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HUMAN ARTERIAL SMOOTH MUSCLE CELLS IN CULTURE: INVERSE RELATIONSHIP BETWEEN PROLIFERATION AND EXPRESSION OF CONTRACTILE PROTEINS

GUNNAR FAGER[1], GÖRAN K. HANSSON, ALLEN M. GOWN, DAVID M. LARSON, OMAR SKALLI, AND GÖRAN BONDJERS

Arterial Biology Group, Wallenberg Laboratory for Cardiovascular Research, Department of Medicine I, University of Göteborg, Göteborg, Sweden (G. F., G. B.); Department of Clinical Chemistry, University of Göteborg, Göteborg, Sweden (G. K. H.); Department of Pathology, University of Washington, Seattle, Washington (A. M. G.); Cardiovascular Pathology, Mallory Institute of Pathology, Boston University School of Medicine, Boston, Massachusetts (D. M. L.); and Department of Pathology, University of Geneva, Geneva, Switzerland (O. S.)

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SUMMARY

Human arterial smooth muscle cells (hASMC) from explants of the inner media of uterine arteries were studied in secondary culture. We had previously found that these cells depend on exogenous platelet-derived growth factor (PDGF) for proliferation in vitro. Deprivation of the serum mitogen(s) by culture in plasma-derived serum or bovine serum albumin (BSA) caused a true growth arrest that was reversible upon reexposure to the mitogen(s). When added to serum-containing medium, heparin caused a reversible growth arrest which could be competed for by increasing concentrations of serum. In the current study we used a set of smooth muscle-specific actin and myosin antibodies to study the expression of contractile proteins in stress fibers under indirect immunofluorescence on hASMC in culture. Even in sparse culture, growth-arrested hASMC expressed stress fibers containing these actin and myosin epitopes. This was true irrespective of whether growth arrest was achieved by culture in media containing only BSA or a combination of heparin and whole blood serum. hASMC proliferating in whole blood serum in sparse culture did not express such stress fibers, as judged by immunofluorescent staining. This was true also for cells that were restimulated to proliferate in serum after a growth arrest. Utilizing a monoclonal antibody against a nuclear antigen expressed in proliferating human cells, we were able to demonstrate an inverse relationship between the expression of this antigen and the SMC-specific contractile proteins, respectively. Under these culture conditions, the reversible transition between dedifferentiated and differentiated hASMC was almost complete and terminated about 1 wk after the change in culture condition. We conclude that hASMC in vitro respond to exogenous PDGF by proliferation and dedifferentiation as a single population of cells. We also conclude that this modulation is reversible, because the cells become uniformly quiescent and differentiated when the mitogenic stimulus is blocked or removed.

Key words: smooth muscle cells; human; vascular; differentiation; cytoskeleton; actin; myosin.

INTRODUCTION

Increased arterial smooth muscle cell (ASMC) mass is typically found in the intimal lesions of advanced atherosclerosis as well as in the medial changes of arterial hypertension. The temporal sequence of events in human atherosclerosis can only be deduced from a few important observations of atheromatous tissue in humans. These are compatible with the concept that a reversible modulation between a differentiated (quiescent) (10,19,27) and a dedifferentiated (proliferative) (21,22) phenotype may occur among ASMC (possibly in response to external stimuli) during the atherosclerotic process. The mechanisms behind this process cannot, however, be studied in detail in man. Conclusions regarding possible mechanisms have instead been derived from extensive experimental studies of animal systems [reviewed in (24,26)]. The results of such studies may not be altogether relevant for man and need to be confirmed by, for example, in vitro studies of human ASMC.

We have previously isolated human ASMC (hASMC) and presented data on their growth control in culture (5). In summary, we found that the hASMC depended on exogenous platelet-derived growth factor (PDGF) for their proliferation in vitro. In plasma-derived serum

[1] To whom correspondence should be addressed at Department of Medicine I, Sahlgren's Hospital, S-413 45 Göteborg, Sweden.

(PDS) or in one percent bovine serum albumin (BSA), hASMC became growth-arrested within 2 d and remained quiescent for up to at least 7 d. This state was, however, reversible upon reexposure to serum. The mitogenic activity of serum was effectively counteracted by anti-PDGF IgG. Under no condition that we studied in vitro could we find any expression of PDGF mRNA in hASMC, as indicated by Northern blots using both A and B chain cDNA probes. When added to the medium, heparin inhibited the mitogenic effect of serum on hASMC in a dose-dependent, reversible and competitive way. Our results suggest that the effect of heparin is due to interference with the binding of PDGF to its cell surface receptor. This conclusion was based on the observation that hASMC expressed PDGF receptors on their surface when heparin was added to a serum-containing medium. In the absence of heparin, the receptor was down-regulated in serum-containing media.

In this study we do not address the well-established differentiation that occurs in spite of the presence of mitogens through contact inhibition in postconfluent SMC cultures (3,23,25). Instead, the current study was aimed at investigating whether the mitogen-dependent and reversible transition between quiescent and proliferating hASMC in sparse culture was associated with a modulation in phenotype, as determined by the expression of SMC-specific contractile proteins in stress fibers at the level of the individual cell.

Materials and Methods

Materials. The basal medium (BM) in this study was Waymouth's MB 752/1 medium containing 10^5 IU/liter penicillin, 100 gm/liter streptomycin, 1 m*M* sodium pyruvate, and 4 m*M* L-glutamine. These components, as well as trypsin (no. 16-893) and Earle's balanced salt solution without Ca^{++} and Mg^{++} (EBSS), were from Flow Laboratories (Irvine, Scotland). Culture flasks (80 cm^2) and petri dishes (10 cm^2) came from Nunclon (Roskilde, Denmark) and fetal bovine serum (FBS) from Tissue Culture Services (Berkshire, UK). Human serum (HS) was obtained from healthy volunteers. Bovine serum albumin (Fraction V, no. A 4503) was provided by Sigma Chemical Company (St. Louis, MO). Heparin sodium powder (Heparin Sodium Pure) from swine mucosa was a gift from Leo Pharmaceutical Products (Ballerup, Denmark).

In the individual experiments, the BM was supplemented with sera, BSA, or heparin as indicated.

Antibodies. A monoclonal antibody against SMC-specific alpha and gamma actin isoforms (CGA7) has previously been characterized (9). CGA7 stains growth-arrested, but not proliferating rat ASMC, and has been found to be a marker of SMC differentiation (23). A second monoclonal antibody against smooth, cardiac, and skeletal muscle alpha actin and smooth muscle gamma actin isoforms (HHF35) (28), as well as a third monoclonal that reacts exclusively with smooth muscle alpha actin (anti-αsm-1) (27), was also used to stain SMC-specific actins. A rabbit antiserum against SMC-specific myosin (ASMM) was used to detect myosin-containing stress fibers in SMC. ASMM antiserum reacts with the heavy chain (200 kDa) of myosin in mature cells in vascular and visceral smooth muscle (18,19). The four antibody preparations were known to react with human actin and myosin epitopes, respectively.

We used a monoclonal antibody to stain proliferating cells (PC) in culture. This PC antibody (Ki-67) (Dakopatts, Copenhagen, Denmark) specifically recognizes an antigen expressed in the nuclear membrane of human cells in G_1 (late), S, G_2, and M phases of the cell cycle (8).

Fluorescein-(FITC) and rhodamine-(TRITC) conjugated rabbit antimouse immunoglobulins, as well as TRITC swine antirabbit immunoglobulins and nonimmune swine serum, were provided by Dakopatts (Copenhagen, Denmark). Para-phenylenediamine (PPD) was purchased from Fluka AG (Buchs, Switzerland).

Cells and cell culture conditions. Primary cultures of hASMC from the inner media of human uterine arteries were established with the explantation technique previously described (5). The studies were carried out using cells in Passages 5 to 8. Bulk preparation of cells for the experiments was made in BM supplemented with HS and FBS at concentrations of 10% (vol/vol) each. This medium was designated S-BM and has been found to induce a rapid proliferation of hASMC in vitro (5). Growth arrest of sparse cells was achieved either by adding heparin (10 g/liter) to S-BM or by replacing the sera with 1% (wt/vol) BSA. These media were designated HEP-BM and BSA-BM, respectively, and their effects on hASMC proliferation have been described (5).

Cells were harvested by trypsinization and passed as previously described (5). The counting of cells was done in an AI Cell Counter 134 (Analys Instrument, Solna, Sweden).

Immunofluorescence studies of cells in culture. In all experiments the cells were seeded in S-BM on gelatin-coated glass cover slips in 10-cm^2 petri dishes. The medium was changed after 16 to 18 h. Thereafter, the medium was changed every 3rd d.

Proliferating cells were studied in sparse cultures seeded at 2×10^3 cells/cm^2 and grown for 4 d in S-BM before preparation for immunofluorescence of contractile proteins. Growth-arrested cells were also studied in sparse cultures. They were seeded at 10^3 cells/cm^2, grown for 4 d in S-BM and for another 6 d in BSA-BM or HEP-BM before immunofluorescence. Restimulated cells were prepared in the same way as growth-arrested cells, except that growth-arrest in BSA-BM or HEP-BM was followed by incubation in S-BM, and cover slips were taken for immunofluorescence of contractile proteins both 3 and 6 d later.

Expression of the PC nuclear antigen and SMC-specific contractile proteins in sparse hASMC cultures was studied in a time-course experiment. hASMC were seeded on gelatin-coated glass cover slips at 10^3 cells/cm^2 as before and grown in S-BM for 2 d. Growth arrest was then induced by culture in BSA-BM for 7 d and, finally, the cells were restimulated to grow in S-BM for another 7 d. During this incubation, triplicate cover slips were taken

at indicated time points for immunofluorescent staining with the Ki-67, the anti-αsm-1 monoclonal antibodies, and the ASMM antiserum, respectively.

The possibility of coexpression of the PC nuclear antigen and SMC-specific α-actin in stress fibers in association with changes in mitogen exposure was also investigated. Cells were seeded on cover slips as before. In one series the cells were preincubated in S-BM for 3 d and then transferred to HEP-BM for another 5 d. In another series cells were growth-arrested by preincubation in HEP-BM for 4 d and then transferred to S-BM for another 5 d. Cover slips were harvested at indicated time points and simultaneously stained with Ki-67 and anti-αsm-1 monoclonals as primary antibodies and TRITC-antimouse immunoglobulins as secondary antibodies. Visualization with immunofluorescence of antigen-antibody reaction was done as described below. The percentages of cells discordantly expressing the PC nuclear or stress fiber SMC-actin antigens as well as the percentages of cells concordantly expressing or not expressing these antigens were determined.

For immunofluorescence studies the cover slips with the hASMC were washed 3 times in ice-cold phosphate buffered saline (PBS) (pH 7.4) containing 1% (wt/vol) BSA (BSA-PBS). Fixation was achieved by treatment with ice-cold methanol (for stainings with CGA7, HHF35, and anti-αsm-1) or acetone (for stainings with Ki-67 and ASMM) for 5 min. Fixated cells were washed 3 times in BSA-PBS, pretreated with nonimmune swine serum (1/50 in PBS, vol/vol), and washed 3 times before immunofluorescent staining.

The antibodies were diluted in BSA-PBS to concentrations (given in parentheses below) which gave optimal fluorescence as evaluated by checker-board titrations. The diluted antibodies CGA7 (1/200, vol/vol), HHF35 (1/200, vol/vol), anti-αsm-1 (1/200, vol/vol), and Ki-67 (1/50, vol/vol), as well as the diluted ASMM antiserum (1/50, vol/vol), were allowed to react with the fixated cells for 45 min at 20° C. Unbound antibodies were removed by 3 washes in BSA-PBS, and the cover slips treated with diluted FITC- or TRITC-rabbit antimouse immunoglobulins (1/50, vol/vol) or TRITC-swine antirabbit immunoglobulins (1/40, vol/vol) for 30 min at 20° C. Unbound antibodies were removed by 3 washes in BSA-PBS and a final wash in distilled water. The cover slips were mounted on microscopic slides in 25 g/liter PPD in glycerol:PBS 9:1 (vol/vol) (pH 8.6) (13).

Control cover slips were prepared exactly as described above, except that the primary antibody or antiserum was replaced by homologous nonimmune IgG or serum, respectively.

Evaluation of results. The microscopic slides were studied with a Zeiss Axiophot microscopy (Carl Zeiss, Oberkochen, West Germany). Immunofluorescence was studied with epifluorescence light and appropriate filters.

Regarding immunofluorescence for SMC-specific contractile proteins, cells with a strong or medium fluorescence and with a clearcut stress fiber distribution were considered positive. The others were held as negative. These were cells with faint fluorescence and without a typical stress fiber distribution, as well as those that were impossible to differentiate from cells on the control slides. Staining with the Ki-67 antibody gave a strong nuclear fluorescence in hASMC that were, consequently, considered proliferative. These cells were easily distinguished from negative cells lacking such fluorescence.

At least 500 cells in at least 10 consecutive visual fields were allocated in this way. Photomicrographs were taken

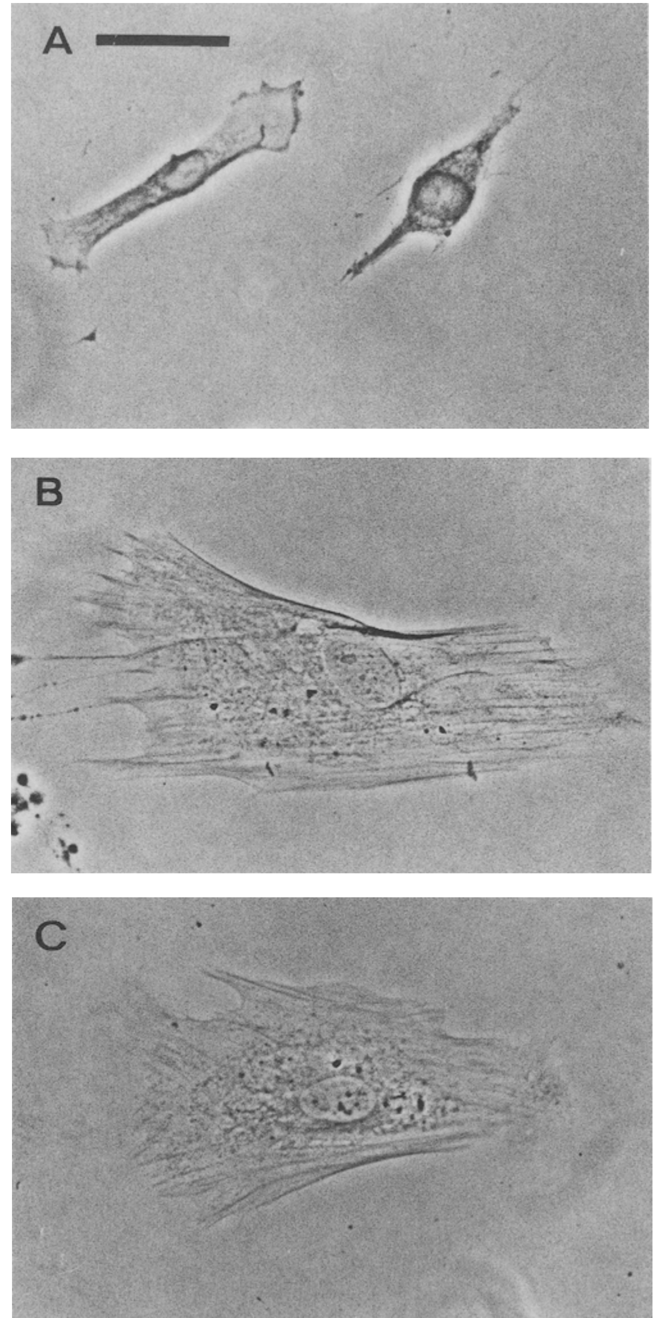
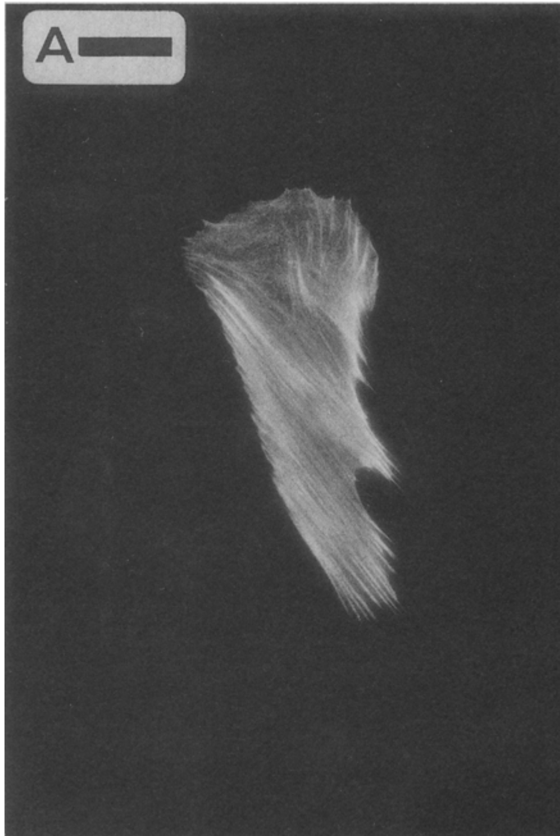


FIG. 1. Phase contrast microscopy of sparsely seeded hASMC after 4 d in S-BM (*A*) (proliferating cells) and after 6 d in HEP-BM (*B*) or BSA-BM (*C*) (growth-arrested cells). *Bar* = 10 μm.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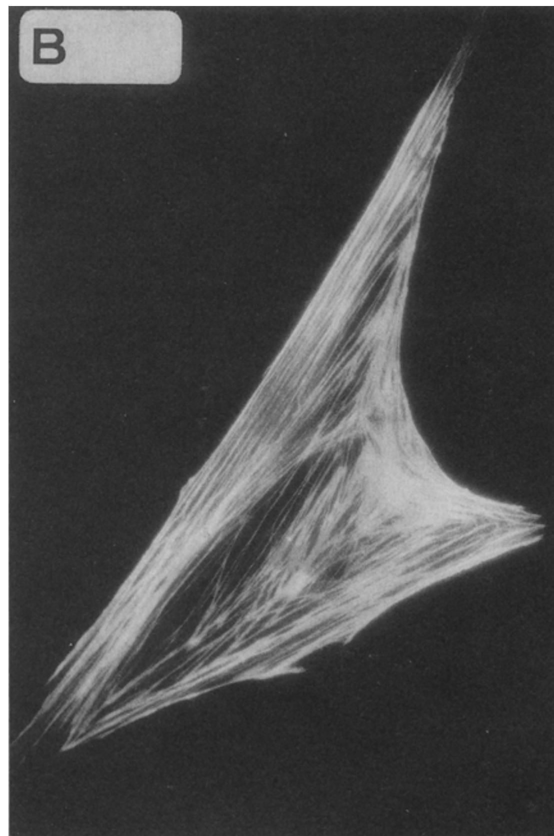
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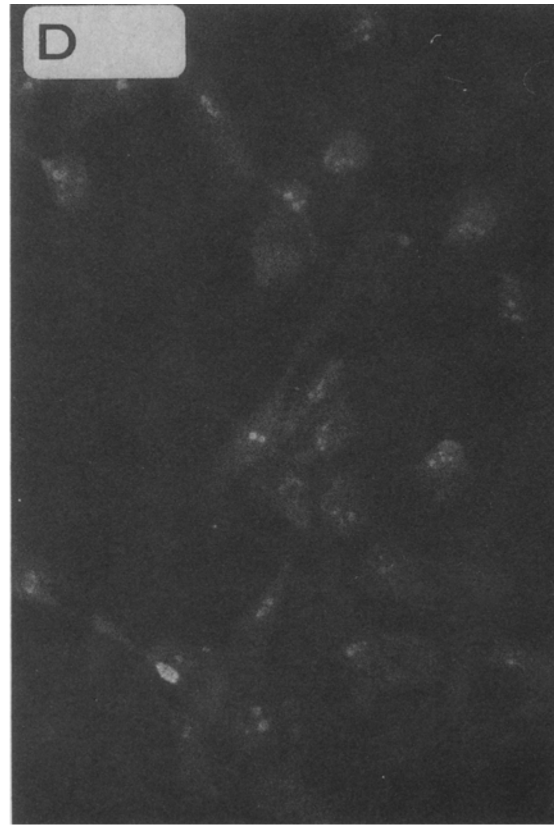
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with exposure times that were found suitable for positive cells. All negative films were copied with the same exposure to yield comparable photomicrographs. The results were evaluated by two-way analysis of variance according to a contingency table. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Phase contrast microscopy. Cells proliferating in S-BM in sparse cultures (Fig. 1 *A*) as well as restimulated cells (not shown) were polygonal and often equipped with long, slender extensions. The nucleus was rather distinct and surrounded by abundant granular material, whereas the peripheral parts of the cytoplasm were less structured.

Growth-arrested, sparse hASMC cultures in HEP-BM (Fig. 1 *B*) or BSA-BM (Fig. 1 *C*) showed cells that were elongated and flattened and which covered a surface about 10 times that of the proliferating cells. The perinuclear area contained little granular material, and the cytoplasm was filled with stress fibers.

Immunofluorescence. Human arterial smooth muscle cells in sparse secondary culture exhibited stress fibers containing smooth muscle actin and myosin isoforms to varying degrees. There was a strong relationship between the degree of filament expression and culture conditions.

The cells that were brought to a growth-arrest (in BSA-BM or in HEP-BM) in sparse culture exhibited stress fibers containing SMC-specific heavy chain myosin (Fig. 2 *A, B*). All of the cells on these slides were considered positive, and the distribution of immunofluorescence was typical of contractile filaments. Only a few cells in proliferating (Fig. 2 *C*) and restimulated (Fig. 2 *D*) cultures were positive however, and these were found in clusters with a high degree of cell-to-cell contact.

When CGA7 (Fig. 3 *A-D*), HHF35 (not shown), or anti-αsm-1 (not shown) were used as a primary antibody, the results of immunofluorescence were very similar. The distribution of fluorescence indicated that the stress fibers of growth-arrested hASMC also in sparse cultures contained SMC-specific actin isoforms. This was true irrespective of whether growth arrest was caused by serum deprivation or by the addition of heparin, as shown for CGA7 in Fig. 3 *A, B*. The majority of proliferating as well as restimulated cells were considered negative (Fig. 3 *C, D*). The positive cells in the latter cultures were seen within occasional clusters of hASMC.

There was general conformance among the results obtained with the four different antibodies. The differences among them were not significant by analysis of variance ($P = 0.068$) (Table 1). However, the differences among culture conditions (proliferation, growth-arrest, and restimulation), as evaluated by analysis of variance, were highly significant ($P = 10^{-11}$). Proliferating cultures and cultures that were restimulated for 6 d in S-BM exhibited a percentage of cells with stress fibers containing smooth muscle actin and myosin that varied between 2 and 20. The corresponding figures were 72-100 for cultures in BSA-BM and 90-100 for those in HEP-BM. Three days of restimulation in S-BM after growth arrest was not sufficient time to attain the basal level of cells expressing smooth muscle actin and myosin in stress fibers, as is evident from the intermediate figures in these cultures (1 to 49%). Although the differences between growth-arrested and proliferating or restimulated cultures were highly significant, there were no consistent differences between cultures grown in BSA-BM and in HEP-BM, or between proliferating cultures and cultures restimulated in S-BM for 6 d after growth arrest.

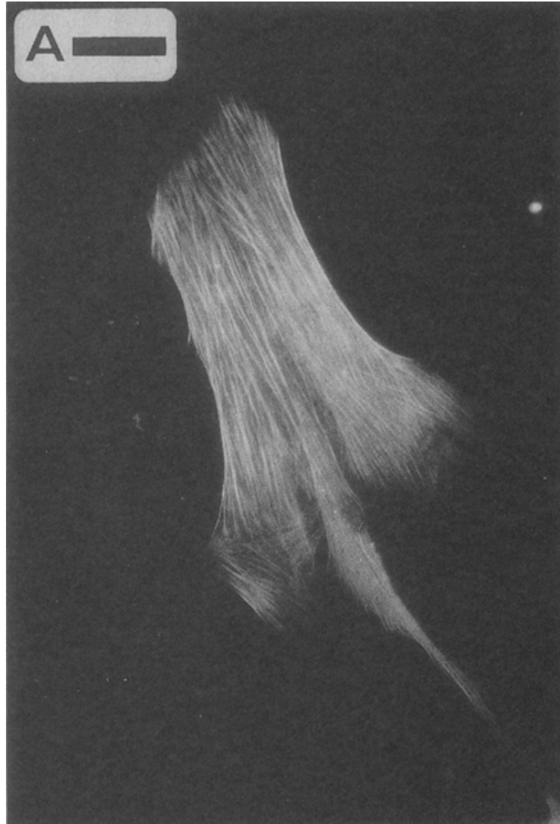
Quiescent hASMC in multilayered confluent cultures uniformly expressed stress fibers containing SMC-specific actin and myosin isoforms, as evaluated with the four antibody preparations (not shown).

Human arterial smooth muscle cells that were grown in S-BM showed a strong nuclear fluorescence at immunofluorescent staining with the Ki-67 monoclonal as the primary antibody (Fig. 4 *A*). Seven days after transfer of these cultures to BSA-BM, almost all cells were negative with regard to such fluorescence (Fig. 4 *B*).

Figure 5 demonstrates the relationship between expression of PC nuclear antigen and SMC-specific contractile proteins, which were evaluated by separate immunofluorescent stainings with the Ki-67 antibody, ASMM antiserum, or anti-αsm-1 antibody, respectively. Cultures grown for 2 d in S-BM exhibited a very low percentage of cells with stress fibers containing these SMC-specific contractile proteins and a high percentage of cells with nuclear fluorescence with the Ki-67 antibody. When transferred to BSA-BM, the cultures showed a gradual increase in the proportion of cells containing SMC-specific contractile proteins in stress fibers and a corresponding decrease in cells with nuclear fluorescence with Ki-67. Seven days after transfer to BSA-BM, nuclear fluorescence was expressed in a very low proportion, whereas stress fiber fluorescence was present in a high proportion of the cells. When the cultures were transferred to S-BM once again, the reverse phenomenon took place with a similar velocity and degree of completeness. These results suggest that the expression of SMC-specific contractile proteins in stress fibers may be confined to hASMC in G_0 or early G_1 phases of the cell cycle and that this expression is subject to the receprocal influence of external mitogen(s).

The time-course relationship between the expression of PC nuclear and stress fiber SMC-specific α-actin antigens was further evaluated by simultaneous costaining with the Ki-67 and anti-αsm-1 monoclonal antibodies. By this technique we found cells within the same culture with

FIG. 2. Indirect immunofluorescent staining with a rabbit antiserum against smooth muscle myosin (ASMM). Growth-arrested cells were cultured in HEP-BM (*A*) or in BSA-BM (*B*) for 6 d. Proliferating (*C*) and restimulated (after growth arrest) (*D*) cells were cultured in S-BM for 4 and 6 d, respectively. *Bar* = 10 μm.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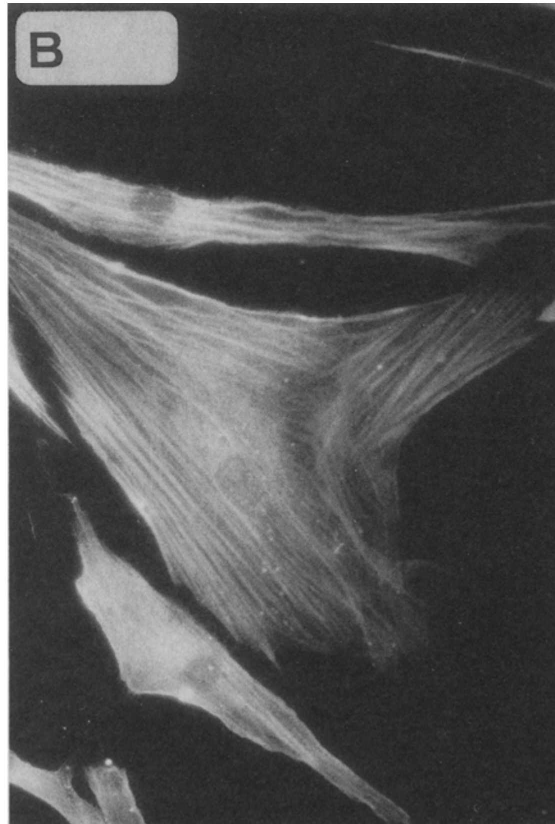
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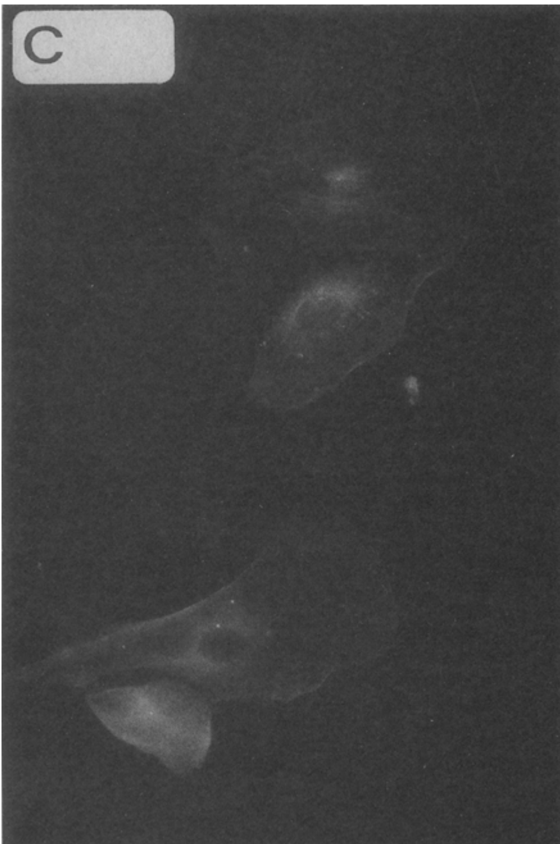
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D

either nuclear fluorescence or stress fiber fluorescence (Fig. 6). Other cells showed nuclear and stress fiber fluorescence simultaneously, and still other cells showed neither nuclear nor stress fiber fluorescence. In cultures that were preincubated for 3 d in S-BM and transferred to HEP-BM to become growth arrested, the percentage of cells positive for the Ki-67 showed a decrease and those positive for anti-αsm-1 antibodies an increase (Fig. 7 *a*), as could be expected from the other experiments (cf. Fig. 5). There was, however, a small percentage of cells that coexpressed the PC nuclear antigen and the SMC-specific α-actin stress fiber antigen simultaneously during the transition from proliferation to growth arrest. When cultures were preincubated in HEP-BM for 4 d to become growth arrested and then restimulated to proliferate in S-BM, the opposite events took place (Fig. 7 *b*). Also in this case a small percentage of the cells showed coexpression of the two antigens during the transition from growth arrest to proliferation. We also found that about 20% of the cells in growth-arrested cultures were negative for both antibodies. This finding however, is consistent with the other experiments showing that Ki-67 does not stain growth-arrested cells and that anti-αsm-1 (in contrast to ASMM) does not stain all growth-arrested cells.

Discussion

While growing under the influence of mitogens in sparse secondary culture with little or no cell-to-cell contact, hASMC do not contain SMC-specific contractile proteins in stress fiber structures. This held true also for cultures that were restimulated to growth for 6 to 7 d after a growth arrest. Such stress fibers were, however, expressed in mitogen-deprived growth-arrested hASMC in sparse secondary cultures, as evaluated from immunofluorescent stainings with antibodies against SMC-specific contractile proteins. The antibodies have been described previously (8,9,18,19,27,28). The Ki-67 monoclonal antibody specifically binds to human cells in late G_1, S, G_2, and M phases of the cell cycle, but not to nonhuman cells or human cells in G_0 and early G_1 (8). In cultures of differentiated rat vascular SMC, the CGA7 antibody (9,23), the anti-αsm-1 antibody (27), and the ASMM antiserum (19) have been found to react with filamentous SMC actin and myosin isoforms, respectively.

The present results are consistent with these conclusions. This time we extended the observations to encompass human ASMC in vitro and the HHF35 and anti-αsm-1 monoclonal antibodies. Furthermore, our results indicate that the epitopes of the four antibody preparations are expressed simultaneously in stress fibers of mitogen-deprived hASMC. We cannot, however, exclude the possibility that proliferating hASMC in culture contain stress fibers with nonmuscle actin and myosin isoforms or monomeric SMC-specific isoforms which have been removed or rendered nonreactive during the staining procedure. In fact, rat vascular SMC in culture do contain accessible SMC myosin as detected by both immunofluorescence and immunoprecipitation with ASMM in a previous study (18). Utilizing cRNA and cDNA clones for α, β, and γ actin mRNA, it has been shown that the expression of actin isoforms varies and that one explanation might be regulation at the transcriptional level in rat aortic SMC (1,16,17).

Modulation toward a differentiated phenotype was obtained concomitantly with growth arrest through deprivation of exogenous growth factors. This was achieved by culture in BSA or by inhibition of mitogenic activity with the addition of heparin to serum-containing medium. We have previously shown that this reversible growth arrest was due to deprivation of PDGF activity in the medium. In addition, we were not able to find any evidence that responsiveness to mitogens was inherent only in a subpopulation of cells (5). In this study, we found that the hASMC population showed an almost complete response toward differentiation upon withdrawal of the mitogenic stimulus. This modulation was, however, reversible upon reexposure to mitogen(s). Under the present culture conditions, the phenotypic modulation was completed in about 6 d.

Thus, we find no evidence of an irreversibly dedifferentiated subpopulation or of a mitogen-responsive proliferation-prone subpopulation among our hASMC in vitro. However, the possibility that our hASMC represent a mitogen-sensitive subpopulation from the arterial media which has been selected by the isolation procedure cannot be ruled out. Were it so, our results would strongly contradict the concept that this subclone is irreversibly dedifferentiated.

The possibility of a controlled modulation between a dedifferentiated-proliferating and a differentiated-quiescent cell has been addressed before in animal SMC cultures (reviews 24,26). It has been argued that SMC from several animal sources with successive subcultivations in sparse cultures in vitro irreversibly lose several properties of differentiated SMC (4). These included microfilaments and specific contractile proteins as well as the ability to redifferentiate and to contract. It has, however, also been shown that some of these properties may be retained during subcultivation (3,6,23,25,26). For instance, Owens et al. (23) found that a dramatic decrease in SMC-specific α-actin preceded proliferation under the influence of mitogens among growth-arrested rat aortic SMC in culture. It was also found that this decrease in SMC-specific α-actin among proliferating rat aortic SMC was partly reversible upon withdrawal of mitogens or upon reaching a multilayered quiescent state. The present results support the concept that phenotypic modulation in

FIG. 3. Indirect immunofluorescent staining with a monoclonal antibody against smooth muscle alpha and gamma actin isoforms (CGA7). Growth-arrested cells were cultured in HEP-BM (*A*) or in BSA-BM (*B*) for 6 d. Proliferating (*C*) and restimulated (after growth arrest) (*D*) cells were cultured in S-BM for 4 and 6 d, respectively. *Bar* = 10 μm.

TABLE 1

PERCENTAGE OF hASMC SHOWING STRESS FIBERS CONTAINING SMOOTH MUSCLE ACTIN AND MYOSIN ISOFORMS

	Growing for 4 d in	Growth Arrested for 6 d in		Growth Stimulated in S-BM for 3 d After Growth Arrest in		Growth Stimulated in S-BM for 6 d After Growth Arrest in	
	S-BM	BSA-BM	HEP-BM	BSA-BM	HEP-BM	BSA-BM	HEP-BM
ASMM	17	100	100	6	18	4	2
CGA7	6	73	90	1	17	1	3
HHF35	10	100	98	28	22	6	20
Anti-αsm-1	4	80	90	24	49	8	8
Mean ± SD[a]	9 ± 6	88 ± 14	94 ± 5	15 ± 13	27 ± 15	5 ± 3	8 ± 8

[a]Mean percentage of all cells with stress fibers containing SMC-specific actin or myosin isoforms.
Key: ASMM = Rabbit antiserum against SMC-specific myosin; CGA7 = monoclonal antibody against SMC-specific alpha and gamma actin isoforms; HHF35 = monoclonal antibody against smooth, cardiac, and skeletal muscle alpha and smooth muscle gamma actin isoforms; Anti-αsm-1 = monoclonal antibody against SMC-specific alpha actin.

response to exogenous mitogens may take place also among human ASMC in vitro.

It has been suggested that only a subset of hASMC in vivo is responsible for proliferation under pathologic conditions, and that these cells are irreversibly dedifferentiated (review; 26). If this were true, differentiated hASMC would not be seen within the atherosclerotic lesions unless they had migrated there without a simultaneous replication. Differentiated hASMC have indeed been observed within advanced atherosclerotic lesions (10,14,15). Our results show that the modulation from a dedifferentiated to a differentiated phenotype during mitogen deprivation is a generalized and reversible response by hASMC, at least in vitro.

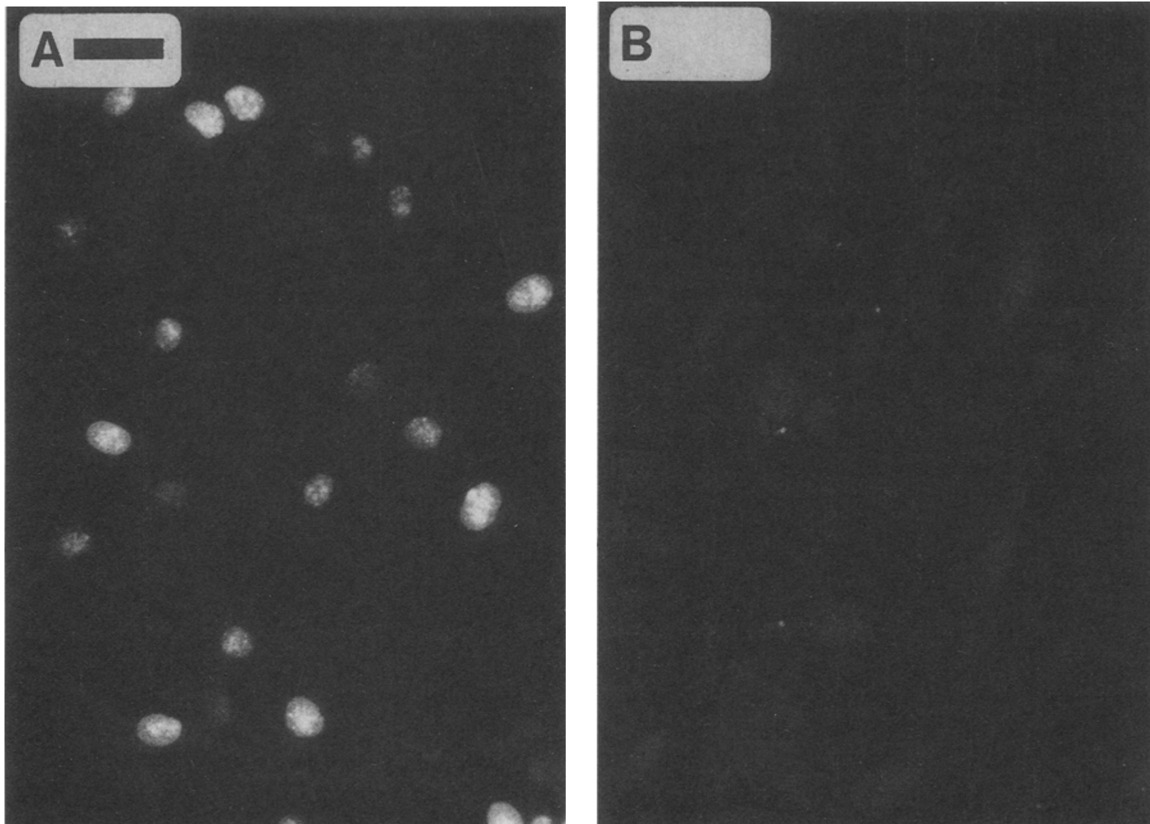

FIG. 4. Indirect immunofluorescent staining with a monoclonal antibody against an antigen expressed in the nuclear membrane of proliferating human cells (Ki-67). Cells were grown in S-BM for 2 d after seeding (*A*) and then growth arrested for another 7 d in BSA-BM (*B*). *Bar* = 10 μm.

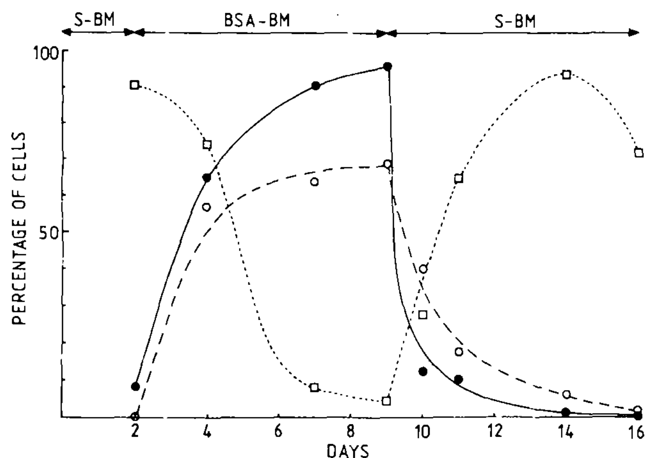

FIG. 5. Effects of exposure and deprivation of serum mitogens on dedifferentiation and differentiation of hASMC in prolonged culture. Proliferation was induced by culture in S-BM for 2 d, growth-arrest by culture in BSA-BM for another 7 d and, finally, proliferation reinduced by culture in S-BM for 7 d. Cover slips were harvested at indicated times and stained for SMC-specific α-actin (anti-αsm-1 monoclonal antibody) (*open circles*), SMC-specific myosin (ASMM rabbit antiserum) (*closed circles*), and PC nuclear antigen (Ki-67 monoclonal antibody) (*squares*).

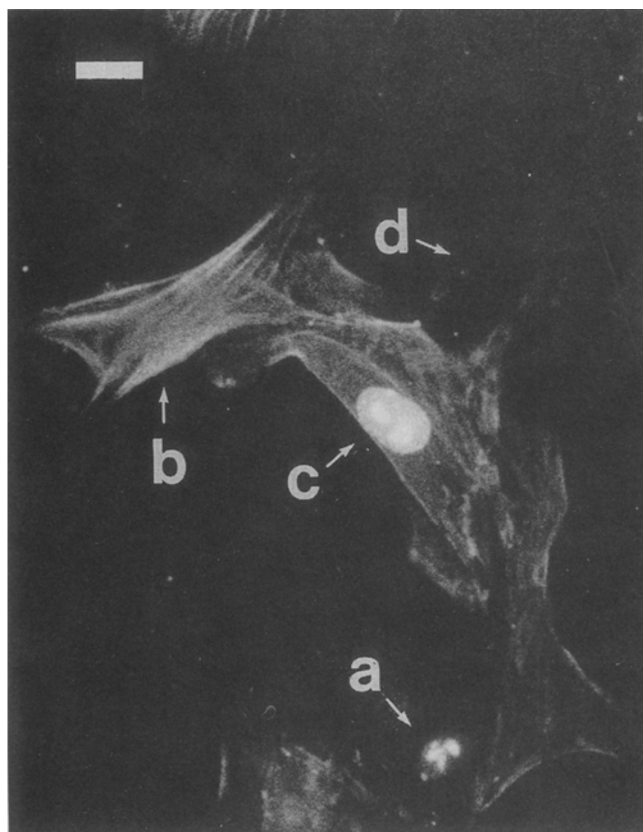

FIG. 6. Simultaneous indirect immunofluorescent staining with the Ki-67 and the anti-αsm-1 monoclonal antibodies. Two days after growth arrest in HEP-BM the hASMC cultures showed cells with either nuclear fluorescence (*a*) or stress-fiber fluorescence (*b*). There were, however, also cells with both nuclear and stress-fiber fluorescence (*c*) as well as cells with neither kind of fluorescence (*d*) *Bar* = 10 μm.

Our results may present a relevant link in the temporal sequence of human atherogenesis, as deduced from observations on human arteries with varying degrees of atherosclerotic involvement. It may seem logical to believe that the differentiated hASMC in the advanced lesion (10) have originated from differentiated ancestors in the normal arterial wall (10,19,27). It also seems logical to believe that the ASMC in early lesions have responded to mitogens in vivo by proliferation and by modulation into a dedifferentiated phenotype (21,22). Such cells may exist in the aortic wall of both rats and humans, as suggested by immunofluorescent studies with the anti-α sm-1 antibody, which failed to stain some cells of the media (27). The current finding that mitogenic stimulation in vitro is associated with proliferation and loss of contractile proteins supports the concept of modulation. It may also seem logical to believe that the proliferative hASMC of the early lesions under certain conditions redifferentiate and become quiescent as observed in the advanced lesions (10). Our results provide one explanation for such a redifferentiation, due to the finding that mitogen deprivation is associated with the reexpression of contractile proteins in vitro.

Heparinlike substances are produced by vascular endothelial (2) and smooth muscle (7) cells and, like heparin, they inhibit SMC proliferation. Our results with heparin may provide a hypothesis for a particularly interesting regulatory system for ASMC modulation. If serum mitogens gain access to the subendothelial space by, for instance, endothelial damage, they may bind to heparinlike substances to form a falling concentration gradient toward the periphery. PDGF is not only a potent mitogen but also a powerful chemoattractant for ASMC, which migrate toward increasing concentrations of the mitogen (11,12). When interacting with heparin, PDGF will not bind to its cell surface receptor to induce proliferation (5) nor serve as a chemoattractant (20). Theoretically, PDGF that is bound and inactivated at the

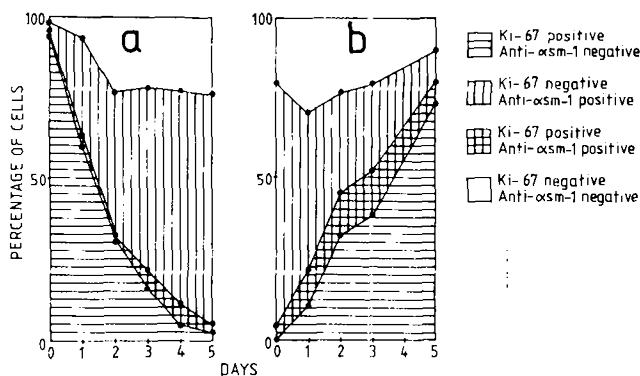

FIG. 7. Time course of coexpression of PC nuclear antigen and SMC-specific α-actin in stress fibers by simultaneous staining with the Ki-67 and anti-αsm-1 monoclonal antibodies. *A*, Sparse cultures preincubated for 3 d in S-BM and then cultured from Days 0 to 5 in HEP-BM. *B*, Sparse cultures preincubated for 4 d in HEP-BM and then cultured from Days 0 to 5 in S-BM. The results are given as percentage of cells positive for Ki-67 only, anti-αsm-1 only, both Ki-67 and anti-αsm-1, and, finally, cells negative for both antibodies. Symbols are indicated in the figure.

site of endothelial damage may however slowly dissociate from heparinlike glycosaminoglycans at the site of endothelial damage, to induce SMC migration as well as proliferation over a prolonged period of time. Such a process may be reinforced by enzymatic degradation of the glycosaminoglycans by endoglycosidases. Fritze et al. (7) have shown that the heparinlike substances produced by bovine ASMC loose their growth-inhibitory property upon enzymatic cleavage.

The dependence of exogenous PDGF and the interaction between PDGF and heparinlike glycosaminoglycans may help to explain not only the ASMC migration into the intima and the delayed proliferative response among SMC after experimental endothelial injury, but provide a basis for the understanding of the regulation of phenotype among ASMC in experimental as well as human atherosclerotic lesions.

REFERENCES

1. Barja, F.; Coughlin, C.; Belin, D., et al. Actin isoform synthesis and mRNA levels in quiescent and proliferating rat aortic smooth muscle cells in vivo and in vitro. Lab. Invest. 55:226–233; 1986.
2. Castellot, J.; Favreau, L. V.; Karnovsky, M. J., et al. Inhibition of vascular smooth muscle cell growth by endothelial cell-derived heparin. Possible role of endoglycosidase. J. Biol. Chem. 257:11256–11260; 1982.
3. 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. Embryol. Exp. Morphol. 32:297–323; 1974.
4. Chamley-Campbell, J. H.; Campbell, G. R. What controls smooth muscle phenotype? Atherosclerosis 40:347–357; 1981.
5. Fager, G.; Hansson, G. K.; Ottosson, P., et al. Human arterial smooth muscle cells in culture. Effects of platelet-derived growth factor and heparin on growth in vitro. Exp. Cell Res. 176:319–335; 1988.
6. Franke, W. W.; Schmid, E.; Vandekerckhove, J., et al. A permanently proliferating rat vascular smooth muscle cell with maintained expression of smooth muscle characteristics, including actin of the vascular smooth muscle type. J. Cell Biol. 87:594–600; 1980.
7. Fritze, L. M.; Reilly, C. F.; Rosenberg, R. D. An antiproliferative heparan sulfate species produced by postconfluent smooth muscle cells. J. Cell Biol. 100:1041–1049; 1985.
8. Gerdes, J.; Lemke, H.; Baisch, H., et al. Cell cycle analysis of a cell proliferation-associated antigen defined by the monoclonal antibody Ki-67. J. Immunol. 133:1710–1715; 1984.
9. Gown, A. M.; Vogel, A. M.; Gordon, D., et al. A smooth muscle-specific monoclonal antibody recognizes smooth muscle actin isozymes. J. Cell Biol. 100:807–813; 1985.
10. Gown, A. M.; Tsukada, T.; Ross, R. Human atherosclerosis. II. Immunocytochemical analysis of the cellular composition of human atherosclerotic lesions. Am. J. Pathol. 125:191–207; 1986.
11. Grotendorst, G. R.; Seppä H. E. J.; Kleinman, H. K., et al. Attachment of smooth muscle cells to collagen and their migration toward platelet-derived growth factor. Proc. Natl. Acad. Sci. USA 78:3669–3672; 1981.
12. Grotendorst, G. R.; Chang, T.; Seppä, H. E. J., et al. Platelet-derived growth factor is a chemoattractant for vascular smooth muscle cells. J. Cell. Physiol. 113:261–266; 1982.
13. Johnson, G. D.; Davidson, R. S.; McNamee, K. C., et al. Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. J. Immunol. Methods 55:231–242; 1982.
14. Jonasson, L.; Holm, J.; Skalli, O., et al. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. Arteriosclerosis 6:131–138; 1986.
15. Kocher, O.; Gabbiani, G. Cytoskeletal features of normal and atheromatous human arterial smooth muscle cells. Hum. Pathol. 17:875–880; 1986.
16. Kocher, O.; Gabbiani, G. Expression of actin mRNA in rat aortic smooth muscle cells during development, experimental intimal thickening, and culture. Differentiation 32:245–251; 1986.
17. Kocher, O.; Gabbiani, G. Analysis of α-smooth-muscle actin mRNA expression in rat aortic smooth-muscle cells using a specific cDNA probe. Differentiation 34:201–209; 1987.
18. Larson, D. M.; Fujiwara, K.; Alexander, R. W., et al. Myosin in cultured vascular smooth mucle cells: immunofluorescence and immunochemical studies of alterations in antigenic expression. J. Cell Biol. 99:1582–1589; 1984.
19. Larsson, D. M.; Fujiwara, K.; Alexander, R. W., et al. Heterogeneity of myosin antigenic expression in vascular smooth muscle in vivo. Lab. Invest. 50:401–407; 1984.
20. Majack, R. A.; Clowes, A. W. Inhibition of vascular smooth muscle cell migration by heparin-like glycosaminoglycans. J. Cell. Physiol. 118:253–256; 1984.
21. Mosse, P. R. L.; Campbell, G. R.; Wang, Z. L., et al. Smooth muscle phenotypic expression in human carotid arteries. II. Comparison of cells from diffuse intimal thickenings adjacent to atheromatous plaques with those of the media. Lab. Invest. 53:556–562; 1985.
22. Orekhov, A. N.; Kosykh, V. A.; Repin, V. S., et al. Cell proliferation in normal and atherosclerotic human aorta. II. Autoradiographic observation on deoxyribonucleic acid synthesis in primary cell culture. Lab. Invest. 48:749–754; 1983.
23. Owens, G. K.; Loeb, A.; Gordon, D., et al. Expression of smooth muscle-specific isoactin in cultured vascular smooth muscle cells: relationship between growth and cytodifferentiation. J. Cell Biol. 102:343–352; 1986.
24. Ross, R. Atherosclerosis: A problem of the biology of arterial wall cells and their interactions with blood components. Arteriosclerosis 1:293–311; 1981.
25. Rovner, A. S.; Murphy, R. A.; Owens, G. K. Expression of smooth muscle and nonmuscle myosin heavy chains in cultured vascular smooth muscle cells. J. Biol. Chem. 261:14740–14745; 1986.
26. Schwartz, S.; Campbell, G. R., Campbell, J. H. Replication of smooth muscle cells in vascular disease. Circ. Res. 58:427–444; 1986.
27. Skalli, O.; Ropraz, P.; Trzeciak, A., et al. A monoclonal antibody against α-smooth muscle actin: a new probe for smooth muscle differentiation. J. Cell Biol. 103:2787–2796; 1986.
28. Tsukada T.; Tippens, D.; Gordon, D., et al. HHF 35, a muscle-actin-specific monoclonal antibody. I. Immunocytochemical and biochemical characterization. Am. J. Pathol. 126:51–60; 1987.

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