PLASMA-DERIVED SERUM AS A SELECTIVE AGENT TO OBTAIN ENDOTHELIAL CULTURES FROM SWINE AORTA

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SUMMARY

Endothelial cell and smooth muscle cell cultures from artery wall provide a potential model system for studying cellular processes involved in atherogenesis. To prepare serial subcultures of swine arterial endothelial cells that are free of smooth muscle cells without either selecting a small population or subjecting the cells to cytotoxic conditions, we used swine plasma-derived serum (SPDS) to establish conditions in which endothelial cells have a growth advantage. Endothelial cells were collected by collagenase digestion and smooth muscle cell cultures were prepared by outgrowth from explants of arterial medial segments. Growth rates were compared when each cell type was maintained on SPDS, or fetal bovine serum (FBS), or swine whole serum (SWS). When 20% FBS or SWS were used the doubling times were <30 h for both endothelial cells and smooth muscle cells. On 20% SPDS the doubling time for endothelial cells was 32 h, but for smooth muscle cells it was at least 168 h. Using SPDS, we prepare endothelial subcultures from swine aorta that express principally polygonal morphology at confluence. Endothelial cell cultures grown on SPDS have higher angiotensin-converting enzyme than those grown on FBS.

Key words: plasma-derived serum; selective agent; swine endothelial cells.

INTRODUCTION

Endothelial cells in culture have a characteristic morphology and growth pattern: They form a monolayer of tightly packed, polygonal cells. Primary cultures that are predominantly endothelial cells can be prepared conveniently by dislodging the cells from the surface of large blood vessels with proteolytic enzymes (1-3). Two difficulties have arisen in the preparation of pure endothelial cell lines from such primary cultures. (a) The primary culture may be contaminated with smooth muscle cells, which may also grow out during subculture, and which have a growth advantage as the cultures become confluent in that they are able to grow as multilayers (4). (b) A morphology other than the typical monolayer of polygonal cells often appears, even in cultures that are apparently free of smooth muscle cells (4). These elongated cells, which can grow in multilayers, superficially resemble smooth muscle cells. However, from the evidence presented by Schwartz (4) and Cotta-Pereira et al. (5) it seems clear that the elongated cells, called "sprouts", are a morphologic variant of endothelial cells, rather than cells derived from smooth muscle cells.

Schwartz (4) has used [3H]thymidine for the selective elimination of smooth muscle cells from cultures of bovine aortic endothelium. Gospodarowicz et al. (6) have selected bovine aortic endothelium by supporting their growth in sparse culture with bovine fibroblast growth factor. It is desirable to have a method for preparing endothelial cell cultures in sufficient mass for enzymatic or biosynthetic studies, which avoids both treatment with cytotoxic agents and selection of a potentially atypical subpopulation.

Ross and Vogel (7) and Ross et al. (8) have shown that smooth muscle cells require a growth factor derived from platelets. Endothelial cells are able to grow without this factor (9-12). Cotta-Pereira et al. (5) have used bovine plasma-derived serum to delay or prevent the overgrowth of bovine endothelial cultures by smooth muscle. Use of plasma-derived serum also delayed the appearance of the sprout morphology. We report
here that the use of swine plasma derived serum enables us to routinely prepare swine arterial endothelial cultures that are apparently free of smooth muscle, and that express principally the polygonal morphology typical of large vessel endothelium.

**MATERIALS AND METHODS**

**Media and solutions.** Fetal bovine serum was obtained from Flow Laboratories, Inc., Rockville, MD, and collagenase was CLS II from Worthington Biochemical Co., Freehold, NJ. All other materials and chemicals for tissue culture were from Grand Island Biological Co., Grand Island, NY.

Tissue culture medium (Dulbecco-Vogt Modified Eagles'-DME) , trypsin suspension (0.05% in versene) , collagenase solution (0.1% in DMEM without serum), and Hanks' balanced salt solution (HBSS) were used according to the method described previously (13). Sera were not heat inactivated.

**Isolation and culture of cells.** Endothelial cells were isolated and cultured according to the methods described previously (13) with the following modifications: Handling of the aortas was less gentle than described previously to increase the initial yield of suspended cells. The vessel was pinched between the fingers, and vigorous mechanical stress was used in pipetting the collagenase solution against the inside vessel wall to dislodge cells. The suspended cells from the collagenase digest were planted in DMEM + 20% FBS containing nystatin in T-25 flasks. However, at the time of the first passage when the cells were transferred to a T-75 flask, and in all subsequent passages, endothelial cells were planted in DMEM + 20% SPDS. Subsequent subcultures were made at a split ratio of 1:2.

Smooth muscle cells were isolated according to the following modifications of the methods of Ross (14) and Augustyn et al. (15). Two hundred, one cubic millimeter segments of middle media from swine thoracic aorta were incubated in an 850 cm² roller bottle with 40 ml DMEM + 10% FBS (containing nystatin). The speed of the roller apparatus was adjusted to 0.5 rpm. Cells appeared after 7 to 10 d. Medium was changed once a week until cells reached confluence (in 2 wk). For experiments, cells were removed by exposure to 0.05% trypsin (Difco Laboratories, Detroit, MI) and the resultant cell suspension planted in T-75 flasks in DMEM + 10% FBS. All subsequent subcultures of smooth muscle cells were maintained on 10% FBS. Confluent cells were passed at a split ratio of 1:3.

**Preparation of SPDS and SWS.** Swine blood (500 ml) was collected in a cold Nalgene beaker containing 50 ml 3.8% sodium citrate in 0.1 M Tris-Ringers solution, pH 7.2, and held on ice until processed (within 1 h). The blood was centrifuged at 1,000 xg for 15 min at 4°C. The cell sediment was discarded and the supernatant was centrifuged at 15,000 xg for 30 min at 4°C in a Nalgene centrifuge bottle. The plasma was diluted with Ringers solution (1 vol Ringers to 6 vol plasma) and made 20 mM in CaCl₂. After 2 h incubation at 37°C, the clotted plasma was stored at −20°C overnight. After thawing, the shrunken clot was discarded and the plasma-derived serum (200 to 300 ml) was filtered and stored in 100 ml aliquots at −20°C. For use, SPDS from at least five animals was pooled. A heavy precipitate forms in this SPDS after freezing and thawing, so it was necessary to filter each freshly prepared solution of DMEM + 20% SPDS. Sera for the experiments described here were prepared in this laboratory. This preparation (SPDS) is now available commercially (Sterile Systems, Logan, UT), and we have found that the commercial preparation gives results comparable to those presented.

For preparation of SWS, swine blood was collected in citrate according to the above method. It was diluted as above with Tris-Ringers solution and made 20 mM in CaCl₂ (on the collection day) and incubated for 30 min at room temperature. After the clot formed the beaker was ringed with a wooden pick and refrigerated at 4°C overnight. The clot was removed, discarded, and the SWS was spun at 15,000 xg for 15 min. Swine whole serum was stored in 100 ml aliquots at −20°C. For use, SWS from two to three hogs was pooled. Medium was made 20% in SWS and filtered as for SPDS above.

**Growth experiment.** Endothelial and smooth muscle cultures were maintained on 20% SPDS and 10% FBS, respectively, until the time of the growth experiment. At that time cells were planted at 1.0 x 10⁶ cells/3.5 cm dish in 15 dishes and fed with 2 ml DMEM containing 20% of the appropriate test serum. Cells from three dishes were counted after trypsinization using a hemocytometer after 24, 48, 72, 96, and 168 h. Cells were fed every other day.