Attachment of smooth muscle cells to collagen and their migration toward platelet-derived growth factor

(type V collagen/substrate adhesion/chemotaxis/mitogens)

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ABSTRACT Smooth muscle cells use fibronectin to bind to type I and type III collagens but bind to type V collagen by a trypsin-resistant intrinsic glycoconjugate. The binding site on type V collagen is located in the αA chain. By using collagen-coated filters in a modified Boyden chamber assay for chemotaxis, it was observed that the platelet-derived growth factor was chemotactic for smooth muscle cells but that several other growth factors were inactive. We suggest that the migration of smooth muscle cells from the media to the intima of a blood vessel, which leads to the formation of an atherosclerotic plaque, may be the result of a chemotactic migration of cells responsive to the platelet-derived growth factor.

Smooth muscle cells are connective tissue cells that are present in blood vessels and other pulsatile tissues. These cells synthesize various matrix proteins including proteoglycan (1), elastin (2), and types I, III, and V collagen (3-6). Because atherosclerotic plaques are composed in large part of smooth muscle cells and the extracellular products they produce (7), there is much interest in factors that regulate the growth and distribution of these cells.

Current theories suggest that the formation of the atherosclerotic plaque is initiated at sites where endothelial cells are lost from the basement membrane (for reviews, see refs. 7-9), leading to an accumulation of platelets and other blood elements at those sites. Under such conditions, platelets release the platelet-derived growth factor (PDGF), a potent mitogen for smooth muscle but not for endothelial cells (10, 11). It is assumed that PDGF causes the proliferation of smooth muscle cells beyond the basement membrane to the inner surface of the blood vessel. Interestingly, Benditt and Benditt (12) found that the cells in many plaques are monoclonal suggesting that a single cell or a single type of cell (13) proliferated to form a plaque.

Recently, we and others have studied the attachment and migration of connective tissue cells. Fibroblasts, for example, are attracted to lymphokines (14), macrophage factors (15), activated serum (16), fibronectin (17), the cell binding fragment of fibronectin (18), and collagen fragments (19). Fibroblasts require a substrate of collagen of types I-IV for attachment and migration in the Boyden chamber assays for chemotaxis (14).

Here, we confirm earlier work on smooth muscle cells that indicates that smooth muscle cells use fibronectin to attach to types I and III collagens (20). We have extended these studies to show that smooth muscle cells attach preferentially to type V collagen without a requirement for fibronectin. When attached to collagen, the cells migrate toward PDGF in the Boyden chamber assay for chemotaxis. The chemotactic activity of

PDGF may be of importance in the early stages of atherosclerosis.

MATERIALS AND METHODS

Materials. Tissue culture media were purchased from GIBCO. Fetal calf serum and bovine serum albumin were obtained from Reheis Chemical (Kankakee, IL). Trypsin and elastase were from Worthington. Neuraminidase and concanavalin A were obtained from Sigma. Collagens were prepared as follows: type I, from rat tail tendon (21); type II, from a rat chondrosarcoma (22); type III, from fetal calf skin (23); type IV, from a murine tumor which produces an extracellular matrix of basement membrane (24); and type V, from human placental membranes (25). The A and B chains of type V collagen were isolated according to the procedure of Bentz *et al.* (26). Fibronectin was prepared from third-passage chicken embryo fibroblasts (27). Electrophoretically homogeneous PDGF was the generous gift of Charles Scher and Charles Stiles (Sidney Farber Cancer Institute, Boston). Fibroblast growth factor (FGF) and epidermal growth factor (EGF) were obtained from Collaborative Research (Waltham, MA). These factors were tested for mitogenic activity by the method described by Scher *et al.* (28).

Cell Culture. Explant cultures of aortic media from fetal sheep of 60-100 days' gestation were prepared by using the methods described by Ross for adult monkey aortic smooth muscle cells (29). The cells were cultured in T-75 flasks in Dulbecco's modified Eagle's medium (DME medium) supplemented with 10% fetal calf serum. Within 4-5 weeks, sufficient cells had migrated from the explant for subculture. These cells exhibited the characteristic hill-and-valley appearance of vascular smooth muscle cells in culture. Also, these cells synthesized collagen types I, III, and V and elastin which is characteristic of smooth muscle cells in culture (J. Davidson and R. Crystal, personal communication).

Cell Attachment. Cell attachment assays were done as described by Kleinman *et al.* (30). Briefly, cells were released from the tissue culture flask by treatment with 0.1% trypsin/0.1% in EDTA for 10-15 min at 37°C. The cells were then suspended in DME medium containing bovine serum albumin at 1 mg/ml (A/DME medium) and centrifuged at 500 $\times g$ for 5 min. The cells were then resuspended in A/DME medium at a concentration of $1-2 \times 10^6$ cells per ml. Cells were added to 17-mm wells of bacteriological plastic Linbro plates (Flow Laboratories, McLean, VA) that were coated with collagen (2 μg per dish) and contained 0.2 ml of A/DME medium and the factors to be assayed for adhesion. The cells were allowed to attach for various times. The unattached cells were removed and the plates were washed three times with phosphate-buffered saline. The at-

tached cells were released with trypsin/EDTA and counted with an electronic cell counter (Coulter, Hialeah, FL). Data are expressed as the percentage of cells that attached; each value represents the mean of triplicate assays which did not differ by more than 10%.

Chemotaxis Assay. Chemotaxis assays were performed by using modified Boyden chambers as described by Postlethwaite *et al.* (14). Nucleopore filters (pore diameter, 8 μ m) were treated with 0.5 M acetic acid overnight and then incubated for 48–72 hr at 25°C in a collagen solution (100 μ g/ml). The coated filters were air dried. The attractant in A/DME medium was added to the lower chamber, beneath the collagen-coated filter, and freshly trypsinized cells (6×10^4 per assay) were added to the upper chamber. The chambers were incubated for 4 hr at 37°C in an atmosphere of 95% air/5% CO₂. The filters were removed, fixed, and stained with hematoxylin, and the number of cells on the lower surface of the filter was determined by counting the number of nuclei per 10 high-power fields ($\times 400$) after removal of the cells from the upper surface. All assays were performed in triplicate and these values did not differ by more than 10%.

RESULTS

Attachment of Smooth Muscle Cells. Previous studies have shown that smooth muscle cells utilize fibronectin to attach to types I and III collagens (20). We confirmed that the attachment of these cells to types I and III collagens was increased in the presence of added fibronectin (Fig. 1) or serum (not shown). The attachment of the cells to type I collagen was inhibited by cycloheximide in the absence but not in the presence of added fibronectin (Table 1). These results confirm those of Gold and Pearlstein (20) and indicate that smooth muscle cells use fibronectin to attach to types I and III collagens.

The attachment of smooth muscle cells to type V collagen was independent of added fibronectin (Fig. 1) or protein synthesis (Table 1). On this substrate, approximately 80% of the cells adhered, and added fibronectin (Fig. 1) or serum (not shown) did not alter this level of attachment. Because type V collagen contains two different chains (α A and α B), we examined the at-

FIG. 1. Attachment of embryonic sheep aortic smooth muscle cells to types I (●), III (▲), and V (■) collagens in the presence of increasing amounts of fibronectin. After preincubation of the substrates for 1 hr with the designated amount of fibronectin, the cells were added and their attachment was determined after an additional hour.

Table 1. Effect of cycloheximide on smooth muscle cell attachment to collagen

Collagen substrate	Cycloheximide (10 μ g/ml)	Fibronectin (10 μ g/ml)	% cells attached*
I	—	—	25
I	+	—	15
I	—	+	75
I	+	+	74
V	—	—	77
V	+	—	73

* Each value represents the mean of triplicate assays which differed by less than 10%.

tachment of the cells to substrates prepared from each of these chains. The cells did not attach to α B but did attach to α A chains (Fig. 2). The number of cells which attached to the α A chain substrate was comparable to that which attached to the denatured type V collagen but was less than that which attached to native type V. These findings suggest that the α A chain contains the binding site for the smooth muscle cells and that the conformation of the site is important in the interaction.

Soluble type V collagen inhibited attachment of the cells to a type V collagen substrate. In contrast, soluble type I collagen had little effect (Fig. 3). These data indicate that the cellular binding sites for type V collagen are distinct from those for type I.

Nature of the Receptor for Type V Collagen. Because attachment occurs after liberation of the cells with trypsin, the binding sites for type V collagen appear to be trypsin resistant. Further incubation of the cells with elastase had no effect on attachment (Table 2). Urea (1 M) extraction, which removes fibronectin from other cells and alters their attachment (27), had no effect on the attachment of smooth muscle cells. Heparinase, which degrades glycosaminoglycans other than keratan sulfate, also was without effect. However, treatment with neuraminidase largely abolished attachment. Concanavalin A inhibited the attachment of the cells when it was present during the attachment assay but not when it was used to pretreat the type

FIG. 2. Attachment of embryonic sheep smooth muscle cells to increasing amounts of native and denatured type V collagen and its purified component chains. Type V collagen was denatured by heating to 56°C for 20 min prior to use for coating the dishes. The number of cells attached to the substrates was measured after 1 hr. ●, Native; ○, denatured; ■, α A; □, α B.

FIG. 3. Effect of soluble types I (○) and V (●) collagens on the attachment of embryonic sheep aortic smooth muscle cells to type V collagen. Varying amounts of soluble type I and type V collagens were added to the attachment assay at the same time as the cells. After 1 hr, the cells attached to the type V collagen substrate were counted.

V collagen substrate. These studies suggest that the type V collagen binding site is an integral component of the cell surface, is stable to proteolytic enzymes, and contains carbohydrate residues, including sialic acid, that are required for activity.

Chemotaxis of Smooth Muscle Cells. Preliminary studies showed that collagen-coated filters were required for the attachment and migration of smooth muscle cells in the Boyden chamber assay. Thorgeirsson *et al.* (31) have recently shown that serum derived from platelet-rich plasma stimulates the random migration of aortic smooth muscle cells over that observed in serum derived from platelet-poor plasma. For this reason, we investigated the chemotactic activity of factors released by aggregating platelets.

The material released from isolated platelets during thrombin-induced aggregation was chemotactic for the cells (Fig. 4). The migration of the smooth muscle cells was greater on type V than on type I collagen. However, in the presence of fibro-

FIG. 4. Migration of smooth muscle cells on substrates of types I and V collagens and on type I collagen with fibronectin (FN) (5 µg/ml) in response to platelet-released material (PRM) (1%, vol/vol) and purified PDGF (1 unit/ml).

nectin the migration on type I collagen was equivalent to that seen on type V. Because the chemotactic factor released from platelets was resistant to boiling, we tested PDGF for chemotactic activity. The smooth muscle cells migrated to the lower surface of the filter only when there was a gradient of PDGF in the Boyden chamber (Fig. 5). When equal concentrations of PDGF were present in both the upper and lower chambers, the cells did not migrate to the lower chamber. Also, a checkerboard analysis of the PDGF-induced smooth muscle cell migration demonstrated that PDGF is a true chemoattractant (data not shown). These data indicate that PDGF at concentrations that induce proliferative responses (0.5 unit/ml) can act as a chemoattractant for smooth muscle cells in the Boyden chamber. Other growth factors such as EGF, FGF, and insulin did not have chemotactic activity when tested at concentrations that would support cell proliferation.

DISCUSSION

Smooth muscle cells attach to different types of collagen by different mechanisms. As previously shown (20), these cells are stimulated to attach types I and III collagens by serum and by purified fibronectin. In addition to the fibronectin-mediated attachment to the interstitial collagens (types I-III), the cells

Table 2. Effect of various treatments on cell attachment to type V collagen

Treatment*	% cells attached†
Phosphate-buffered saline	77
Urea (1 M)	75
Elastase (1 mg/ml)	78
Heparinase (1 mg/ml)	76
Neuraminidase (4 units/ml)	21
Concanavalin A (20 µg/ml)‡	16

* Cells were first treated with trypsin/EDTA and washed in A/DME medium containing 1 mg of soybean trypsin inhibitor per ml. The cells were then resuspended in phosphate-buffered saline and exposed to the indicated secondary treatment for 15 min at 37°C. The cells were washed with A/DME medium and their attachment to type V collagen was assayed.

† Results are from duplicate experiments which varied by less than ±5%.

‡ Concanavalin A was used at a concentration below that which causes agglutination. Pretreatment of the type V collagen substrate with concanavalin A followed by washing to remove the unbound concanavalin A had no effect on cell attachment (≈82% of the cells attached).

FIG. 5. Effect of increasing amounts of PDGF on smooth muscle cell migration. ●, PDGF in lower chamber only; ○, PDGF in both upper and lower chambers.

appear to attach directly to type V collagen via a component of the smooth muscle cell surface. This cell surface component is not sensitive to trypsin, elastase, heparinase, or urea extraction. However, it is inactivated by treatment with neuraminidase and is blocked by concanavalin A, suggesting that it may be an integral membrane glycoprotein or glycolipid.

Other cell types have been shown to possess unique attachment factors for specific collagens. For example, chondrocytes attach to type II collagen by a serum factor, termed "chondronectin," which is distinct from fibronectin (32). The attachment of epithelial and endothelial cells to type IV collagen is mediated by laminin (33). Unlike the above cells, smooth muscle cell attachment to type V collagen is not mediated by a soluble factor. In fact, soluble type V collagen but not soluble type I collagen blocked adhesion of smooth muscle cells to substrates of type V collagen, suggesting further that these cells contain a surface ligand for type V collagen. In addition, type V collagen appears to be located primarily at the cell surface rather than only in the extracellular matrix (34, 35), and it may be considered to be a membrane-bound collagen.

PDGF is chemotactic for smooth muscle cells. It is unlikely that the movement of cells to the lower surface of the filter is a result of cell division because the time course is too short and PDGF alone is not sufficient for multiple cell doublings (36). Also when PDGF is present on both sides of the filter, no migratory response is seen. The finding that the smooth muscle cells migrate equally well on types I and V collagens, if fibronectin is present, indicates that the substrate for migration need not be of a specific type as long as it supports adequate adhesion for the cells. The ability to adhere to various types of matrices enables smooth muscle cells to migrate in a heterogeneous matrix, such as that of the arterial wall, with a high degree of efficiency.

We have also found that PDGF is chemotactic for fibroblasts (18). This suggests that PDGF may function to regulate the wound-healing response in the body. PDGF appears to act by attracting connective tissue cells to sites of injury and activating them for the proliferative response needed to repair the damage. Altered levels of PDGF or changes in the target cells' sensitivity to PDGF may be responsible for many fibrotic as well as wound healing disorders.

Our results suggest that the initial accumulation of smooth muscle cells in the intima could be due to their chemotactic response to PDGF. This hypothesis would agree with studies which indicated the need for functional platelets in atherogenesis (37, 38). In addition, the chemotactic response of the smooth muscle cells to PDGF may work as part of a selective mechanism in the formation of monoclonal plaques. According to Chamley *et al.* (39), only "modulated" smooth muscle cells can migrate or divide. These cells would make up only a small part of the smooth muscle cell population in the artery wall and may be the only cells responsive to the PDGF chemotactic signal. Any factor that might increase the percentage of these modulated cells in the population may cause an increased risk of atherosclerosis.

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