Inhibition of Endothelial Cell Movement by Pericytes and Smooth Muscle Cells: Activation of a Latent Transforming Growth Factor-β1-like Molecule by Plasmin during Co-culture

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Abstract. When a confluent monolayer of bovine aortic endothelial (BAE) cells is wounded with a razor blade, endothelial cells (ECs) spontaneously move into the denuded area. If bovine pericytes or smooth muscle cells (SMCs) are plated into the denuded area at low density, they block the movement of the ECs. This effect is dependent upon the number of cells plated into the wound area and contact between ECs and the plated cells. Antibodies to transforming growth factor-β1 (TGF-β1) abrogate the inhibition of BAE cell movement by pericytes or SMCs. TGF-β1, if added to wounded BAE cell monolayers, also inhibits cell movement. When cultured separately, BAE cells, pericytes, and SMCs each produce an inactive TGF-β1-like molecule which is activated in BAE cell-pericyte or BAE cell-SMC co-cultures. The activation appears to be mediated by plasmin as the inhibitory effect on cell movement in co-cultures of BAE cells and pericytes is blocked by the inclusion of inhibitors of plasmin in the culture medium.

THE organization of the cellular elements of the microvasculature is relatively simple since most capillaries and postcapillary venules consist only of endothelial cells (ECs)[1] and pericytes. In these vessels, the pericytes are located on the abluminal side of the ECs and appear to make direct contact with the ECs. In larger blood vessels, the two major cell types, the EC and the smooth muscle cell (SMC), are separated by the basal lamina which delineates the intima from the media.

While there is some data available on the effects of in vivo interactions between ECs and SMCs (Reidy et al. 1982, 1983), there is increasing evidence that pericytes may be negative regulators of EC proliferation and neovascularization in vivo (Ausprunk and Folkman, 1977; Crocker et al., 1970; De Olivera, 1966; Feldman et al., 1978; Kuwabara and Cogan, 1963). Recently Orlidge and D'Amore (1987) published experiments demonstrating that pericytes or SMCs suppressed EC division when co-cultured with ECs. The mediators and mechanisms for these in vitro and in vivo interactions have not been defined.

We have recently shown that when a confluent EC monolayer is wounded with a razor blade, the ECs move rapidly into the denuded space (Sato and Rifkin, 1988). This process requires basic fibroblast growth factor (bFGF) and is blocked by neutralizing antibodies to bFGF or by transforming growth factor-β1 (TGF-β1). In this paper, we report that EC movement into a denuded area is blocked by pericytes or SMCs. This process appears to involve the activation of latent TGF-β1-like molecules produced by the cells. Moreover, the conversion of the latent molecule to its active form is mediated by the protease plasmin.

1. *Abbreviations used in this paper*: BAE, bovine aortic endothelial; bFGF, basic fibroblast growth factor; EACA, e-amino-n-caproic acid; EC, endothelial cell; MEM, minimal essential medium; PA, plasminogen activator; SMC, smooth muscle cell; TGF-β1, transforming growth factor-β1.

Materials and Methods

Cell Culture

Bovine aortic endothelial (BAE) cells were isolated as described previously (Gross et al., 1982), and were cultured on gelatin-coated dishes in alpha minimal essential medium (MEM) containing 10% calf serum. Pericytes, isolated from bovine retinae, were the generous gift of Dr. Patricia A. D'Amore, (Harvard Medical School, Boston, MA) and grown in alpha MEM containing 10% calf serum.

SMCs were explanted from bovine or rat aortas. Bovine SMCs were grown in alpha MEM containing 10% calf serum, while rat SMCs were grown in DME containing 10% FCS. Fibroblasts were explanted from human foreskins or bovine embryonic skin and grown in DME containing 10% calf serum. NIH 3T3 cells were grown in DME containing 10% calf serum.

Wound Assays for BAE Cell Migration

Wound assays were performed as previously described (Sato and Rifkin, 1988). Briefly, confluent monolayers of BAE cells in 35-mm dishes were wounded with a razor blade. After wounding, the cells were washed with PBS and further incubated in alpha MEM containing 0.1% gelatin for 20 h at 37°C. The cells were fixed with absolute methanol after the incubation and stained with Giemsa. Cells that had migrated from the edge of the wound were counted in successive (seven) 125-μm increments at 100× using a light microscope with an ocular grid. The cell numbers represent the mean from at least four different fields.

Cell Migration in the Presence of a Second Cell Type

Immediately after wounding a BAE monolayer as described above, the second cell type was inoculated into the culture dish usually at 15,000 cells per dish, and incubated in alpha MEM containing 0.1% gelatin. After 20 h, the number of BAE cells that had migrated across the original wounded edge were counted at 100×. The values represent the mean from four different fields. BAE cells were distinguished from other cell types by shape, size, and nuclear morphology. In some experiments, the confluent BAE cells were prelabeled with 10 μg/ml of 1,1'-dioctodecyl-3,3,3',3' -tetramethylindocarbocyanine perchlorate-labeled acetyl LDL (DiI-acetyl-LDL) (Biomedical Technologies Inc., Stoughton, MA) for 4 h at 37°C. The cells were then washed three times with PBS, and the monolayer wounded. The cells were washed again with PBS and co-cultured with BAE cells which were plated at a density of 15,000 cells per 35-mm dish in alpha MEM containing 0.1% gelatin. After 20-h incubation, the culture was washed three times with PBS and fixed in 3% formaldehyde in PBS. Labeled cells were visualized by fluorescence microscopy using standard rhodamine excitation. Labeled cells that had migrated from the edge of the wound were counted at 400×. The values represent the mean from 10 different fields.

Additional wound assays were performed under conditions in which contact between the wounded monolayer and the second cell type was prevented by using 60 × 15-mm Cooper dishes (Falcon Labware, Oxnard, CA). BAE cells were grown to confluence in 60-mm dishes. Pericytes (1×10^5), BAE cells (4×10^5) or combinations of pericytes (1×10^5) and BAE cells (4×10^5) were plated on the inverted upper side of a Cooper dish and allowed to attach to the surface at 37°C. After 4 h, the upper side of the Cooper dish was washed with PBS to remove unattached cells. The BAE cell monolayer was wounded, covered with medium, and the upper side of the Cooper dish was placed over the BAE culture so that the monolayer of pericytes rested above the wounded BAE cells. After 20 h of incubation, the BAE cells that migrated were counted as before.

Preparation of Conditioned Medium from BAE Cells and Pericytes

BAE cells and pericytes were seeded separately in 100-mm plastic dishes with MEM containing 10% calf serum. When the cells had reached confluence, the cultures were rinsed with PBS and fresh serum-free media added. After 24-h incubation, media were collected and centrifuged to remove cell debris. Medium from co-cultures of BAE cells and pericytes was prepared as follows. 1.6×10^6 BAE cells and 4.0×10^5 pericytes were seeded in the same 100-mm plastic dish with MEM containing 10% calf serum, and incubated for 4 h at 37°C. The cells were rinsed with PBS and fed with serum-free medium. After 24 h, the medium was collected and centrifuged to remove debris. The conditioned media were acidified to pH 2.0 with HCl, and after 1 h neutralized with NaOH at room temperature.

Proteinase Inhibitors and Antisera

$Alpha_2$ plasmin inhibitor was a generous gift from Dr. P. Harpel (Cornell University Medical School, New York). Aprotinin and e-amino-n-caproic acid (EACA) were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-porcine TGF-β1 IgG was purchased from R & D Systems (Minneapolis, MN). This antibody has previously been shown to neutralize porcine and human TGF-β1 (Keski-Oja et al., 1987). According to the manufacturer this antibody does not cross-react with either acidic or bFGFs, EGF, or platelet-derived growth factor. This antibody neutralizes the TGF-β stimulation of anchorage-independent growth of normal rabbit kidney cells as well as TGF-β1 binding to its receptor. Nonimmune serum was prepared from normal rabbits.

Results

On one occasion in the course of analyzing the requirements for bFGF in BAE cell migration (Sato and Rifkin, 1988), we observed that at the site of wounding of the BAE cell monolayer the migration of the ECs appeared to be inhibited by two cells whose morphology was quite different than that of the surrounding cells (Fig. 1 *A*). The normal migration of the BAE cells in this experiment can be seen at the edges of Fig. 1 *A*. Since Orlidge and D'Amore (1987) have demonstrated that pericytes and/or SMCs could block EC division and since the major contaminant of BAE cell cultures might be expected to be SMCs, we tested, in a separate experiment, the effects on BAE cell migration of pericytes and SMCs plated directly into wounded cultures. As can be seen in Fig. 1, *B* and *C*, the presence of pericytes or SMCs inhibited the movement of the BAEs into the wound area. It appears that the BAE cells were able to migrate a short distance and then ceased moving. The migration of the control BAE cells in this experiment can be seen in Fig. 1 *D*. The total distance migrated by the cells in this experiment was not as great as that observed in the experiment illustrated in Fig. 1 *A*. This may result from the fact that the cells used in the experiment illustrated in Fig. 1 *A* were from a lower passage than those used in the experiment depicted in Fig. 1, *B–D*.

When the cell migration was quantitated by counting the number of BAE cells that had moved into the denuded area, the inhibition of movement was between 40 and 60% (Fig. 2). This experiment has been repeated over 25 times and the degree of inhibition has averaged 47%. In this assay all cells that had either partially or completely moved across the initial wound boundary are scored as positive. Thus, the value obtained is weighted towards a lack of inhibition, since cell movement which occurs before inhibition is established is in-

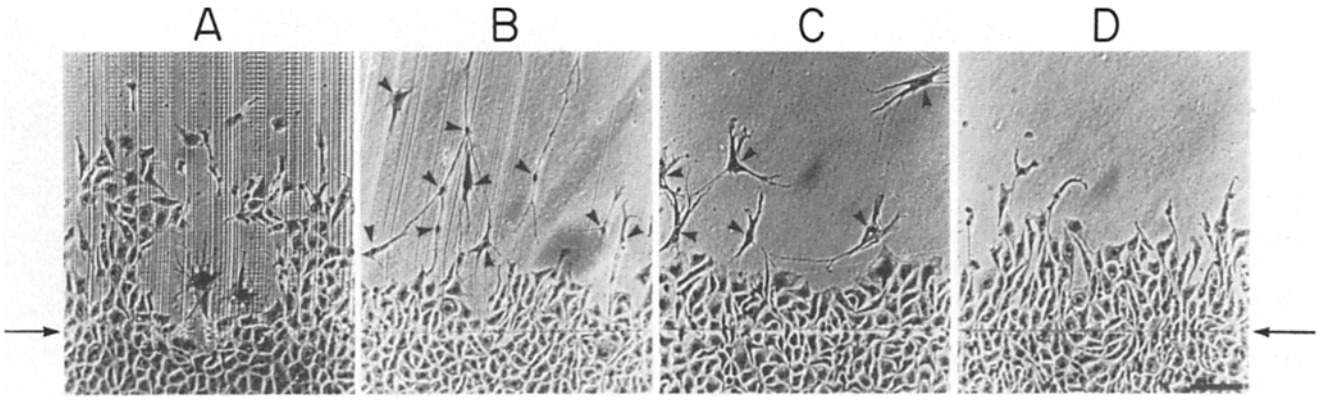

Figure 1. BAE cell migration in the presence of pericytes or SMCs. Confluent monolayers of BAE cells were wounded with a razor blade as described in Materials and Methods. The cells were then either incubated overnight (*A* and *D*) or co-cultured with SMCs (*B*) or pericytes (*C*) as described in Materials and Methods. After 20-h incubation the cells were fixed, stained, and photographed. The arrows point to the original edge of the wound. The arrowheads in *B* and *C* identify the SMCs and pericytes added to the wounds. Bar, 200 μm.

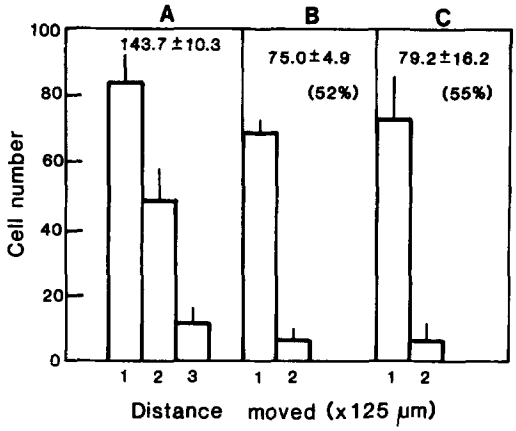

Figure 2. Quantitative inhibition of BAE cell migration by pericytes or SMCs. Confluent cultures of BAE cells were wounded as described in Materials and Methods. Immediately thereafter 1.5×10^4 pericytes or SMCs were added to the cultures and the cells incubated for 20 h, fixed, and stained. Migration was quantitated by observing the cells with a grid marked in increments of 125 μm. The number of cells within each 125 × 875 μm area in four fields was counted using the original mark made by the razor blade as the origin. The results are presented as the average number of cells per field. At least four fields were counted in each sample. *A*, control; *B*, SMCs; *C*, pericytes. The total number of cells in each sample was (*A*) 143, (*B*) 75, and (*C*) 79. The number of cells that had moved >125 μm was (*A*) 60, (*B*) 6, and (*C*) 6.

cluded in the calculation. However, if the number of cells present in the first 125-μm segment is excluded and only those cells that had moved 125 μm or more from the wound edge are scored, an even greater inhibition of movement was observed. For example, in Fig. 2 the inhibition by SMCs increases from 48 to 88%, and that by pericytes from 45 to 88%. While the experiment reported in Fig. 2 was performed in the absence of serum, similar results were obtained in the presence of 10% calf serum (data not shown).

The cell specificity of the inhibition was explored by seeding different types of cells after wounding the BAE monolayer (Table I). Bovine SMCs and pericytes inhibited movement as did bovine embryonic skin fibroblasts. Human fibroblasts did not inhibit nor did NIH 3T3 cells or rat SMC. BAE cells did not block the movement of ECs from the wound edge. This was shown by first labeling the BAE cells in the monolayer with DiI-acetyl-LDL, wounding and seeding fresh BAE cells which were not labeled with DiI-acetyl-LDL. Cells that had migrated were scored using a fluorescence microscope to distinguish between the original cells and the freshly plated cells. BAE cells were capable of blocking the movement of pericytes when a pericyte monolayer was wounded and BAE cells plated into the denuded area (data not shown). These results indicate that there is a cellular specificity to the inhibition. In addition, the lack of an effect of BAE cells plated into the wound area of a BAE culture indicates that the decrease in cell movement is not simply a result of contact inhibition of movement since BAE cells are known to be contact inhibited in a homotypic fashion (Schwartz et al., 1980). The inhibitory effect of bovine pericytes on BAE cell migration was density dependent (Fig. 3). Some inhibition was observed with as few as 5×10^3 cells. The degree of inhibition increased with increasing cell number and reached a maximum at 10^5 cells per dish.

In an earlier paper (Sato and Rifkin, 1988), we demonstrated that TGF-β1 blocked cell migration when added to cultures of wounded BAE cells. Because of this result and the fact that most cells produce TGF-β, we investigated the effect of a neutralizing anti-TGF-β1 IgG on pericyte and SMC inhibition of BAE cell migration. Fig. 4 shows the results obtained when wounded BAE cell monolayers were incubated with pericytes in the presence of anti-TGF-β1 IgG or nonimmune IgG. Incubation of the BAE cells with pericytes resulted in an inhibition of cell movement by 36% (68% if only cells that had moved 125 μm were counted) (Fig. 4 *D*). The inclusion of nonimmune IgG or anti-TGF-β1 IgG induced a small increase in the movement of control BAE cells (Fig. 4, *B* and *C*), but anti-TGF-β1 IgG completely neutralized the inhibition mediated by the pericytes (Fig. 4 *E*). A slight stimulation of migration was observed with the nonimmune serum. The reason for this is unknown but has been described previously (Sato and Rifkin, 1988). When the migration assays were conducted in the presence of nonimmune IgG and pericytes (Fig. 4 *F*), pericyte inhibition of cell migration was relatively unaffected when compared to the immune control (Fig. 4 *E*) but was inhibited when compared to the movement of BAE cells in nonimmune serum (Fig. 4 *C*). Similar results were obtained with SMCs (data not shown).

These results suggested that the co-culture of BAE cells and pericytes caused the release of a molecule that was inhibitory for cell movement and whose activity was eliminated by neutralizing antibodies to TGF-β1. Under normal conditions, many cultured cells release TGF-β1 in an inactive or latent form (Lawrence et al., 1984; Kryceve-Martinerie et al., 1985; Lyons et al., 1988). This inactive form can be converted into an active form by acid or denaturing agents. Indeed, both BAE cells and pericytes released a molecule into their culture medium which after acid treatment inhibited BAE cell migration and whose activity was abolished by anti-TGF-β1 IgG (Fig. 5). The conditioned medium without acid treatment was noninhibitory as was acid-treated MEM (Fig. 5). However, conditioned medium collected from co-cultures of BAE cells and pericytes inhibited BAE cell movement without acid pre-treatment (Fig. 5). This inhibition was abrogated by the anti-TGF-β1 IgG (Fig. 5).

The results of the experiment illustrated in Fig. 5 indicated that in co-cultures of BAE cells and pericytes a diffusible in-

Table I. Specificity of Inhibition of BAE Cell Movement

Cell type added	Degree of inhibition
	%
–	0
SMCs	48
Pericytes	45
BAE	0
Human foreskin fibroblasts	7
Bovine embryonic skin fibroblasts	53
3T3	12
Rat smooth muscle cells	9

Confluent monolayers of BAE cells were wounded as described in Materials and Methods. The cell type to be tested was added to the cultures at a density of 1.5×10^4 cells/dish, incubation continued overnight, and the cell movement quantitated as described in Materials and Methods.

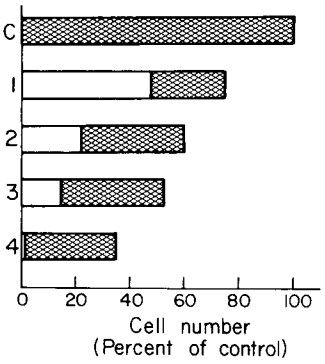

Figure 3. Cell number-dependent inhibition of BAE cell movement by pericytes. Confluent cultures of BAE cells were wounded as described in Materials and Methods. Increasing numbers of pericytes were plated into wounded BAE cell cultures, the cells incubated for 20 h, and the cells fixed, stained, and counted. The results are presented as the percent of cells which had moved in the control cultures. *C*, control; *1*, BAE cells plus 0.5×10^4 pericytes; *2*, BAE cells plus 1.5×10^4 pericytes; *3*, BAE cells plus 3.0×10^4 pericytes; *4*, BAE cells plus 1.0×10^5 pericytes. ▩, total cells that had moved across wound margin; □, cells that had moved further than 125 μm. The absolute number of cells counted in the control was 97, while the number of cells that had moved >125 μm in the control was 27.

hibitory substance is liberated, with the properties of TGF-β1, and suggested that if heterotypic co-cultures of BAE cells and pericytes were positioned in close proximity to a wounded BAE cell monolayer, inhibition of movement should be observed. We attempted to demonstrate this by the following experiment (Fig. 6). Either homotypic cultures of BAEs or pericytes or heterotypic co-cultures of BAE cells and pericytes were plated on the upper surfaces of Cooper dishes, allowed to attach, and the tops inverted and placed over wounded monolayers of BAE cells. In this conformation the two surfaces are separated by <1.5 mm. After 20-h incubation, the BAE cell monolayer was fixed, stained, and the cells that had migrated counted. Under these conditions, homotypic co-cultures of BAE cells or pericytes positioned above the wounded BAE cells had no effect on migration of cells, while the heterotypic co-cultures of BAE cells and pericytes inhibited BAE cell movement by 50%. The activity in the co-culture medium was stable to freezing and thawing and could be used to inhibit migration of target cells in the absence of the activating cells (data not shown). The effect of the heterotypic co-cultures was blocked by anti-bFGF-β1 IgG, whereas the inclusion of nonimmune IgG did not affect the inhibition of migration (data not shown). This indicates that the inhibitory effect of the co-cultures is mediated through the release of a soluble active TGF-β1-like molecule and that this requires heterotypic cell contact or very close apposition of the two cell types.

Therefore, it appeared that when cultured separately both BAE cells and pericytes produced latent TGF-β1, while when the two cell types were cultured together an active TGF-β1-like molecule was formed. It has been shown that one mechanism for the activation of latent TGF-β1 is by proteolysis by plasmin (Lyons et al., 1988). Both BAE cells and pericytes produce plasminogen activators (PA) (Gross et al., 1982; Sato, Y., unpublished data), and, therefore, both are capable of generating plasmin, which, in turn, might activate latent TGF-β1. To test whether plasmin mediated the conversion of the latent TGF-β1-like molecule to an active form, BAE cell migration was monitored in the presence of inhibitors of plasmin (Fig. 7). While both pericytes or TGF-β1 inhibited cell movement (Fig. 7, *Expt. 1, rows 2 and 3*), inclusion of the serine protease inhibitor aprotinin had little effect on the normal movement of endothelial cells (Fig. 7, *Expt. 1, row 4*). However, when migration in the presence of pericytes was monitored with aprotinin included in the culture medium, BAE cell migration was no longer suppressed (Fig. 7, *Expt. 1, row 5*). The effect of the aprotinin was not directed against TGF-β1, since added active TGF-β1 fully suppressed cell migration in the presence of aprotinin (Fig. 7, *Expt. 1, row 6*). Two other plasmin inhibitors, EACA, which is a broad spectrum serine protease inhibitor, and α_2 plasmin inhibitor, which is a rather specific plasmin inhibitor, blocked the effect of pericytes on BAE cell migration (Fig. 7, *Expt. 2 rows 3 and 4*) but had no effect on normal BAE cell movement. Preliminary experiments using bovine anti-urokinase PA antibodies also neutralized the inhibition (data not shown). Thus, plasmin appears to be required for the inhibition of BAE cell movement by pericytes. Similar results were obtained with co-cultures of BAE cells and SMCs (data not shown).

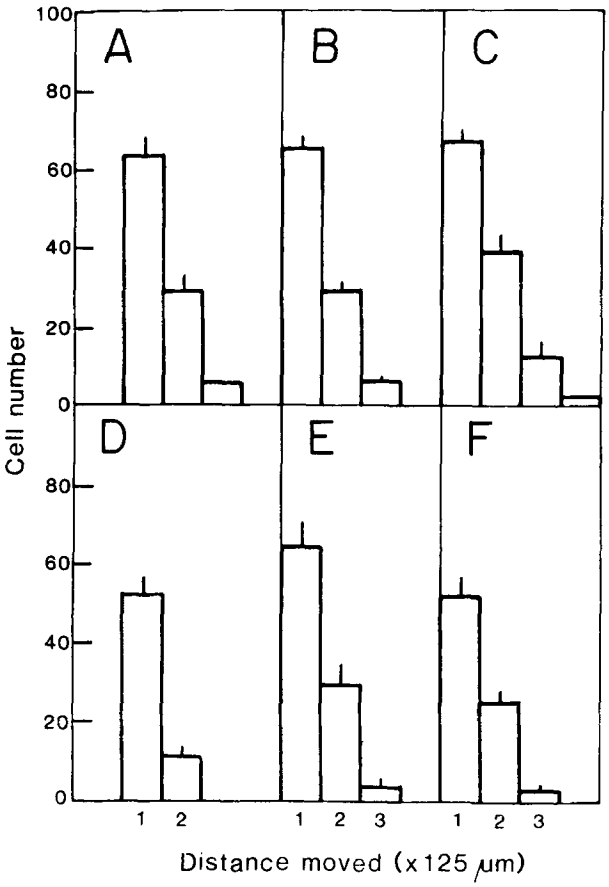

Figure 4. Effect of anti-TGF-β1 IgG on pericyte inhibition of BAE cell movement. Confluent cultures of BAE cells were wounded with a razor blade as described in Materials and Methods. The cells were incubated 20 h with pericytes and/or the indicated additions, fixed, stained, and the number of cells that had moved quantitated as described in the legend to Fig. 2. *A*, control; *B*, BAE cells plus anti-TGF-β1 IgG (100 μg/ml); *C*, BAE cells plus nonimmune IgG (100 μg/ml); *D*, BAE cells plus pericytes (1.5×10^4); *E*, BAE cells plus pericytes (1.5×10^4) plus anti-TGF-β1 IgG (100 μg/ml); *F*, BAE cells plus pericytes (1.5×10^4) plus nonimmune IgG (100 μg/ml). The number of cells that had moved from the origin was (*A*) 97, (*B*) 100, (*C*) 118, (*D*) 62, (*E*) 96, and (*F*) 75. The percent inhibition was (*A*) 0, (*B*) 0, (*C*) 0, (*D*) 36, (*E*) 4, and (*F*) 23.

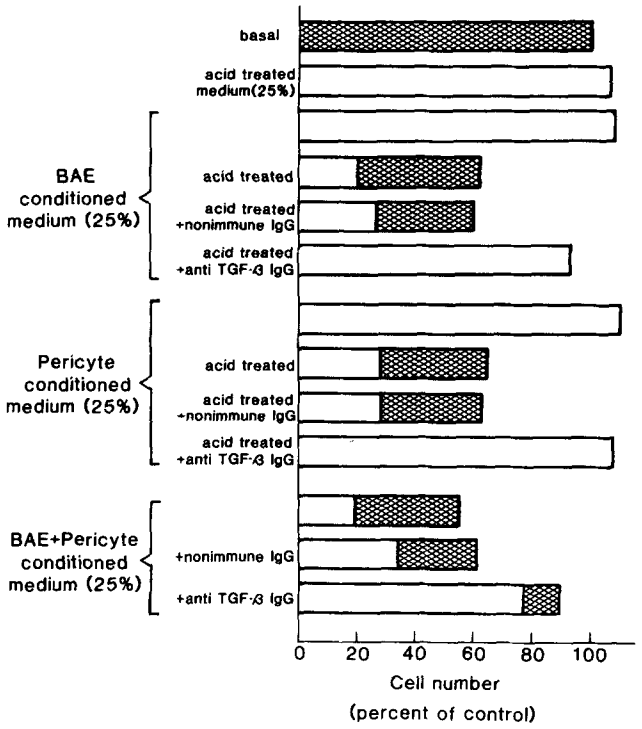

Figure 5. TGF-β1 production by BAE cells and pericytes. Serum-free conditioned medium was collected from cultures of BAE cells, pericytes, or mixtures of BAE cells plus pericytes as described in Materials and Methods. The medium was treated as indicated and placed on wounded monolayers of BAE cells in the presence or absence of either immune or nonimmune anti-TGF-β1 IgG. The cultures were treated as described in the legend to Fig. 2 and the number of cells that had moved quantitated. The results are presented as a percent of the control culture. The total number of cells that had moved in the control culture was 98, while the number that had moved >125 μm was 16. ▩, all cells; □, cells that had moved >125 μm.

derives from the generation of TGF-β1, the following indicate that this is the most likely possibility. First, the inhibition is abrogated by a neutralizing antiserum to TGF-β1. Two, TGF-β1 added to wounded BAE cell cultures blocks migration (Sato and Rifkin, 1988). Three, inhibitors of plasmin prevent the inhibition, and plasmin has been shown previously to be capable of activating latent TGF-β (Lyons et al., 1988). Fourth, preliminary experiments have demonstrated the presence of active TGF-β in concentrated media from co-cultures of BAE cells and pericytes but not concentrated media from BAE cultures using a receptor inhibition assay (Lyons, R., personal communication) (Tucker et al., 1984). However, unambiguous identification of the active species will require a more extensive characterization.

TGF-β1 was first described as a protein that promoted the proliferation of fibroblasts in soft agar, but more recently it has been shown to have various effects on a variety of cell types (for review see Sporn et al., 1987). Frater-Shroeder et al., (1986) and Baird and Durkin (1986) demonstrated that TGF-β inhibited the proliferation of ECs. Heimark et al. (1986) and Sato and Rifkin (1988) demonstrated that TGF-β blocked EC movement in wounded monolayers. The alleviation of the pericyte-mediated inhibition of BAE cell migration by neutralizing antibodies to TGF-β1 is consistent with these earlier reports on the action of TGF-β on ECs and implies that when ECs and pericytes are co-cultured active TGF-β1 may be produced.

The mechanism for the formation of the active TGF-β1-like molecule in the co-cultures remains unclear. Several studies have demonstrated that TGF-β is released by various cell types in a biologically inactive form, termed latent TGF-β (Lawrence et al., 1984; Kryceve- Martinerie et al., 1985; Lyons et al., 1988). While latent TGF-β is unable to bind to

Discussion

These experiments demonstrate that the migration of BAE cells can be severely impaired by bovine pericytes, SMCs, and fibroblasts but not by bovine ECs, human or mouse fibroblasts, or rat SMCs. Thus, there appears to be both a species and cell specificity to the inhibition. The effect appears to be the result of the formation of an active TGF-β1-like molecule via the action of plasmin on latent TGF-β1 produced by the cells. The activation of the latent TGF-β1-like molecule appears to require cell-cell contact or very close apposition of the cells. However, once the active species is formed, it is freely diffusible. Heimark and Schwartz (1985) reported that cell membrane fractions from confluent ECs inhibited both the migration and proliferation of ECs. Our results differ somewhat from these results since we observed no inhibition of movement in homotypic co-cultures of BAE cells. Orlidge and D'Amore (1987) also described the contact-mediated inhibition of EC division by pericytes or SMCs. Our results are quite consistent with their results.

The mechanism of this inhibition appears to be via the action of TGF-β1 or a molecule which is immunologically and functionally similar to TGF-β1. While we have not rigorously proven that the inhibition of migration that we have observed

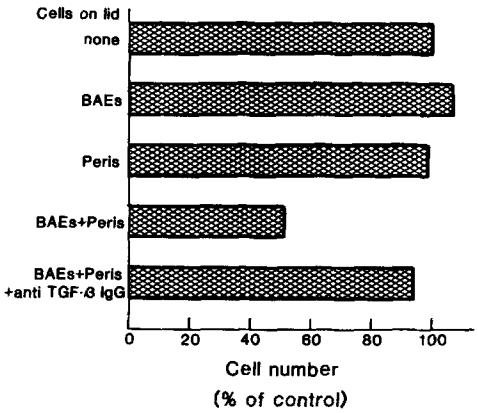

Figure 6. Release of a diffusible inhibitor in co-cultures of pericytes and BAE cells. BAE cells (4×10^5), pericytes (1×10^5), or BAE cells (4×10^5) and pericytes (1×10^5) were allowed to attach to the tops of Cooper dishes as described in Materials and Methods. After attachment was completed, the tops were inverted and placed so that the adherent cells were located above a wounded BAE cell monolayer. The cultures were incubated overnight and the next day the number of cells which had moved quantitated as described in the legend to Fig. 2. The results are presented as percent of the control culture which had no cells on the top of the Cooper dish. The total number of cells moved in the control culture was 73.

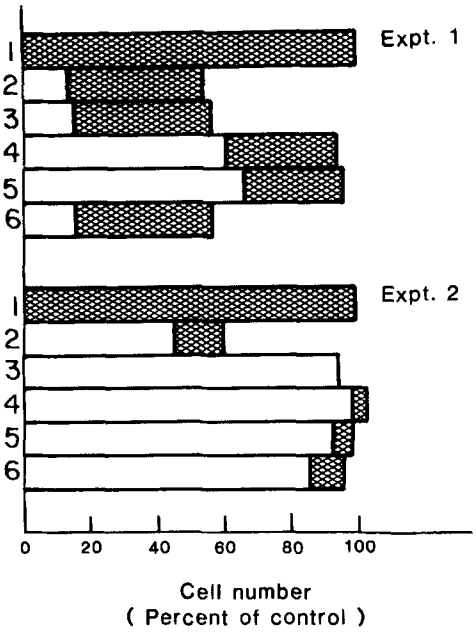

Figure 7. Effect of plasmin inhibitors on pericyte-dependent inhibition of BAE cell movement. Confluent monolayers of BAE cells were wounded with a razor blade as described in Materials and Methods. The BAE cells were incubated with pericytes and/or the indicated additions for 20 h, fixed, stained, and the number of cells that had moved quantitated as described in the legend to Fig. 2. Experiment 1 (*Expt. 1*): *1*, control; *2*, pericytes (1.5×10^4); *3*, TGF-β1 (4 ng/ml); *4*, aprotinin (200 μg/ml); *5*, pericytes (1.5×10^4) plus aprotinin (200 μg/ml); *6*, TGF-β (4 ng/ml) plus aprotinin (200 μg/ml). Experiment 2 (*Expt. 2*): *1*, control; *2*, pericytes (1.5×10^4); *3*, pericytes (1.5×10^4) plus α_2 plasmin inhibitor (2 μg/ml); *4*, pericytes (1.5×10^4) plus EACA (50 μg/ml); *5*, α_2 plasmin inhibitor (2 μg/ml); *6*, EACA (50 μg/ml). The total number of cells that had moved in *Expt. 1*, *row 1*, 113, and in *Expt. 2*, *row 1*, 109. The number of cells that had moved >125 μm were *Expt. 1*, *row 1*, 58; and *Expt. 2*, *row 1*, 30; ▩, all cells; □, cells that had moved >125 μm.

the TGF-β receptor (Wakefield et al., 1987), it can be converted to an active form by transient acid treatment (Lawrence et al., 1985; Lyons et al., 1988). Our data indicate that both pericytes and BAE cells release latent TGF-β1 when cultured separately, but the latent TGF-β1 does not inhibit migration when incubated with wounded monolayers of BAE cells. Co-cultivation of BAE cells and pericytes appears to result in the activation of a latent TGF-β1-like molecule. Lyons et al. (1988) have demonstrated that latent TGF-β can be activated by the serine protease plasmin in a cell-free system, and they have proposed this as a physiological mechanism for activation. Our observation that inhibitors of plasmin abrogate the inhibitory effects of TGF-β1 in heterotypic co-cultures of BAE cells and pericytes is consistent with this hypothesis. To our knowledge, our results may be the first indication of the possible activation of latent TGF-β1 by a serine protease under cell culture conditions. Proof of activation of latent TGF-β1 under these experimental conditions will require the isolation of the active species.

Why latent TGF-β may be activated under these co-culture conditions and not when the cells are grown separately is not clear. Both BAE cells and pericytes produce PA when cultured separately (Sato, Y., unpublished observations). Therefore, co-cultivation does not result simply in the induction of the PA-plasmin system. It appears more likely that co-cultivation results in a more subtle modulation of this proteolytic cascade and its interaction with latent TGF-β1. The activation of plasminogen probably occurs while the zymogen is bound to cell surface receptors (Hajjar et al., 1986; Miles and Plow, 1985, 1987; Plow et al., 1986; Vali and Patthy, 1984). PA, itself, is also bound to a specific receptor (for review see Blasi et al., 1987). Therefore, the availability of specific receptors or the modulation of receptor number upon the establishment of cell-cell contact may have dramatic effects on subsequent proteolytic events. An elegant example of the interaction of two cell types in plasminogen activation comes from the experiments of Huarte et al. (1987). They demonstrated that when spermatocytes mature they have no intracellular PA but express the PA receptor on their membranes. As the spermatocytes migrate through the ductus deferens, they bind proPA which has been secreted into the lumen by the epithelial cells of the duct, thereby initiating the activation of the PA-plasmin system. It is also possible that in our co-culture system changes in PA inhibitor levels may regulate plasmin formation or that activation requires that the latent TGF-β1 be localized via binding molecules whose synthesis is induced by cell-cell contact. The molecular details of the activation reaction of TGF-β1 in these co-cultures is currently being studied.

At first it may seem odd that interactions of cells in the intima and cells in the media are mediated by contact. However, there is increasing evidence that contacts between ECs and SMCs do occur. Electron microscopic studies have shown long processes extending from SMCs to ECs with the formation of junctions between the two cell types (Huttner et al., 1973*a*,*b*; Spagnoli et al., 1982). Our results are easier to reconcile with observations of pericytes and ECs, since the cells are in close proximity and junctions between pericytes and ECs have been described (Bruns and Palade, 1968; Matsusaka, 1970; Spitznas and Reale, 1975). In fact, Larson et al. (1987) have demonstrated the junctional transfer of small molecules between microvascular ECs and bovine pericytes in vitro. Furthermore, Guinan et al. (1988) recently described the intracellular communication of human ECs and human lymphocytes but not of human lymphocytes and Madin-Darby canine kidney cells. These results demonstrate that ECs are able to communicate not only with pericytes and SMCs but also other cell types in the same species. The functional significance of junctional communication is, perhaps, best characterized in a number of developing vertebrate organisms (see Schultz, 1985, for review). However, there are several reports of the in vitro arrest of growth of transformed cells upon contact with normal cells (Stoker, 1964; Stoker et al., 1966; Borek and Sachs, 1966; Eagle et al., 1968). Recently Mehta et al. (1986) have confirmed that growth inhibition of transformed cells is mediated by their junctional communication with normal cells. It would be interesting to determine if anti-TGF-β1 IgG neutralized this effect.

The proof that EC-pericyte or EC-SMC contacts have relevance in vivo will require model systems in which the function of one cell type is ablated or provided by specific chemical mediators. While the potential role of this type of cell-cell contact in endothelial processes such as neovascularization have been considered, it seems appropriate to

speculate as to whether processes involving SMCs may be regulated by EC contact. The proliferation of SMCs in atherosclerosis and pulmonary hypertension may be such conditions.

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