Developmental Changes in Isoactin Expression in Rat Aortic Smooth Muscle Cells in Vivo

RELATIONSHIP BETWEEN GROWTH AND CYTODIFFERENTIATION*

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There is an inverse relationship between cellular proliferation and smooth muscle α-isoactin expression in cultured vascular smooth muscle cells (SMCs) (Owens, G. K., Loeb, A., Gordon, D., and Thompson, M. M. (1986) J. Cell Biol. 102, 343–352). In the present studies, changes in isoactin expression were studied during developmental growth of rat aortic SMCs (ages 1–180 days) to better understand interrelationships between growth and cytodifferentiation in these cells in vivo. Actin expression (i.e., content and synthesis) was evaluated by one- and two-dimensional gel electrophoresis and using isoactin-specific antibodies. The major actin present in cells from newborn rats was nonmuscle β-actin (56% of total actin), whereas cells from adult animals contained principally smooth muscle α-actin (Sm-α-actin) (76% of total actin). Increases in Sm-α-actin content with increasing age were due, in part, to an increase in Sm-α-actin synthesis. However, in SMCs from 90- and 180-day-old rats, the fractional content of Sm-α-actin exceeded its fractional synthesis at a time when total Sm-α-actin content was increasing. This suggests that Sm-α-actin turns over more slowly in mature animals. Decreases in the frequency of SMCs undergoing DNA synthesis with age could not account for increases in Sm-α-actin expression with age. However, combined immunocytochemical and [3H] thymidine autoradiographic studies demonstrated that nearly 50% of the medial derived cells from newborn rat aortas did not show detectable staining with a monoclonal antibody to smooth muscle-specific isoactins, and the replicative frequency was much higher in these cells than in cells that contained Sm-α-isoactins. Taken together, the results of the present studies and previous studies in cultured SMCs support the hypothesis that cessation of proliferation during development is associated with the induction of Sm-α-actin expression, but that factors other than cellular growth state play an important role in determining the level of Sm-α-actin expression in fully differentiated SMCs.

Actin is a major cytoskeletal component of all eukaryotic cells and serves as an integral part of the contractile apparatus in muscle cells (Pollard, 1981). Whereas all actins appear to be remarkably similar in their physical properties (Pollard, 1981), amino acid sequence analysis has shown that higher vertebrates may express as many as six different actin polypeptides or isoactins (Garrels and Gibson, 1976; Vandekerckhove and Weber, 1978). The six actins can be grouped into three classes, α-, β-, and γ-actin, based on their migration on isoelectric focusing (IEF) gels (Garrels and Gibson, 1976; Wallevik and Jensening, 1982).

The distribution of actin isoforms exhibits striking tissue and cell specificity. Three distinctly different α-isoactins predominate in fully differentiated skeletal, cardiac, and vascular smooth muscle, whereas the major form in chicken gizzard and visceral smooth muscle is a smooth muscle-specific γ-isoactin form (Garrels and Gibson, 1976; Vandekerckhove and Weber, 1979, 1981). Two nonmuscle β- and γ-isoactins predominate in nonmuscle tissues but can also be found in large amounts in undifferentiated skeletal muscle cells (Devlin and Emerson, 1978; Whalen et al., 1976). In addition, the nonmuscle isoactins comprise about 28% of total actin in differentiated vascular smooth muscle cells (SMCs) (Fatigati and Murphy, 1984; Vandekerckhove and Weber, 1979).

The present study was prompted, in part, by observations in skeletal myoblasts that during differentiation profound alterations occur in isoactin expression (Devlin and Emerson, 1978; Whalen et al., 1976). Rapidly dividing pre-fusion skeletal myoblasts contain mostly Nm-β- and γ-isoactins. Cessation of growth and cell fusion is associated with an abrupt switch in actin synthesis to the muscle-specific skeletal and cardiac α-forms characteristic of differentiated skeletal muscle (Devlin and Emerson, 1978; Minty et al., 1982; Mohun et al., 1984; Whalen et al., 1976). There is good evidence to suggest that similar alterations in isoactin expression occur in association with growth and differentiation of SMCs. Saborio et al. (1979) studied the variant forms of gizzard actin present during chick embryogenesis and found an increase in Sm-γ-isoactin and a decrease in Nm-β-isoactin with development of the embryo and showed that this differential expression of actins was controlled at the transcription level. Studies by Kocher et al. (1985) have shown that marked increases in Sm-α-actin expression occur in rat aortic SMCs during development, whereas marked decreases in Sm-α-actin expression occur in SMCs from atheromatous lesions in humans or experimental animal models (Gabbiani et al., 1984). We have recently demonstrated an inverse relationship between Sm-α-actin expression and cellular proliferation in cultured rat aortic SMCs (Owens et al., 1986). Density arrest of growth or growth arrest in serum-free medium was associ-

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1The abbreviations used are: IEF, isoelectric focusing; HBSS, Hank's balanced salt solution; Nm, nonmuscle; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Sm, smooth muscle; SMCs, smooth muscle cells.
ated with increased Sm-α-actin expression, whereas serum stimulation of growth resulted in a dramatic decrease in Sm-α-actin expression. Similar observations have been reported by Straugh and Rubenstein (1984) in BC3H1 cells, a smooth muscle cell-like line derived from a mouse brain tumor.

The present studies represent an extension of our earlier cell culture studies to include examination of the interrelationship between vascular SMC growth and cytodifferentiation in vivo during developmental growth of the rat thoracic aorta. To do this, we have examined changes in isoactin synthesis and content in relation to changes in SMC proliferation; and by using a monoclonal antibody to the smooth muscle isoactin (Gown et al., 1985) combined with [3H] thymidine autoradiography, these changes were directly related to the population of proliferating cells.

MATERIALS AND METHODS

Tissue Preparation—All rats used in these studies were purchased from Hilltop Lab Animals, Inc. (Scottsdale, PA). Pregnant females were obtained, and litters were raised in our animal facility for all studies involving rats 1 month of age or less. All other rats were used within 2 weeks of purchase.

Animals were killed with CO2, and the thoracic aorta to the diaphragm was isolated and placed in Hank's balanced salt solution (HBSS, GIBCO). Adhering fat and connective tissue were removed by blunt dissection. The adventitia was carefully removed under a dissecting microscope, and the luminal surface was scraped with forceps to remove endothelial cells. Several preparations were fixed and processed for light microscopy to confirm that removal of the adventitia and endothelium was complete. Medial preparations were subsequently processed for biochemical evaluations of actins or for cell dispersions as described below. Aortas from litter mates were pooled for biochemical determinations at the 1-, 9-, and 16-day time points in order to have sufficient samples for analyses. At all other time points, aortas were analyzed individually for each rat. Cardiac and skeletal muscle (leg muscle) for immunoblot analyses were obtained by running lanes of known amounts of purified rabbit 

Antibody Staining — The specificity of the smooth muscle-specific isoactin monoclonal antibody (designated CGA7) and the polyclonal actin antibody (designated PAA) used in these studies has been reported previously (Gown et al., 1985). CGA7 is specific for the Sm-α- and Sm-γ-isoactins but does not react with either cardiac or skeletal muscle α-isoactins or with Nm-β- and -γ-actins. The PAA antibody, made in rabbits against purified chicken gizzard actin, reacts with both muscle and nonmuscle actins and was used as a positive control in these experiments. Antibodies were generously supplied by Drs. A. Gown and D. Gordon (University of Washington Medical School).

Antibody staining was done using an avidin/biotin/peroxidase procedure (Vectastain, Vector Laboratories, Inc., Burlingame, CA). CGA7 was used as ascites fluid diluted 1:100 in phosphate-buffered saline (pH 7.4). PAA was used at a dilution of 1:200. Stained cells were fixed in 2% glutaraldehyde plus 1% paraformaldehyde in HBSS, and processed for autoradiography as previously described (Owens, 1985).

The following controls for specificity of staining were done: 1) substitution of the primary antibody with nonimmune mouse serum, preimmune rabbit serum, or an antineurofilament monoclonal antibody unreactive to smooth muscle; 2) exclusion of both primary and secondary antibodies; or 3) exclusion of the primary antibody alone. In addition, medial derived cell dispersions were also stained with Factor VIII antibody (rabbit anti-human, Behring Diagnostics) (Schwartz, 1978) to determine whether any endothelial cells were present in cell smears. Freshly obtained rat aortic endothelial cells (harvested by scraping a thoracic aorta of a rat with a scalpel blade) were also stained with the smooth muscle-specific isoactin antibody as a negative control.

Evaluation of stained cell smears for the frequency of stained and unstained cells and for the frequency of [3H]thymidine-labeled cells was carried out without knowledge of the experimental group (e.g. PAA versus CGA7). At least 400 cells were analyzed per experimental group. Fragmented cells were excluded from evaluations.

Methionine Incorporation and Sample Preparation—Freshly excised thoracic aortas were incubated in medium 199 (GIBCO) containing 90 μCi/ml [35S]methionine (986–1103 Ci/mmol, New England Nuclear) for 4 h at 37°C in an atmosphere of 95% air and 5% CO2. The vessels were then washed in cold phosphate-buffered saline, and medial preparations were prepared as described above, lightly blotted, weighed, and homogenized on ice in phosphate-buffered saline at 10 mg of tissue/ml. Aliquots were taken for protein (Lowery et al., 1951) and DNA (Labarca and Piegen, 1980) assays, as well as for dilutions in Laemmli (1970) sample buffer (final concentrations: 50 mM Tris (pH 6.8), 1% sodium dodecyl sulfate (SDS), 10% glycerol. 0.001% bromphenol blue, 25 mM dithiothreitol) or IEF homogenization medium (final concentrations: 1% SDS, 10% glycerol, 20 mM dithiothreitol) (Fatigati and Murphy, 1984) for one- and two-dimensional polyacrylamide gel electrophoresis (PAGE). All electrophoretic samples were stored at -80°C.

Measurement of Actin/DNA Ratios: One-dimensional PAGE—The actin content of medial homogenates was determined by densitometric evaluation of Coomassie Blue-stained gels following SDS-PAGE (Larsen et al., 1984). Tissues were solubilized in sample buffer, heated to 100°C for 5 min, run on a 2% Laemmli SDS-polyacrylamide gel with a 4% stacking gel, and stained with Coomassie Brilliant Blue R (Fatigati and Murphy, 1984). After destaining, the total absorbance of each lane was determined by comparison to standards obtained by running lanes of known amounts of purified rabbit skeletal muscle actin on the same gel slabs as the medial samples. Two-dimensional IEF/SDS analyses of these samples showed no other major proteins with the same molecular weight as actin. Therefore, it is reasonable to assume that our densitometric analysis of the 45,000-dalton band on one-dimensional gels reflects predominantly actin and not other proteins. Correlation coefficients for standard curves were routinely greater than 0.99. The actin content of a sample was expressed as the ratio of micrograms of actin/microgram of DNA. All samples were analyzed by triplicate. The final results were obtained by running lanes of known amounts of purified rabbit skeletal muscle actin on the same gel slabs as the medial samples.

Fractional Isoactin Content: Two-dimensional PAGE—A modification of O’Farrell’s (1975) two-dimensional IEF/SDS electrophoresis technique described by Fatigati and Murphy (1984) was used for resolving isoactins, on a 4.0–6.5 or 5.0–6.0 pH gradient (Pharmalyte, Pharmacia P-L Biochemicals), as previously described (Owens et al., 1986). Samples contained between 7 and 20 μg of total protein in IEF homogenization medium. After completion of electrophoresis in the second dimension, the gels were stained with Coomassie Brilliant Blue R, destained, and analyzed by densitometry (Fatigati and Murphy, 1984). Isoactins were identified by 1) co-electrophoresis of our samples with purified skeletal muscle actin, chicken gizzard actin, and endothelial lysates; 2) immunoblotting with actin antibodies; and 3) comparison to published data (Fatigati and Murphy, 1984; Garrels and Gibson, 1976; Vande Voorde and Weber, 1979).

Fractional isoactin contents were determined by densitometric evaluation as described by Fatigati and Murphy (1984). Multiple scans in both electrophoretic dimensions were made to determine maximal optical densities. This method gave linear results over the harvest range of protein loadings used and repeated measurements on the same sample showed a coefficient of variation for α-actin of less than 3%.

Fractional Isoactin Synthesis—After scanning for isoactin content, the gels were impregnated with 2,5-diphenyloxazole in dimethyl sulfide (Cleland and Lane, 1984), and the dried gels were scanned on a Kodak X-Omat AR or K film at -80°C for 1–3 weeks. Developed fluorographs were scanned with an Optronics P-1000 densitometer, and the relative [35S]methionine incorporation into each isoactin was quan-
Isoactin Expression in Vascular Smooth Muscle

**RESULTS**

Developmental Changes in Actin Expression: Isoactin Content—Our initial objective was to determine whether changes in isoactin expression occur in vascular smooth muscle during the normal developmental growth of the rat thoracic aorta. Figs. 1 and 2 show the results of two-dimensional gel electrophoretic analysis of isoactins in rat thoracic aorta SMCs at different ages. The isolectric variants of actin (i.e., α-, β-, and γ-) are clearly resolved, although the γ-component includes both the Sm- and Nm-γ-actins which are not resolved by this technique (Garrels and Gibson, 1976; Vandekerckhove and Weber, 1978, 1979). Two-dimensional gel electrophoresis also does not distinguish the three α-variants of actin (i.e. smooth muscle, skeletal, and cardiac types). Sequence analysis by others has established that adult rat aortic SMCs contain only the smooth muscle variant of the α-isotype (Vandekerckhove and Weber, 1979, 1981), but such analyses has not been performed on vascular smooth muscle from newborn or immature rats. This distinction may be important since developing skeletal myocytes are known to express cardiac α-actin (Minty et al., 1982; Mohun et al., 1984). To determine whether skeletal or cardiac α-actin is expressed in SMCs from newborn rats, Western blot analyses were done on isoactins resolved by two-dimensional gel electrophoresis using α skeletal/cardiac α-actin-specific S-α-N antibody (Bulinski et al., 1983). Neither the α-actin from newborn rat or from adult rat aortas showed reactivity with the S-α-N antibody to skeletal and cardiac α-actins (Fig. 3). As reported by Bulinski et al. (1983), the S-α-N antibody did not react with β- or γ-actins but did react with skeletal and cardiac α-actins (Fig. 3).

The relative proportions of the various isoactins changed with age (Figs. 1 and 2). Nm-β-actin was the most abundant actin present in aortic SMCs from newborn rats, whereas Sm-α-actia was the major actin present in cells from 90- and 180-day-old rats. Most apparent was the large increase in fractional α-actin content and the decrease in Nm-β-actin that occurred between birth and 30 days of age. Fractional contents of Sm-α-actin also increased moderately (p < 0.05, analysis of variance) between 30 and 180 days of age, whereas Nm-β-actin was unchanged during this interval.

To assess whether changes in fractional isoactin content with age were due to increases and/or decreases in the absolute amounts of each isoactin, the data were also expressed as isoactin content/DNA. Since by far the majority of SMCs are diploid (Owens, 1985) and nondividing at all ages studied, this is a relatively good index of isoactin content per cell. These analyses showed (Fig. 4) that the increase in fractional Sm-α-actin content and the decrease in Nm-β-actin content were due principally to an absolute increase in Sm-α-actin. In fact, the absolute amounts of Nm-β- and γ-actin also increase with age but not nearly as much as Sm-α-actin. Total actin/DNA ratios increased more than 9-fold between 1 and 180 days of age.

**Fig. 1.** Coomassie Blue-stained, two-dimensional IEF (x axis)/SDS (y axis) gels showing the fractional content of isoactins in aortic SMCs at various ages: a, 1 day; b, 9 days; c, 16 days; d, 30 days; e, 90 days; f, 180 days. Samples at 30, 90, and 180 days of age are from medial preparations of thoracic aortas from individual rats, whereas samples at 1, 9, and 16 days of age represent pooled samples from litter mates. α, Sm-α-actin; β, Nm-β-actin; γ, Sm-γ-actin + Nm-γ-actin.

**Fig. 2.** Changes in fractional isoactin content (as a percentage of total actin) with age as determined by densitometric evaluation of Coomassie Blue-stained two-dimensional polyacrylamide gels such as those illustrated in Fig. 1. Values represent means ± S.E. Sample number (indicated in parentheses) at 30 days and older are for individual animals, whereas those at 16 days and younger are for the number of litters. All samples were analyzed at least in duplicate. The coefficient of variation for repeated evaluations of the same sample was less than 3%. Note that γ-actin is the sum of Sm-γ-actin + Nm-γ-actin.
Fractional Isoactin Synthesis and Turnover—The observed changes in isoactin contents with age could reflect differences in rates of isoactin synthesis and/or turnover. This question was addressed by measuring the fractional incorporation of [35S]methionine into the isoactins (Fig. 5). Results demonstrated that the increase in α-actin and the decrease in Nm-β-actin contents between 1 and 30 days of age were associated with corresponding changes in fractional isoactin synthesis. Thus, the increase in α-actin content with age are due, at least in part, to increased synthesis. An interesting and surprising observation was that the fractional synthesis of Sm-α-actin was significantly lower than content (p < 0.001, analysis of variance) at 90 and 180 days of age (Table I). Conversely, the fractional synthesis of Nm-β-actin was significantly greater (p < 0.001) than content at these ages. Since Sm-α-actin content was increasing during this age interval (Figs. 1, 2, and 4), these data suggest that Sm-α-actin turns over more slowly than Nm-β-actin.

We were concerned that the observed decreases in Sm-α-actin synthesis relative to content may have been an artifact of our in vitro incubation assay. For example, there may be differences in lability of mRNAs for different isoactins during the 4-h incubation. We therefore compared synthesis values in vessels incubated with [35S]methionine 0–30 min following isolation versus 210–240 min following isolation. Values were identical for the two groups (e.g. fractional Sm-α-actin synthesis was 64.8 ± 2.3 (S.E.) for the 0–30 min group versus 64.8 ± 0.7 (S.E.) for the 210–240 min group, n = six rats per group). Attempts to compare fractional synthesis values of animals pulsed with [35S]methionine in vivo versus vessels incubated in vitro were unsuccessful in adult animals because of low activity in samples. However, nearly identical values for fractional isoactin synthesis were obtained in vessels from 1-day-old rats pulsed in vivo as compared to vessels incubated in [35S]methionine containing medium in vitro (e.g. fractional Sm-α-actin synthesis values were 36.7 ± 2.1 and 38.0 ± 1.0 for the in vivo and in vitro determinations, respectively). Taken together, these experiments provide good evidence that our in vitro incubation assay provides a valid index of fractional isoactin synthesis in vivo.

Relationship of Isoactin Expression to SMC Proliferation:
TABLE I
Comparison of fractional isoactin synthesis and content in rat aortic smooth muscle in 90- and 180-day-old rats

| Actin | Fractional isoactin synthesis | Fractional isoactin content |
|-------|-------------------------------|----------------------------|
|       | %                             | %                          |
| 180-day-old rats (n = 5)       |                               |                            |
| Sm-α  | 76.5 ± 0.7                    | 51.4 ± 1.2                 |
| Nm-β  | 19.2 ± 0.7                    | 37.5 ± 0.6                 |
| Sm-γ + Nm-γ                      | 4.3 ± 0.2                    | 11.1 ± 0.9                 |
| 90-day-old rats (n = 5)         |                               |                            |
| Sm-α  | 73.3 ± 0.5                    | 65.9 ± 1.1                 |
| Nm-β  | 18.6 ± 0.4                    | 28.1 ± 1.1                 |
| Sm-γ + Nm-γ                      | 7.1 ± 0.3                    | 6.0 ± 0.5                  |

*Fractional isoactin synthesis is significantly different than content (p < 0.001, analysis of variance).

FIG. 6. Fraction of thoracic aortic SMCs undergoing DNA replication at various ages as determined by [3H]thymidine autoradiography. Animals were pulsed with [3H]thymidine 1 h prior to death. Labeling indices were determined on light micrographs stained with hematoxylin. The number of cells counted varied between 5,000 and 40,000 with the larger number being evaluated in older animals which had lower labeling frequencies. Values represent means ± S.E. n = number of animals.

Combined [3H]Thymidine Autoradiographic and Immunocytochemical Studies with Isoactin Antibodies—Studies thus far have demonstrated that major changes in isoactin expression occur in vascular SMCs during normal developmental growth. As a first step in determining whether an increase in Sm-α-actin expression was associated with cessation of cellular proliferation, the frequency of SMCs undergoing DNA replication was determined for each age group studied (Fig. 6). As expected, these analyses showed a decreased frequency of replicating cells with increasing age. These data represent the fraction of cells undergoing DNA synthesis during a 1-h pulse with [3H]thymidine and thus provide a direct measurement of the frequency of cells in the S-phase of the cell cycle.

In general, decreases in SMC replicative frequency appeared to parallel increases in Sm-α-actin expression. Whereas these results obviously provide only indirect evidence regarding the interrelationship between isoactin expression and cytodifferentiation, two observations were significant. 1) The percentage of labeled cells was very low even in newborn rats. 2) The absolute change in the frequency of labeled cells with increasing age was very small compared to changes in Sm-α-actin content. For example, between 50 and 180 days of age, the [3H]thymidine labeling index decreased by less than 0.2% (Fig. 6), whereas Sm-α-actin content (Fig. 4) increased more than 400%. Thus, it is extremely unlikely that a decrease in the fraction of replicating cells alone can account for the increase in Sm-α-actin expression with age. However, the data do not exclude the possibility that initial induction of Sm-α-actin expression in a given cell is associated with withdrawal from the cell cycle and that Sm-α-actin expression is low or absent in cells that are part of the growth fraction, as observed in cell culture studies (Owens et al., 1986; Strauch and Rubenstein, 1984). The following experiment was done to specifically explore this possibility in vivo. Four-to-five-day-old rat pups from a single litter were randomly assigned into two groups and given either: 1) multiple pulses of [3H]thymidine at 6-h intervals beginning 24 h prior to death or 2) a single pulse 1 h prior to death. Since S-phase is approximately 7.6 h in vascular smooth muscle cells (Ross et al., 1979), the multiple pulse regimen should label any cell that initiated DNA synthesis within 24–32 h prior to death, whereas the single pulse should only label cells in S-phase at the time of death. SMCs were then enzymatically dissociated from medial preparations of thoracic aortas and fixed in methanol (4 °C), and cell smears were prepared. Cells were then immunoperoxidase-stained with the smooth muscle-specific isoactin CGA7 monoclonal antibody or the polyclonal actin antibody (PAA) (Gown et al., 1985) fixed in 2% glutaraldehyde in HBSS, and processed for autoradiography. Attempts to do this same experiment in tissue sections were unsuccessful. The major problem was the very limited cell-to-cell resolution in frozen sections (formaldehyde fixatives destroy the antigenicity for CGA7, thereby prohibiting paraffin embedding, etc.). An additional problem was the relatively low frequency of labeled cells and the limited number of nucleated cellular profiles in cross-sections.

Cells derived from medial preparations of rat thoracic aortas showed marked heterogeneities in staining with the CGA7 smooth muscle isoactin antibody (Fig. 7 and Table II). Nearly one-half of the cells from 4–5-day-old rat pups did not show detectable staining above controls with CGA7 (Fig. 7a and b, and Table II). However, all cells were stained with PAA (Fig. 7c and d). Control studies showed no staining with nonimmune mouse IgG, with preimmune rabbit serum, with an antinucleofilament monoclonal antibody (Fig. 7c), or with secondary antibody alone. Less than 1% of the cells stained with Factor VIII antibody (Fig. 7e), a marker for endothelial cells. Freshly isolated rat aortic endothelial cells (obtained by scraping rat aortas with a scalpel blade) did not stain with the smooth muscle isoactin CGA7 antibody but did stain with the polyclonal actin antibody and with the Factor VIII antibody.

In rats given one pulse of [3H]thymidine 1 h prior to death, 93.3% of the [3H]thymidine-labeled cells did not show detectable staining with the smooth muscle-specific isoactin antibody, whereas in rats given multiple doses of [3H]thymidine, 78.6% of labeled cells showed no staining above background (Table II). Thus, by far the majority of proliferating aortic SMCs in newborn rat pups did not stain with a monoclonal antibody to smooth muscle isoactins. However, there was a
small fraction of replicating cells that did stain with CGA7 (Fig. 7b). The frequency of [3H]thymidine-labeled cells which stained with the CGA7 smooth muscle isoactin antibody appeared to be greater in pups given multiple pulses of [3H] thymidine than in pups given a single pulse (Table II).

In contrast to observations in cells from 4–5-day-old rats, 99.4% (i.e. 1988/2000) of cells derived from medial preparations of thoracic aortas from 3-month-old rats stained with the smooth muscle isoactin antibody. Due to the extremely low frequency of unstained cells, we cannot at present exclude the possibility that these represent non-SMC contaminants (i.e. endothelial cells or fibroblasts).

DISCUSSION

The shift from primarily nonmuscle- to smooth muscle-specific actins (Figs. 1 and 2) during development of the rat aorta is similar to the results of Saborio et al. (1979) who studied changes in isoactin expression in the chicken gizzard and confirm and extend recent reports by Kocher et al. (1985). A significant new observation in the present study was that the increase in fractional Sm-α-actin content with development was due to an absolute increase in Sm-α-actin rather than to a decrease in Nm-β- and γ-actin. In fact, the absolute amount of Nm-β-actin (per DNA or cell) also increased somewhat during development (Fig. 4). The predominance of nonmuscle actins in aortic SMCs in newborn animals is consistent with the morphologic appearance of these cells. SMCs in the aorta of newborn rats are ultrastructurally very similar to fibroblasts (i.e. they are deficient in myofilaments but contain abundant polyribosomes, rough endoplasmic reticulum, and Golgi bodies) and only gradually acquire the features of adult cells (Cliff, 1967; Gerrity and Cliff, 1975). Aortas from newborn rats also show diminished force-developing capabilities (Seidel and Allen, 1979). Taken together, these results indicate that aortic SMCs from newborn rats are not fully differentiated and that expression of Sm-α-isoactin is a useful biochemical marker of cytodifferentiation in these cells.

Our immunolabeling studies showed that there were marked heterogeneities in staining between individual SMCs from newborn rats using a monoclonal antibody to the smooth muscle isoactins. In fact, nearly 50% of the cells derived from thoracic aortic medial preparations from 4–5-day-old rats showed little or no staining with the smooth muscle isoactin antibody (Table II). Less than 1% of these unstained cells were endothelial cells as identified by Factor VIII staining. Although a fraction of the unstained cells may be adventitial

SMCs were enzymatically dispersed from medial preparations of rat thoracic aortas. Vessels were pooled from litter mates. Cell smears were prepared, fixed in methanol, stained with antibodies using an avidin/biotin/peroxidase procedure, and then processed for autoradiography. Silver grains, indicating [3H]thymidine uptake, appear as black dots which are out of focus since they lie in a plane above the cells. Cells in a–c were stained with hematoxylin. a, medial derived cells stained with the smooth muscle-specific CGA7 isoactin antibody. The two cells labeled with arrows showed little or no staining above controls. Magnification ×1326. b, medial derived cells stained with the smooth muscle-specific CGA7 isoactin antibody. Negatively stained cells are labeled with large arrows. One of these (lower left) has incorporated [3H]thymidine. Note that the cell indicated by the small arrow (upper right) represents one of the few cells that stained with CGA7 and also incorporated [3H]thymidine. Magnification ×1235. c, medial derived cells stained with an antineurofilament monoclonal antibody unreactive to SMCs (i.e. negative control for a and b). Magnification ×1690. d, medial derived cells stained with a rabbit anti-chicken gizzard actin polyclonal antibody (PAA) that reacts with muscle and nonmuscle isoactins (positive control). Magnification ×1265. e, medial derived cells stained with Factor VIII antibody. Magnification ×1040. Magnification bars = 10 μm.
fibroblasts, it is very unlikely that fibroblasts could account for all unstained cells since extreme care was taken to dissect away the adventitia in vessels used in these studies and removal of the adventitia was confirmed by light microscopic evaluation of several dissected vessels. It also seems unlikely that the failure of the smooth muscle isoactin antibody to stain a portion of medial derived cells relates to an alteration in the conformational state of actin or to the method of fixation since this antibody does show reactivity to denatured smooth muscle isoactins in Western blots (Gown et al., 1985) and greater than 99% of cells derived from adult aortas react with the antibody using an identical fixation and staining protocol. Our interpretation of these data is that there is a subpopulation of relatively undifferentiated SMCs in the thoracic aorta of the newborn rat, at least in terms of actin expression. Significantly, results of combined [3H]thymidine autoradiographic and immunocytochemical studies demonstrated that the frequency of proliferating cells was much higher in these relatively undifferentiated cells, suggesting an inverse relationship between growth state and Sm-α-actin expression. This idea is consistent with results of our previous studies (Owens et al., 1986) and those of Strauch and Rubenstein (1984) demonstrating that cessation of proliferation in cultured vascular smooth muscle cells is associated with induction of Sm-α-actin expression, whereas growth initiation is associated with increased expression of nonmuscle actins. It is important to note, however, that we did observe a number of proliferating cells that stained with the smooth muscle isoactin antibody in the present study, indicating that cell proliferation and expression of smooth muscle isoactins (at least at the protein level) are not mutually exclusive. This is not surprising since in cultured SMCs, Sm-α-actin synthesis is very low but not absent in exponentially growing cells (Owens et al., 1986). Furthermore, we found (Owens et al., 1986) that α-actin content remained high for some time following serum stimulation of growth despite the fact that Sm-α-actin synthesis declined to extremely low levels. Thus, it is not clear whether the CGA7-positive cells that incorporated [3H]thymidine represent cells that had temporarily withdrawn from the cell cycle, differentiated, and then reinitiated cell proliferation or whether these cells express some smooth muscle isoactin while part of the growth fraction. It is important to emphasize that our data clearly indicate that increases in Sm-α-actin content with age cannot be explained simply by a reduction in the fraction of replicating cells since absolute changes in replicative frequency were extremely small compared to increases in Sm-α-actin content. Furthermore, whereas Sm-α-actin content increases following growth arrest in cultured SMCs, growth arrest alone does not increase the level of Sm-α-actin expression to that found in fully differentiated SMCs in vivo (Owens et al., 1986; Strauch and Rubenstein, 1984). Taken together, these data support the hypothesis that cessation of SMC proliferation during development is associated with initial induction of Sm-α-expression but that factors other than growth state are also important in determining the level of Sm-α-actin expression in fully differentiated SMCs. This may be analogous to what occurs in skeletal myoblasts where growth arrest leads to initial differentiation, but not to maturation to the fully differentiated state characteristic of myocytes in vivo (Minty et al., 1982; Mohun et al., 1984).

The functional significance of alterations in isoactin expression during development is unclear. However, there is considerable presumptive evidence implicating functional differences between different isoactins. In skeletal muscle, shifts in isoactin expression from nonmuscle forms to the skeletal muscle α-form during differentiation are accompanied by extensive changes in the structure and function of actin in these cells (Devlin and Emerson, 1978; Wallevik and Jensen, 1982). The increases in Sm-α-actin in aortic SMCs observed in the present study appear to correlate very closely with increases in force-generating capacity (Seidel and Allen, 1979). Furthermore, our observations of differential turnover of isoactins (Table I) imply differences in structure and/or intracellular processing (e.g. control of polymerization, reaction with actin-binding proteins, etc.) of the different isoactins. Immunocytochemical studies by Paro et al. (1983) have demonstrated differential subcellular localization of muscle and nonmuscle actins in skeletal muscle cells. Rubenstein et al. (1982) showed that muscle and nonmuscle isoactins were released differentially from developing quail myoblasts, again indicating a differential utilization of these isoactins in the cell. Whereas these studies support the idea that isoactins have specialized roles within the cell, the precise nature of purported functional differences are unclear since muscle and nonmuscle actins appear to have similar physical properties.

**Table II**

| Group | Antibody | [3H]Thymidine labeling index | Cells stained* | [3H]Thd-labeled cells unstained | [3H]Thd labeling index in unstained cells | [3H]Thd labeling index in stained cells |
|-------|----------|----------------------------|---------------|---------------------------------|------------------------------------------|---------------------------------------|
| Single [3H]Thd pulse 1 h prior to death | CGA7 (n = 2) | 1.07 ± 0.63 | 57.0 ± 4.5 | 93.3 ± 11.5 | 2.18 ± 0.90 | 0.21 ± 0.36 |
| | PAA (n = 2) | 1.51 ± 0.28 | 100 | 0 | 1.51 ± 0.28 | |
| | Nonimmune mouse IgG control (n = 3) | 1.27 ± 0.38 | 100 | 1.27 ± 0.38 | |
| Multiple [3H]Thd pulses during the 24 h prior to death | CGA7 (n = 2) | 6.85 ± 0.96 | 58.5 ± 4.0 | 78.6 ± 13.6 | 13.73 ± 5.54 | 2.41 ± 1.40 |
| | PAA (n = 2) | 9.25 ± 0.36 | 100 | 0 | 6.25 ± 0.35 | |
| | Nonimmune mouse IgG control (n = 2) | 5.97 ± 0.47 | 100 | 5.97 ± 0.47 | |

*Based on evaluation of immunoperoxidase staining with a monoclonal antibody specific for smooth muscle isoactins (CGA7) and with a rabbit anti-chicken gizzard polyclonal actin antibody (PAA) that reacts with muscle and nonmuscle isoactins.
(Pollard, 1981). However, physical studies are compromised by the fact that the normal cellular controls over actin polymerization and depolymerization are lacking.

Convincing evidence has been presented showing that marked decreases in Sm-α-actin expression occur in SMCs from human atheromatous lesions (Gabbiani et al., 1984) or intimal lesions from experimental animal models (Kocher et al., 1984).

Our recent studies in cell culture demonstrating that stimulation of growth in quiescent SMCs is associated with a dramatic decrease in Sm-α-actin synthesis (Owens et al., 1986) and the present studies showing that proliferating cells in the newborn rat aorta are deficient in smooth muscle isoactins support the idea that decreases in smooth muscle isoactins in SMCs in atheromatous lesions may be secondary to growth stimulation. However, an alternative possibility is that SMCs within the atheromatous lesion are derived from a subpopulation of smooth muscle "stem" cells which are relatively undifferentiated. Results of the present studies demonstrate that examination of the isoactin expression of cells may be a very useful index to explore this possibility.

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