CINEMATOGRAPHIC ANALYSIS OF VASCULAR SMOOTH MUSCLE CELL INTERACTIONS WITH EXTRACELLULAR MATRIX

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SUMMARY

The interactions of vascular smooth muscle cells with growth modulators and extracellular matrix molecules may play a role in the proliferation and migration of these cells after vascular injury and during the development of atherosclerosis. Time-lapse cinematographic techniques have been used to study cell division and migration of bovine carotid artery smooth muscle cells in response to matrix molecules consisting of solubilized basement membrane (Matrigel) and type I collagen. When cells were grown adjacent to Matrigel, both migration and cell proliferation were increased and interdivision time was shortened. Cells grown in Matrigel or in type I collagen had markedly reduced migration rates but interdivision time was not altered. Further, diffusible components of the Matrigel were found to stimulate proliferation of the smooth muscle cells.

Key words: smooth muscle cells; cell migration; cell division time; extracellular matrix; cinematography.

INTRODUCTION

Vascular smooth muscle cell (SMC) proliferation and migration in response to injury is considered to be an important factor in the development of arteriosclerotic lesions (8,18). In vitro studies have provided information on proliferative and migratory behavior of cultured SMCs in response to a variety of mediators that may be involved in vascular injury and repair. Cultured SMCs proliferate in response to a variety of growth factors including epidermal growth factor (EGF) (16), platelet derived growth factor (PDGF) (4), and fibroblast growth factor (FGF) (9). PDGF alone stimulates SMC migration, whereas proliferation of SMCs requires additional growth factors (4).

The interactions of growth factors and extracellular matrix may also play an important role in the response of SMCs to injury and subsequent reconstruction of vessels. In vitro studies have shown that SMC proliferation is modulated in the presence of extracellular matrix. Gospodarowicz and Ill (9), demonstrated that SMCs grown on extracellular matrix (ECM) proliferate in the presence of plasma equally as well as in the presence of serum. Thrombospondin, a glycoprotein component of the extracellular matrix, acts synergistically with EGF to stimulate SMC proliferation; an effect which is inhibited by heparin (16). Heparin also inhibits smooth muscle migration (15).

In the studies reported here, time-lapse cinematography was used to determine proliferation and migration of individual bovine carotid artery SMCs cultured in the presence of either a basement membrane extracellular matrix, Matrigel, or type I collagen.

MATERIALS AND METHODS

Isolation of SMCs. Smooth muscle cells were isolated from bovine carotid arteries as previously described (1,20). Intact arteries were dissected clean and cut into cylindrical segments of approximately 7 cm. One end was tied off and a solution of elastase, collagenase, and DNAase in low calcium (0.5 mM) physiologic saline was introduced through the open end to pressurize the segment, after which the segment was tied off. The pressurized segments were then incubated in physiologic saline at 37 °C for 90 min. The enzyme solution from this first incubation contained endothelial cells and was discarded. The segments were refilled with fresh enzyme solution and incubated an additional 90 min at 37 °C. The vessels were then gently flushed several times with physiologic saline containing 0.5 mM calcium to remove SMCs.

Cell culture. The isolated SMCs were seeded at 1.0 × 10^4 cells/cm² in a complete growth medium containing Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and incubated in an atmosphere of 10% CO₂, balance air at 37 °C. Stock cultures were subcultured at weekly intervals. To subculture, cell monolayers were rinsed in 0.53 mM EDTA, then dispersed with 0.25% trypsin in calcium-free and magnesium-free phosphate buffered saline (CMF-PBS). Cells were enumerated by hemacytometer counts and the number of population doublings was calculated for each subculture.

Culture of SMCs in basement membrane and collagen. To study the effect of Matrigel (Collaborative Research, Bedford, MA), a solubilized basement membrane containing laminin, type IV collagen, heparan sulfate proteoglycan, and entactin (13) on SMC cultures, SMCs were either seeded onto the surrounding solidified Matrigel or were mixed with Matrigel which had been diluted in complete growth medium. A frozen sample of Matrigel was allowed to thaw overnight at 4 °C, then 50-µl samples were placed in a 60-mm dish and allowed to solidify 30 min at 37°C. SMCs were suspended in 5.0 ml complete medium and layered over the solidified gel.
before filming. To prepare SMCs cultured in diluted Matrigel, cells were mixed in 5.0 ml complete medium containing 0.25 ml Matrigel and placed in a 60-mm dish. In another experimental series, the effect of a collagen matrix on SMC behavior was studied by mixing cells in complete medium with a solubilized preparation of type I collagen from rat-tail tendon (Serva, Feinbiochemica, Heidelberg, NY). The collagen was mixed with complete growth medium at a final concentration of 0.75 mg/ml. SMCs were added to the mixture at a concentration of $4 \times 10^4$/ml. One milliliter of the SMC-collagen mixture was placed in a 60-mm dish and allowed to gel at $37^\circ$ C for 30 min (19), after which 5.0 ml of complete medium was layered over the gel. For filming, culture dishes were sealed and films were made as described below.

**Growth of SMC in Matrigel-conditioned medium.** To determine if mitogens exist within Matrigel, conditioned medium was prepared in the following manner. One milliliter of Matrigel was placed in a 60-mm culture dish and allowed to solidify, after which 5.0 ml complete growth medium was added. Control dishes contained 5.0 ml complete growth medium only. Control and Matrigel-containing dishes were incubated at $37^\circ$ C in 10% CO$_2$ for 24 and 48 h, after which the conditioned medium was removed and filtered through a 0.45-µm membrane (Gelman). To evaluate growth effects of the conditioned medium, SMCs were cultured for 3 d in the presence of Matrigel-conditioned medium or control medium. Cell proliferation was assessed by uptake of methylene blue using the method of Oliver, et al. (17).

**Time-lapse cinematography.** SMCs were seeded at $1.0 \times 10^5$ into 60-mm petri dishes in 5.0 ml complete medium and allowed to attach for 2 h. The dishes were fitted with a Cooper dish lid (Falcon) and sealed with sterile Vaseline. The cinematography apparatus consisted of a Sage time-lapse unit, 16mm Bolex camera, and Zeiss standard microscope with long working distance condenser and 10X phase objective. The stage was warmed with an airstream incubator (Nicholson Precision). Kodak Plus X reversal film was used and the cultures were filmed at a rate of one frame/min, for 3 to 6 d. Analysis of cell division and migration activity of individual cells was analyzed in two ways. In one method, an analytical projector (NAC) was used and the film was projected onto a screen. Data were obtained by an operator manually following the cells from division to division. Cell cycle (interdivision) times were calculated from the filming rate and the number of elapsed frames between mitotic events. In the second method, the films were converted to video tapes. Video images were digitized by a personal computer, and the cell division and migration determined via a specially designed interactive computer program. Cells were analyzed for time spent in motion, total micrometers moved per hour, direction of movement, and the number of transitions of direction (i.e. angular change of trajectory path).

**Data analysis and statistics.** Data obtained from analysis of films included total number of cell divisions, interdivision time, migration rate, and migration trajectories. Natural logarithms were used to transform the interdivision time (IDT) and migration rate (MIG) values to obtain a more normal distribution (14). Measurements of cell size (cross-sectional area of cell spreading) were made on fixed and stained cells using a Zeiss Videoplan image analyzer. Analysis of variance was used to determine significance of effects of experimental treatments on cell division and migration behavior. All values are presented as the mean and standard error with significance at $P < 0.05$.

**RESULTS**

**Cell division and interdivision time.** Cell division in cultures of SMCs growing adjacent to solidified Matrigel was significantly higher than in control cultures grown on plastic, $P = 0.004$ (Fig. 1 A). The proliferation of SMCs cultured in a mixture of Matrigel diluted 1:20 in complete growth medium was similar to cells grown on plastic. Cell IDT was distinctly affected in cells growing proximate to Matrigel. IDT distributions are plotted as cumulative percents in Fig. 1 B. The median IDT for SMCs growing near Matrigel...