mg wet weight intima-media per month and a correlation coefficient of -0.92 ($P < 0.0001$); the y intercept would indicate 111.3 clones/mg at birth and the x intercept would indicate 0 clones/mg at 60 months of age (Figure 5, Group II, $CB6F_1$).

Comparable experiments with *P leucopus* were carried out within 2 days of the Group II $CB6F_1$ sacrifices and with the use of identical incubator conditions for culture; the linear regression data are also plotted on Figure 2. It gives a regression coefficient of -2.64 ± 0.12 (SE) clones/mg wet weight intima-media per month and a correlation coefficient of -0.98 ($P < 0.0001$). The y intercept would indicate 195.4 clones/mg at birth, and the x intercept would indicate 0 clones per milligram at 74 months of age.

Both parametric and nonparametric tests show that the regression coefficients (slopes) of *M musculus* and *P leucopus* are significantly different ($P < 0.01$).

Secondary Cloning

For the case of the Group II $CB6F_1$ animals, the more densely seeded primary cultures were trypsinized, cell counts were made, and cloning efficiencies were determined upon replating ("secondary" cloning efficiencies). The results, given in Figure 6, show cloning efficiencies varying from a mean of 2.2% with cultures from 6-month-old animals to a mean of 0.77% with cultures from 30-month-old animals. A linear regression analysis gave a regression of -0.06 ± 0.007 (SE) percent cloning efficiency per month and a correlation coefficient of -0.90 ($P = < 0.0001$).

Discussion

The present results establish, on a firm statistical basis, certain of our previously published results with the aging aorta of randomly bred *M musculus*.[12] As a mouse ages, the number of cells from the aorta capable of initiating primary clones declines in a linear fashion and with a slope that may be characteristic of the species. In addition, as the mouse ages, electron-microscopic analysis of intact aorta reveals increasing cell degeneration, accumulation of cell debris, and deposition of an extracellular matrix, findings comparable to those previously described in the arterial wall of aging rats.[25-28] This decline in primary cloning, however, can be observed during the early postmaturational period (between 6 and 12 months), before any morphologic alterations become detectable with the electron microscope. That such declines can be observed even in *secondary* cloning experiments indicates that this decline in replicative potential is intrinsic to the cell population and is not

Table 2—Total Body Weights and Wet Weights of Thoracic Aortas after Adventitial Stripping—*Peromyscus leucopus*, Ages 8 Months, 24 Months, and 69 Months

	8 months	24 months	69 months
Body weight (g)			
M	24.1	23.8, 22.4	22.8
F	24.2		21.1
$\bar{X}$	24.2	23.1	22.0
Thoracic aorta weight (stripped) (mg)			
$\bar{X}$	10.0	11.0	16.5

An average weight for thoracic aorta intimal-medial segment was calculated by dividing by 2 the wet weight of a pool of 2 specimens for each age group. M, male, F, female.

merely attributable to alterations in the extracellular matrix which might influence the yield of clonable cells obtainable with our technique of enzymatic digestion.

Although we cannot rule out some degree of contamination by adventitial fibroblasts, we believe that the predominant cell type whose replication potential is being assayed is the medial smooth muscle cell, because 1) the adventitial layer was mechanically removed prior to enzymatic digestion, 2) Factor VIII antigen could not be detected in any of 96 randomly sampled clones, and 3) the morphologic features of confluent cultures were characteristic of other smooth muscle cultures established in this laboratory and were not characteristic of endothelial cells.[19] It would, of course, be of great interest to carry out comparable studies of endothelial cells with the use of more suitable methods. Autoradiographic studies of DNA synthesis in organoid cultures, in fact, suggest that *all* cell types of the vascular wall, although with different intrinsic labeling indices, show declines in replicative potential with age.[12]

Using comparable methods with thoracic aortas from a comparatively long-lived murine species, *P leucopus* (having an MLSP twice that of *M musculus*), we have demonstrated that the total yield of clones from young sexually mature animals is about twice that of young sexually mature *M musculus*. The rate of decline, however, is somewhat greater, as confirmed by two types of statistical analysis, parametric and nonparametric. Methodologic variations, such as the batch of elastase employed, the conditions of digestion, and the culture environment, could obviously influence the total yield of clones. It is likely that such variables, rather than cohort effects, explain the differences in yield achieved with the two independent groups of genetically identical

Table 3—DNA Measurements of Thoracic Aortas of *Mus musculus* (Male of Strain AB6F1) and *Peromyscus leucopus* (Males)

	Age (months)	Specimen	N	DNA
M musculus				
	6	T	6	0.32 ± 0.02
	6	P	5	0.18 ± 0.02
	27-28	T	6	0.33 ± 0.04
	27-28	P	5	0.20 ± 0.03
P leucopus				
	6	T	3	0.26 ± 0.02
	24	T	3	0.28 ± 0.06

T, micrograms DNA per mg wet weight of intima-media.

P, micrograms DNA of pellet of elastase-derived cell suspension per milligrams wet weight of intima-media.

Results are expressed as the means ± standard deviations of assays of individual specimens from single animals.

N, number of animals.

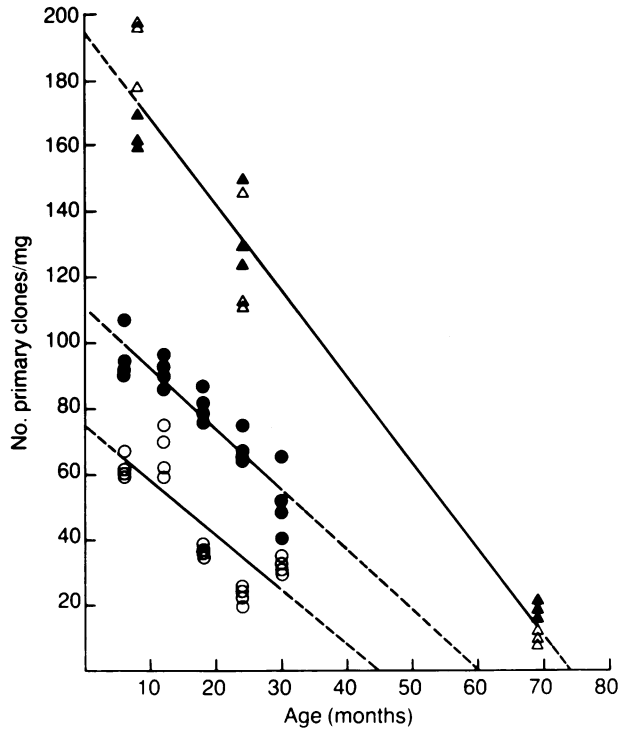

Figure 5—Primary cloning (number of smooth muscle clones per milligram wet weight) of the elastase-dissociated medial-intimal segments of thoracic aorta as functions of donor age. *M musculus* ($CB6F_1$), Group I (O—O) (linear regression coefficient = −1.69 ± 0.27 [SE] clones per milligram wet weight intima-media per month; correlation coefficient = −0.83; $P = < 0.0001$). *M musculus* ($CB6F_1$), Group II (● — ●) (linear regression coefficient = −1.88 ± 0.19 [SE] clones/milligram wet weight intima-media per month; correlation coefficient = −0.92; $P = < 0.0001$). *P leucopus* (△ = innoculum of 0.05 ml cell suspension per Petri dish; ▲ = innoculum of 0.1 ml cell suspension per Petri dish) (linear regression coefficient = −2.64 ± 0.12 [SE] clones/milligram wet weight intima-media per month; correlation coefficient = −0.98; $P = < 0.0001$).

M musculus (Figure 5) animals, especially because different batches of elastase were employed for the two experiments. It seems probable, however, that the differences in total clonal yields observed between *M musculus* and *P leucopus* are due, to a substantial degree, to intrinsic biologic differences, since the culture conditions and the batch of elastase used for *P leucopus* were identical to those used for the Group II *M musculus*; in model experiments with *M musculus*, the slight variations in the digestion protocol necessitated by the limited amounts of *P leucopus* tissue did not appear to account for the observed differences in yield. This conclusion is reinforced by the observation of much more substantial differences in clonal yield from other species (see below). Moreover, the rate of decline in clonal yield as a function of age appears to be quite reproducible in independent experiments with tissue from the same species (Figure 5). We therefore assume that these parameters are characteristic for the species. Thus, it

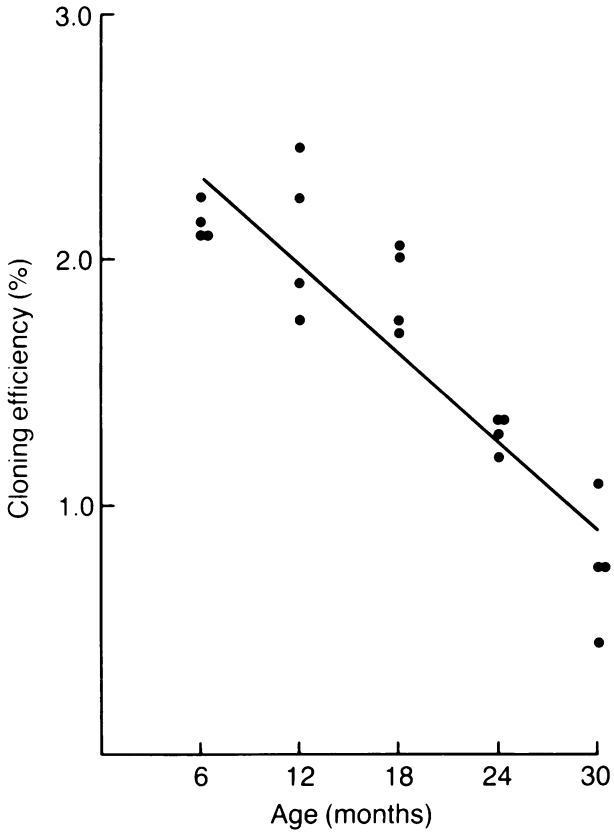

Figure 6—Secondary cloning efficiencies (%) from primary cultures of elastase-dissociated medial-intimal segments of thoracic aorta as a function of donor age. *M musculus* ($CB6F_1$) Group 1 (linear regression coefficient = −0.06 ± 0.007% (SE) per month; correlation coefficient = −0.90; $P = < 0.0001$).

seems possible that there are sets of genes that determine both the initial proportions of cells capable of replication at sexual maturity and the rates at which these proportions decline with age. It is already clear, however, that there is no simple correlation in this tissue of such assays of replicative potential with MLSP of the donor species. Unpublished experiments from our laboratory indicate, for example, that elastase digests of the intima-media of the mid-thoracic aorta of young (4-year-old) rhesus monkeys (*Macaca mulatta*) yield only about 1-3 primary clones per milligram wet weight. Different assays of replicative potential—for example, the cumulative population doublings of the primary clones or of the mass cultures—might give a different picture. It is thus likely that proliferative homeostasis and its rate of alteration with age[29] are under complex genetic control. This control could explain, in part, the varying susceptibilities of mammalian species to age-related degenerative and proliferative vascular disorders.[30]

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