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

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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...
(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.

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 G1 (late), S, G2, 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 (Analyt 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-cm² 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 × 10⁴ cells/cm² 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 10² cells/cm², 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 10⁴ cells/cm² 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 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.

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-sm-1) (27), was also used to stain SMC-specific actins. A rabbit antiserum against SMC-specific myosin (ASMM) was used to detect myosin-

MATERIALS AND METHODS

Materials. The basal medium (BM) in this study was Waymouth's MB 752/1 medium containing 10⁶ IU/liter penicillin, 100 gm/liter streptomycin, 1 mM sodium pyruvate, and 4 mM L-glutamine. These components, as well as trypsin (no. 16-893) and Earle's balanced salt solution without Ca²⁺ and Mg²⁺ (EBSS), were from Flow Laboratories (Irvine, Scotland). Culture flasks (80 cm²) and petri dishes (10 cm²) came from Nunclon (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).

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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.

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