DIFFERENTIAL EFFECTS OF SOLUBLE AND IMMOBILIZED FIBRONECTINS ON AORTIC ENDOTHELIAL CELL PROLIFERATION AND ATTACHMENT

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SUMMARY

We studied the effects of soluble and immobilized forms of plasma fibronectin on bovine aortic endothelial cell (AEC) proliferation and attachment. Soluble fibronectin stimulated AEC growth at 10 μg/ml, but at higher concentrations of soluble fibronectin AEC growth was progressively inhibited. The growth rates of arterial smooth muscle cells (ASMC) and dermal fibroblasts (DF) were not altered by soluble fibronectin concentrations of 10 to 100 μg/ml. Plasma fibronectin, immobilized by attachment to culture dish surfaces, had no significant effects on the proliferation of any of the cell types examined. The attachment rates of AEC were decreased in the presence of 50 μg/ml soluble fibronectin. Immobilized fibronectin increased the rate of AEC attachment, but had no significant effects on ASMC or DF attachment; however, 12 h after plating there was nearly 100% attachment in all groups, whether or not fibronectin was present in the system. That soluble and immobilized fibronectins elicit disparate cellular responses is consistent with published reports of different cell surface receptors for different forms of the protein; in this manner, cells ensheathed in an interstitial matrix containing immobilized fibronectin could still respond to soluble fibronectin in the extracellular milieu.

Key words: fibronectins; endothelium; cells, cultured; proliferation.

INTRODUCTION

Fibronectin, a plasma glycoprotein and a major constituent of cell surfaces and extracellular matrices, has multiple effects on the expression of cellular functions. The adhesion, spreading, and migration of a variety of normal cell types are modulated by fibronectin, as are the morphology, spreading, and attachment of several transformed cell lines [reviewed in (18,19)]. Considerable effort has been devoted to elucidating differences between plasma and cellular forms of fibronectin, although the activities of both forms appear equal in assays of cell adhesion and spreading (27), as well as in fibronectin-mediated phagocytosis (23). It has been reported, however, that cellular fibronectin is more effective in restoring a "normal" morphology to transformed cells and in effecting hemagglutination, when compared to the plasma form (32). Furthermore, minor structural differences between fibronectins of plasma and cellular origins have been elucidated by biochemical (13) and immunologic methods (2). More recent evidence indicates that plasma and cellular fibronectins are derived from a single gene, with posttranscriptional modifications leading to minor differences in the primary structure of the two forms (18).

One of the most demonstrable differences between the plasma and cellular forms of fibronectin is the solubility of the protein at physiologic pH. Whereas plasma fibronectin is readily soluble at pH 7.4, the cellular form has been reported to be soluble only in alkaline solutions (1,33). Recently it was shown that plasma fibronectin was incorporated into tissues in vivo (26) and into the matrices of cultured cells (14,24), suggesting that the source of fibronectin may not be as critical a determinant of cell function as is the form in which it interacts with cells. Thus, soluble plasma fibronectin may exert different effects on cell metabolism from those elicited by fibronectin in an immobilized form.

Vascular endothelial cells are unique in that their luminal surfaces are exposed to high concentrations (approximately 300 μg/ml) of plasma fibronectin in presumably soluble form, whereas abluminal surfaces are tightly anchored in basement membranes and interstitial matrices containing a number of connective tissue proteins, including immobilized fibronectin. During angiogenesis normally "sedentary" endothelial cells become highly invasive as they form new blood vessels. Capillary sprouts develop from endothelial cells that have detached from existing blood vessels in a complex process requiring directed migration, proliferation, and reorganization of cells into a functional structural element. We previously reported that soluble plasma fibronectin stimulates chemotaxis in aortic
endothelial cells (5). It seemed likely that fibronectin modulates other cellular responses in angiogenesis as well. Indeed, as we describe in this report, soluble and immobilized forms of plasma fibronectin have disparate effects on the proliferation and attachment of cultured aortic endothelial cells.

MATERIALS AND METHODS

Cell cultures. Bovine aortic endothelial cells (AEC) and arterial smooth muscle cells (ASMC) were obtained from the thoracic aortae of 2 to 3-d-old calves as previously described (20). In addition, single cell-derived clones of AEC were prepared by diluting suspensions of AEC from the second in vitro subculturing to a concentration of 10 cells/ml of media and placing 100 μl of the cell suspension in 16-mm tissue culture wells. The wells were then examined by phase contrast microscopy and only those wells containing a single cell were maintained. Aortic endothelial cells grew in a characteristic cobblestone morphology and reacted to anti-Factor VIII antibody (20).

Bovine dermal fibroblasts (DF) were cultured from finely minced explants of auricular dermis from which the epidermis had been removed by gentle scraping. AEC and ASMC cultures were maintained in RPMI 1640 medium containing 10 to 20% fetal bovine serum (FBS) (Irvine Scientific Co., Irvine, CA), 50 μg/ml gentamicin, and 5 μg/ml amphotericin B. DF were maintained in Dulbecco’s minimum essential medium (DMEM) containing 10% FBS and antimicrobials at the concentrations used for AEC and ASMC cultures. Cells were routinely subcultured by trypsinization upon reaching confluence. In these experiments, only cells from the 4th through 8th in vitro subculturings were used. Cells were split at a 1:4 ratio for subculturing.

Preparation of fibronectin. Human and bovine plasma fibronectins were prepared by the method of Engvall and Ruoslahti (9) after prechromatographing 50 ml of plasma through a 15 × 2.5-cm column of Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, NJ). Human and bovine fibronectins were also purchased from Collaborative Research, Inc. (Waltham, MA), Bethesda Research Laboratories (Gaithersburg, MD) and Calbiochem-Behring, Inc. (La Jolla, CA). The purity of the fibronectin preparations was assessed by two dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5). Fibronectin was stored lyophilized at −20°C until just before use, when it was dissolved in 50 mM 3-(cyclohexylamino) propane-sulfonic acid (CAPS) buffer, pH 8.6 (Calbiochem-Behring, Inc.). Fibronectin concentrations were determined by turbidometric immunoassay (Boehringer Mannheim Biochemicals, Indianapolis, IN) and by enzyme-linked immuno assay (ELISA) (6).

Cell proliferation. Confluent monolayers of cells were dissociated with 0.05% trypsin, 0.02% EDTA at 4°C for 3 to 5 min with gentle agitation. The trypsin was then inactivated by adding a threefold molar excess of soybean trypsin inhibitor (Worthington Biochemical Co., Freehold, NJ) to the cells; the cells were centrifuged at 700 ×g for 5 min, then washed twice in RPMI 1640 or DMEM containing 5% FBS. The cells were suspended at a final concentration of 50 000 cells/ml in media containing 5% FBS and plated into 16-mm tissue culture wells (Falcon Plastics, Inc., Oxnard, CA). In some experiments the dishes were precoated with fibronectin by placing 1 ml of a 2-mg/ml fibronectin solution in the empty wells. The covered wells containing the fibronectin solution were left at room temperature (approximately 23°C) for 12 h. The wells were then washed extensively with phosphate buffered saline before being plated with cells.

Twenty-four hours after the cells were plated, the medium was replaced with fresh medium containing 5% FBS and soluble fibronectin. We found that a small amount of FBS in the medium was necessary for the exponential proliferation of AEC in our assays. Medium containing 5% FBS had a fibronectin concentration of 1.4 ± 0.4 μg/ml when no additional fibronectin was added. Cell counts were determined daily using an electronic cell counter after cells were dissociated by trypsinization. Cell viability was measured by trypan blue dye exclusion.

![Fig. 1. Proliferation of cells in the presence of soluble fibronectin. Aortic endothelial cells (A), arterial smooth muscle cells (B), and dermal fibroblasts (C) were grown in the presence of 50 μg/ml soluble fibronectin (●). Controls consisted of cells grown in medium containing 5% FBS only (○). To account for attachment differences, data are expressed as the percentage of cells present on Day 1 ± SD from three separate experiments, each with quadruplicate observations.](image)