Induction of Polyploidy in Cultures of Neonatal Rat Aortic Smooth Muscle Cells

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Arterial smooth muscle cells become tetraploid with age and hypertension. To further study this phenomenon, neonatal rat aortic smooth muscle cells were placed in cell culture and studied over time. Numerous cells with tetraploid and even octaploid DNA content appeared beginning in primary cultures. These increases in DNA content per cell were determined by quantitative fluorescence microscopy and flow cytometry, and true polyplody was confirmed by chromosome counts. In contrast, cells from adult rat aortas failed to produce significant polyplody cells over time in culture. In vitro culture of neonatal aortic cells may therefore be a model system for studying the initiation of polyplody in arterial smooth muscle. (Circulation Research 1986; 59:633–644)

Two distinct forms of replication of arterial smooth muscle cell DNA occur as responses to arterial injury or aging. An increase in total cell number is a common response of the vessel wall to numerous stimuli such as mechanical trauma. There is evidence that this response may be mediated by release of platelet-derived growth factor at sites of endothelial denudation. In contrast, DNA synthesis associated with larger cells with tetraploid and octaploid DNA content follows hypertensive stimuli in the rat. This hyperplastic replication is also a part of the normal maturation of the arterial tree in rat and man with increases in frequency of polyplody cells occurring with age in both species. The response is a true and permanent increase in the amount of DNA per cell since Goldberg et al were able to show that tetraploid cells from hypertensive vessels retain their polyploid DNA content even when isolated by cell sorter and passaged in cell culture. The mediator of this response is unknown.

The observations reported here began as an effort to study a particular instance of cell replication, that is, the changes in growth control that occur during normal development and aging. We attempted to explore an analogy to the development of striated muscle. In that analogy to striated muscle suggests that we might be able to devise an in vitro system to study development of smooth muscle cells and also suggested that control of smooth muscle cell growth might be developmentally regulated. We have shown that cultured rat pup smooth muscle cells, but not adult cells, make platelet-derived growth factor, perhaps representing a developmentally controlled autocrine mechanism during fetal or neonatal development. About the same time, Kocher et al showed that neonatal cells in vitro were relatively undifferentiated, that is, lacking or having a lower content of some of the contractile proteins characteristic of mature smooth muscle. Thus, while the analogy is obviously weak, the smooth muscle cell in the newborn aorta of the rat is by several criteria still a relatively undifferentiated cell. While it would be desirable to study growth control in a more primitive vascular anlage, we began to study the growth properties of cultured smooth muscle cells obtained from the still-replicating and still-differentiated smooth muscle population of the newborn rat aorta.

We report here that polyploidy occurs at early time points in cultures of smooth muscle cells from newborn rats but not in cultures from adult rats. In vitro culture may therefore be a model system for studying arterial smooth muscle development, including the induction of polyploidy.

Materials and Methods

Aortas from male and female rats, ages 12–19 days, 3 months, and retired breeders, were used from the spontaneously hypertensive rat (SHR) and Wistar-Kyoto (WKY) rat strains, both obtained from Charles River and Taconic Farms. Culture preparation was the same for each rat strain. After sacrifice by ether and/or cervical dislocation, the thoracic aorta was removed...
and placed in Waymouth's culture medium with added penicillin (100 U/ml) and streptomycin (0.1 mg/ml). For the neonatal aortas and with the aid of a dissecting microscope, a straight pin was inserted into the lumen of each aortic specimen to remove the endothelial cells by direct abrasion. Then with a scalpel and watchmaker's forceps, the adventitial layer was removed. For the adult aortas, which were larger and easier to handle, each aorta was opened longitudinally, and the endothelium was scraped away with a teflon spatula. Then the vessel was subjected to a 30-minute enzymatic treatment (see below), after which the adventitia of these adult aortas could be more easily removed with forceps. In both neonatal and adult smooth muscle cell preparations the adequacy of adventitial removal was verified by elastin staining of representative medial segments showing that all cells used for culture came from areas between elastic laminae.

Using a procedure similar to Chamley-Campbell et al., the aortic segments for culture were then subjected to enzymatic dissociation by mincing in 0.1% collagenase (Type I, 146 U/mg; Worthington) and 0.05% elastase (Type I, 32 U/mg; Sigma Chemical Company) in Waymouth's medium (pH 7.4), with added soybean trypsin inhibitor (0.375 mg/ml) and bovine serum albumin (2 mg/ml). These mixtures were then incubated at 37°C under a 5% CO₂, 95% O₂ atmosphere for 1-2 hours, with gentle pipetting using a wide-mouth pipette to aid the tissue dissociation. After adequate dispersion, enzymes were inactivated by adding bovine whole blood serum (20% final concentration). The cells were then centrifuged (200 g for 7 minutes), and the cell pellet was resuspended in calcium and magnesium-free phosphate-buffered saline with 0.5 mM EDTA, and washed twice with this medium before placement in final culture.

The cell yield from enzymatic dissociation averaged approximately 10,000 cells per neonatal aorta and approximately 1.5 x 10⁶ cells per adult aorta. Given a total aortic DNA content averaging 4.55 µg and an estimate of 7.1 pg DNA/cell, this represents an average 1.6% cell yield from each neonatal aorta. Previous estimated yields from adult aortas have averaged 20%. The viability of the enzymatically dispersed cells averaged 70% as measured by trypan blue exclusion. Two methods of primary culture were used. In the first and most used method, cells from the same rat strain were pooled and plated in Waymouth's medium with 20% bovine whole blood serum at approximately 10⁵-10⁶ cells per "35-mm" plastic petri dish, giving a plating density of approximately 1.3-13 x 10⁴ cells/cm². Estimated plating efficiencies ranged from 30 to 80%. Primary cultures were grown to postconfluence and studied at or near time of passaging, at which time the cell number averaged 7.5 x 10⁶ cells per 35-mm dish. After primary culture each 35-mm dish of cells was transferred into a 75-cm² plastic tissue culture flask (Corning, N.Y.). At subsequent passages, postconfluent cultures with approximately 7-12 x 10⁶ cells/flask were split 1:10, again into 75-cm² flasks. This was true for both the neonatal and adult cultures.

In the second method, the cells obtained from each aorta were plated in small "24-multiwell" or "96-multiwell" plates with similar cell densities and with the same media as described above. Subsequent passaging was done into 35-mm dishes and later into 75-cm² flasks at similar densities and passaging ratios as described above. There was no difference in plating density between neonatal and adult cells.

**Quantitative Fluorescence Microscopy**

For measurements of DNA/cell, duplicate cultures were trypsinized at various times and subsequently fixed in suspension with 70% ethanol at 0°C for one-half hour and stored as such until ready for analysis. The cells were then spun down (200 g for 7 minutes), and the cell pellet was resuspended in 1 ml of a phosphate-buffered saline solution containing 0.2% RNase (Sigma) and 25 µg/ml of the nucleic acid-specific compound propidium iodide (Calbiochem) for 30 minutes at room temperature. Then using quantitative fluorescence microscopy (Leitz MPV Compact microscope) cell number vs. DNA/cell histograms were generated based on 200 cell readings per sample. Chicken erythrocytes were used as DNA standard (2.5 pg DNA/cell). From such histograms the frequency of cells with 2c DNA (diploid G₀/G₁ cells), 4c cells (diploid G₀/G₁ cells or tetraploid in G₁), and 8c cells (tetraploid G₀/G₁ cells or octaploid cells in G₁) could be determined. The fluorescent microscope used allowed visualization of individual intact cells by phase microscopy and only individual, single cells were measured. This same microscope also allowed direct clear visualization of the nucleus (nuclei) within the cell during the fluorescent measurement. In this way the mononucleate vs. binucleate status of the cell could be ascertained. The quality of nuclear staining was very good and comparable to preparations using Triton X-100 and DAPI staining (done for flow cytometry below). Additionally, after each measurement, if any uncertainty remained regarding the nucleus, the fluorescent light was put back on for more careful examination of the nucleus, focusing up and down.

**Thymidine Labelling Time-Course Trials**

Forty-six neonatal WKY rat aortas were subjected to enzymatic dissociation as described above, and the resultant cells were pooled. Representative portions of some isolations were fixed in suspension with 70% ethanol for initial quantitative fluorescence microscopy determinations of DNA/cell, also as described above. The remaining cells were then placed in cell culture on either glass coverslips or on multiwell plastic petri dishes at a density of approximately 7 x 10⁴ cells/cm² with added tritiated thymidine (0.001 µCi/ml; New England Nuclear, specific activity = 6.7 mmol/mCi of tritiated thymidine). Using similar unlabelled cultures as controls, this low dose of tritiated thymidine was determined as one in which no significant effect on cell number was apparent at 4 days due to this chronic exposure. Also the ability of the media from such chronically labelled cultures to label-grow-
ing cells was confirmed by incubating some adult growing cultures with this conditioned medium. The cells were harvested after 2 and 4 days of continuous tritiated thymidine exposure, fixed either on a glass coverslip or in suspension in 70% ethanol, later treated with RNase as above, and then dried on glass coverslips. These coverslips were later dipped in autoradiographic emulsion (Kodak NTB-2 Nuclear Track Emulsion, at a 50/50 dilution with distilled water) and exposed in the dark for two weeks before developing (Kodak D-19 developer, Kodak Rapid Fix) as done previously in this laboratory. 21

For quantitative fluorescence microscopy, these coverslips were stained with a propidium iodide staining solution described above for 30 minutes and then inverted over glass coverslips and sealed. This technique produced good quality fluorescence DNA/cell histograms. Additionally, since the autoradiographic grains were within the radiographic emulsion immediately adjacent to the cells and since the coverslip was inverted during the measurements on our epifluorescent microscope, the grains could thus be visualized immediately below each labelled cell and did not interfere with the fluorescence reading (from fluorescent light directed back up into the photomultiplier tube). Thus, we were able to determine the DNA content of each cell, as well as its thymidine labelling status. Only cells with 5 grains or greater over the nucleus were considered positively labelled.

**Flow Cytometry**

Flow cytometry on selected cultures was performed as previously described. 5 Briefly, after trypsinization of the culture, cells were centrifuged at 1,500g rpm for 8 minutes, and the cell pellet was suspended in 1.0 ml of nuclear isolation medium (Tris-buffered isotonic saline, pH 8.0, with 0.6% Nonidet P-40, 1.0 mM CaCl2, 0.5 mM MgSO4, 106 mM MgCl2 containing diamidinophenylindole at 10 μg/ml; NIM-DAPI) for 10 minutes on ice. The nuclei were then syringed three or more times through a 26-gauge needle to ensure only single nuclei being measured by the machine. The lack of nuclear clumps was verified by direct fluorescence microscopy on the sample before being run on the flow cytometer. Histograms of DNA/nucleus were generated as previously described, 5,20 with cell cycle compartments being estimated by an adaptation of the method of Dean and Jett, 22 and nonlinear least-squares fitting being done by the method of Marquardt. 23

**Chromosome Counts**

Karyotyping was performed using standard methods. 24 Growing cells were treated with 0.05 μg/ml colcemid for 30 minutes, then trypsinized, pelleted, and resuspended in hypotonic KCl (0.075 M) for 30 minutes. The cells were then fixed in ice-cold 3:1 methanol:acetic acid for 30 minutes and finally dropped onto glass slides and air dried. The slides were then stained with Diff-Quick Solution II (Dade Diagnostics, Inc.) for 1 minute and then coverslipped. Photomicrographs of metaphase spreads were obtained, on which the chromosome counting was performed.

**Cell Cycle Arrest**

Cell cycle arrest was achieved by a combination of thymidine block and low serum. 5,20 since low serum alone failed to arrest these cells. 16 Briefly, cells were switched for 2-3 days into culture medium containing 0.5% CM-Sephadex-treated plasma-derived serum. 25 The cells were then trypsinized and replated at 5 × 10^4/cm² in 75-cm² flasks, with 0.5% plasma-derived serum and 2.5 mM thymidine for 48 hours. Control cultures were either replated in 0.5% plasma-derived serum alone or 5% whole bovine serum for 48 hours. The cells were finally trypsinized and prepared for flow cytometry.

**Immunocytochemistry**

Actin immunocytochemistry was performed on frozen sections of cleaned neonatal thoracic aorta, as well as on enzymatically dispersed cells fresh from the neonatal aorta. We have developed a monoclonal anti-actin antibody, raised to a preparation of chicken gizzard actin, which is smooth muscle cell specific and which recognizes a determinant common to smooth muscle alpha- and smooth muscle gamma-actin isotypes. 28 A monoclonal anti-desmin antibody (OncoGene Science, N.Y.) was also used. Both tissue sections and slides of dispersed cells were fixed in ice-cold methanol for 5 minutes, washed in phosphate-buffered saline, and then reacted with the monoclonal antibody (1:100 dilution of mouse ascites fluid for 30 minutes). This was followed by counterstaining with FITC-conjugated goat anti-mouse IgG and IgM (1:40 dilution; Tago Inc, Burlingame, CA). The specimens were studied on a Leitz MPV Compact fluorescence microscope. Anti-von-Willebrand’s factor staining (Dakopatts, Denmark) was also performed to assess the degree of endothelial cell contamination of the freshly dispersed cells.

**Results**

**Histology and Immunocytochemistry**

Histologic sections of whole pup aortas revealed the expected morphology for this tissue. The intima was composed of a monolayer of endothelial cells, media was composed of smooth muscle cells scattered between the elastic laminae, and there was a scant outer adventitial layer. Representative sections of vessel cleaned for use in culturing medial smooth muscle cells confirmed the absence of adventitia and endothelium.

Actin immunohistochemistry was also performed on these neonatal and adult aortas. For this purpose, we used a monoclonal antibody that is specific for smooth muscle actin as demonstrated immunocytochemically and in immunotransfer. 28 Sections reacted with this monoclonal anti-actin antibody showed uniform staining of all medial cells without evident heterogeneity. Endothelial cells and adventitial cells, as expected, did not stain. Immunocytochemistry of representative dis-
persed cells from either young or old animals used for culture revealed greater than 98% of the cells reacting with our smooth muscle actin monoclonal antibody (Figure 1, top) and none reacting with an anti-von-Willebrand's factor antibody.

Culture Morphology and Immunohistochemistry

Culture morphology was the same with cultures derived from individual aortas or from several pooled aortas. Although the cells were initially rounded-up and refractile immediately after enzymatic dissociation, most cells were well spread and proliferating by the second to fifth day in culture. Variable amounts of cell death were evident in all cultures in the first two days, with lesser amounts through the primary culture and occasionally into the first passage.

Although those cells from neonatal rat aortas that attached to the petri dishes in primary culture spread in 1–2 days, the density of this spread population was less than that of the adult cultures, and such neonatal cultures took approximately 3–4 weeks to reach confluence. The adult cultures took only approximately 2 weeks to reach similar confluence. Thereafter, both types of cultures grew at similar rates, achieving confluence 7–10 days after trypsinization, passing with a ratio of 1:10. Thus, the growth differences in the primary cultures appeared to be related to the initial spread cell density and not to some intrinsic difference.

FIGURE 1. Top: Immunofluorescence of enzymatically dispersed cells from a 2-week-old SHR rat thoracic aorta, using the anti-smooth-muscle actin monoclonal antibody. The bar shown represents 10 μ. Bottom: Control of immunofluorescence of dispersed cells using normal mouse serum. Same magnification as in "top."
between adult and neonatal aortic smooth muscle cells. All cultures grew well for greater than 10 passages without any period of marked slowing of growth or extensive cell death. Specifically, although the cultures commonly contained a few floating cells, these comprised less than 5% of the total cells present; the rest were well spread. No culture exhibited cell death of nearly all the cells present.

The primary and subsequent cultures consisted of spindled or branched mesenchymal-appearing cells often overlapping each other and forming typical "hill and valley" patterns similar to other smooth muscle cell cultures we have seen from adult rat aortas (Figure 2, top). Occasional binucleate cells were also seen, especially after the first passage, but comprised less than 3% of the cells present (assayed by fluorescence microscopy as described in the "Materials and Methods" section). In all of the neonatal cultures and some of the adult cultures an additional morphologic appearance was noted, intermixed with the above-described mesenchymal-appearing cells. This consisted of a cobblestoned, monolayer pattern, somewhat resembling endothelial cells (Figure 2, bottom). The extent of this second pattern varied from culture to culture. These cells were negative for von Willebrand's factor, using an antibody that recognizes rat and bovine endothelial cells. Both adult and neonatal smooth muscle cells lost their reactivity with the smooth muscle-spe-

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**Figure 2.** Phase micrographs of cultured neonatal rat aortic smooth muscle cells. Top: Representative WKY culture in the fifth passage displaying the spindle-shaped "mesenchymal" morphology. Bottom: Representative WKY culture in the fifth passage, focally displaying a flat "cobblestone" morphology in one area (arrow).
cific anti-actin antibody with passage in culture while at subconfluent densities. This is similar to the findings of Gröschel-Stewart et al\textsuperscript{29} using a smooth muscle-specific antimyosin antibody. This marker was therefore not useful for identifying individual cells in culture as being of smooth muscle origin. However, after reaching confluence, focal reactivity with this antibody was noted in the "hill" regions. Similarly, these passaged cells did not react with a monoclonal anti-desmin antibody despite previous desmin positivity being demonstrated in the aortic tissue. The cobblestoned pattern was more apparent in cultures at low density than in cultures at confluence. Moreover, Walker et al, in our department, have recently observed a similar phenotype in cultured smooth muscle cells obtained from the thickened intima of adult rat carotid arteries following endothelial denudation with a balloon catheter (unpublished observations). In that case there is no source of cells other than the tunica media, and we have already shown, as noted above, that all cells in the tunica media stain positively for smooth muscle actin. Thus, this phenotype can appear even when there is no apparent source of endothelium.

The eventual loss of monolayer, the absence of von Willebrand's factor antigenicity, the presence of smooth muscle actin in essentially all the freshly isolated cells, and the procedure used to remove endothelium from the vessels prior to culture all argue that this second phenotype is not derived from contaminating endothelial cells.

Cultures from both WKY and SHR rat strains showed similar morphologies without distinguishing characteristics.

**Quantitative DNA Fluorescence Measurements**

Quantitative fluorescence microscopy confirmed our earlier results for freshly isolated rat aortic cells.\textsuperscript{6} Neonatal cells fixed immediately after enzymatic dissociation from the aorta had a diploid DNA content (Figure 3, top). Rare cells (0-3\%) with a 4c DNA content were present with a frequency small enough to be considered as most likely cycling G\textsubscript{2} diploid cells given the proliferative state of the aorta in this age animal. No 8c cells were seen. Thus, neonatal rat aortic smooth muscle cells enter cell culture in the diploid state.

The presence of some cells with enlarged nuclei (nuclei covering approximately twice the spread area of the majority of the cell nuclei present and with four or more nucleoli as visualized by phase microscopy) led us to suspect that hyperploid cells were present in primary cultures derived from the neonatal aortas. This was confirmed by karyotyping (see below) and strongly suggested by quantitative DNA/cell measurements and flow cytometry.

With time in culture, and often beginning during the primary culture, an increasing percentage of the cells were found with 4c and even 8c DNA content (see Figure 3, middle and bottom). Again, almost all of these cells were mononucleate, and these high DNA contents per nucleus were confirmed on selected cultures subjected to flow cytometry. Thus, these higher DNA values do not represent simply the few binucleate cells seen in these cultures. Cells with 4c DNA content could represent diploid cells in the G\textsubscript{2} or late S phases of the cell cycle. The presence of 8c cells, however, implies that there are at least some true tetraploid cells present, at least in G\textsubscript{2}, if not octaploid cells in G\textsubscript{3}.

As increasing numbers of cells became available for study at later passages, flow cytometry was performed. This provided DNA/cell histograms similar to the quantitative fluorescence microscopy histograms described above (see Figure 4, top). Here we wished to eliminate the possibility of diploid cells in late S or G\textsubscript{2} phases of the cell cycle being interpreted as "tetraploid." Thus, when cultures with high 4c and 8c values were arrested in their growth by a combination of low serum concentration and a thymidine block to eliminate the presence of cycling G\textsubscript{2} cells (Figure 4, middle...
and bottom), we noticed that significant numbers of cells with 4c and 8c DNA values remained. Freshly isolated aortic smooth muscle cells were added to the cultured samples to define the diploid DNA content peak position. These cells produced a prominent peak at the same 2c position as the few 2c cells of the polyploid cultures, with similar coefficients of variation (data not shown).

DNA per cell histograms of rat aortic cultures, generated by quantitative fluorescence microscopy, were compared as a function of passage number. Figures 5-7 display representative cultures followed over time from 6 neonatal WKY isolations, 8 neonatal SHR isolations, 8 three-month rat isolations (3 WKY and 5 SHR), and 5 retired breeder isolations (3 WKY and 2 SHR). To minimize the number of actively cycling cells, the cultures were passaged approximately every 28–30 days, well after cultures had reached confluence. The data from representative cultures are presented in Figures 5–7 as plots of the frequency of cells with 4c or greater DNA content versus time in culture. Numerous “4c and above” cells were produced over time in nearly all neonatal cultures, occasionally reaching 50–100% of the culture population. There was much variability in the percentage of such “4c and above” cells from one culture to the next even within the same rat strain. The proportion of such cells within a given culture also fluctuated over time. In the later passages, some cultures had a small percentage of cells in this category (Figure 5, top right), whereas others displayed a predominantly diploid DNA content (Figure 5, top left). Some of this dispersion may represent different proportions of cells actively growing at various passage times. Despite this dispersion, significant hyperploid cellular DNA content was produced and maintained in these cultures over time.

No consistent differences in DNA/cell measurements were evident between cultures of cells derived from SHR or WKY aortas nor did the level of DNA content appear to be associated with the presence or absence of the cell culture “cobblestone” morphology. Cultures derived from adult rat aortas produced different data. Since this laboratory had previous experience with freshly isolated adult SHR and WKY aortic smooth muscle cells, only a few representative fresh isolations were studied by quantitative DNA fluorescence microscopy. Consistent with the previously reported data, the initial 4c DNA percentages ranged from 4 to 30% in these 3-month and older animals. At early time points in culture, cycling G2 cells increased the 4c DNA population. In contrast to the neonatal cultures, however, cells derived from adult aortic smooth muscle cultures all tended towards diploidy with passage in culture (Figures 6 and 7). Again, this study revealed no discernible differences in polyploidy potential between the SHR and WKY rat strains.

**Thymidine Labelling Time-Course Trials**

The aim of these experiments on neonatal aortic smooth muscle cells with chronic tritiated thymidine labelling for up to 4 days was to look for evidence of significant cell fusion in the formation of “4c and above” DNA content cells. Given that the freshly isolated neonatal aortic smooth muscle cells had little to no 4c DNA cells, we hypothesized that if cell fusion was occurring before DNA synthesis, then several unlabelled 4c DNA cells would be found. Unfortunately the results were inconclusive. Using neonatal WKY aortas, quantitative fluorescence microscopy of two initial isolates revealed only 2% of the cells as having
4c DNA content (9/420 cells total), a frequency similar
to that previously described. Then during the first 2
days of adaptation to culture conditions, very little cell
proliferation occurred associated with some cell death.
Seven coverslips of cells subjected to 2 days of chronic
thymidine labelling were pooled and revealed a total
labelling index of only 1% (22/2,314 cells). The label-
ing index of the 2c cells was less than that of the 4c
cells (0.5% vs. 9.6%). In particular, the percent of
total cells which were 4c and unlabelled was 4%, as
compared to 2% in the initial isolate. A histogram from
a representative coverslip is shown in Figure 8. Addi-
tionally in this pooled 2-day labelling group, 5 binucle-
ate cells were found, none of them labelled. This com-
 pared with no binucleate cells being present in the
initial isolate.

By 4 days in culture almost all of the cells had
undergone replication. The labelling index on two
pooled 4-day chronically labelled coverslips was 95%
(295/312 cells) with that of the 2c cells and 4c cells
being 93 and 98%, respectively. By this time the per-
cent of all cells which were unlabelled 4c cells was
only 0.6%. No binucleate cells were seen. Given the
low frequency of unlabelled 4c cells, it is at least
possible that these represent persistent arrested G
2
cells
or a small percentage of tetraploid cells already present
in the neonatal rat aorta.

Karyotyping

Chromosome counts were performed on 174 meta-
phase spreads of selected neonatal rat aortic cultures.
Distinct diploid, tetraploid, and higher ploidy meta-
phase spreads were clearly present (diploid = 42 chro-
mosomes\(^{29}\)) but with much dispersion in the actual
counts. More specifically, 93 spreads (53% of the to-
tal) were in the diploid (37–47 chromosome count)
cluster, 20 spreads (11% of the total) were in a second,
tetraploid (74–94 count) cluster, and 5 spreads (3% of
the total) were in the greater than 94 chromosome
count range. The remaining spreads fell outside of
these ranges. Given the flow cytometry data with far
better coefficients of variation, we suspect that random
losses and redistributions of chromosomes among the
metaphase spreads were responsible for the spread in
counts observed. Polytene chromosomes and other ob-
vious gross anomalies were not seen.

Discussion

We have reported above our initial observations on
culturing neonatal vs. adult rat aortic smooth muscle
cells. Diploid smooth muscle cells from the neonatal
aortas of SHR and WKY rats attain a tetraploid or
octaploid DNA content soon after being placed in cell
culture, and these high DNA/cell levels generally per-
sist through multiple passages. In contrast, cultured
cells derived from SHR and WKY adult rat aortas
show, if anything, a decrease in the frequency of high
DNA content cells with passage. This has been demon-
strated here primarily through the use of quantitative
fluorescence microscopy. Flow cytometry was used in
representative cases to confirm the percentages of high
DNA content cells by this more precise technique
(which analyzes many more cells than does quantita-
tive fluorescence microscopy), as well as to monitor
the effects of low serum and thymidine blocking. Fi-
nally, chromosome counts were performed to verify
that true tetraploidy had been produced in at least many
of these cells with high DNA content. The appearance

\[\text{Figure 5. Graphs of the percent of cells}
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\[\text{with 4c or greater DNA content in cultured}
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\[\text{cells derived from neonatal rat aortas versus}
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\[\text{time. These measurements were obtained by}
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\[\text{the quantitative fluorescence microscopy tech-
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\[\text{nique, and each point represents a determina-
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\[\text{tion from a sample of at least 200 cells. Determin-
}\]

\[\text{ations were usually done on postconfluent}
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\[\text{cultures. X axis: passage number with subdivi-
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\[\text{sions indicating the time within each passage}
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\[\text{level (each small division represents 10 days).}
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\[\text{Y axis: percent of cells with 4c or greater DNA}
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\[\text{content. Left three: Representative WKY neo-
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\[\text{natal cultures (from a total of 6 different isola-
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\[\text{tions). Right three: Representative SHR neo-
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\[\text{natal cultures (from a total of 8 different}
\]

\[\text{isolations).}
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of 4c DNA content cells within the first passage, the low coefficients of variation of the peaks by flow cytometry, the quantal doubling of the DNA content, and the absence of obvious karyotypic derangements argue that this phenomenon is analogous to the physiologic increase in ploidy seen in animals\textsuperscript{6} rather than an aneuploid change associated with transformation in culture.\textsuperscript{3,12}

Polyploidy is found as a normal change in certain types of cells as animals get older.\textsuperscript{33} It is also typically seen in certain types of cells at late passage in cell culture.\textsuperscript{34,35} These latter changes are generally not seen until after many passages of the cultured cells, in contrast to the data presented here. Moreover, the phenomenon reported here is characteristic of cells from young animals and not of cells from adult animals. This contrasts with the usual in vitro cell aging phenomenon.\textsuperscript{36,37}

Neither the full nature of the high DNA content cells nor the events leading to their formation are clear. By chromosome counts, however, we do know that a significant number of these cells are hyperploid. Considering the 4c DNA content cells, which comprised the majority of our increased DNA content cells, only a small proportion of these cells appear to be simply cycling diploid cells in the late S or G\textsubscript{2} phases of the cell cycle, since halting cell growth by low serum and thymidine block hardly changed the ratio of these cells to 2c cells (e.g., see Figure 4, middle and bottom). Additionally in those cultures in which the majority of the cells had 4c and above DNA content, the flow cytograms frequently displayed an S phase plateau between 4c and 8c, consistent with cycling tetraploid cells (e.g., Figure 4, middle). Also as mentioned before, direct visualization of the fluorescing nuclei during the quantitative microscopy allowed us to deter-

**Figure 6.** Graphs of the percent of cells with 4c or greater DNA content in cultured cells derived from 3-month-old adult SHR aortas versus time. These measurements were obtained by the quantitative fluorescence microscopy technique, and each point represents a determination from a sample of at least 200 cells. Determinations were usually done on postconfluent cultures and are abstracted from a total of three WKY isolations and five SHR isolations. X axis: passage number with subdivisions indicating the time within each passage level (each small division represents 10 days). Y axis: percent of cells with 4c or greater DNA content. Three separate representative cultures from three separate adult SHR rat aortas are displayed.

**Figure 7.** Graphs of the percent of cells with 4c or greater DNA content in cultured cells derived from retired breeder SHR and WKY aortas versus time. These measurements were obtained by the quantitative fluorescence microscopy technique, and each point represents a determination from a sample of at least 200 cells. Determinations were usually done on postconfluent cultures. X axis: passage number with subdivisions indicating the time within each passage level (each small division represents 10 days). Y axis: percent of cells with 4c or greater DNA content. Three separate cultures from three separate retired breeder rat aortas are displayed with “top” and “middle” from representative WKY cultures (from a total of three isolations) and “bottom” from a representative SHR culture (from a total of two isolations).
mine the frequency of binucleate cells among the 4c DNA cells, and this accounted for only up to 3% of such cells. The remaining two possibilities for the majority of the 4c cells observed, therefore, are cells arrested in G2, and true tetraploid cells.

It is difficult to determine the exact frequency of the tetraploid cells vs. the nontetraploid, 4c DNA diploid cells in G2 or late S phase. This information cannot be obtained from flow cyotograms, since one does not really know how many 4c cells to expect from a given number of 8c cells (which presumably include cycling tetraploid or even octaploid cells). Additionally, although the chromosome counts establish the presence of hyperploid cells, various factors in the technique such as a probable strong sampling bias against intact tetraploid metaphase spreads vs. diploid spreads and low number of counts compared to the thousands available with flow cytometry make this a very imprecise tool for measuring tetraploid frequency. Such frequency estimates must await the availability of reliable markers of tetraploidy which can be used on a cell-by-cell basis with interphase nuclei.

We have somewhat equivocal information about the mechanism of production of these cells with high DNA content. One possibility is that polyploidy occurs via cell fusion, as is typical of striated muscle, followed by nuclear fusion. Data which suggest that cell fusion is not occurring to a significant degree include the following: First of all, there was a slight increase in the percent of unlabelled 4c cells after 2 days of chronic thymidine labelling. Second, none of the binucleated cells in our primary cultures treated continuously with tritiated thymidine were labelled. If these unlabelled binucleate cells represent fusion of separate cells, then we could imagine nuclear fusion occurring, perhaps during a subsequent mitosis, with the formation of cells with 4c or higher DNA content. Thus, the hypothesis of cell fusion contributing to the production of cells with high DNA content must remain viable. Since the data presented here suggest that there is continued new production of high DNA content cells throughout culture, other experiments are possible to assess better the degree of possible cell fusion. Cells can now be followed by more detailed videomicroscopy to look for fusion vs. failure of karyokinesis or cytokinesis. Additionally, separate cultures can be labelled with different fluorescent beads and then combined to see if a few cells containing both types of beads are found, suggesting significant fusion.

We do not understand why neonatal cultures appear to generate high DNA content cells in high frequency, while the adult cultures do not. In addition, adult cells appear to lose the high DNA content cells they had initially. Certainly our data and those of Goldberg et al. demonstrate that smooth muscle cells with tetraploid DNA content can proliferate as tetraploid cells. It is possible that in these adult cultures there is some increased relative cell death of such high DNA content cells or a proliferative advantage exists for the diploid cells. It does suggest an inherent difference between the neonatal and adult cultures. It is interesting that these cultures also differ with respect to growth factor production. Passaged cells from young animals secrete platelet-derived growth factor; cells from adults do not. Further investigations of such differences are currently underway in our laboratory.

The analogy of smooth muscle development and maturation to that of striated muscle is intriguing. During differentiation in vivo or in vitro, skeletal muscle...
cells increase their DNA content by cell fusion and, at the same time, rapidly express a characteristic set of muscle-specific contractile proteins.\textsuperscript{9,13,40,41} Similarly, the ploidy of arterial smooth muscle in vitro increases after birth and with age or hypertension.\textsuperscript{5,6} Also, the expression of smooth muscle-specific proteins in vitro correlates the animal's age and cell proliferative status.\textsuperscript{17,42,43} Our in vitro polyploidization observations are not directly analogous to studies of skeletal muscle, however, since studies using our anti-smooth-muscle actin antibody as well as an anti-desmin antibody show little to no reactivity of either diploid or polyploid neonatal cells (data not shown). Indeed, despite the initial reactivity of neonatal aortic smooth muscle cells to both of these antibodies, with time in culture, nearly all of these cells appear to lose their $\alpha$-actin and desmin content, both by immunocytochemistry and gel electrophoresis (data not shown). This is in agreement with studies of other investigators, showing variable-to-total loss of immunocytochemical detectability of muscle-specific cytoskeletal proteins once smooth muscle cells have been in culture for a few doublings, including those obtained from adult animals (e.g., smooth muscle myosin\textsuperscript{29,44,45}; desmin: Giulio Gabriani, private communication). Thus, in contrast to skeletal muscle, in our system the development of polyploidy appears associated with decreased-to-absent expression of muscle-specific proteins. This suggests that for arterial smooth muscle, muscle-specific cytoskeletal expression and polyploidization are separable phenomena. Interestingly, these two phenomena can be separated in skeletal muscle cultures, since cell fusion with subsequent polyploidy is not required for the expression of M-CPK\textsuperscript{46} or of skeletal muscle-specific myosin heavy chain mRNA.\textsuperscript{47}

Another initial reason for studying SHR vs. WKY animals was based on the in vitro observation that significantly more high DNA content cells develop in the SHR aorta than in the WKY aorta.\textsuperscript{6} While there appeared to be a correlation with blood pressure, we were interested in the hypothesis that if such cellular DNA and cytoplasmic increases were occurring at the resistance arteriole level, this change might conceivably be causative of the hypertension seen in SHR vs. WKY animals. Thus, we looked for evidence that SHR cells were somehow intrinsically more prone to undergo polyploid change than the WKY cells. However, the variation in our present culture system would seem to indicate that there is no such obvious species predisposition at the smooth muscle cell level.

In summary: We have shown that neonatal rat aortic cells, when placed in cell culture, become hyperplloid. Since arterial smooth muscle cells naturally become hyperplloid in vivo, in vitro cell culture may be a controllable model system for studying the induction of polyploid replication and possibly differentiation in arterial smooth muscle.

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References


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