A large accumulation of non-muscle myosin occurs at first entry into M phase in rat vascular smooth-muscle cells

David J. GRAINGER,* T. Robin HESKETH,* James C. METCALFE*† and Peter L. WEISSBERG†

*Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K., and †School of Medicine, University of Cambridge, Hills Road, Cambridge CB2 2QQ, U.K.

Vascular smooth-muscle cells (VSMCs) from rat aortae contained very little non-muscle myosin heavy chain (MHC) immediately after dispersal, and the protein did not accumulate if the cells were held in G₀/G₁ phase by withholding serum or were held in first S phase by the addition of bromodeoxyuridine (BrdU). However, non-muscle MHC accumulated by > 20-fold per cell during first M phase, when over 80 % of the cells divided between 48 h and 72 h after addition of serum. Delaying the addition of serum caused a delay in the accumulation of the non-muscle MHC until the cells subsequently entered M phase. If the cells were held in M phase at the metaphase/anaphase boundary by nocadazole, the accumulation of non-muscle myosin still occurred, although division was blocked. When the cells were pulse-labelled with [³⁵S]methionine, it was found that non-muscle MHC was one of the major proteins being made and that its synthesis occurred at similar rates throughout the cell cycle. This implied that the rate of degradation of the protein before first M phase was much faster than in M phase, when the protein accumulated rapidly. This was confirmed by direct measurements of the rate at which [³⁵S]methionine-labelled non-muscle MHC disappeared from the cells, which gave a half-life for the protein of about 8 h before M phase but about 5 days in post-mitotic cells, i.e. an increase of approx. 15-fold. These data are consistent with the hypothesis that there is a mechanism in VSMCs which shortens the half-life of the protein before first M phase and that the accumulation of non-muscle MHC which results from the increase in half-life at first M phase may be necessary for division of these cells.

INTRODUCTION

In early studies of cultured VSMCs, a progressive change in cell phenotype was observed as the cells underwent proliferation. The change was defined mainly by morphological criteria: freshly dispersed VSMCs have a high volume fraction of myofilaments, few biosynthetic organelles [1,2], and retain the ability to contract in response to vasoconstrictors [3]. In contrast, proliferating VSMCs have a low volume fraction of myofibrils, a high proportion of biosynthetic organelles [1,2], and have lost the ability to contract in most [4–6], but not all, studies [7,8]. Underlying these changes in myofibril content and contractility, it was found that the ‘contractile’ phenotype predominantly expresses the isoforms of MHC (SM-1 and SM-2) [9–14] and α -actin [15–18] that are specific to smooth-muscle cells, whereas the ‘proliferative’ phenotype expresses non-muscle forms of these proteins (non-muscle MHC and β - and γ -actin) [9–18] that are found in many types of cells.

In some studies it has been observed that the morphological change occurs before the cells reach mitosis in the first cell cycle [19–22], from which it has been suggested that this change is essential before division can occur [4]. More specific molecular changes that occur before DNA synthesis or first division have also been identified. In particular, it has been proposed that the appearance of the non-muscle β - and γ -actins in VSMCs may be obligatory for the first cell division, because actomyosin is required to form the cleavage furrow at cytokinesis. However, any obligatory requirements for changes in the expression of any of the isoforms of actin or myosin for passage through the cell cycle remain undefined. In particular, it is not known whether

either the expression of the non-muscle isoforms of the proteins or the de-differentiation step marked by the loss of the smooth-muscle isoforms that are required for contraction must occur before the cells can achieve first division.

We have examined the expression of both non-muscle and smooth-muscle-specific MHCs in primary cultures of rat aorta VSMCs to determine whether the expression of these proteins is correlated with the first cycle of cell division, or whether their expression can be uncoupled from cell division.

MATERIALS AND METHODS

Cell culture and [³H]thymidine incorporation

The aortae were removed from 250–300 g Wistar rats killed by asphyxiation with CO₂. The tunica media was separated from the adventitia and endothelium by collagenase treatment, and the cells were dispersed as previously described [23] in collagenase and elastase (both from Sigma Chemical Co.) in Medium 199 for 2 h at 37 °C. The cells were suspended in medium [Medium 199 supplemented with 100 mM-NaHCO₃, 2 mM-glutamine, penicillin (100 units/ml) and 10 % foetal-calf serum (FCS; from ICN Flow)] to give 4 × 10⁶ cells/ml. The cell suspension was plated on to 35 mm-diam. sterile culture dishes (ICN Flow) at 2.0 ml/dish and incubated in 5 % CO₂ in air (pH 7.3) at 37 °C. In all time-course experiments the start of this incubation was timed as 0 h. The cells were given fresh medium at 24 h and thereafter at 48 h intervals. More than 99 % of the cells stained positively for smooth-muscle-specific MHC isoforms at 48 h when an anti-serum to chicken gizzard smooth-muscle myosin (aChGM) was

Abbreviations used: aChGM, antiserum to chicken gizzard smooth-muscle myosin; BrdU, bromodeoxyuridine; FCS, foetal-calf serum; MHC, myosin heavy chain; PBS, phosphate-buffered saline; SM-1 and SM-2, 204 kDa and 200 kDa smooth-muscle-specific MHC isoforms respectively; TBS, Tris-buffered saline; VSMC, vascular smooth-muscle cell.

† To whom correspondence should be addressed.

used as described [6]. The incorporation of [^3H]thymidine (Amersham International) was assayed by incubation with $2.0\ \mu\text{Ci/ml}$ in 5% CO_2 in air at 37°C for 2.0 h. The medium was aspirated and the cells were washed twice in ice-cold phosphate-buffered saline (PBS), containing $137\ \text{mM-NaCl}$, $2.7\ \text{mM-KCl}$, $8.1\ \text{mM-Na}_2\text{HPO}_4$, $0.9\ \text{mM-CaCl}_2$, $0.5\ \text{mM-MgCl}_2$ and $1.5\ \text{mM-KH}_2\text{PO}_4$ (pH 7.2), and lysed in $0.5\ \text{ml}$ of 1.0% SDS. The lysate was treated with an equal volume of 15% (w/v) trichloroacetic acid on ice for 20 min before filtering through Whatman GF/C paper and washing with $4\ \text{ml}$ of 7.5% trichloroacetic acid and then with $4\ \text{ml}$ of 96% ethanol. After drying in air, the filters were scintillation-counted. DNA synthesis was also assayed by BrdU incorporation by using the cell-proliferation kit (Amersham International).

Cell counting

Cells were detached by incubation with trypsin/EDTA solution (Gibco) for 10 min. The suspension of released cells was centrifuged ($900\ g$, 4 min), and the pelleted cells were resuspended in $1.0\ \text{ml}$ of PBS at room temperature and counted by haemocytometer. All cell counts were obtained from cells from triplicate dishes, and are plotted as means $\pm$ S.D.; all experiments were repeated on at least two separate cultures as indicated.

Analysis of MHC isoforms

Samples of 4×10^5 cells were prepared in suspension in PBS as for counting, centrifuged ($900\ g$, 4 min) and lysed in gel sample buffer [24] after removing the supernatant. The lysed cell samples were boiled for 4 min, stored at -20°C , and re-boiled for 4 min before analysis. PAGE was run as described by Laemmli [24] except that a $3\text{--}5\%$ acrylamide gradient gel was used, SDS was replaced by lithium dodecyl sulphate (Sigma Chemical Co.), and piperazine diacrylamide (Bio-Rad) was used as the cross-linker. Gels were run at constant $100\ \text{V}$ for 15 h at 4°C and silver-stained as described by Oakley *et al.* [25]. For Western blotting, gels prepared as described above were soaked in blot buffer, containing $39\ \text{mM-glycine}$, $48\ \text{mM-Tris}$, 0.0375% SDS and 20% methanol in water, for 30 min, and then blotted on to nitrocellulose by using a Novablot semi-dry blotter at $150\ \text{mA}$ constant current for 90 min. The membrane was rehydrated in Tris-buffered saline (TBS), containing $137\ \text{mM-NaCl}$ and $50\ \text{mM-Tris/HCl}$, pH 7.4, and blocked by incubation with 3% (w/v) BSA in TBS for 30 min. The aChGM antiserum was used to detect smooth-muscle MHC [26], and antiserum to human platelet myosin [11] was used to detect the non-muscle MHC isoform. The antisera containing the primary antibodies were added at a final concentration of about $1\ \mu\text{g/ml}$ in TBS containing 3% BSA and incubated at room temperature with agitation for 12–16 h. The anti-(smooth-muscle MHC) antibody was labelled by an 8 h incubation with goat anti-(rabbit IgG) Fab fragment (ICN Flow), followed by an 8 h incubation with sheep anti-goat IgG conjugated to peroxidase (Sigma Chemical Co.). The anti-(non-muscle MHC) antibody was labelled directly by an 8 h incubation with sheep anti-rabbit IgG-peroxidase conjugate (Sigma Chemical Co.). The membranes were developed with $500\ \mu\text{g}$ of 4-chloro-1-naphthol (Sigma Chemical Co.)/ ml in 20% methanol in TBS. H_2O_2 was added at $0.02\ (\text{v/v})$ to activate the reaction, which was terminated by washing with excess water.

Non-muscle MHC synthesis and half-life determination

The rate of synthesis of the MHC proteins was determined by pulse-labelling the cells at various times by adding $150\ \mu\text{Ci}$ of L-[^{35}S]methionine (Amersham International)/ ml to the medium for 2.0 h. The cells were harvested and the MHC isoforms resolved by lithium dodecyl sulphate/PAGE as described above. The gel

was blotted on to nitrocellulose and the MHC isoforms were detected by Western blotting using both antisera. The [^{35}S]methionine-labelled proteins were detected by autoradiography of the blots at -80°C for 5 days. Overlaying the autoradiograph on the blot allowed the [^{35}S]methionine-labelled proteins corresponding to the MHC isoforms to be identified. The half-life of the non-muscle MHC was determined by pulse-labelling the cells with [^{35}S]methionine at $25\ \mu\text{Ci/ml}$ for 24 h, after which the medium was replaced with fresh medium containing $35\ \mu\text{M-cycloheximide}$ (Sigma Chemical Co.). Cell samples were analysed at various times after the pulse-labelling, and the amount of labelled non-muscle MHC was determined from the blots with a t.l.c. linear analyser (Bertholdt).

RESULTS

Myosin isoforms through the first cell cycle

Freshly dispersed VSMCs (0 h) in culture began to enter S phase after 30 h and to divide after 48 h (Fig. 1). At 48 h, direct immunocytochemistry with an anti-(smooth-muscle MHC)-specific antibody showed that $> 99\%$ of the cells were VSMCs [6]. A logarithmic increase in cell number was maintained until 144 h, with a population doubling time of $34.9 \pm 1.8\ \text{h}$ (mean $\pm$ S.D.; $n = 5$). At this time the cells became confluent and the rate of division decreased. The cells ceased dividing and entered a stationary phase between 144 h and 168 h.

Cell samples from the experiment shown in Fig. 1 were analysed by silver staining after lithium dodecyl sulphate/PAGE. In the freshly dispersed cells, only the smooth-muscle MHC isoforms could be detected as a doublet [9] at $204\ \text{kDa}$ (SM-1) and $200\ \text{kDa}$ (SM-2) (Fig. 2a). The intensity of the smooth-muscle MHC doublet decreased with time, and after 72 h the $196\ \text{kDa}$ non-muscle MHC isoform was observed, which became the major MHC species by 96 h (Fig. 2a). The SM-1 isoform disappeared with a half-time of approx. 60 h, estimated from the integrated intensity of the SM-1 band (see Figs. 2b and 2c). After the cells ceased dividing, the non-muscle MHC band decreased in intensity and the smooth-muscle MHC doublet increased in intensity to give a pattern similar to that in the freshly dispersed cells (results not shown). The identities of the MHC components were confirmed by Western blotting with specific antibodies (see below).

Fig. 1. DNA synthesis and proliferation of primary VSMC cultures

The cell suspensions ($4 \times 10^5/\text{ml}$) were plated on to culture dishes at $t = 0\ \text{h}$. The incorporation of [^3H]thymidine ($2.0\ \mu\text{Ci/ml}$) was assayed by incubation for 2.0 h (O), and cells from triplicate dishes were counted after trypsin treatment (●) as described in the Materials and methods section. Values are means of five separate cells cultures analysed in triplicate; error bars are S.D.

Fig. 2. Lithium dodecyl sulphate/PAGE analysis of silver-stained total VSMC lysates

(a) Equal numbers of VSMCs (4×10^6) harvested from cell culture at various times were lysed and the lysates analysed by lithium dodecyl sulphate/PAGE on 3–5 % gradient gels (see the Materials and methods section). The MHC isoforms are indicated: SM-1 (204 kDa) and SM-2 (200 kDa) are smooth-muscle-specific MHC isoforms; NM (196 kDa) (◀) is the non-muscle MHC isoform. An additional band (approx. 210 kDa), which did not react with the anti-myosin antibodies, is also indicated. (b) Densitometric traces of the lanes in (a). Peaks corresponding to the smooth-muscle MHC isoforms (SM-1 and SM-2) and non-muscle MHC isoform (NM) are indicated. (c) MHC isoform content per cell determined from the traces in (b). The 204 kDa SM-1 isoform (◆) and non-muscle MHC (◻) are shown. Non-muscle MHC accumulation coincides with the onset of cell division at 48 h.

Fig. 3. MHC isoform analysis during first mitosis

(a) Cell number at 6 h intervals over the period in which first mitosis occurs. (b) Cells were pulsed with L-[35 S]methionine (150 μ Ci/ml) for 2 h at 6 h intervals between 44 h and 74 h. Total cell lysates from 4×10^6 cells were analysed by lithium dodecyl sulphate/PAGE and Western-blotted with antisera to both smooth-muscle myosin (aChGM) and non-muscle human platelet myosin to identify the MHC isoforms. (N.B. the autoradiograph obtained from the same Western blot is shown in Fig. 5).

The non-muscle MHC appeared soon after the onset of cell division, and therefore a more detailed analysis was made of the MHC isoforms through first mitosis from 48 h to 72 h (Fig. 3a). The cells were sufficiently synchronous to show that over 80 % of the total cell population in the culture had divided during this period (see Fig. 3a). Western blotting for both smooth-muscle and non-muscle MHCs at 6 h intervals showed that there was a marked increase in the amount of non-muscle MHC between 56 h and 62 h during the first M phase (Fig. 3b). The amount of non-muscle MHC continued to increase thereafter until the cells became confluent (Fig. 2b). It is emphasized that this was not due to the increase in cell number over this period, since the samples at each time point were prepared from the same number of cells.

To estimate the magnitude of the increase in the amount of non-muscle MHC between 56 h and 62 h, separate experiments were performed in which lysate samples from cells at 62 h were serially diluted to determine the lowest relative concentration of non-muscle MHC in the cells which could be detected by Western blotting under the experimental conditions used. The non-muscle MHC was just detectable at 10-fold dilution of the cells, indicating that there is at least a 10-fold increase in the amount of non-muscle MHC per cell between 50 h and 62 h.

Effect on the myosin isoforms of blocking the cells within the first cell cycle

The above data showed that the large increase in non-muscle MHC occurred in M phase. To determine whether this occurred before mitotic metaphase, the cells were treated with nocodazole (0.05 μ g/ml) from 48 h to 72 h to prevent entry into mitotic anaphase and completion of division by disrupting microtubule

Fig. 4. Effect on cell number and MHC isoforms of blocking the first cell cycle at M, G₀/G₁ or S phase

Lysates from 4×10^5 cells were analysed in each lane. (a) Lane 1 shows a cell sample at 48 h before M phase. Addition of nocodazole ($0.05 \mu\text{g/ml}$) at 48 h blocked the increase in cell number at 72 h (◆) compared with an untreated control sample (□) by holding the cells in M phase, but did not affect the accumulation of non-muscle MHC (NM) at 72 h (lane 2) compared with an untreated control sample at the same time (lane 3). Wash-out of nocodazole at 72 h allowed the cells to complete M phase and divide (◆). (b) Cells were cultured for 72 h in the absence of serum on dishes pre-treated with bovine plasma fibronectin ($50 \mu\text{g/cm}^2$). Under these conditions the cells did not enter S phase or divide. FCS (10%) was then added to stimulate entry into the cell cycle. Non-muscle MHC accumulation, assayed by Western blotting with anti-(human platelet myosin) antiserum, did not occur until cell division had occurred at 120 h. (c) Addition of BrdU ($10 \mu\text{M}$) before S phase (at 24 h) prevented first cell division (○), but addition after S phase (at 48 h) had no effect on first cell division (◆) compared with an untreated control sample (■). Non-muscle MHC accumulation was analysed by Western blotting. Lanes 1 and 2 were lysates from untreated control cells at 48 h and 72 h respectively; lane 3 was from cells treated with $10 \mu\text{M}$ -BrdU at 24 h and analysed at 72 h; lane 4 was from cells treated with BrdU at 48 h and analysed at 72 h.

reorganization [27,28]. Cell division ceased while nocodazole was present and resumed when it was washed out (Fig. 4a). However, the presence of nocodazole had no significant effect on the appearance of the non-muscle MHC, which therefore preceded mitotic anaphase (Fig. 4a).

It remained a possibility that the timing of the accumulation of non-muscle MHC depended on the particular culture conditions, and therefore on the time the cells had been in culture, rather

than their progression through the first cell cycle. Two types of experiments were performed to examine this.

In VSMCs cultured in the presence of serum, over 80% of the cells had passed through the cell cycle and divided by 72 h (see Fig. 3a). In contrast, when VSMCs were cultured on fibronectin in the absence of serum, less than 5% of the cells entered S phase, as assayed by an antibody to BrdU incorporated into DNA (see the Materials and methods section), and there was no increase in

Fig. 5. Analysis of non-muscle MHC synthesis

The autoradiograph obtained by exposure for 5 days of the Western blot shown in Fig. 3(b).

Fig. 6. Determination of the half-life of non-muscle MHC

Cells were labelled by a 24 h pulse of L-[35 S]methionine at 30 μ Ci/ml. [35 S]Methionine was washed out and cycloheximide (10 μ g/ml) was added, which completely inhibited further protein synthesis. Cell lysates were analysed by lithium dodecyl sulphate/PAGE and the non-muscle MHC band was identified by Western blotting. The percentage of radioactivity remaining in the non-muscle MHC band at various times after the pulse was determined with a t.l.c. linear analyser. The half-life for non-muscle myosin in cells labelled from 0 h to 24 h before first mitosis was 8 h ($\square$), and from 72 h to 96 h after first mitosis it was approx. 5 days ($\blacklozenge$). The 100% values were 3981 c.p.m. at 24 h and 9602 c.p.m. at 96 h, consistent with the slower rate of protein synthesis in cells in the G_0/G_1 phase compared with proliferating cells (see the text).

cell number after 72 h. If serum was then added, the cells divided within the next 48 h (Fig. 4b). Western blotting demonstrated that non-muscle MHC did not accumulate in cells arrested in G_0/G_1 for 72 h in the absence of serum, although the intensity of the smooth-muscle MHC doublet decreased normally. However, 48 h after the addition of serum, when 80% of the cells had divided (Fig. 4b), the non-muscle MHC had accumulated. This showed clearly that the appearance of the non-muscle MHC was correlated directly with the passage of the cells through the cell cycle and was not determined merely by the time the cells had been in culture. The appearance of the non-muscle MHC during M phase under the defined culture conditions is not therefore coincidental.

This conclusion was tested further in separate experiments on VSMCs cultured in the presence of serum in which the cells were arrested in S phase by addition of BrdU (10 μ M) after 24 h in culture. After 72 h there was no increase in cell number, and no accumulation of non-muscle MHC was observed, although the smooth-muscle MHC had decreased in intensity normally. If BrdU was added after the completion of S phase at 48 h, 90% of the cells subsequently divided, and the normal accumulation of non-muscle MHC was observed (Fig. 4c). These data confirmed that the large increase in non-muscle MHC shown in Fig. 2 did not occur in G_1 or S phase.

Rates of synthesis and degradation of MHC isoforms

The large accumulation of the non-muscle MHC during M phase must have resulted from either an increase in the rate of its synthesis or a decrease in the rate at which the protein was degraded. The rate of non-muscle MHC synthesis was determined by a series of 2 h pulses with [35 S]methionine between 44 h and 74 h, i.e. before and through first M phase. The autoradiograph in Fig. 5 shows that the non-muscle MHC was one of the major proteins being synthesized in this period, and that its rate of synthesis varied by less than $\pm 25\%$ as determined by densitometry. It was therefore clear that the rate of non-muscle MHC synthesis was not changing significantly over a period in which the amount of the non-muscle MHC in the samples used in Fig. 3(b) was increasing by more than 10-fold, as estimated by Western blotting of the same samples (Fig. 3). This implied that the half-life of the non-muscle MHC must have increased substantially at first M phase to cause the protein to accumulate, and its half-life was therefore estimated before and after first mitosis. The cell proteins were labelled with [35 S]methionine at 24 h and at 96 h, and cycloheximide (35 μ M) was added to prevent further incorporation of [35 S]methionine into the protein. The rate of decrease in the amount of [35 S]methionine-labelled non-muscle MHC (Fig. 6) gave a half-life for the protein of approx. 8 h before first mitosis and, by extrapolation, approx. 5 days afterwards, i.e. an increase of about 15-fold. This was sufficient to account for the observed accumulation of the protein through mitosis.

DISCUSSION

The results show that there is a greater than 10-fold increase in the amount of non-muscle MHC in M phase before cytokinesis. The accumulation occurs in the presence of nocodazole and therefore precedes mitotic metaphase; it does not occur if the cells are retained in G_0/G_1 in the absence of serum or in S phase by the addition of BrdU. Once the cells have completed S phase, addition of BrdU in G_2 has no effect either on subsequent cell division or on the accumulation of non-muscle MHC. Releasing the cells from a prolonged G_0/G_1 phase into the cell cycle by addition of serum confirmed that the appearance of the non-muscle MHC is directly correlated with the entry of the cells into M phase, rather than the time they have been in culture. The coincident time course of the non-muscle MHC accumulation and the increase in cell number indicates that the major accumulation to levels of non-muscle MHC observed in other types of proliferating cells does not occur in G_2 . It should be emphasized, however, that our data do not preclude a small increase in non-muscle MHC before first M phase, below the amount which would be detected at the cell protein concentrations used for Western blotting. The limit to detection of non-muscle MHC by Western blotting is about 10% of the amount observed in the post-mitotic cells at 72 h. This accumulation does not occur if the cells are prevented from leaving S phase by BrdU, and therefore the switch in half-life does not occur before the end of S phase. However, there is an increase in the rate of general protein synthesis between 24 h and 48 h of about 2-fold, and a similar increase in the rate of incorporation of [35 S]methionine into non-muscle MHC over the same period. This corresponds to the general stimulation of protein synthesis observed in other types of somatic cells through G_1 to M phase, and could account for an increase of approx. 2-fold in the amount of non-muscle MHC in the cells from late G_1 to late G_2 .

Our data show that the main mechanism for the accumulation of non-muscle MHC through M phase is an increase in half-life of the protein and that there is little variation (less than $\pm 25\%$) over this period in the rate of its synthesis. Amounts of non-

muscle MHC similar to those observed in post-mitotic VSMCs have been detected in other proliferating cell types, including Swiss 3T3 fibroblasts (D. J. Grainger, unpublished work), where the half-life of the non-muscle MHC is comparable with that estimated here for the post-mitotic cells. The novel observation that there is a mechanism in VSMCs which substantially shortens the half-life of the non-muscle MHC before first mitosis raises the question of whether this increase in half-life of non-muscle MHC is necessary for the VSMCs to divide. Since actomyosin is directly implicated in the formation of the cleavage furrow required for cytokinesis, the accumulation of non-muscle MHC in M phase may be essential for cell division if the amount of the protein present in the freshly dispersed VSMCs is too small to sustain this function.

The conclusion that the large accumulation of non-muscle MHC is coupled to first mitosis is in marked contrast with the inference which can be drawn from the observations by Seidel *et al.* [12], that the timing of the accumulation of non-muscle MHC depends on the culture conditions and can therefore be uncoupled from the cell cycle. They found that the first cell cycle of dog saphenous-vein VSMCs had a similar time course to the rat aorta VSMCs used in our study, but they observed accumulation of non-muscle MHC in G₁/S phase. Furthermore, the protein did not accumulate until after first mitosis if the cells were held in serum-free medium for 48 h before addition of serum. The latter observation implies that cytokinesis can occur without the accumulation of non-muscle MHC in M phase. It would be of interest to block the expression of non-muscle MHC immediately before first M phase by transfecting the VSMCs with an inducible construct containing the antisense non-muscle MHC sequence and to determine whether this blocks cytokinesis after the cells enter M phase.

In marked contrast with the correlation between the cell cycle and the accumulation of non-muscle MHC, the decrease in smooth-muscle MHC occurs at a similar rate in the presence or absence of serum, is not blocked by holding the cells in G₀/G₁, S or M phase, and is not therefore correlated with passage through the cell cycle. The mechanism that triggers this de-differentiation of the cells independently of exogenous mitogens is not known, but, since the smooth-muscle MHC isoforms reappear at stationary phase, regulation of their expression is clearly an intrinsic property of VSMCs. It is therefore possible that the loss of cell-cell contact in the dispersed cell preparation is a major determinant of the loss of smooth-muscle MHC before confluence is reached. However, it is also possible that other signals, for example from fibronectin [29], are necessary to activate de-differentiation.

It is unlikely that the decrease in smooth-muscle MHCs is necessary for the VSMCs to pass through first division. Only about 50 % of these proteins present in freshly dispersed cells are lost before the cells divide, and some studies have shown that VSMCs retain a contractile response at first mitosis [7,8], which presumably requires a functional complement of smooth-muscle myofibrils. This suggests that the functional de-differentiation of the cells marked by the loss of smooth-muscle MHC and the eventual loss of contractility occurs in parallel with the proliferative response, but may be independent of it. This would imply that the contractility of the cells at first division may depend on the time taken in culture to reach first mitosis. Where this occurs relatively rapidly (42 h in this study and in that by Seidel *et al.* [12]) sufficient smooth-muscle myosin may be present to sustain a contractile response. If the conditions are such that the cells reach first mitosis more slowly, so that the cell number does not increase until 144 h [18,23,30] and the smooth-muscle myosin has decreased to about 10 % of the amount in freshly dispersed cells, the contractile response may be lost before first

mitosis. It therefore remains an open question as to which, if any, of the VSMC-specific proteins which characterize the contractile phenotype must be down-regulated before proliferation can occur.

In summary, the data suggest two working hypotheses for further examination. (i) The mechanism causing rapid breakdown of the non-muscle MHC before first M phase is specific to smooth-muscle cells and must be turned off before the cells can complete the first cell cycle. (ii) Functional de-differentiation of the cells marked by the loss of smooth-muscle MHC and the contractile response is not necessary for the first cell cycle to be completed. In this view, the functional de-differentiation is a process parallel to, but independent of, the mitogenic pathway, although this does not preclude the possibility that the two processes are initially triggered by a common set of signals.

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REFERENCES

- Ross, R., Wight, T. N., Strandness, E. & Thiele, B. (1984) *Am. J. Pathol.* **114**, 79–93
- Thyberg, J., Palmberg, L., Nilsson, J., Ksaizek, T. & Sjolund, M. (1983) *Differentiation* **25**, 156–167
- Hermesmeier, K., deCino, P. & White, R. (1976) *In Vitro* **12**, 628–634
- Chamley-Campbell, J. H., Campbell, G. R. & Ross, R. (1979) *Physiol. Rev.* **59**, 1–61
- May, J. F., Paule, W. J. & Blankenhorn, D. H. (1974) *Anat. Rec.* **178**, 413
- Chamley, J. H., Campbell, G. R., McDonnell, J. D. & Gröschel-Stewart, U. (1977) *Cell Tissue Res.* **177**, 503–509
- Murray, T. R., Marshall, B. E. & Macarack, E. J. (1990) *J. Cell. Physiol.* **143**, 26–38
- Chamley, J. H. & Campbell, G. R. (1974) *Exp. Cell Res.* **84**, 105–110
- Rovner, A. S., Murphy, R. A. & Owens, G. K. (1986) *J. Biol. Chem.* **261**, 14740–14745
- Gaylinn, B. D., Eddinger, T. J., Martino, P. A., Monical, P. L., Hunt, D. F. & Murphy, R. A. (1989) *Am. J. Physiol.* **257**, C997–C1004
- Kawamoto, S. & Adelstein, R. S. (1987) *J. Biol. Chem.* **262**, 7282–7288
- Seidel, C. L., Wallace, C. L., Dennison, D. K. & Allen, J. C. (1989) *Am. J. Physiol.* **256**, C793–C798
- Gröschel-Stewart, U., Chamley, J. H., Campbell, G. R. & Burnstock, G. (1975) *Cell Tissue Res.* **165**, 13–22
- Sartore, S., De Marzo, N., Borriore, A.-C., Zanellato, A.-M. C., Saggini, L., Fabbri, L. & Schiaffino, S. (1989) *Eur. J. Biochem.* **179**, 79–85
- Gabbiani, G., Kocher, O., Bloom, W. S., Vandekerckhove, J. & Weber, K. (1984) *J. Clin. Invest.* **73**, 148–152
- Corjay, M. H., Thompson, M. M., Lynch, K. R. & Owens, G. K. (1989) *J. Biol. Chem.* **264**, 10501–10506
- Owens, G. K., Loeb, A., Gordon, D. & Thompson, M. M. (1986) *J. Cell Biol.* **102**, 343–352
- Campbell, J. H., Kocher, O., Skalli, O., Gabbiani, G. & Campbell, G. R. (1989) *Arteriosclerosis* **9**, 633–643
- Chamley, J. H., Campbell, G. R. & Burnstock, G. (1973) *Dev. Biol.* **33**, 344
- Campbell, J. H. & Campbell, G. R. (1987) in *Hypertrophic Response of Smooth Muscle* (Seidel, C. L., ed.), pp. 127–149, CRC Press, London
- Campbell, G. R. & Campbell, J. H. (1987) in *Vascular Smooth Muscle in Culture* (Campbell, J. H. & Campbell, G. R., eds.), vol. 1, pp. 39–55, CRC Press, London

22. Thyberg, J., Nilsson, J., Palmberg, L. & Sjolund, M. (1985) *Cell Tissue Res.* **239**, 69–76
23. Chamley-Campbell, J. H., Campbell, G. R. & Ross, R. (1981) *J. Cell Biol.* **89**, 379–383
24. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
25. Oakley, B. R., Kirsch, D. R. & Morris, N. R. (1980) *Anal. Biochem.* **105**, 361–363
26. Gröschel-Stewart, U., Magel, E., Paul, E. & Neidlinger, A.-C. (1989) *Cell Tissue Res.* **257**, 137–139
27. Hesketh, T. R., Beaven, M. A., Rogers, J., Burke, B. & Warren, G. B. (1984) *J. Cell Biol.* **98**, 2250–2254
28. Zieve, G. W., Turnbull, D., Mullins, J. W. & McIntosh, J. R. (1980) *Exp. Cell Res.* **126**, 397–405
29. Hedin, U., Bottger, A., Forsberg, E., Johansson, E. & Thyberg, J. (1988) *J. Cell Biol.* **107**, 307–319
30. Ang, A. H., Tachas, G., Campbell, J. H., Bateman, J. F. & Campbell, G. R. (1990) *Biochem. J.* **265**, 461–469

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