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Comparison of Vascular Smooth Muscle Cells from Adult Human, Monkey and Rabbit in Primary Culture and in Subculture*

Julie H. Chamley**, Gordon R. Campbell*** and Janet D. McConnell
Department of Anatomy and Embryology, University College, London, England

Ute Gröschel-Stewart****
Institut für Zoologie, Technische Hochschule, Darmstadt, Germany

Summary. A method is presented for growing large numbers of pure isolated smooth muscle cells from adult human, monkey, and rabbit blood vessels in primary culture.

In the first few days in culture these cells closely resembled those in vivo and could be induced to contract with angiotensin II, noradrenaline and mechanical stimulation. They stained intensely with antibodies against smooth muscle actin and myosin. Fibroblasts and endothelial cells did not stain with these antibodies thereby allowing the purity of each batch of cultures to be monitored. This was consistently found to be better than 99%. The smooth muscle cells modified or "dedifferentiated" after about 9 days in culture to morphologically resemble fibroblasts. At this stage cells could no longer be induced to contract and did not stain with the myosin antibodies. Intense proliferation of these cells soon resulted in a confluent monolayer being formed at which stage some differentiated characteristics returned. The modification or "dedifferentiation" process could be inhibited by the presence of a feeder layer of fibroblasts or endothelial cells, or the addition of cAMP to the culture medium.

Smooth muscle cells which had migrated from explants in primary culture, and cells in subculture, had morphological and functional properties of "dedifferentiated" cells at all times.

The advantages of differentiated rather than "dedifferentiated" smooth muscle cells in culture for the study of mitogenic agents in atherosclerosis is discussed.

Send offprint requests to: Prof. Dr. rer. nat. Ute Gröschel-Stewart, Institut für Zoologie, Technische Hochschule, Darmstadt, Federal Republic of Germany

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Introduction

A number of studies have centred on the involvement of smooth muscle cell proliferation in the formation of atherosclerotic lesions (Thomas et al., 1971; Geer and Haust, 1972; Ross and Glomset, 1973; Fischer-Dzoga et al., 1976), and the relationship of this phenomenon to some of the theories of atherosclerotic plaque formation has recently been reviewed (Ross and Glomset, 1976).

The response of cells to any atherogenic factor is extremely difficult to assess in vivo, and therefore the growth of smooth muscle cells in culture is a useful method for studying their involvement in this disease. Two methods of culture are employed in the majority of reports. The first involves placing small pieces of artery media into a culture chamber for several weeks until the outgrowth of cells becomes confluent. The cells are then trypsinized and serially subcultured (Ross, 1971). Cells from the 9th subculture or more are studied as they are said to form a homogenous population, do not contain any extracellular material carried over from the original tissue and are in large enough numbers to enable biochemical analysis (Ross, 1971). The second method of culture also involves the use of explants, but in this case the outgrowth of the cells in primary culture is studied (Fischer-Dzoga et al., 1976). However, several studies have indicated that isolated vascular and visceral smooth muscle cells in culture must first "dedifferentiate" to morphologically resemble fibroblasts before proliferation will occur (Chamley et al., 1974; Chamley and Campbell, 1975a; Gröschel-Stewart et al., 1975a; Campbell and Chamley, 1976). A similar modification has been observed in smooth muscle cells migrating from explants (Chamley et al., 1974; Fritz et al., 1970). The question therefore arises as to whether outgrowth and subcultured aortic smooth muscle cells are phenotypically representative of those present in vivo.

This communication describes a method for obtaining large numbers of isolated smooth muscle cells from enzyme dispersed adult human, monkey and rabbit blood vessels in primary culture. These cells are compared with those of subcultures and primary cultures derived from the outgrowth of explants of arterial media. A method for positively distinguishing smooth muscle cells from other cell types by the use of FITC-labelled antibodies against contractile proteins is also described.

Materials and Methods

The tissues used were saphenous vein (approx. 2 inches) obtained from aortacoronary artery bypass graft operations on male humans aged between 50 and 60 years, and the entire thoracic aorta from 1 year old Rhesus monkeys and 5–6 month old New Zealand white albino rabbits. Immediately upon dissection the tissue was placed into cold balanced salt solution (BSS) with 10% serum, and blood, fat and connective tissue removed. It was then treated in either of two ways:

1. The blood vessel, still as a tube, was placed into 4 ml of 1 mg/ml collagenase (Worthington Biochemical Corp.) and 0.5 mg/ml elastase (Sigma Chemical Co.) in BSS in a small Petri dish at 37° C for 30 min. The adventitia (plus a small amount of outer media) was cleanly stripped off with watchmaker forceps and discarded. The remaining tissue was placed into 4 ml of fresh collagenase and elastase for 2 h at 37° C with occasional gentle agitation. After this time almost the whole media had been dispersed into single cells and small clumps, with only a thin tube of intima remaining.

2. The vessel was everted so that the endothelium formed the outside layer of cells. The open ends of the tube were sealed with sterile clips and the tissue placed in collagenase (1 mg/ml) for 30 min then two changes of 1.25 mg/ml trypsin (1.300 Nutritional Biochemicals Corp.) for 20 min each at 37° C. This completely removed the endothelium which could be grown in culture. The vessel was then placed in 4 ml collagenase (1 mg/ml) and elastase (0.5 mg/ml) for 2 h with occasional gentle agitation. After this time the major part of the media had been dispersed into single cells and small clumps with only a small rim of media plus all the adventitia remaining as a tube.

In both cases, the suspension of pure smooth muslce cells was centrifuged at 900 rpm for 3 min, resuspended in medium 199 with 5 or 10% foetal calf or rabbit serum, and injected into modified Rose chambers. When the cultures had become confluent, some were trypsinized (1.25 mg/ml) and subcultured (1:4) a total of seven times.

Explants of media from monkey and rabbit blood vessels were also grown in culture and the cells which migrated out serially subcultured a total of seven times upon reaching confluence.

Primary cultures of enzyme-dispersed cells and explant-migrated cells, and subcultures derived from both types of primary culture, were studied with phase-contrast microscopy, time-lapse microcinematography, and electron microscopy (Chamley et al., 1973, 1974; Campbell et al., 1971) and stained with FITC-labelled antibodies against native actin (Gröschel-Stewart et al., 1976; Chamley et al., 1976) and myosin (Gröschel-Stewart et al., 1975a, b; 1976) obtained from the smooth muscle of the chicken gizzard.

Results

Primary Culture of Enzyme-Dispersed Vascular Tissue

Isolated cells from the media of blood vessels of adult animals attached to the glass coverslip during the first 24 h in culture, but did not fully flatten until after 2–3 days. They were spindle or ribbon-shaped (1500–200 μm × 10–15 μm) with a sausage-shaped nucleus and phase dense cytoplasm with few visible inclusions (Fig. 1A). Spontaneous contractions were only rarely observed in isolated smooth muscle cells from adult blood vessels, but contraction could always be elicited when angiotensin II (10^{-7}–10^{-5} g/ml) was injected into the chamber. The contraction was usually so strong that parts of the cell pulled away from the coverslip giving the cell an irregular shape (Fig. 5A, B). Noradrenaline (10^{-5} g/ml) produced a similar but less pronounced contraction.

Within the first few days in culture isolated smooth muscle cells ultrastructurally resembled those of the adult vascular wall (see Cliff, 1976) with their cytoplasm occupied almost exclusively with tracts of myofilament bundles and associated dark bodies. Between the tracts were organelles such as mitochondria, free ribosomes, rough endoplasmic reticulum (ER) and Golgi apparatus. Both thick (120–180 Å) and thin (60–80 Å) filaments were observed in the myofilament bundles (Fig. 2). When thick myofilaments were observed, they were found in the majority of cells of that culture. However, in some cultures thick myofila-

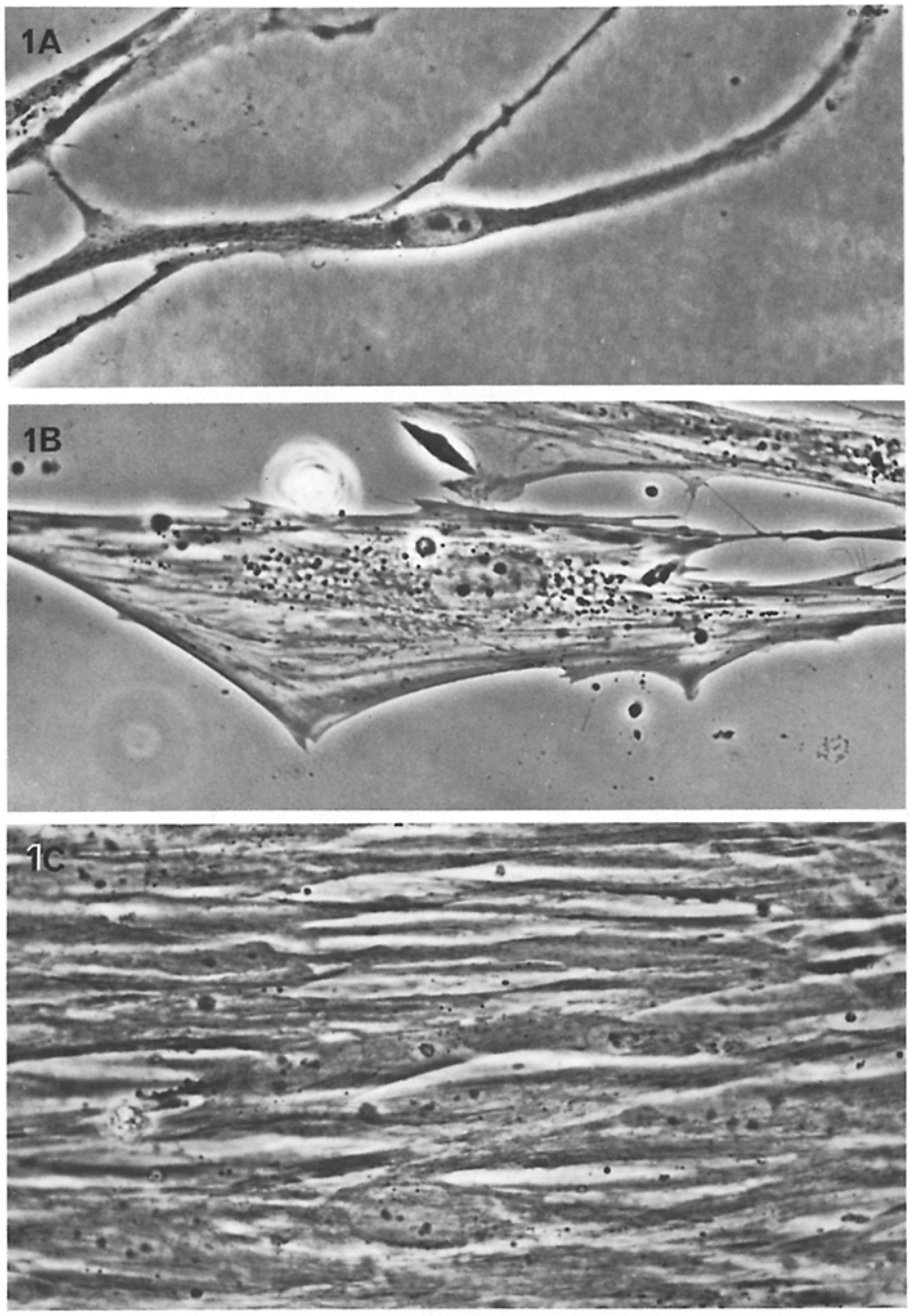

Fig. 1. A Smooth muscle cell from the monkey aortic media 3 days in culture. **B** "Dedifferentiated" smooth muscle cell from the monkey aortic media 10 days in culture. **C** Confluent monolayer of partially redifferentiated smooth muscle cells from the monkey aortic media 13 days in culture. × 1000

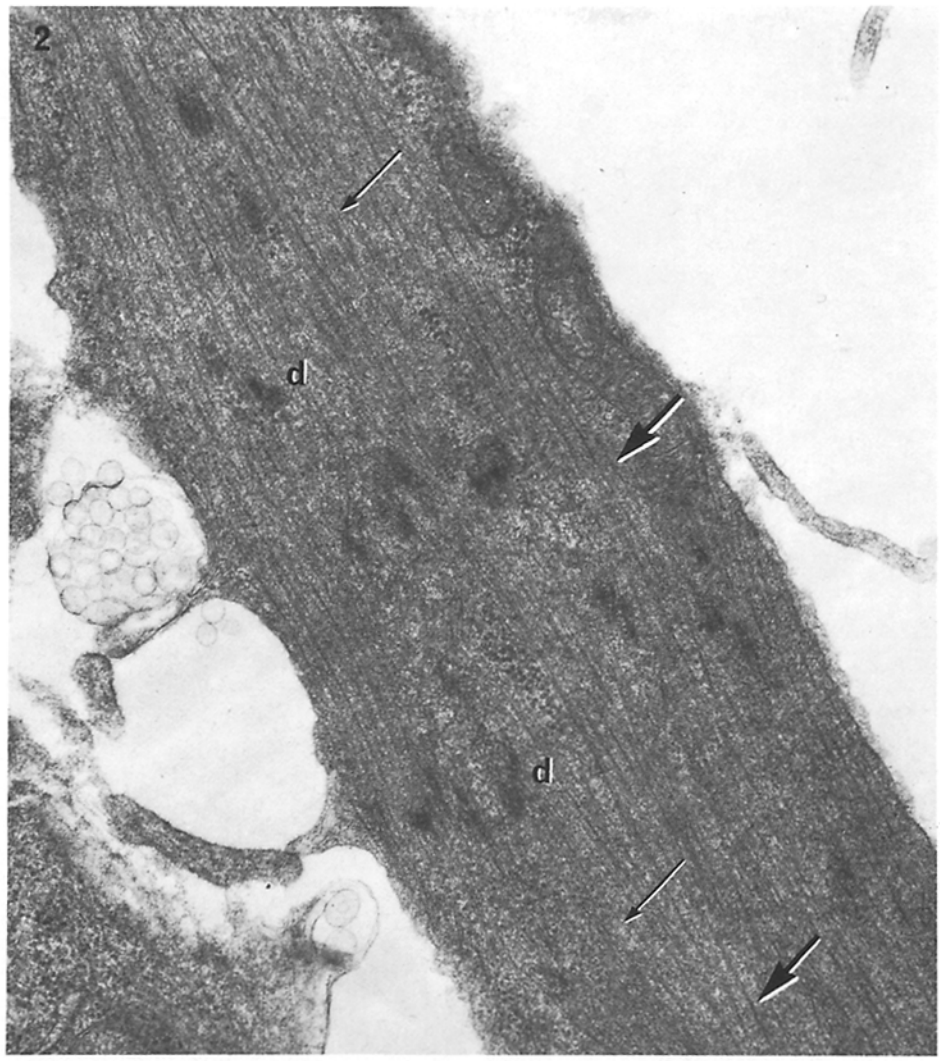

Fig. 2. Smooth muscle cell from the monkey aortic media 4 days in culture. The cytoplasm in this region is largely filled with myofilaments both thick (large arrows) and thin (small arrows) and associated dark bodies (*d*). ×24,500

ments were not observed within any cells. This inconsistency can almost certainly be explained in terms of the lability of the myosin-containing filaments of smooth muscle and their ability to alter in response to ionic changes and different conditions of chemical fixation (Campbell and Chamley, 1975). A basal lamina was not present around muscle cells during the first few days in culture and was presumably lost during the enzyme treatment. It was, however, generally reconstituted by day 5.

The cells stained intensely with FITC-labelled antibodies against smooth muscle actin (Fig. 3A). The fluorescence was exclusively in long, straight, non-interrupted fibrils scattered densely throughout the cytoplasm. There was no staining in the cytoplasm between the fibrils and no nuclear staining.

The cells also stained intensely with FITC-labelled antibodies against smooth muscle myosin (Fig. 3B, C). Usually the reaction was so intense and densely distributed that its precise localisation within the cytoplasm could not be distinguished. However, in cells which were flatter, and thus thinner, many fluorescent "myosin aggregates" and a small number of non-interrupted fibrils (Gröschel-Stewart et al., 1975a) could be observed. The "myosin aggregates" were approximately 0.5 mμ in diameter and were often aligned giving the cell a striated appearance (Fig. 3C). There was no nuclear staining.

It was possible to monitor the cellular purity of the cultures using the very specific reactions of the smooth muscle antibodies. Any contamination with fibroblasts from the adventitia could be readily observed, for apart from their distinctive appearance under phase contrast microscopy (Fig. 4A; see also Chamley et al., 1973, 1974), they did not stain with the anti-actin (Fig. 4C) and only very weakly with the anti-myosin (Fig. 4E). Similarly, endothelial cells (Fig. 4B) did not stain with anti-actin (Fig. 4D; see also Chamley et al., 1976), and only very weakly with anti-myosin (Fig. 4F). Whole cultures containing up to 10^6 cells could thus be scanned for the presence of fibroblasts and endothelial cells by combined phase contrast-fluorescence microscopy. Using the cell dispersal procedures described in "Materials and Methods", the purity of the cultures was always found to be considerably better than 99% smooth muscle.

The isolated smooth muscle cells maintained their "differentiated" morphology and contractility for about 9 days in culture. During this time, only five smooth muscle cells from the many cultures studied were observed in the state of division. These cells stained intensely with the FITC-labelled antibodies against smooth muscle myosin (Fig. 5c).

After 9 days in culture, the cells began to undergo morphological changes to resemble fibroblasts both with phase contrast microscopy and electron microscopy (Fig. 1B). That is, they became broad and flat and contained an increased number of organelles, particularly free ribosomes, but the number of thin filaments appeared to decrease and the thick filaments had disappeared completely (see also Chamley et al., 1974; Chamley and Campbell, 1976; Campbell and Chamley, 1976). The cells still contained a considerable amount of stainable actin (Fig. 6A) in the form of fibrils as before, but the myosin first became clumped (Fig. 6B) and then almost completely disappeared from the cells with only a few fibrils still faintly visible and no stainable "myosin aggregates" (Fig. 6C).

It was at this stage that the cells spontaneously began to divide. The proliferation was so intense that a confluent monolayer of these modified or "dedifferentiated" cells usually resulted after about 12–14 days in culture. A couple of days after confluence, the cells became parallel-aligned (Fig. 1C), and then arranged into clusters.

Extracellular material could often be observed between cells at this stage.

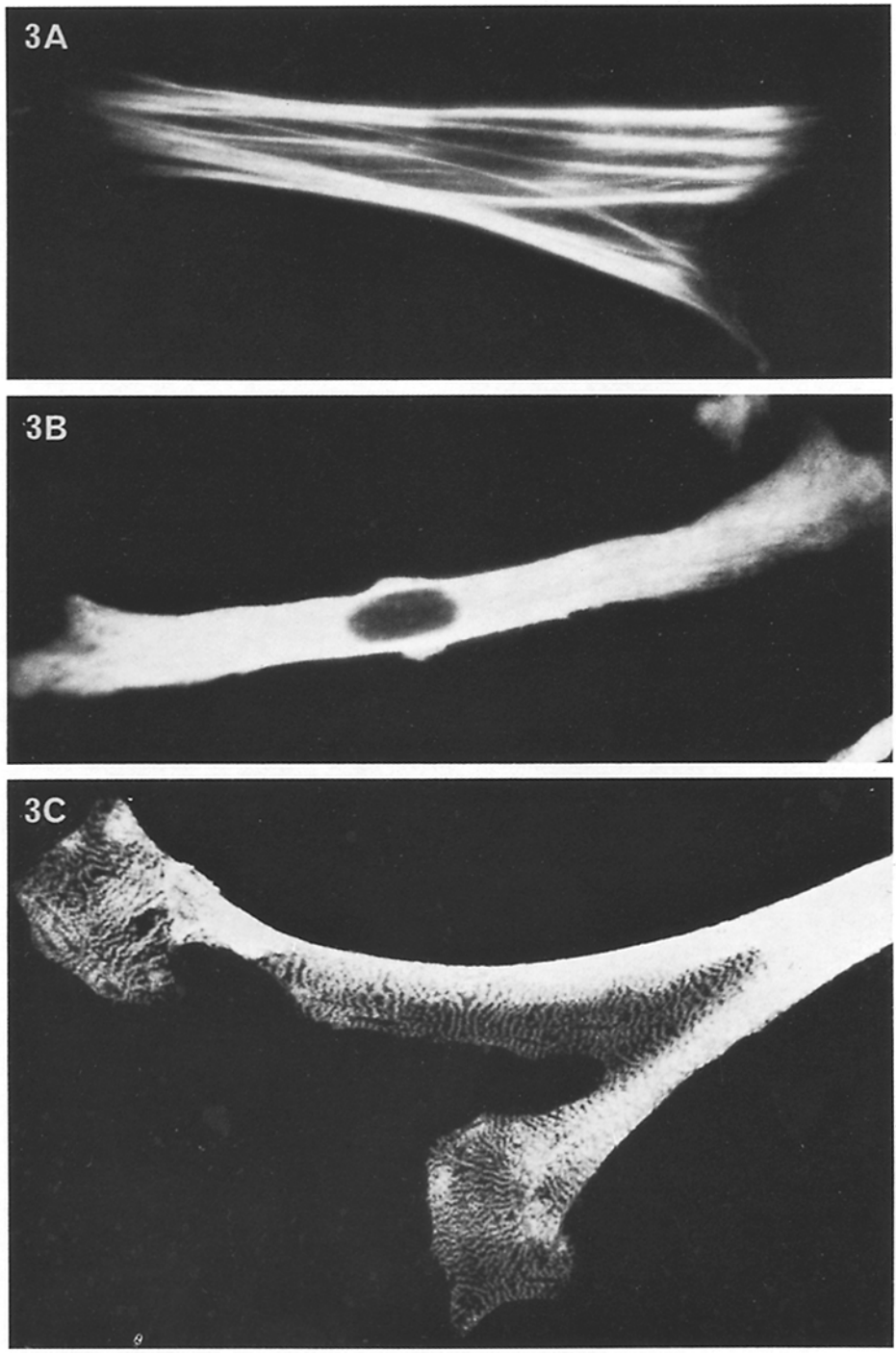

Fig. 3. A Smooth muscle cell from the rabbit aortic media 3 days in culture stained with FITC-labelled antibodies against smooth muscle actin. × 1000. **B** Smooth muscle cell from the human saphenous vein 4 days in culture stained with FITC-labelled antibodies against smooth muscle myosin. × 1000. **C** Higher magnification (× 1800) of a flattened region of a smooth muscle cell from the human saphenous vein 4 days in culture stained with anti-myosin. Note the presence of "myosin aggregates" aligned across the cell

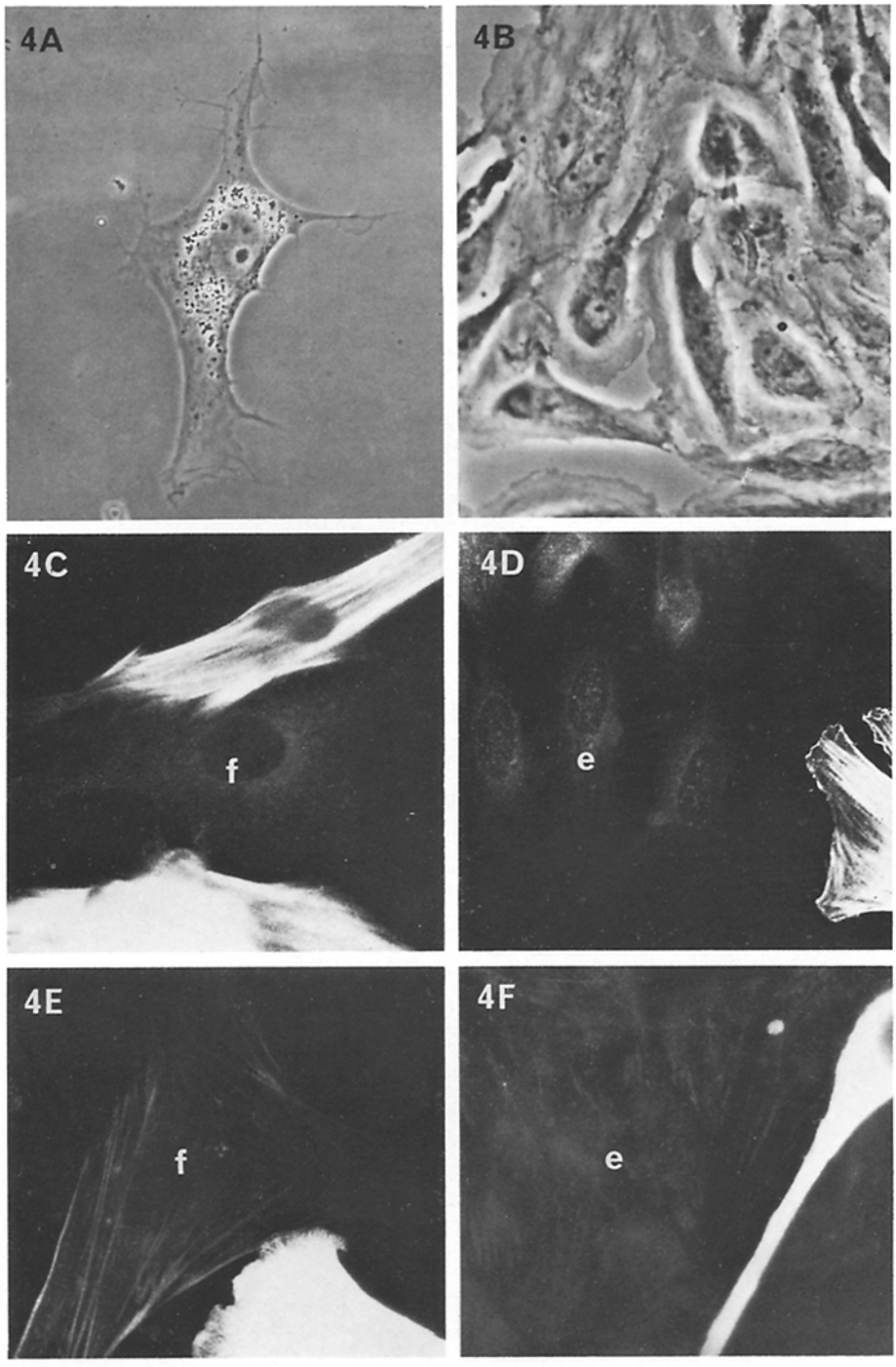

Fig. 4. A Fibroblast from the adventitia of the monkey aorta 3 days in culture. **B** Sheet of endothelial cells from the rabbit aorta 8 days in culture. **C** Fibroblast (*f*) between smooth muscle cells from the monkey aorta 6 days in culture stained with anti-actin. **D** Endothelial cells (*e*) with part of a smooth muscle cell from the rabbit aorta 4 days in culture stained with anti-actin. **E** Fibroblast (*f*) with part of a smooth muscle cell from the monkey aorta 6 days in culture stained with anti-myosin. **F** Endothelial cells (*e*) with part of a smooth muscle cell from the monkey aorta 6 days in culture stained with anti-myosin. × 700

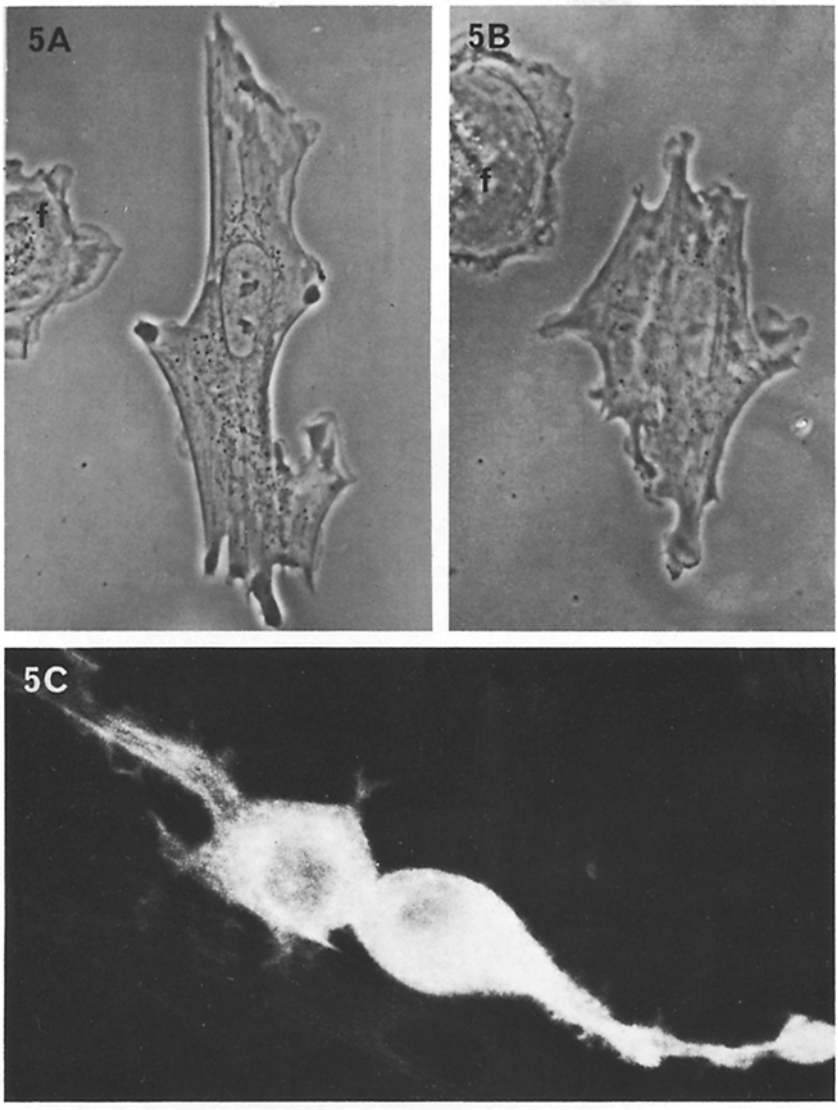

Fig. 5. A Smooth muscle cell from the rabbit aortic media 3 days in culture. **B** Same cell as in **A** after the addition of 10^{-7} g/ml angiotensin II. *f* ruffled membrane of a migrating fibroblast. ×800. **C** Dividing "differentiated" smooth muscle cell from the monkey aortic media 7 days in culture stained with anti-myosin. ×1000

"Myosin aggregates" began to reappear in some cells and myosin staining in the fibrils became more intense but never approached pre-dedifferentiation levels. Ultrastructurally, the cells contained more thin filament bundles, but thick myofilaments were only rarely observed. Spontaneous contraction of adult cells at this stage has only been observed once. This was in a 41 day culture of human saphenous vein. Induced contraction at this stage is rare, that is, has only been observed in three clumps from the many studied. In sparsely-

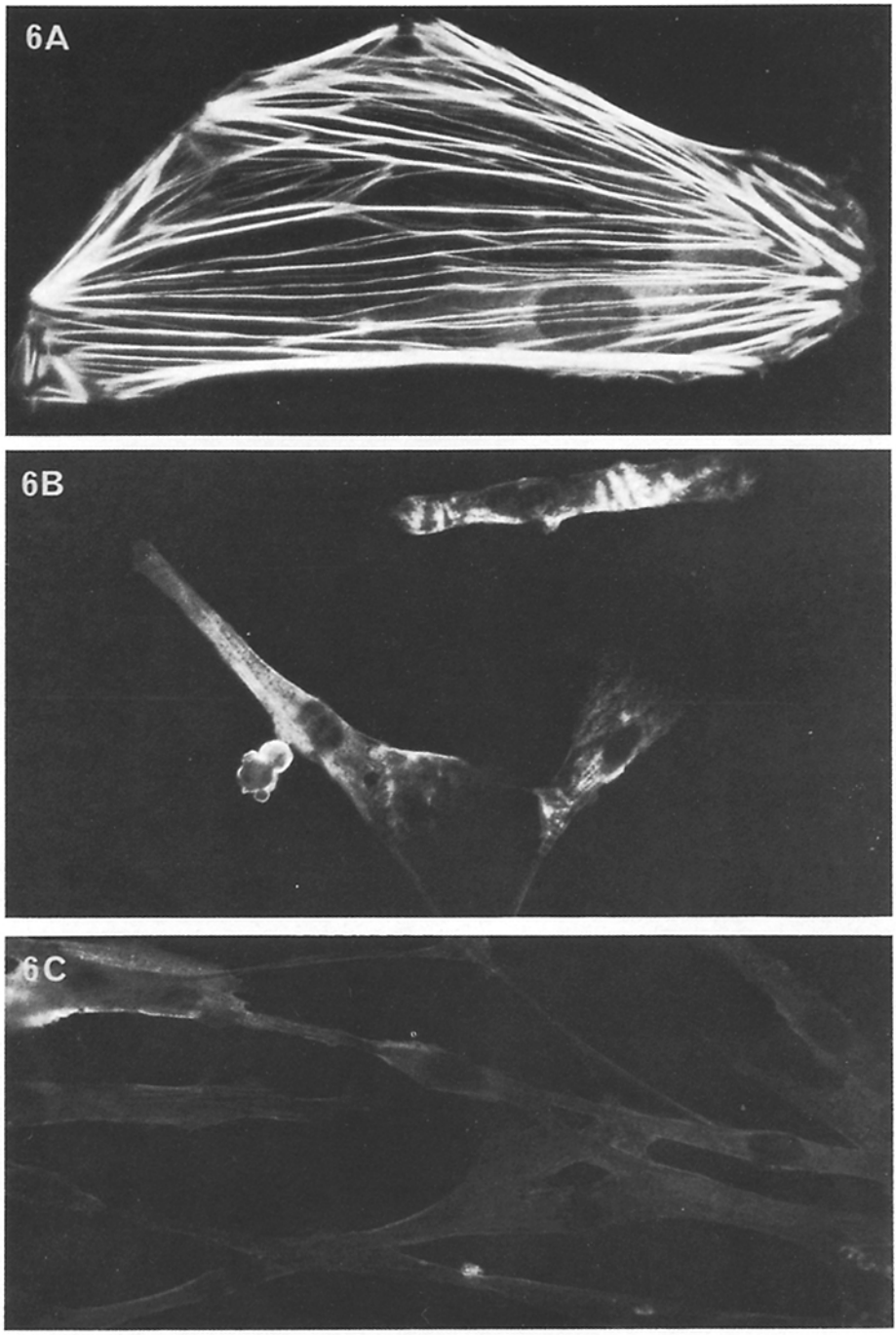

Fig. 6. A "Dedifferentiated" smooth muscle cell from the monkey aortic media 11 days in culture stained with anti-actin. ×1000. **B** Three partly "dedifferentiated" smooth muscle cells from the rabbit aortic media 9 days in culture stained with anti-myosin. ×600. **C** Fully "dedifferentiated" smooth muscle cells from the rabbit aortic media 12 days in culture stained with anti-myosin. ×600

seeded cultures, confluence was not achieved until after at least three weeks and the cells remained morphologically and functionally "dedifferentiated".

A small number of vascular smooth muscle cells (< 5%) within each culture did not undergo the changes after 9 days described above but maintained their "differentiated" ultrastructure and contractile protein staining reactions over several weeks. It is not known whether these cells are derived from the same cells which undergo division in the differentiated state. Smooth muscle cells which do not proceed through the same phenotypic changes as the vast majority of their fellows have also been observed in cultures of newborn guinea-pig vas deferens (Chamley et al., 1974; Gröschel-Stewart et al., 1975a) and in anterior eye chamber transplants of adult guinea-pig vas deferens (Campbell et al., 1971b).

In the presence of a confluent monolayer of endothelial cells or fibroblasts, either on the same or opposite coverslip, many more smooth muscle cells maintained their "differentiated" morphology and myosin immunofluorescence and did not undergo division over several weeks in culture. Similarly, in the presence of 0.05 mM dibutyryl cyclic AMP + 0.05 mM theophylline many more cells maintained their differentiation than in control cultures.

Subculture

When 13–16 day confluent monolayer primary cultures were trypsinized and subcultured (1:4), the cells attached and flattened within the first 12 h. The shape of the cells was variable with some cells ribbon or more rarely spindle but most irregular in shape (Fig. 7A). They began to proliferate within the first 2 days, becoming confluent after 5–7 days. The confluent cells were serially subcultured a total of seven times over a period of 7 months. With each subsequent subculture, the period of time taken to achieve confluency became longer and for the last subculture was 6 weeks.

At no time, either during subconfluency or confluency, did the cells of any of the seven subcultures undergo spontaneous contraction, nor could they be stimulated to contract with angiotensin II, noradrenaline, or mechanical stimulation with a microelectrode.

With the electron microscope, subcultured smooth muscle cells were observed to contain filament bundles interspersed with areas of free ribosomes and other organelles (Fig. 8). No thick myofilaments (120–180 Å) were observed in these cells at any time under a variety of fixation conditions (see Campbell and Chamley, 1975). More 100 Å filaments (Uehara et al., 1971) appeared to be present in subcultured smooth muscle cells than in muscle cells of primary cultures.

Fibrils within the subcultured cells stained intensely with anti-actin (Fig. 7B) and non-fluorescent structures thought to be assembly or attachment sites for actin filaments (see Lazarides, 1975) were frequently seen. However, there was very little staining with anti-myosin similar to that in "dedifferentiated" cells (see Fig. 6C) More myosin staining occurred in later subcultures than in the first 2 or 3, but it was always weak and present in fibrils only, never in "myosin aggregates" (Fig. 7C).

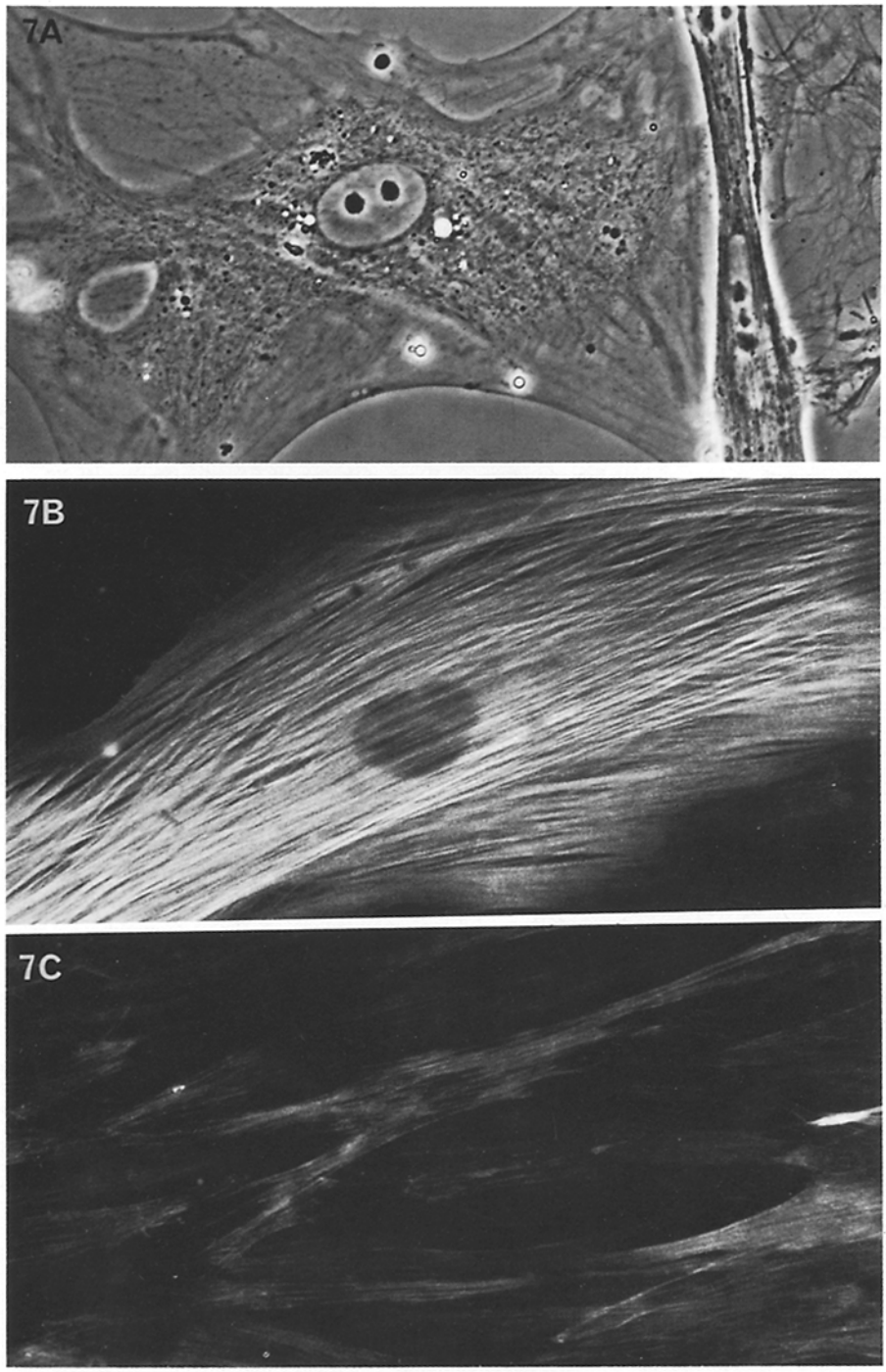

Fig. 7. **A** One irregular-shaped and one ribbon-shaped smooth muscle cell from the 5th subculture of monkey aortic media. ×800. **B** Smooth muscle cell from the 7th subculture of monkey aortic media stained with anti-actin. ×800. **C** Smooth muscle cells in a confluent monolayer from the 7th subculture of monkey aortic media stained with anti-myosin. ×500

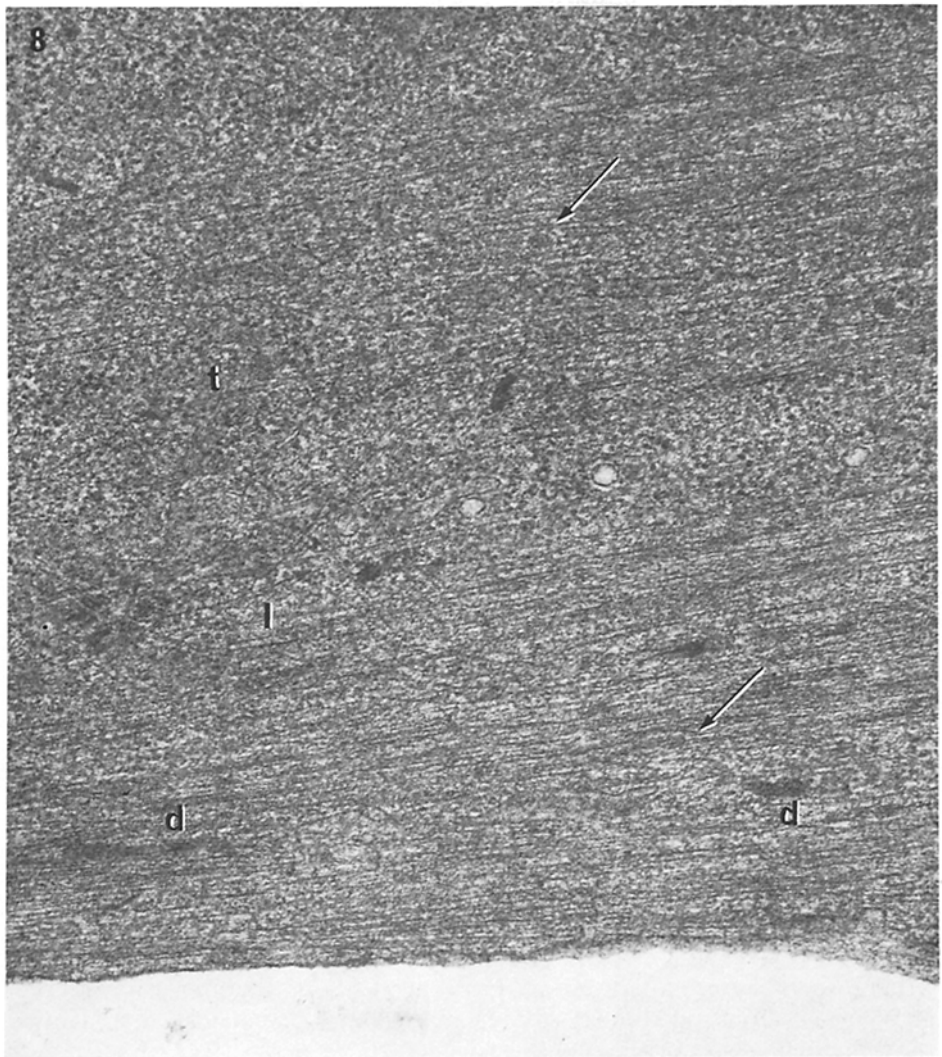

Fig. 8. Smooth muscle cell from the 4th subculture of the monkey aortic media, 4 days in culture. Bundles of thin filaments (small arrows), 60–80 Å in diameter, run parallel to the long axis of the cell and have associated dark bodies (*d*). A large number of free ribosomes are distributed throughout the cytoplasm between these bundles. Microtubules (*t*) and 100 Å filaments (*l*) can also be observed. ×34,000

The addition of dibutyryl cyclic AMP and theophylline (both 0.05 mM) to the culture medium inhibited the proliferation of the cells but did not alter their morphology with phase-contrast or electron microscopy, nor did it increase their staining with anti-myosin or make them responsive to drugs or mechanical stimulation. Similarly the presence of a feeder layer of endothelium or fibroblasts had no "differentiating" effects on the cells.

A small contamination of most subcultures with fibroblasts was discovered by their lack of staining with FITC-labelled antibodies against smooth muscle actin. The level of this contamination was always less than 1% of the total cells, that is, about the same as in the original primary cultures.

Outgrowth from Explants of Media

Cells did not migrate from explants of rabbit or monkey media until after 1 week in culture, and it was 3–4 weeks before the area of the culture had become confluent from continued migration of cells and division.

Fibrils within the cells stained intensely with anti-actin but at no time before confluence was anti-myosin staining in the form of either "aggregates" or fibrils observed, and ultrastructurally the cells appeared "dedifferentiated". At confluence some myosin appeared, but the staining was weak and only in the fibrils.

Subculturing the cells produced the same results as described in the previous section, except that the amount of myosin at confluence of each of the seven subcultures was less than in the same subculture of the originally enzyme-dispersed tissue. At no time during sub-confluency or confluency could the migrated cells in primary culture or in subculture be stimulated to contract with angiotensin, noradrenaline or mechanical stimulation, and thick myofilaments were never observed.

Discussion

The differentiation of a cell can be defined as the change in morphology and function which occurs during development and usually involves the cell transforming from an initial general type into the specialized form of the adult. This process occurs not only during embryonic development but also in regeneration and repair. Dedifferentiation is the reversal of differentiation and involves the loss of adult phenotypic traits.

The concept of a differentiated cell is complicated when applied to vascular smooth muscle as these cells appear to be capable of both contraction and synthesis. The notion that smooth muscle is capable of synthesis was originally proposed on the basis that it is the only cell type present in the media of mammalian arteries and must therefore be responsible for the maintenance of the connective tissue elements (Pease and Paule, 1960; Karrer, 1961; Paule, 1963). Associated with this argument is the large amount of rough ER and Golgi apparatus present in these cells during development at the stage when much of the intercellular elastin and collagen is being formed (Cliff, 1967; Gerrity et al., 1975). Rough ER and Golgi apparatus are generally associated with protein synthesis, transport and secretion (Zeigel and Dalton, 1962; Warshawsky et al., 1963; Caro and Palade, 1964; Jamieson and Palade, 1966, 1967). The synthetic activities of smooth muscle cells in vivo have been confirmed

more recently with autoradiographic studies (Jurukova and Rohr, 1968; Rohr and Jurukova, 1968; Tokuoka, 1968; Ross and Klebanoff, 1971).

Similar morphological features are also observed in smooth muscle cells undergoing proliferation. Although both vascular and visceral smooth muscle is capable of division while in a contractile form (Chamley and Campbell, 1974), the majority of smooth muscle cells observed in mitosis are at least partially modified, containing few myofilament bundles and a large number of organelles such as free ribosomes and rough ER (Thomas et al., 1963; Scott et al., 1967; Thomas et al., 1968; Imai et al., 1970; Campbell et al., 1971b; Poole et al., 1971; Gabella, 1973; Stary, 1974; Glagov and Ts'ao, 1975). It would appear therefore that in most cases modification of smooth muscle cells from a contractile state in which the cell's cytoplasm is filled with myofilaments, is a pre-requisite for proliferation. Therefore a non-contractile smooth muscle cell with few myofilaments and a large number of organelles may either be actively involved in synthesis, proliferation, or both. A number of terms such as "modified smooth muscle" (Thomas et al., 1963) have been employed to describe those cells modified from a contractile function to one of synthesis and for proliferation. However, we have chosen the terms "differentiated" to represent the smooth muscle cell most commonly found in the adult vascular media whose major function is contraction, and "dedifferentiated" to represent the smooth muscle cell actively involved in synthesis and/or proliferation.

The present communication describes a method for the growth of large numbers of isolated smooth muscle cells from adult human, monkey and rabbit blood vessels in pure primary culture, and compares the properties of these cells with cells which have migrated from explants and those in subculture.

It is stated that the advantage of using subcultured cells is that large numbers of pure, homogenous cells can be obtained with no extra-cellular material carried over from the media to interfere with the biochemical analyses of their synthetic properties (Ross, 1971). With the present method of isolating cells, large numbers (at least 15×10^6) of viable smooth muscle cells can be obtained from the thoracic aorta of an adult rabbit or monkey. Ultrastructural observations of these cells indicate that all extracellular material has been removed from their surface, including the basal lamina (see also Hinek and Konwiński, 1975). The basal lamina is replaced on the smooth muscle cells after about 5 days in culture.

The purity and homogeneity of the cells was confirmed on each batch of cultures using the very specific staining reactions of FITC-labelled antibodies against smooth muscle actin and myosin. Smooth muscle and fibroblast myosins are not identical (Burridge, 1974), and antibodies directed against the heavy chain of smooth muscle myosin stain "differentiated" smooth muscle cells intensely but only very weakly stain fibroblasts, endothelial cells or "dedifferentiated" smooth muscle cells (see also Gröschel-Stewart et al., 1975a, b). It is also now widely accepted that muscle and non-muscle actins differ (Booyse et al., 1973; Gruenstein and Rich, 1975; Elzinga et al., 1976; Anderson, 1976; Storti and Rich, 1976). Antibodies directed against native actin from smooth muscle stain both "differentiated" and "dedifferentiated" smooth muscle cells but not fibroblasts or endothelial cells (see also Chamley et al., 1976). Any

contamination of cultures with these non-muscle cells was therefore readily determined by a scan of the culture with combined phase contrast-fluorescence microscopy. In this way, it was observed that the primary cultures were always considerably better than 99% pure smooth muscle.

The isolated smooth muscle cells retain the characteristic features of the in vivo cells. They contain thick (120–180 Å) myofilaments and can be induced to contract with drugs such as angiotensin II. They do not migrate readily and only rarely undergo mitosis. They stain intensely with FITC-labelled antibodies against smooth muscle actin and myosin. The myosin staining is mainly in small aggregates 0.5 μm in diameter which are often aligned across the cell to give it a striated appearance (see also Gröschel-Stewart et al., 1975a, b). The actin staining is localized in fibrils scattered densely throughout the cytoplasm, and is continuous along the fibril (see also Chamley et al., 1976).

These cells maintain their "differentiated" appearance for about 9 days in culture after which the majority begin to modify or "dedifferentiate". They become very broad and flat, and contain an increased number of organelles such as ribosomes, rough ER and Golgi apparatus, but thick myofilaments can no longer be demonstrated. In addition, they no longer stain with antibodies against smooth muscle myosin, although they still react intensely with antibodies against smooth muscle actin. They are now capable of mitosis, and undergo intense proliferation to form a confluent monolayer. If the cells are densely seeded, confluence occurs after about 12 days in culture, and the cells begin to redifferentiate back towards their in vivo morphology. Antibody staining of "myosin aggregates" begins to reappear, but thick myofilaments and spontaneous or induced contractions are only rarely observed. In more sparsely-seeded cultures where confluence is not achieved until after at least three weeks, there is very little change in the morphology of the cells from the "dedifferentiated" state. It would appear that either the large number of mitoses these cells have to undergo to achieve confluence, or the long period of time they remain "dedifferentiated" in culture, or both of these factors, affect them in such a way that they can no longer regain differentiated characteristics.

Cells which migrate from explants and finally become confluent remain in a similar morphological state to smooth muscle cells from sparsely seeded cultures. During migration these cells contain little myosin as observed with FITC-labelled antibody. At confluence a small amount of myosin immunofluorescence is regained, however, there are no thick myofilaments in these cells and they can not be induced to contract. Similar results were found by Mauger et al. (1975) who studied the response to angiotensin II (10^{-5} M), noradrenaline (10^{-5} M), 5-hydroxytryptamine (10^{-5} M), potassium chloride (50 mM) and electrical stimulation of cells which migrated from explants of young rabbit aorta. In one experiment only could confluent cells be stimulated to contract with noradrenaline and potassium chloride while subconfluent cells never responded. Blose et al. (1975) also examined a confluent monolayer of cells which had migrated from explants of one month old guinea-pig aorta media and were unable to demonstrate thick myofilaments under identical conditions to those in which thick myofilaments could be observed in guinea-pig vas deferens cells in culture.

Subcultured smooth muscle cells from adult monkey and rabbit still contain large amounts of actin as demonstrated with immunofluorescence. Their smooth muscle myosin immunofluorescence completely disappears in the first subculture, but does return very weakly after the fourth subculture. This staining is exclusively in fibrils, with "myosin aggregates" never being observed. Thick myofilaments are not observed in these cells under a variety of different conditions (see Campbell and Chamley, 1975) and the cells can not be induced to contract either with drugs or mechanical stimulation. Mauger et al. (1975) also found that subcultured cells from young rabbit aorta, either confluent or sub-confluent, never responded to drugs or electrical stimulation. The addition of dibutyryl adenosine 3′:5′ monophosphate (cyclic AMP) and theophylline, or a feeder-layer effect of endothelial cells or fibroblasts, which inhibited the "dedifferentiation" and proliferation of the isolated smooth muscle cells in primary culture (see also Chamley and Campbell, 1975b; Campbell and Chamley, 1976), had no effect on the state of differentiation of cells which had migrated from explants or those in subculture.

One of the primary events in atherosclerosis is the intense proliferation of medial or subendothelial smooth muscle cells (Ross and Glomset, 1973) with the subsequent accumulation of extracellular matrix components including collagen, elastic fibers and proteoglycans (Ross and Glomset, 1976). However, it must be remembered that before they can divide or synthesize, the majority of smooth muscle cells must modify or "dedifferentiate" (see Campbell et al., 1974). As has been shown in this communication, cells in the outgrowth or in subculture are already in this "dedifferentiated" state. We have presented a method for obtaining pure cultures of large numbers of isolated smooth muscle cells from adult blood vessels and shown that they are phenotypically more representative of the cells in vivo. It will therefore be of interest to determine whether agents such as platelet factors (Rutherford and Ross, 1976) and hypercholesterolemic serum (Fischer-Dzoga et al., 1976) that have been shown to be mitogeneic for smooth muscle cells which have migrated from explants or those in subculture, are also involved in the initial modification of differentiated cells to the phenotypic state in which they are capable of proliferation and synthesis.

The culture method described in this paper also allows smooth muscle cells to be selectively isolated from specific zones of the vascular wall. This will enable variation in responses of individual muscle cells from different regions to be monitored, a factor which may prove particularly useful in studies of the involvement of the autonomic nervous system in hypertension. A report of the interaction of sympathetic nerves and isolated aortic and ear artery medial cells has recently been published (Chamley and Campbell, 1976).

References

Anderson, P.J.: The actin of muscle and fibroblasts. Biochem. J. **155**, 297–316 (1976)

Blose, S.H., Lazarides, E., Chacko, S.K.: Loss of differentiated phenotype in the progeny of aortic smooth muscle cells. J. Cell Biol. **67**, 34a (1975)

Booyse, F.M., Hoveke, T., Rafelson, M.: Human platelet actin, isolation and properties. J. biol. Chem. **248**, 4083–4091 (1973)

Burridge, K.: A comparison of fibroblast and smooth muscle myosins. Febs Letters **45**, 14–17 (1974)

Campbell, G.R., Chamley, J.H.: Thick filaments in vertebrate smooth muscle. Cell Tiss. Res. **156**, 201–216 (1975)

Campbell, G.R., Chamley, J.H.: Ultrastructure of differentiating vascular smooth muscle. Vascular neuroeffector mechanisms. 2nd Int. Symp., Odense 1975, pp. 1–9. Basel: Karger 1976

Campbell, G.R., Chamley, J.H., Burnstock, G.: Differentiation and dedifferentiation of arterial smooth muscle cells. In: Functional and comparative anatomy of the artery (C.J. Schwartz, ed.). (In press) 1977

Campbell, G.R., Uehara, Y., Malmfors, T., Burnstock, G.: Degeneration and regeneration of smooth muscle transplants in the anterior eye chamber. An ultrastructural study. Z. Zellforsch. **117**, 155–175 (1971 b)

Campbell, G.R., Uehara, Y., Mark, G., Burnstock, G.: Fine structure of smooth muscle cells grown in tissue culture. J. Cell Biol. **49**, 21–34 (1971 a)

Caro, L.G., Palade, G.E.: Protein synthesis, storage, and discharge in the pancreatic exocrine cell. An autoradiographic study. J. Cell Biol. **20**, 473–495 (1964)

Chamley, J.H., Campbell, G.R.: Mitosis of contractile smooth muscle cells in tissue culture. Exp. Cell Res. **84**, 105–109 (1974)

Chamely, J.H., Campbell, G.R.: Trophic influences of sympathetic nerves and cyclic AMP on differentiation and proliferation of single smooth muscle cells in culture. Cell Tiss. Res. **161**, 497–510 (1975a)

Chamley, J.H., Campbell, G.R.: Isolated ureteral smooth muscle cells in culture. Including their interaction with intrinsic and extrinsic nerves. Cytobiol. **11**, 358–365 (1975b)

Chamley, J.H., Campbell, G.R.: Tissue culture: Interaction between sympathetic nerves and vascular smooth muscle. Vascular neuroeffector mechanisms. 2nd Int. Symp. Odense 1975, pp. 10–18. Basel: Karger 1976

Chamley, J.H., Campbell, G.R., Burnstock, G.: An analysis of the interactions between sympathetic nerve fibers and smooth muscle cells in tissue culture. Develop. Biol. **33**, 344–361 (1973)

Chamley, J.H., Campbell, G.R., Burnstock, G.: Dedifferentiation, redifferentiation and bundle formation of smooth muscle cells in tissue culture: The influence of cell number and nerve fibres. J. Emb. exp. Morph. **32**, 297–323 (1974)

Chamley, J.H., Gröschel-Stewart, U., Campbell, G.R., Burnstock, G.: Distinction between smooth muscle, fibroblasts and endothelial cells in culture by the use of fluoresceinated antibodies against smooth muscle actin. Cell Tiss. Res., **177**, 445–457 (1977)

Cliff, W.J.: The aortic tunica media in growing rats studied with the electron microscope. Lab. Invest. **17**, 599–615 (1967)

Cliff, W.J.: Blood vessels. London: Cambridge University Press 1976

Elzinga, M., Maron, B.J., Adelstein, R.S.: Human heart and platelet actins are products of different genes. Science **191**, 94–95 (1976)

Fischer-Dzoga, K., Fraser, R., Wissler, R.W.: Stimulation of proliferation in stationary primary cultures of monkey and rabbit aortic smooth muscle cells. I. Effects of lipoprotein fractions of hyperlipemic serum and lymph. Exp. molec. Path. **24**, 346–359 (1976)

Fritz, K.E., Jarmolych, J., Daoud, A.S.: Association of DNA synthesis and apparent dedifferentiation of aortic smooth muscle cells in vitro. Exp. molec. Path. **12**, 354–362 (1970)

Gabella, G.: Fine structure of smooth muscle. I. Cellular structures and electrophysiological behaviour. Phil. Trans. B **265**, 7–16 (1973)

Geer, J.C., Haust, M.D.: Smooth muscle cells in atherosclerosis. Monogr. Atheroscler. **2**, 1–88 (1972)

Gerrity, R.G., Adams, E.P., Cliff, W.J.: The aortic tunica media of the developing rat. II. Incorporation by medial cells of ^{3}H-proline into collagen and elastin: autoradiographic and chemical studies. Lab. Invest. **32**, 601–609 (1975)

Glagov, S., Ts'ao, C.: Restitution of aortic wall after sustained necrotizing transmural ligation injury: Role of blood cells and artery cells. Amer. J. Path. **79**, 7–30 (1975)

Gröschel-Stewart, U., Ceurremans, S., Lehr, I., Mahlmeister, Ch., Paar, E.: Production of specific antibodies to contractile proteins, and their use in immunofluorescence microscopy. II. Species-

specific and species-non-specific antibodies to smooth and striated chicken muscle actin. Histochemistry, in press (1976)
Gröschel-Stewart, U., Chamley, J.H., Campbell, G.R., Burnstock, G.: Changes in myosin distribution in dedifferentiating and redifferentiating smooth muscle cells in tissue culture. Cell Tiss. Res. **165**, 13–22 (1975a)
Gröschel-Stewart, U., Chamley, J.H., McConnell, J.D., Burnstock, G.: Comparison of the reaction of cultured smooth and cardiac muscle cells and fibroblasts to specific antibodies to myosin. Histochemistry **43**, 215–224 (1975b)
Gröschel-Stewart, U., Schreiber, J., Mahlmeister, Ch., Weber, K.: Production of specific antibodies to smooth and striated chicken muscle myosin. Histochemistry **46**, 229–236 (1976)
Gruenstein, E., Rich, A.: Non-identity of muscle and non-muscle actins. Biochem. biophys. Res. Commun. **64**, 472–477 (1975)
Hinek, A., Konwiński, M.: Electron microscopic studies on cells isolated from aorta of newborn and adult rabbits. Pol. med. Sci. Hist. Bull. **15**, 9–22 (1975)
Imai, H., Lee, K.J., Lee, S.K., Lee, K.T., O'Neal, R.M., Thomas, W.A.: Ultrastructural features of aortic cells in mitosis in control and cholesterol-fed swine. Lab. Invest. **23**, 401–415 (1970)
Jamieson, J.D., Palade, G.E.: Role of the Golgi complex in the intracellular transport of secretory proteins. Proc. natl. Acad. Sci. (Wash.) **55**, 424–431 (1966)
Jamieson, J.D., Palade, G.E.: Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex. J. Cell Biol. **34**, 577–596 (1967)
Jurukova, Z., Rohr, H.P.: Beitrag zur Bildung bindegewebiger Matrix in glatten Muskelzellen. Elektronenmikroskopisch-autoradiographische Untersuchungen mit ^{35}S-Sulfat an glatten Muskelzellen nach Doppelligatur der Arteria carotis. Path. europ. **3**, 551–570 (1968)
Karrer, H.E.: An electron microscope study of the aorta in young and aging mice. J. Ultrastruct. Res. **5**, 1–27 (1961)
Lazarides, E.: Immunofluorescence studies on the structure of actin filaments in tissue culture cells. J. Histochem. Cytochem. **23**, 507–528 (1975)
Mauger, J.P., Worcel, M., Tassin, J., Courtois, Y.: Contractility of smooth muscle cells of rabbit aorta in tissue culture. Nature (Lond.) **255**, 337–338 (1975)
Paule, W.J.: Electron microscopy of the newborn rat aorta J. Ultrastruct. Res. **8**, 219–235 (1963)
Pease, D.C., Paule, W.J.: Electron microscopy of elastic arteries. The thoracic aorta of the rat. J. Ultrastruct. Res. **3**, 469–483 (1960)
Poole, J.C.F., Cromwell, S.B., Benditt, E.P.: Behaviour of smooth muscle cells and formation of extracellular structures in the reaction of arterial walls to injury. Amer. J. Path. **62**, 391–414 (1971)
Rohr, H.P., Jurukova, Z.: Beitrag zur Bildung bindegewebiger Matrix in glatten Muskelzellen. (Elektronenmikroskopisch-autoradiographische Untersuchungen mit ^{3}H-Prolin und ^{35}S-Sulfat am Oestrogen-stimulierten Mäuseuterus.) Path. europ. **3**, 571–579 (1968)
Ross, R.: The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibers. J. Cell Biol. **50**, 172–186 (1971)
Ross, R., Glomset, J.A.: Atherosclerosis and the arterial smooth muscle cell. Science **180**, 1332–1339 (1973)
Ross, R., Glomset, J.A.: The pathogenesis of atherosclerosis. New Engl. J. Med. **295**, 420–425 (1976)
Ross, R., Klebanoff, S.J.: The smooth muscle cell. I. In vivo synthesis of connective tissue proteins. J. Cell Biol. **50**, 159–171 (1971)
Rutherford, R.B., Ross, R.: Platelet factors stimulate fibroblasts and smooth muscle cells quiescent in plasma serum to proliferate. J. Cell Biol. **69**, 196–203 (1976)
Scott, R.F., Jones, R., Daoud, A.S., Zumbo, O., Coulston, F., Thomas, W.A.: Experimental atherosclerosis in Rhesus monkeys. II. Cellular elements of proliferative lesions and possible role of cytoplasmic degeneration in pathogenesis as studied by electron microscopy. Exp. molec. Path. **7**, 34–57 (1967)
Stary, H.C.: Proliferation of arterial cells in atherosclerosis Adv. exp. Med. Biol. **43**, 59–65 (1974)
Storti, R.V., Rich, A.: Chick cytoplasmic actin and muscle actin have different structural genes. Proc. nat. Acad. Sci. (Wash.) **73**, 2346–2350 (1976)
Thomas, W.A., Florentin, R.A., Nam, S.C., Kim, D.N., Jones, R.M., Lee, K.T.: Preproliferative phase of atherosclerosis in swine fed cholesterol. Arch. Path. **86**, 621–643 (1968)

Thomas, W.A., Florentin, R.A., Nam, S.C., Reiner, J.M., Lee, K.T.: Alterations in population dynamics of arterial smooth muscle cells during atherogenesis I. Activation of interphase cells in cholesterol-fed swine prior to gross atherosclerosis demonstrated by "postpulse salvage labelling". Exp. molec. Path. **15**, 245–267 (1971)

Thomas, W.A., Jones, R., Scott, R.F., Morrison, E., Goodale, F., Imai, H.: Production of early atherosclerotic lesions in rats characterised by proliferation of "modified smooth muscle cells". Exp. molec. Path., Suppl. **1**, 40–61 (1963)

Tokuoka, S.: Response of the uterine smooth muscle cell to oestrogen stimulation: an electron microscopic and autoradiographic study. Acta path. jap. **18**, 417–423 (1968)

Uehara, Y., Campbell, G.R., Burnstock, G.: Cytoplasmic filaments in developing and adult vertebrate smooth muscle. J. Cell Biol. **50**, 484–497 (1971)

Warshawsky, H., Leblond, C.P., Droz, B.: Synthesis and migration of proteins in the cells of the exocrine pancreas as revealed by specific activity determination from radioautographs. J. Cell Biol. **16**, 1–28 (1963)

Zeigel, R.F., Dalton, A.J.: Speculations based on the morphology of the Golgi systems in several types of protein secreting cells. J. Cell Biol. **15**, 45–54 (1962)

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