Cytoskeletal Features of Rat Aortic Cells during Development
An Electron Microscopic, Immunohistochemical, and Biochemical Study
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SUMMARY. Actin, vimentin, desmin, and tropomyosin distribution in rat aortic endothelial and smooth muscle cells has been studied during development using fetal (18 to 20 days of gestation), and 5- and 14-day-, and 5- and 12-week-old rats. Endothelial cells of newborn animals actively replicate and contain many actin stress fibers, whereas, in adult animals, replication is minimal and actin stress fibers are rare. The actin, vimentin, desmin, and tropomyosin content of smooth muscle cells increases gradually from fetal to adult animals. The number of desmin-containing cells also increases from 13% in fetal rats to 51% in adult rats. The α-actin isoform is predominant in fetal and newborn animals, but gradually the α-isoform becomes quantitatively the most important, as seen by bidimensional polyacrylamide gels. Several analogies exist between the features of developing smooth muscle and what is known for developing striated muscle cells. The evolution of cytoskeletal features from fetal to adult animals is remarkably the opposite of what takes place in: (1) rat aortic smooth muscle cells proliferating after an endothelial injury, (2) human arterial smooth muscle cells present in atheromas, and (3) actively growing rat aortic smooth muscle cells in vitro. Thus, the assumption that pathological or cultured smooth muscle cells are "dedifferentiated" is supported by our biochemical observations. (Circ Res 56: 829-838, 1985)

ALTHOUGH several features of embryonic and neonatal arterial endothelial (EC) and smooth muscle (SMC) cells are well documented (Stein et al., 1969; Gerrity and Cliff, 1975; Gerrity et al., 1975; Cliff, 1976; Schwartz and Benditt, 1977; Olivetti et al., 1980), little is known about their cytoskeletal composition. Quantitative and qualitative changes of cytoskeletal elements have been shown to represent useful markers for the study of differentiation phenomena (Moll et al., 1982; Osborn and Weber, 1982) and, in some cases, of pathological phenomena (Osborn and Weber, 1983; Rungger-Brandle and Gabbiani, 1983). Our laboratory has previously reported that during endothelial regeneration and smooth muscle proliferation in the rat aorta following an experimental endothelial injury, typical morphological and biochemical changes of cytoskeletal elements develop (Gabbiani et al., 1984; Kocher et al., 1984), which vary with the evolution of the lesions (Kocher et al., 1984). SMC of the human atheromatous plaque show a pattern of cytoskeletal composition similar to that of proliferating rat SMC (Gabbiani et al., 1984). We undertook the present study to learn more about the cytoskeletal features (and their evolution) of aortic EC and SMC in embryonic, neonatal, and adult rats and to compare these features with those previously described in the same cells of rats and humans under pathological conditions.

Methods

Experimental Procedures
A total of 190 fetal (18-20 days of gestation), 5- and 14-day-old, and 5- and 12-week-old female Wistar rats were used. The rats were killed by perfusion through the left ventricle of the heart with 0.9% NaCl when the aortas were used for biochemical studies (70 rats), and by enzymatic digestion (70 rats) with 2% paraformaldehyde when the aortas were used for immunofluorescent staining (40 rats) or with 2% glutaraldehyde when the aortas were used for electron microscopy (10 rats). For the biochemical studies and SMC isolation, the aorta was opened on its dorsal aspect, the endothelium was gently scraped with a scalpel, and the media was carefully dissected from the adventitia under a magnifying glass with fine forceps (Kocher et al., 1984). The completeness of the dissections was controlled by light microscopic examination of hematoxylin and eosin or van Gieson elastin-stained sections, or of toluidine blue-stained sections of Epon-embedded tissue.

SMC were harvested from enzymatically dissociated rat aortic media according to the method described by Ives et al. (1978) for normal rabbit aorta. After 2 hours of digestion at 37°C in minimal Eagle medium (MEM), pH 7.4, containing 0.2% collagenase (Clostridium histolyticum, Sigma Chemical Co., type I, 125-250 U/mg), 0.05% elastase (pancreatopeptidase, Sigma Chemical Co.) type III, 70 U/mg, 0.1% soybean trypsin inhibitor (Boehringer Co., Mannheim), 25 mM HEPES (Sigma Chemical Co.), SMC were centrifuged at 300 g, washed, and resuspended in
MEM and were used for immunofluorescence or electro-
photometric analysis. The percent of cell recovery was 32.5 ±
5.5 (mean of three experiments ± se) in adult animals and
29.0 ± 4.0 (mean of three experiments) in newborn ani-
mals. In both cases, the dead cells (measured by Trypan
blue dye exclusion) were less than 5%.

Electron Microscopy

The fetal aorta was fixed by immersion in glutaralde-
hyde (2% in 0.1 m cacodylate buffer, pH 7.4), and the
same fixative was perfused through the left ventricle of
the heart at a constant pressure of 30 mm of Hg for 5-
and 14-day-old rats and of 100 mm of Hg for 5- and 12-
week-old rats. The thoracic aorta was isolated and pro-
cessed as previously described (Gabbiani et al., 1979).
Sections 1 mm thick were cut on a Reichert ultramicrotome
and stained with toluidine blue; from selected areas, thin
sections were cut and examined with a Philips 300 or 400
electron microscope.

EC Thymidine Index, Density, and En Face Staining with Anti-Actin Antibodies (AAA)

Five-day-old (20) and 12-week-old (20) Wistar rats were
used for this experiment. For the study of EC thymidine
index and density, we followed essentially the protocol
described by Schwartz and Benditt (1977). Each rat re-
ceived intraperitoneally tritium-labeled thymidine ([³H]-
Tdr, The Radiocchemical Center, specific activity 5 Ci/
mol) dissolved in saline at the dose of 5 μCi of [³H]Tdr
per 10 g body weight 3 times at 8-hour intervals. One
hour after the third injection, perfusion with 2% glu-
taraldehyde in 0.1 m cacodylate buffer (pH 7.4) was carried
out through the left ventricle of the heart for 10 minutes.
Thoracic aortas were then isolated, cleaned, opened on
the dorsal aspect, and cut into blocks, about 5 mm long,
which were prepared for autoradiography on en face
preparation, as previously described (De Chastonay et al.,
1983). Cells were counted using a Zeiss light microscope
equipped with a square-shaped reticulum in a 10X eye-
chamber, specific filters for fluorescein and rhodamine, generally using
a plan apo-chromate ×63/1.4 objective on 640-T color
slide film (3M Company).

DNA and Protein Determination, Sodium Dodecyl
Sulfate Polyacrylamide Gels (SDS-PAGE), and
Densitometry

We determined DNA content by the method of Burton
(1968), and of protein content by the method of Bradford
(1976). For SDS-PAGE, tissues or isolated cells were dis-
solved directly in sample buffer containing 1 mm phenyl-
methylsulfonylfluoride, sonicated (3 times, 20 seconds, at
setting 7 of a Branson sonifier) and boiled for 3 minutes.
We used 10% resolving gels (Laemmli, 1970) which were
stained with Coomassie blue. For quantification of actin,
vimentin, desmin, and tropomyosin, gels were scanned
with a high-resolution laser beam densitometer built from
readily obtainable precision optical, electronic, and me-
chanic components essentially according to Brayden and
Halpem (1983) with few modifications. The densitometer
was connected by means of an analog-to-digital converter
to a computer (microcomputer based on the S-100 bus
running under CP/M operating system, Digital Research
Inc.) programmed for scanning and quantifying one-di-
dimensional Coomassie blue or silver-stained gels and one-
dimensional autoradiographic film profiles generated in
gel electrophoresis of radioactive proteins or nucleic acids.

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The BASIC-program developed in our laboratory was
based on previous work (Schumaker, 1978; Yakin et al.,
1982): it corrects the baseline and minimizes the back-
ground noise. Areas of selected peaks are integrated and
given as percentage of the total area of the scan. Raw
densitometric data (Fig. 1a) are smoothed with a triangu-
larly weighted average of neighboring samples with the
same algorithm used by Yakin et al. (1982). Every density
sample taken within the scanning line is replaced by the
weighted mean of an area of a predetermined length (or

830


(Geisler and Weber, 1981) or desmin from chicken gizzard
(Geisler and Weber, 1980). For immunofluorescence stain-
ing, we used the indirect method (Gabbiani et al., 1981)
with: (1) the antibody containing whole sera (a drop of 40
μl diluted 1:20), (2) IgG fractions (a drop of 40 μl contain-
ing 0.5 mg of protein/ml for antidesmin IgG and 0.9 mg/
ml for antivimentin IgG), and most frequently (3) the
affinity-purified antibodies (a drop of 40 μl containing
0.05 mg of protein/ml for antidesmin and 0.18 mg/ml of
antivimentin antibodies). For the second step, we used
goat antibodies to guinea pig and rabbit IgG (Behring-
werke, both diluted 1:20 in PBS). To avoid cross-reactions,
the second antibodies were passed on a solid immuno-
absorbent made with glutaraldehyde-coupled rat IgG
(Avrameas and Ternynck, 1969). Isolated SMC were cy-
tocentrifuged with a Shandon cytocentrifuge (Shandon
Scientific Co, Ltd.) at 125 g, fixed in methanol at −20°C
for 5 minutes, and double stained with desmin and vi-
mintin antibodies. For fetal, 5-day- and 14-day-old rats,
we have pooled cells from five animals per determination,
and, for 5-week-old rat, we have pooled cells from three
rats per determination. Results are given as mean ± se
of at least three determinations. The percentage of cells pos-
itive for vimentin, desmin, or both were counted 3 times
independently by two researchers without knowledge of
the condition examined. Fragmented cells were excluded
from the evaluation.

Photographs were taken with a Zeiss (Carl Zeiss, Inc.)
photomicroscope equipped with epi-illumination and spe-
cific filters for fluorescein and rhodamine, generally using
a plan apo-chromate ×63/1.4 objective on 640-T color
slide film (3M Company).

Immunofluorescent Staining for Vimentin and
Desmin

Vimentin antibodies were raised in guinea pigs against
the 57-kD band of a 7% preparative sodium dodecyl
sulfate-polyacrylamide gel (SDS-PAGE) loaded with a
Triton X-100 high-salt extract (Franke et al., 1979) of
human embryo lung fibroblasts (Azzarone et al., 1983).
Desmin antibodies were raised in rabbits against a 53-kD
band of a 7% preparative SDS-PAGE loaded with chicken
gizzard acetone powder highly enriched in desmin (Hub-
bard and Lazarides, 1979). An IgG fraction of both sera
was obtained by passing them on a column of sepharose
covalently linked with protein A (protein-A sepharose CL-
4B, Pharmacia). Affinity-purified antibodies were then
prepared by passing these fractions through a column of
ultragel AcA22 (LK8 Co.) covalently linked (Nagle et al.,
1983), respectively, with pure vimentin from bovine lens
FIGURE 1. Programmed background reduction and baseline correction on a densitometric profile obtained by scanning a Coomassie blue-stained SDS-PAGE of a total extract of 3-week-old rat aortic media. Panel a: sketch of the absorbance along the migration path of proteins. Panel b: profile developed from panel a, after processing by the program for smoothing and baseline evaluation. Panel c: automatic enlargement of the area under the profile in panel b from the vimentin (V) peak to the tropomyosin (T) peak and arbitrary tracing of the limits of vimentin and desmin (D) peaks. Panel d: enlargement of tracings obtained from gels in which pure vimentin is mixed together with pure desmin; left: 1 μg of vimentin plus 0.5 μg of desmin; right: 2 μg of vimentin plus 0.5 μg of desmin. The surface calculated in internal units of the computer is: Left: vimentin = 8200, desmin = 4150; and right: vimentin = 16500, desmin = 4200, thus showing that increasing the vimentin peak does not alter the surface of the desmin peak and that doubling the amount of vimentin results in doubling the peak area. Panel e: enlargement from the V-peak to the T-peak of a tracing of normal adult rat aortic media. Panel f: enlargement of the tracing of the same normal adult rat aortic media to which 2 μg of vimentin have been added. Here again, addition of vimentin to the preparation produces an increase of the peak area very close to the expected value, whereas the desmin peak does not change. Quantification of peak areas is given in Table 1. A = actin.

The window size has been adjusted to provide the best noise filtration without modifying protein peaks. Baseline evaluation is derived from the method of Schumaker (1978) which breaks up the total profile into smaller regions; these are used to determine line segments giving the background. We use a window of predetermined length which moves along the total profile. For every point located in the middle of the window, we obtain a background which is equal to the minimal value in the window. The resulting background profile is subtracted from the smoothed profile, giving the final profile as shown in Figure 1b. Single peaks are integrated and given as a percentage of the total area of the scan. For determination of overlapping peaks, these are arbitrarily delimited by tracing a diagonal line connecting the presumed peak limit (in general, at a shoulder of the slope) with the baseline. For practical reasons, this is made on an enlargement of the relevant area (Fig. 1c represents an enlargement of the area containing the peaks of vimentin, desmin, actin, and tropomyosin in Fig. 1b). Care should be taken that the two lines delimiting each peak (here, vimentin and desmin) do not overlap. The delimited peaks are integrated and given as percentage of the total area of the scan.

Calibration experiments were done with purified cytoskeletal proteins (actin, vimentin, and desmin), alone, or mixed in different proportions, and by adding different amounts of each of these proteins to preparations of media of normal adult rats. In all cases, the addition of known amounts of actin, vimentin, and desmin, in the range of the amounts tested, resulted in linear increases of the areas of the peaks studied. Special care was taken in controlling the arbitrary delimitation of vimentin- and desmin-overlapping peaks by calculating the area of peaks under the tracing of known amounts of purified vimentin and desmin mixed together in different proportions (Fig. 1d) or added to preparations of media of normal adult rats (Figs. 1, e and f). The results thus obtained showed a good correlation between the area of peaks and the amounts of the two proteins charged on the gels. Table 1 shows an example of the results obtained by adding known amounts of vimentin to a preparation of normal adult rat aorta. In further control experiments, we verified that, over the concentration range studied, Coomassie blue staining is directly proportional to the amount of vimentin, actin, and desmin, and that the same amounts of these proteins stain with comparable intensity, thus allowing direct visual or densitometric comparison on the same gel. Results are expressed as means ± SEM. The number of samples utilized for each analysis is indicated in the tables. The evaluation of the results was done by means of Student's t-test and Bonferroni method (Wallenstein et al., 1980).

Bidimensional gels were performed according to the method of O'Farrel (1975) with minor modifications in sample preparation (Kelly and Cotman, 1978).

Results

Morphology, Thymidine Index, and Cell Density of EC

At electron microscopic examination, EC of newborn animals were in general similar to those of adult rats, as previously described (Schwartz and Benditt, 1972; Cliff, 1976). However, EC of newborn animals contained larger amounts of rough endoplasmatic reticulum than did those of adult animals (Schwartz and Benditt, 1972), and, in addition, contained many cytoplasmic microfilament bundles, localized mainly at the abluminal periphery of the cell (Fig. 2), which showed dense areas distributed throughout their length. These bundles or "stress fibers" (Burridge, 1981; Gabbiani et al., 1983; White et al., 1983; Wong et al., 1983) were similar to those previously described by means of electron microscopy in aortic EC of hypertensive adult animals.
TABLE 1  
Densitometric Evaluation of Vimentin and Desmin in Preparations of Normal Adult Rat Aorta  
with or without Addition of Known Quantities of Vimentin  

<table>
<thead>
<tr>
<th>Description</th>
<th>Vimentin</th>
<th>Desmin</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of total proteins</td>
<td>µg</td>
<td>% of total proteins</td>
</tr>
<tr>
<td>Normal adult rat aorta</td>
<td>1.89 ± 0.24</td>
<td>0.95 ± 0.12</td>
</tr>
<tr>
<td>Normal adult rat aorta + 0.5 µg of vimentin</td>
<td>2.91 ± 0.17</td>
<td>1.46 ± 0.09</td>
</tr>
<tr>
<td>Normal adult rat aorta + 1 µg of vimentin</td>
<td>3.80 ± 0.30</td>
<td>1.90 ± 0.15</td>
</tr>
<tr>
<td>Normal adult rat aorta + 2 µg of vimentin</td>
<td>5.97 ± 0.24</td>
<td>2.99 ± 0.12</td>
</tr>
</tbody>
</table>

In all cases, 50 µg of proteins of normal adult rat aorta were loaded on the gel. Values in µg are calculated from the % of total proteins. The expected values are in parentheses.

(Gabbiani et al., 1975, 1979). After immunofluorescent staining of en face preparations in 5-day-old rats, stress fibers were very evident, averaging 3–4/ cell, and being present in practically all EC (Fig. 3a). No differences in stress fibers distribution were seen in EC located close to the ostia of intercostal arteries compared to EC located in the ventral part of the aorta. Thymidine index in en face preparations of newborn rat EC was 0.130 ± 0.004 and cell density was 130 ± 2.4. In adult rats, endothelial cells contained stress fibers only exceptionally and preferentially in areas immediately below the ostia of intercostal arteries (Gabbiani et al., 1983), but showed a faint accumulation of actin at their periphery (Fig. 3c). Thymidine index was 0.007 ± 0.0007 and cell density was 75 ± 1.

Morphology of SMC

By means of electron microscopy, we examined the SMC of fetal, 5-day-, 14-day-, 5-week-, and 12-week-old rats. As previously described (Cliff, 1976; Gerrity and Cliff, 1975; Gerrity et al., 1975; Stein et al., 1969), fetal and newborn rat aortas contained 6–7 layers of SMC. In contrast to adult animals, they showed prominent endoplasmic reticulum and Golgi apparatus (Fig. 4a). In the extracellular space, elastic laminae were present but discontinuous, and very few collagen fibers were seen. In older rats, the appearance of SMC changed; in particular, they developed an important microfilamentous apparatus, distributed throughout the cytoplasm (Fig. 4b) whereas endoplasmic reticulum and Golgi apparatus became minor cytoplasmic components and were confined to the perinuclear space. Intermediate filaments were present at all times, distributed throughout the cytoplasm. With increasing age, the layers of SMC became separated by much wider elastic laminae, and collagen fibers increased in quantity, as previously described (Stein et al., 1969; Gerrity and Cliff, 1975; Gerrity et al., 1975; Cliff, 1976).
Kocher et al. / Cytoskeleton of Developing Rat Aortic Cells

**FIGURE 3.** Actin distribution in EC of newborn and adult rats. The en face preparation of EC in a newborn rat (panel a) shows the presence of many actin-positive intracellular stress fibers. These are absent in EC of an adult animal (panel c). Panel e shows a control en face preparation of EC in an adult animal incubated with normal human IgG instead of AAA. Panels b, d, and f are Nomarski optics micrographs corresponding to panels a, c, and e, respectively. Note that the cell density is higher in the newborn than in the adult animal; the EC of the adult animal also appears flatter than those of the newborn animal. 400X.

**Immunofluorescent and Biochemical Characterization of Cytoskeletal Elements in SMC**

Double immunofluorescence staining with anti-vimentin and antidesmin antibodies was applied on isolated SMC from the media of fetal, 5-day-old, 14-day-old, and 5-week-old rats (Fig. 5). In fetal rats, 87.0 ± 1.9% (mean ± se) of the isolated cells showed a positive staining for vimentin alone, 13.2 ± 0.9% for both vimentin and desmin, and none for desmin alone. The same results were obtained with 5-day-old rats. In 14-day-old rats, 76.7 ± 2.3% of the cells showed a positive staining for vimentin alone and 22.7 ± 1.5% for both vimentin and desmin. In 5-week-old rats, 61.3 ± 1.1% of the isolated SMC showed positive staining for vimentin alone and 39.3 ± 1.4% for both vimentin and desmin. In all cases, none of the isolated SMC examined was positive for desmin alone. We knew from previous experiments (Kocher et al., 1984) that in adult (12-week-old) rat aortic media, 51% of the isolated cells were positive for vimentin alone, 48% for both vimentin and desmin, and only 1% for desmin alone.

For the biochemical studies, we first determined the DNA and protein content per mg of tissue in the aortic media of fetal, 5-day-old, 14-day-old, 5-week-old, and adult (12-week-old) rats, as well as the DNA and protein content in enzymatically isolated SMC from the media of 5-day- and 5-week-old rats. These procedures allowed us to calculate, after densitometric estimation of actin, vimentin, desmin, and tropomyosin on the gels as percentage of total pro-

**FIGURE 4.** Morphological features of aortic SMC in a newborn (panel a) and a 5-week-old (panel b) rat. The cytoplasm of the SMC in the newborn rat (panel a) contains a well-developed endoplasmic reticulum and Golgi apparatus and many mitochondria. Microfilaments are barely visible, whereas they become the major cytoplasmic component in cells of a 5-week-old animal (panel b). 22,000X.

**FIGURE 5.** Vimentin and desmin distribution in aortic SMC. After double immunofluorescent staining for vimentin (panel a) and desmin (panel b) in cytocentrifuged SMC isolated from a 5-week-old rat aortic media, all cells appear positive for vimentin, but only some are positive for desmin. 630X.
The quantity of DNA per mg of tissue (Table 2) was found to be 8.7 μg in fetal rat aortic media; it did not change significantly (P > 0.05) in 5-day-old the results were werrat aortic media in which it was 7.9 μg, but decreased progressively to 4.0 μg in 14-day-old rat aortic media, 2.7 μg in 5-week-old, and 1.6 μg in adult rats [see, for comparison, the results obtained by Stein et al. (1969) in 1- and 3-month-old rats].

The DNA content of isolated cells was similar in 5-day- and 5-week-old aortic SMC: 6.0 pg of DNA per cell (Table 3).

The protein content of the aortic media was about 56 μg protein/mg tissue in fetal and 5-day-old rat, decreased to 37.5 μg in 14-day-old and 5-week-old rats, and to 41.8 μg in adult rats (Table 2). In isolated cells, the protein content was 33.6 pg/cell in 5-day-old, and almost doubled to 54.9 pg/cell in 5-week-old rat SMC (Table 3). We know from previous experiments that SMC isolated from adult rat aorta contain, under our conditions, about 110 pg protein/cell (Kocher et al., 1984).

Table 2

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>DNA*</th>
<th>Protein*</th>
<th>Actin†</th>
<th>Vimentin†</th>
<th>Desmin†</th>
<th>Tropomyosin†</th>
<th>Vimentin: actin</th>
<th>Desmin: actin</th>
<th>Tropomyosin: desmin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal</td>
<td>10</td>
<td>8.7±0.1</td>
<td>56.2±0.6</td>
<td>3.6±0.08</td>
<td>1.25±0.18</td>
<td>0.44±0.10</td>
<td>0.72±0.12</td>
<td>0.34±0.02</td>
<td>0.12±0.02</td>
</tr>
<tr>
<td>5 days</td>
<td>10</td>
<td>7.9±0.4</td>
<td>56.3±0.8</td>
<td>4.0±0.30</td>
<td>1.26±0.07</td>
<td>0.49±0.02</td>
<td>0.89±0.13</td>
<td>0.31±0.01</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>14 days</td>
<td>10</td>
<td>4.0±0.2</td>
<td>37.5±1.4</td>
<td>6.4±0.28</td>
<td>1.56±0.09</td>
<td>0.72±0.09</td>
<td>1.99±0.22</td>
<td>0.24±0.02</td>
<td>0.11±0.01</td>
</tr>
<tr>
<td>5 weeks</td>
<td>8</td>
<td>2.7±0.1</td>
<td>37.3±2.8</td>
<td>10.23±0.34</td>
<td>1.80±0.16</td>
<td>1.20±0.03</td>
<td>3.91±0.27</td>
<td>0.18±0.01</td>
<td>0.12±0.02</td>
</tr>
<tr>
<td>Adult</td>
<td>8</td>
<td>1.6±0.1</td>
<td>41.8±1.3</td>
<td>20.04±0.32</td>
<td>2.96±0.38</td>
<td>2.01±0.09</td>
<td>7.04±0.67</td>
<td>0.15±0.02</td>
<td>0.10±0.01</td>
</tr>
</tbody>
</table>

* pg/mg of tissue.
† pg/cell.

The results are summarized in Tables 2 and 3.

The results were converted to amounts per cell according to the measurements of DNA and protein by densitometric analysis of SDS-PAGE, and the results were converted to amounts per cell according to the measurements of DNA and protein per milligram of tissue (Table 2). The vimentin:actin, desmin:actin, tropomyosin:actin, and vimentin:desmin ratios were also calculated (Table 2). Actin, as percentage of total protein in the aortic media, did not change between fetal (9.4 ± 0.2) and 5-day-old (9.5 ± 0.7) rats, but increased in 14-day- (11.4 ± 0.5) and 5-week- (12.7 ± 0.5) old rats, and then remained unchanged in adult rats (12.8 ± 0.2). Vimentin progressively decreased from fetal (3.23 ± 0.46) to adult (1.89 ± 0.24) rat aortic media. Desmin increased slightly but not significantly in all groups studied, whereas tropomyosin progressively increased from fetal (1.86 ± 0.02) to adult (4.5 ± 0.43) aortic media. The actin content per cell (Table II) did not change between fetal and 5-day-old rat (P > 0.05), but increased progressively from 5-day-old to adult rats with a significant difference between all the age groups. The changes were statistically significant, using both Student's t-test (P < 0.001 in every comparison) and the Bonferroni method. The vimentin content per cell did not change significantly between fetal and 14-day-old rats, and between 14-day- and 5-week-old rats, but increased significantly between 5-day- and 5-week-old rats (P < 0.01) and between 5-week-old and adult rats (P < 0.001). The desmin content per cell did not change significantly between fetal and 14-day-old rats, but increased progressively from 14-day-old to adult rats (P < 0.001 between the different age groups).

The tropomyosin content per cell did not change from fetal to 5-day-old rats, but increased progressively from 5-day-old to adult rats (P < 0.001 between the different age groups). The tropomyosin content per cell did not change from fetal to 5-day-old, but increased progressively from 5-day-old to adult rats (P < 0.001 between the different age groups). The vimentin:actin ratio decreased significantly from 5-day- to 14-day-old rats (P < 0.001), and from 14- to 5-week-old rats. There was no significant change of this ratio between fetal and 5-day-old rats, both of which had a high vimentin:actin ratio, and there also was no significant change between 5-week-old and adult rats, which, on the contrary, had a low vimentin:actin ratio compared to the other groups. The desmin:actin ratio did not change, under any of the conditions studied. Fetal and 5-day-old rat aortic...
Kocher et al. / Cytoskeleton of Developing Rat Aortic Cells

Figure 6. Protein profile of isolated aortic SMC by means of SDS-PAGE. Panel a shows a cytoskeletal preparation of baby hamster kidney cells containing vimentin (V), desmin (D), and actin (A) as markers. SMC from 5-day-old (panel b) and 5-week-old (panel c) rats differ clearly in their content of vimentin, actin, and tropomyosin (T).

SMC had a low tropomyosin:actin ratio compared to 14-day-old, 5-week-old, and adult rat SMC. The vimentin:desmin ratio did not change between fetal and 5-day-old rats, but decreased progressively from 5-day-old to 5-week-old rat SMC. There was no difference in the vimentin:desmin ratio between 5-week-old and adult rat aortic SMC.

We controlled the results obtained with total extracts of media using enzymatically isolated SMC (from 5-day- and 5-week-old rats) (Fig. 6). The results were similar to those obtained in tissues (Table 3). Furthermore, we dissolved whole tissues and isolated cells in a buffer composed of Tris-HCl 50 mM, EGTA 5 mM, and KCl 100 mM containing 1% Triton X100, in order to recover cytoskeletal elements and to obtain gels with a low background. Under these conditions, the relative proportions of actin, vimentin, and desmin were similar to those obtained with whole tissue extracts (data not shown).

IEF, as well as bidimensional gel electrophoresis (Fig. 7), showed that the SMC of fetal and 5-day-old rats had a predominance of β-nonmuscle actin, whereas α- and γ-actins were present in small amounts. This tended to change in 14-day-old rat SMC which showed approximately equal amounts of α- and β-actins. Five-week-old rat VSMC showed a clear α-actin predominance and a decrease of the β-actin practically similar to the α-actin predominance previously described in the SMC of the adult rat aortic media (Gabbiani et al., 1981, 1984; Kocher et al., 1984).

Discussion

It is well accepted that arteries, aorta in particular, become less and less cellular during development, due to a relative increase of extracellular tissue (Stein et al., 1969; Gerrity and Cliff, 1975; Gerrity et al., 1975; Cliff, 1976; Schwartz and Benditt, 1977; Olivetti et al., 1980). EC and SMC have a high replicating activity during fetal and early neonatal life, but show a limited degree of replication in normal adult animals (Berry et al., 1972; Schwartz and Benditt, 1977). Developing SMC are ultrastructurally very similar to fibroblasts and only gradually acquire the features typical of adult cells (Hoyes et al., 1974; Gerrity and Cliff, 1975; Chamley-Campbell et al., 1979).

Our results agree with these previous findings, and furnish new information concerning the morphological and biochemical characteristics of developing aortic cells. Stress fibers, i.e., bundles of microfilaments such as those present in cultured cells, are common organelles in EC of developing animals, whereas they are rare in EC of adult animals (Gab-
In the aorta of adult animals, stress fibers are numerous only in EC located in areas submitted to turbulent flow (Gabbiani et al., 1983; Wong et al., 1983) or during pathological situations such as hypertension (Gabbiani et al., 1975; White et al., 1983) or regeneration after injury (Gabbiani et al., 1983). These conditions are characterized by an increased degree of replicating activity and cell density (Haudenschild and Schwartz, 1979; De Chastanoy et al., 1983) which is also true for developing endothelial cells (Schwartz and Bendi, 1977; see our Results). However, previous studies have shown that stress fibers persist in vivo long after regeneration and replication have ceased (Gabbiani et al., 1983). Moreover, in vitro, the presence of stress fibers is probably related more to attachment to the substrate and isometric contraction than to replication and/or cell movement (Burridge, 1981; Herman et al., 1981). Further studies are needed in order to define the function of endothelial stress fibers, but it is noteworthy that these organelles are particularly numerous in vivo both in the normal aorta of developing rats and during pathological conditions such as hypertension (Gabbiani et al., 1975) and regeneration (Gabbiani et al., 1983) in adult animals.

It has been shown that in the developing rat aorta, SMC undergo two different phenomena. Early in life, hyperplasia is the most important phenomenon: between the first and the fifth day of life, the number of SMC in the aortic media doubles (Olivetti et al., 1980). The DNA synthesis drops progressively up to 1 year of age (Looker and Berry, 1972), and during that time, SMC undergo hypertrophy. According to our results, the amount of total protein per cell triples from the neonatal period to the adult state (see Results). For this reason, we have expressed our protein measurements as amounts of protein per cell, as well as ratios between two given proteins, in order to be able to understand how one protein is changing compared to the others.

The gradual increase of actin, tropomyosin, vimentin, and desmin content per cell in developing SMC correlates well with the previously described morphological changes accompanying SMC maturation (Hoyes et al., 1974; Gerrity and Cliff, 1975; Chamley-Campbell et al., 1979). It appears that the increase in actin (which, in adult SMC, becomes by far the most prevalent protein) is the major factor contributing to the classical morphological aspect of adult SMC. Concurrent with an increase of actin content, there is also a switch from the predominance of the β-isotype to the predominance of the α-isotype, typical of the adult vascular SMC (Gabbiani et al., 1981). Previous morphometric studies have indicated that the proportion of SMC cytoplasm occupied by myofilaments does not change between birth and 11 days of age, although the absolute volume of microfilaments increases with age (Olivetti et al., 1980). However, in 14-day-old rats, we see a significant increase in the actin content per cell, compared to both fetal and 5-day-old rats. These apparent discrepancies may be due to the difficulties of evaluating morphometrically the space occupied by various cytoplasmic filaments, or, alternatively, to the fact that at these periods an important proportion of actin is present in the form of oligomers or short filaments.

The present work, as well as previous studies (Gabbiani et al., 1981; Frank et al., 1982), have shown that SMC of the arterial wall are heterogeneous as far as their contents of vimentin and desmin are concerned. This heterogeneity is not apparent from ultrastructural studies (Gerrity and Cliff, 1975; Olivetti et al., 1980; this work). The functional significance of this SMC heterogeneity is presently not known.

Although quantification of proteins by means of immunocytochemistry and gel scanning should be interpreted with caution, our results suggest several general considerations. Vimentin and desmin represent an important component of SMC cytoplasm. From fetal to adult animals, the vimentin content per cell appears to increase about 2.3 times (this increase being less important than the increase of total cellular proteins), whereas desmin increases about 5 times. However, desmin increase probably reflects more an increase in the number of desmin positive cells than a real increase of desmin per cell. We have previously shown that, contrary to striated or cardiac muscle, adult aortic SMC contain large amounts of vimentin (Gabbiani et al., 1981). Our quantitative data show that, even in adult life, the rat aortic media contains vimentin as the prevalent intermediate filament protein. However, from fetal to adult life, the percentage of SMC containing desmin increases from 13 to 51. In striated and cardiac muscle cells, the appearance of α-actin isotype, as well as the appearance of desmin, coincides with the appearance of differentiated functions (Buckingham et al., 1982; Caplan et al., 1983). The same is true for aortic SMC with the exception that, in animal adult, there remains still a high proportion of vimentin-positive SMC (Gabbiani et al., 1981; Kocher et al., 1984; see Results). In the normal aorta of fetal and newborn rats (Berry et al., 1972; Olivetti et al., 1980) and in arterial intimal thickening after endothelial injury of adult rats (Björkén and Bondjers, 1973; Clowes et al., 1983), the replicative activity of SMC is high, and most SMC contain only vimentin as judged by immunofluorescent staining (Kocher et al., 1984; see Results), suggesting that vimentin-positive SMC possess a higher mitotic potential than those also containing desmin during development as well as in adult life.

It is remarkable that during proliferation after endothelial injury in experimental animals and in human atheromatosis, SMC undergo a series of morphological and biochemical changes concerning their cytoskeletal elements which are practically the reverse of what we have described for developing SMC: (1) they lose microfilaments and acquire a...
prominent rough endoplasmic reticulum and Golgi apparatus (Ghidoni and O’Neal, 1967; Poole et al., 1971; Chamley-Campbell et al., 1979); (2) the actin content per cell decreases and there is a switch from the predominance of the α-isotype to the predominance of the β-isotype (Gabbiani et al., 1984; Kocher et al., 1984); (3) the number of vimentin-positive cells, as well as the content of vimentin per cell, increases (Gabbiani et al., 1981; Kocher et al., 1984); and (4) the number of desmin-positive cells decreases (we do not know about the content of desmin per cell) (Kocher et al., 1984). Similar changes are seen in actively replicating SMC in vitro (Gabbiani et al., 1984). These observations suggest that the term “dedifferentiation,” which was based only on morphological features of pathological SMC (Fritz et al., 1970; Blosse et al., 1975; Chamley et al., 1977), and hence was considered controversial (Chamley-Campbell et al., 1979), is supported by the biochemical changes of proliferating and atheromatous SMC, compared to their normal adult counterparts, at least as far as the cytoskeletal elements are concerned.

In conclusion, our study has illustrated the progressive changes of cytoskeletal components in developing rat aortic EC and SMC, and has indicated an analogy between the cytoskeletal features of SMC cells during early development and SMC during proliferation after an experimental lesion, atheromatous plaque formation, or active growth in vitro.

References


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INDEX TERMS: Actin • Vimentin • Desmin • Endothelium • Smooth muscle
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