

Hyaluronan-binding Protein in Endothelial Cell Morphogenesis

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Abstract. Previous studies from several laboratories have provided evidence that interaction of hyaluronan (HA) with the surface of endothelial cells may be involved in endothelial cell behavior. We have recently characterized a mAb, mAb IVd4, that recognizes and neutralizes HA-binding protein (HABP) from a wide variety of cell types from several different species (Banerjee, S. D., and B. P. Toole. 1991. *Dev. Biol.* 146:186–197). In this study we have found that mAb IVd4 inhibits migration of endothelial cells from a confluent monolayer after “wounding” of the monolayer. HA hexasaccharide, a fragment of HA

with the same disaccharide composition as polymeric HA, also inhibits migration. In addition, both reagents inhibit morphogenesis of capillary-like tubules formed in gels consisting of type I collagen and basement membrane components. Immunocytology revealed that the antigen recognized by mAb IVd4 becomes localized to the cell membrane of migrating cells, including many of their lamellipodia. Treatment with high concentrations of HA hexamer causes loss of immunoreactivity from these structures. We conclude that HABP recognized by mAb IVd4 is involved in endothelial cell migration and tubule formation.

IT is well established that the extracellular matrix plays an important role not only in tissue structure but also in cell behavior and differentiation, and that many of the effects of matrix macromolecules are mediated by cell surface binding sites or receptors (e.g., Hay, 1991). Endothelial cell morphogenesis, as occurs in angiogenesis or embryonic vasculogenesis, involves a series of events that include cell migration, division, and tubule formation (Folkman and Haudenschild, 1980; Folkman and Klagsbrun, 1987; Poole and Coffin, 1989). Many experimental studies have indicated that interaction of endothelial cells with the extracellular matrix plays a key role in these processes (Madri and Pratt, 1986; Furcht, 1986; Folkman et al., 1988; Ingber, 1991). The integrin family of matrix receptors and a 67/69 kD laminin receptor appear to mediate at least part of this influence of matrix on the behavior of endothelial cells (Charo et al., 1987; Dejana et al., 1988; Grant et al., 1989; Basson et al., 1990; Defilippi et al., 1991). In this study we address the potential function of interactions between the matrix polysaccharide, hyaluronan (HA)¹, and cell surface HA-binding protein (HABP) in endothelial cell behavior.

Based on histochemical evidence, Ausprunk et al. (1981) originally proposed that the pericellular region surrounding migrating tips of newly forming capillaries is enriched in HA. It was also found by this group that capillaries of the chorioallantoic membrane begin to form in HA-rich areas but the level of HA surrounding the capillaries rapidly decreases thereafter (Ausprunk, 1986). In other laboratories

it was shown that blood vessel formation does not occur in HA-rich zones (Feinberg and Beebe, 1983) and that endothelial cell growth is inhibited by polymeric HA (West and Kumar, 1989). However, oligomers of HA containing 3–16 disaccharide repeats were shown to be angiogenic (West et al., 1985) and to stimulate endothelial cell growth (West and Kumar, 1989). Taken together these results suggest the possibility that, in vivo, degradation of HA by hyaluronidase leads to formation of oligosaccharides that promote vessel formation, whereas regions where high concentrations of HA polymer persist would be inhibitory.

Although the above hypothesis is attractive, little information has been forthcoming on the production of HA oligomers in vivo or their mechanism of action, with respect to the above conclusions. On the contrary, there are very few reports showing extracellular action of hyaluronidases in vertebrates. HA-degrading activities with near neutral pH optima have been detected in some systems (Bernfield et al., 1984; Nakamura et al., 1990), but most vertebrate hyaluronidases so far characterized are lysosomal and have acidic pH optima (Roden et al., 1989). Furthermore, lysosomal degradation of HA has been shown to lead to complete degradation to monosaccharides and smaller products (Roden et al., 1989). Even though it is not clear that HA oligomers themselves play a physiological role, investigation of the biochemical effect of exogenously added HA oligomers is likely to give insight into the potential role of endogenous interactions between polymeric HA and HABPs.

One mechanism for the angiogenic action of exogenously added HA oligomers might be to disrupt or modify an endogenous interaction of HA polymer with an endothelial cell

1. *Abbreviations used in this paper:* HA, hyaluronan; HABP, HA-binding protein.

surface HABP. Indeed, HA-binding sites have been detected on the surface of vascular endothelial cells (McGuire et al., 1987), and are greater in number in sparse as compared to confluent cells (Antonelli and D'Amore, 1991). The specialized endothelial cells of liver sinusoids also exhibit HA-binding sites; however, these sites are known to be involved in clearance of HA from the circulation (Laurent et al., 1986; McGary et al., 1989) and are probably distinct from the HA receptor(s) involved in mediating effects of HA on cell behavior (Raja et al., 1988).

We have raised a mAb, IVd4, that recognizes HABP present in many embryonic tissues and on the surface of cultured cells from a variety of species. The expression and localization of this HABP correlate with morphogenetic events during the development of embryonic tissues (Banerjee and Toole, 1991; and unpublished data). mAb IVd4 blocks HA binding to soluble and cell surface-bound HABP (Banerjee and Toole, 1991) and inhibits formation of HA-dependent pericellular matrices (Yu et al., 1992). Thus we have used this antibody to probe further the possible role of HA-HABP interactions in endothelial cell behavior. In this study we show that the antibody and HA oligomers inhibit endothelial cell migration and formation of capillary-like tubules. These results strongly imply that interaction of endogenous HA polymer with endothelial cells is essential to endothelial morphogenesis, rather than antagonistic as suggested by some of the results from other laboratories described above. We propose that these apparently contradictory results may be explained on the basis of opposing effects of low vs high concentrations of HA in the pericellular milieu of the endothelial cell.

Materials and Methods

mAbs and HA Hexamer

The mAbs, IVd4 and IIIg1, were produced and characterized as described previously (Banerjee and Toole, 1991). mAb IVd4 recognizes HABP from several cells and tissues, especially embryonic, and from a variety of species. mAb IIIg1 was prepared from the same series of hybridomas as mAb IVd4 and recognizes an unknown antigen present in the mixed antigen preparation used for immunization of the mice. Both antibodies are of the IgM class.

HA hexasaccharide was prepared from testicular hyaluronidase digests of HA by Sephadex G50 gel exclusion chromatography, as described previously (Banerjee and Toole, 1991).

Endothelial Cell Culture

Endothelial cells were obtained from two sources. Bovine aortic endothelial cells were isolated by standard techniques (Yannariello-Brown et al., 1988) from aortas collected fresh from a local slaughterhouse and used between passage 4 and 10. Bovine pulmonary artery cells were from American Type Culture Collection (CCL 209) and were used between passage 16 and 30. The cells were maintained in 75-cm² tissue culture flasks in DME containing 5% calf serum (aortic endothelium) or MEM with 20% FBS (pulmonary artery endothelium) plus antibiotic/antimycotics. The cultures were routinely monitored with antibodies to the LDL receptor or factor VIII, and for acquisition of cobblestone morphology at confluence.

For the experiments described herein, the cells were harvested in 0.6 mM Versene in PBS at 37°C, transferred to 35-mm dishes, and allowed to attach in the same media as above. Sparse cultures are defined as cultures containing $\sim 5 \times 10^4$ cells per 35-mm dish, in which cell contact is rare. Confluent cultures were used 3–4 d beyond the time when they first appeared confluent by microscopic observation, and contained $\sim 2 \times 10^6$ cells per dish.

Experiments testing the inhibitory effects of antibody or HA oligomers on cell migration or tubule formation were performed in serum-free media

to avoid possible interference from HABPs present in serum (Underhill, 1982; Yoneda et al., 1990). To ensure that the cells remained healthy during the course of these experiments, reversal controls were carried out where the cells were washed subsequent to the experiment to remove the agent and reversal of inhibition was then monitored.

Wounded Cultures

Confluent cultures, as described above, were used for establishment of "wounded" cultures by a modification of previously published techniques (Sato and Rifkin, 1988; Hooock et al., 1991). Before wounding, the cells were washed extensively with serum-free DME (aortic) or MEM (pulmonary) containing 0.1% BSA and incubated at 37°C in this medium for 2 h. After this they were incubated at 4°C for 30 min with or without experimental reagents (i.e., antibody or HA hexamer) and washed again in serum-free medium with 0.1% BSA. A scratch was then made in the monolayer using a pasteur pipette tip that had been smoothed by flaming. The monolayer was washed to remove debris and fresh serum-free medium containing 0.1% BSA, with or without test substances was added. The cultures were then incubated for 8, 17, or 30 h at 37°C, after which the degree of emigration of endothelial cells from the wound edges was observed by microscopy and the cultures were processed for photography and/or immunocytochemistry.

In some cases the cultures were washed, scratched, and incubated for 8 h before addition of antibody or HA hexamer, and then re-incubated for an additional 17 h in the presence of the particular reagent. These cultures were then processed for photography and/or immunocytochemistry.

To ensure that the cells were viable during the course of the experiments, recovery controls were performed. Subsequent to incubation with antibody or HA oligomer as described above, the wounded monolayers were incubated in three changes of reagent-free media at 37°C for 15 min, reincubated for several hours in the absence of reagent, and then monitored for migration from the wound edge.

For quantitation of migration of the cells in the wounded cultures, photographs were taken at various time intervals using an ocular grid. The photographs were then analyzed for the number of cells migrating from the wound edge within a defined field (Sato and Rifkin, 1988).

Capillary-like Tubule Formation

The culture conditions used for production of capillary-like tubules were modified from a combination of previously published methods (Montesano et al., 1983; Madri et al., 1983; 1988; Kubota et al., 1988). Type I collagen (2 mg/ml; UBI, Lake Placid, NY; or Collaborative Research Inc., Bedford, MA) and a mixture of basement membrane proteins (50 µg/ml ECL from UBI) were mixed with DME (aortic) or MEM (pulmonary), with or without experimental reagents (i.e., antibody or HA hexamer), at 4°C. Confluent endothelial cells from bovine aorta or pulmonary artery were then harvested by scraping in medium containing 1% BSA, washed by centrifugation, incubated with or without the experimental reagents at 4°C for 30 min, and resuspended as clumps while mixing with the collagen-ECL mixture at 4°C. The mixture was allowed to gel at 37°C for 30 min, and then diluted with serum-free medium and incubated for 48 h under culture conditions.

In experiments where recovery subsequent to treatment with antibody or hexamer was examined, the cultures were first incubated for 48 h in the presence of the reagent as described above. They were then incubated in three changes of reagent-free medium containing 2% BSA at 37°C for 15 min, and reincubated in the absence of reagent for a further 48 h.

Immunocytochemistry

The cultures were examined for localization of HABP with mAb IVd4 by routine immunocytochemical methods. Briefly, the cells were washed with PBS, fixed with 3.7% paraformaldehyde in saline for 15 min at room temperature, washed, and quenched with 0.1 M NH₄Cl or 0.25 M glycine in PBS for 30 min. Non-specific reactions were blocked by incubating for 30 min with a cocktail containing 10 mg/ml BSA, 10 mg/ml dried non-fat milk and 100 µg/ml rabbit IgG. The cells were then washed, incubated with 20 µg/ml primary antibody (mAb IVd4) overnight at 4°C, washed, incubated with 4 µg/ml rhodamine-conjugated rabbit anti-mouse Ig for 30 min, washed, and mounted in gel-mount media (Biomedica, Foster City, CA).

Gel Electrophoresis and Western Blotting

A subconfluent culture of bovine aortic endothelial cells was harvested by scraping in 0.6 mM Versene in PBS containing 1 mM PMSF and extracted by sonication in 0.25 M sucrose/40 mM Tris-HCl, pH 7.4 buffer containing

protease inhibitors (Banerjee and Toole, 1991). After centrifugation, the supernatant was assayed for protein content (Bradford, 1976), and 20 μg protein per lane separated by SDS-PAGE and Western blotted, using a 1:100 dilution of QAE-purified mAb IVd4, as described previously (Banerjee and Toole, 1991), except that the secondary antibody was adsorbed with a suspension of lyophilized endothelial cells ($1 \times 10^6/\text{ml}$ in 0.1% Tween in TBS) and centrifuged before use. Also, an additional sample was electrophoresed by the same procedure as described above except that the reaction with mAb IVd4 was omitted to control for residual reactivity of the proteins with the adsorbed secondary antibody.

Results

Immunocytochemical Localization of HABP

Sparse, confluent, and wounded endothelial cell cultures were examined by immunocytochemistry using mAb IVd4 to establish the presence in endothelial cells of HABP recognized by this antibody and to determine whether the localization of HABP changes in motile vs. sessile cells.

In confluent cultures of arterial endothelial cells that had not been permeabilized, immunoreactivity was mainly localized to patches beneath the monolayer (Fig. 1 B). On treatment with EDTA to remove the cells, much of this immunoreactive material remained attached to the substratum, and is presumably present in the extracellular matrix or in membrane fragments attached to the matrix (Fig. 2). When the cells were permeabilized, strong immunofluorescence was observed within the cytoplasm, mainly in a perinuclear location (data not shown).

When the cultures were wounded, however, most of the cells emerging from the edges of the confluent monolayer exhibited strong immunoreactivity associated with various regions of their plasma membrane (Fig. 3). In $\sim 50\%$ of these cells, reactivity was mainly present in lamellipodia at their leading edges (Fig. 3 D). Patches of subcellular reactivity, similar to those seen beneath the cells in non-wounded confluent cultures, were present in association with the motile cells emerging from the edges of the wounded monolayer as well as the confluent cells remaining behind the edges. On

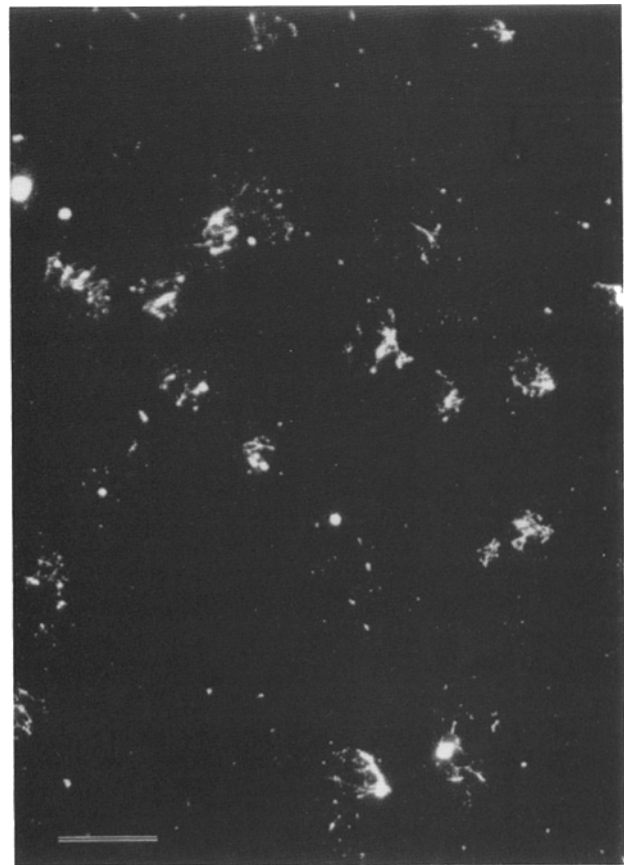


Figure 2. mAb IVd4 immunoreactivity of substratum-attached material. The substratum-attached material was processed for immunocytochemistry subsequent to removal of a confluent monolayer of pulmonary artery endothelial cells by treatment of the culture with 10 mM EDTA. Bar, 25 μm .

permeabilization, it was apparent that intracellular reactivity was reduced in the motile cells in comparison to the confluent cells behind the wound edges.

A similar pattern of immunoreactivity to the above was

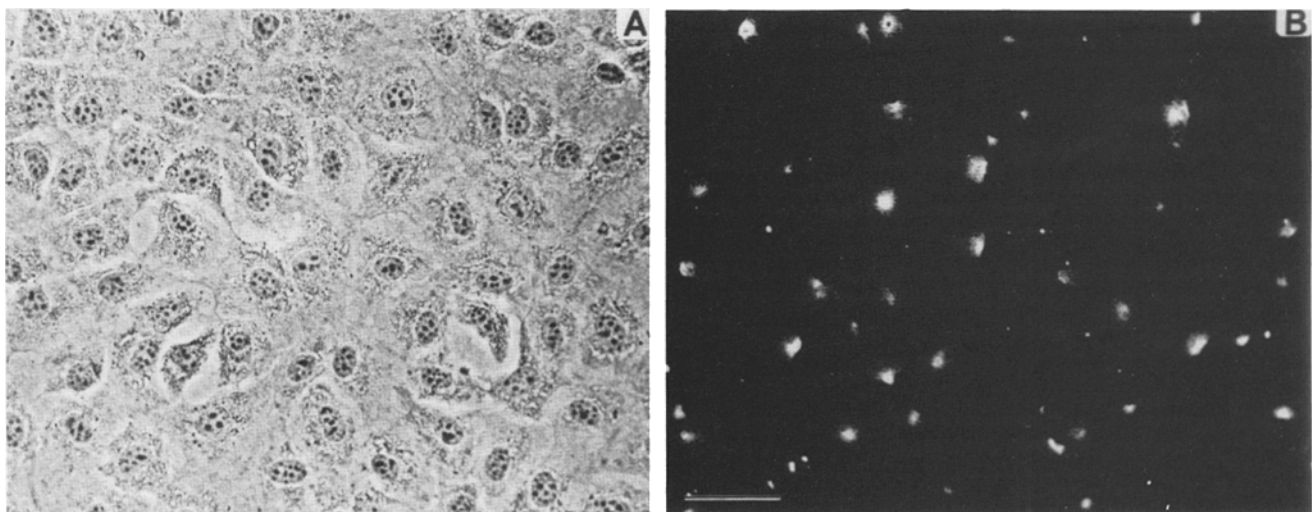


Figure 1. mAb IVd4 immunoreactivity of pulmonary artery endothelial cells in culture. The cultures were processed for immunocytochemistry without permeabilization as described in the Materials and Methods. (A) Phase contrast; (B) mAb IVd4 immunoreactivity. Immunoreactivity is mainly seen in subcellular patches. Bar, 40 μm .

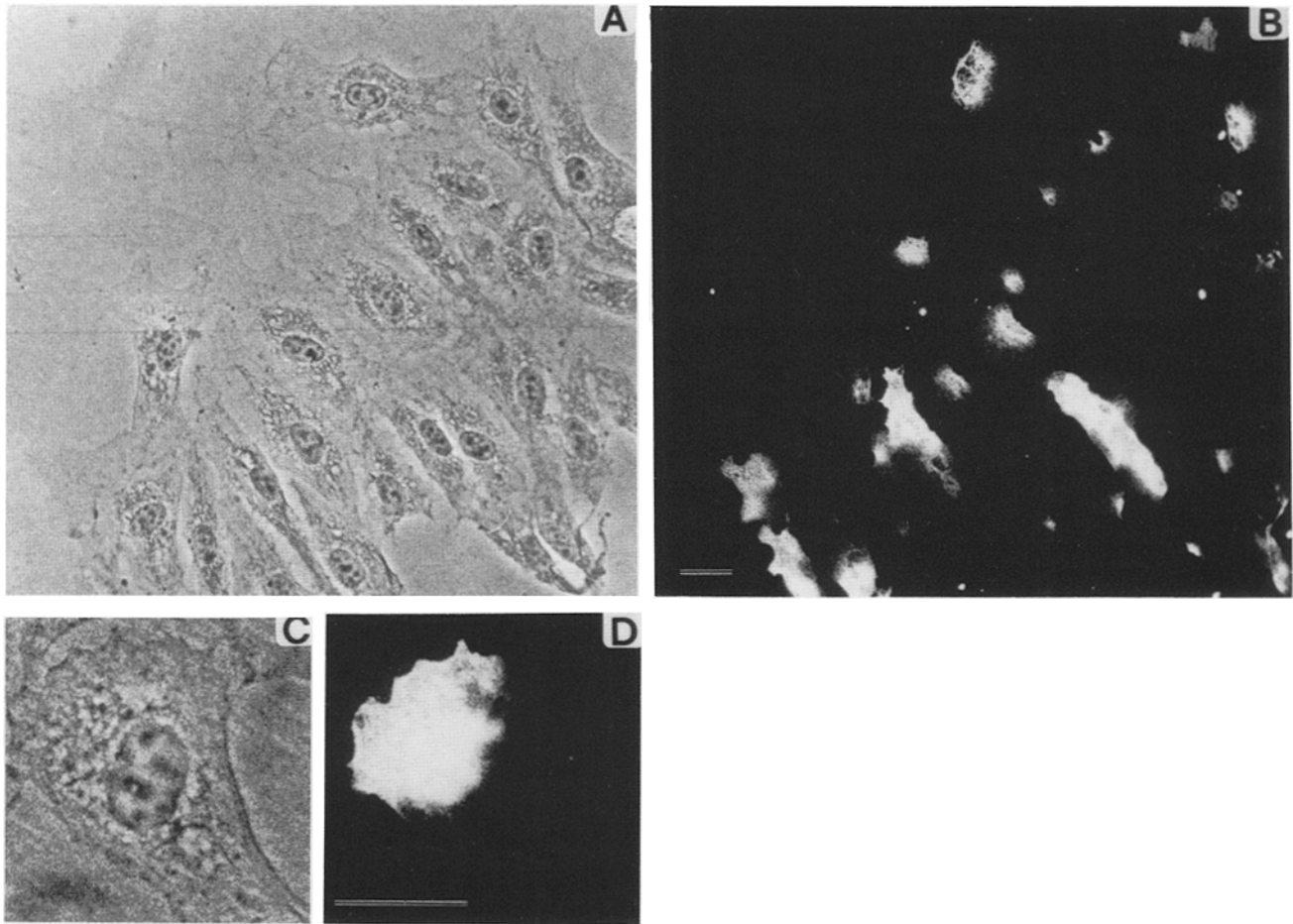


Figure 3. mAb IVd4 immunoreactivity of a wounded monolayer of pulmonary artery endothelial cells. (*A* and *C*) Phase contrast; (*B* and *D*) mAb IVd4 immunoreactivity. A confluent monolayer was wounded, and then incubated for 8 h before processing for immunocytochemistry as described in the Materials and Methods. (*A* and *B*) Immunoreactivity is seen in the cell membranes, including several of the lamellipodia, of the cells emigrating from the edge of the wound. Most of the cells behind the original wound edge show identical distribution of reactivity to those in an undisturbed confluent monolayer, i.e., in subcellular patches, as shown in Fig. 1 *B*. (*C* and *D*) Higher magnification of a cell showing immunoreactivity in its lamellipodium. Bars, 18 μm .

seen in sparse, non-wounded cultures of endothelial cells in that many of the cells, without permeabilization, showed variable regions of membrane staining. Trypsinization of these cells removed the membrane reactivity, confirming that it is localized at the outer cell surface. However, these sparse cultures also contained numerous cells that resembled confluent cells in that they only exhibited patches of subcellular reactivity (data not shown).

The pattern of immunoreactivity was indistinguishable in the bovine aortic and pulmonary artery endothelium. We also examined sparse cultures of endothelial cells from human umbilical vein, human aorta, human omental microvasculature (from Dr. R. Orkin, Vascular Surgery, Massachusetts General Hospital, Boston, MA), and rat epididymal microvasculature (from Dr. Castellot, Tufts Medical School, Boston, MA) and observed a similar pattern to that seen for the bovine aortic and pulmonary artery endothelium (data not shown).

Immunoblot of Endothelial Cell Extract

An extract of growing endothelial cells was electrophoresed and Western blotted using mAb IVd4. As shown in Fig. 4, three major bands were obtained, with mol wts of ~ 95 , 77, and 50 kD.

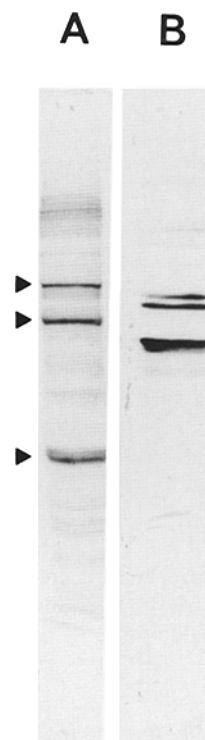


Figure 4. Western blot of endothelial cell extract with mAb IVd4. (*A*) extract of subconfluent bovine aortic endothelial cells (arrowheads indicate major protein bands at 95, 77, and 50 kD); (*B*) partially purified preparation of HABP from chick embryo brain, with major bands at 93, 90, and 69 kD (Banerjee and Toole, 1991).

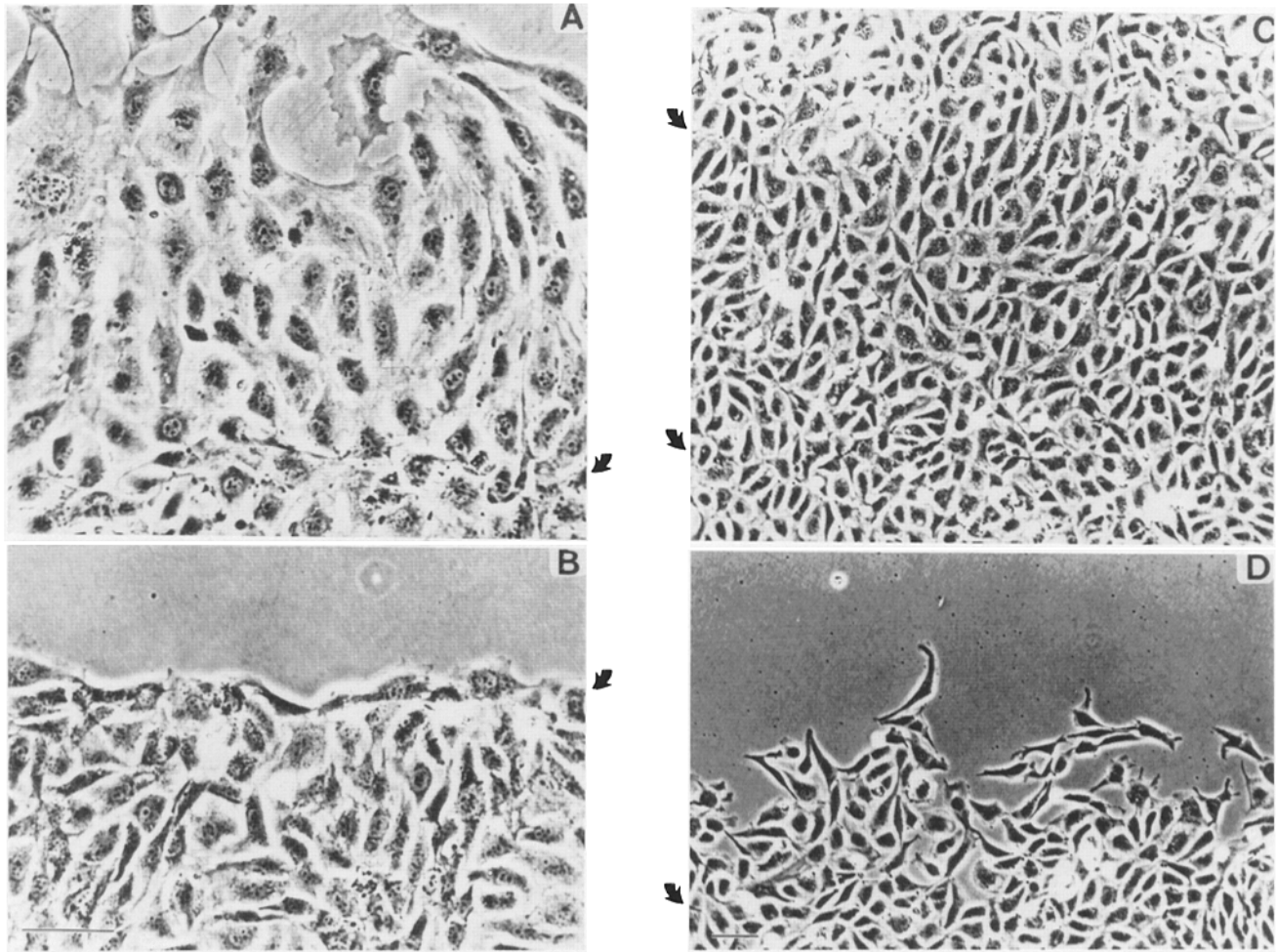


Figure 5. mAb IVd4 inhibition of endothelial cell migration. Confluent monolayers of bovine aortic endothelial cells were wounded by scratching with a smoothened pasteur pipette and then incubated for 8 h (A and B) or 30 h (C and D) in the presence of 280 $\mu\text{g/ml}$ mAb IVd4 (B), 50 $\mu\text{g/ml}$ mAb IVd4 (D), or in the absence of the antibody (A and C) (see Materials and Methods). The curved arrows in the margin indicate the approximate positions of the cut edges immediately after wounding. In the absence of antibody, the cells emigrated into the space between the wound edges, partially occupying this space within 8 h (A) and completely filling the space within 30 h (C). Incubation with 280 $\mu\text{g/ml}$ completely blocked the migration occurring over 8 h (B) or longer intervals (see Table I), whereas 50 $\mu\text{g/ml}$ caused partial inhibition (D). Bars, 60 μm .

Effect of mAb IVd4 on Endothelial Cell Migration

To determine whether HA-HABP interactions might be important in endothelial cell migration, mAb IVd4 was added to wounded cultures and emigration of cells into the space between the cut edges was analyzed. The antibody was found to inhibit emigration of the cells (Fig. 5); 50–100 $\mu\text{g/ml}$ of mAb IVd4 caused partial inhibition whereas 250–300 $\mu\text{g/ml}$ was completely inhibitory. A series of experiments was performed in which concentrations of 50 and 280 $\mu\text{g/ml}$ of mAb IVd4 were added to aortic or pulmonary artery cells. As can be seen from Table I, the lower concentration of antibody gave rise to an average of 70% inhibition of emigration of the endothelial cells and the higher concentration caused complete inhibition. The results obtained with the aortic endothelium were indistinguishable from those obtained with the pulmonary artery cells. On removal of mAb IVd4 from the cultures subsequent to treatment, the cells initiated migration in similar fashion to controls. Also, addition of 250–500 $\mu\text{g/ml}$ of mAb IIIg1, which is of the same Ig class

Table I. Effects of mAb IVd4 and HA Hexamer on Endothelial Cell Migration

	Number of cells migrating per field	
	Mean	SD
Controls		
No mAb	93.8	12.3
mAb IIIg1	96.0	5.8
mAb IVd4		
50 $\mu\text{g/ml}$	30.2	6.5
280 $\mu\text{g/ml}$	0	0
Hexamer		
75 $\mu\text{g/ml}$	33.7	6.7
500 $\mu\text{g/ml}$	0	0

The data represent nine experiments, in each case performed with both bovine aortic and pulmonary artery endothelial cells cultured for 17 h after wounding. In three of these experiments, control cultures included 250 $\mu\text{g/ml}$ mAb IIIg1; in the other six experiments the control cultures contained no antibody; in all nine experiments cultures treated with the two indicated concentrations of mAb IVd4 and HA hexamer were included. Fields along the cut edge of wounded cultures were selected randomly and analyzed for the numbers of cells that had migrated from the wound edge (Sato and Rifkin, 1988).

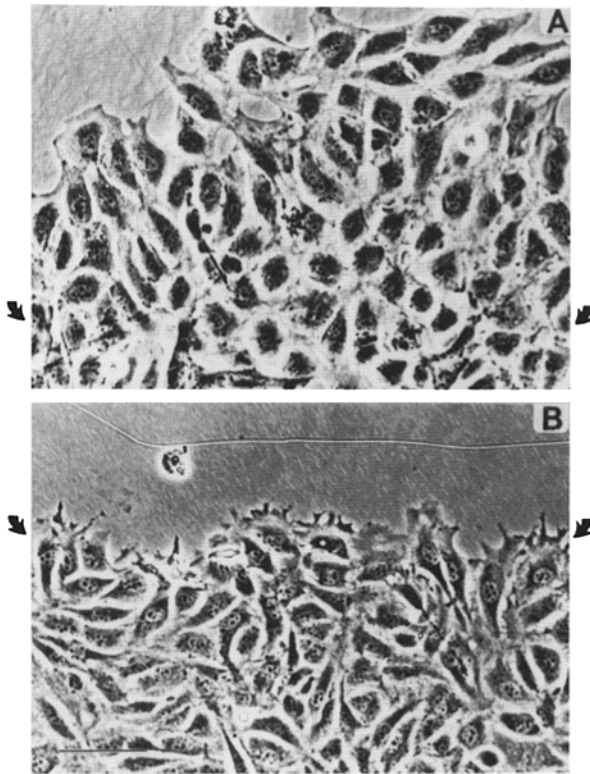


Figure 6. Inhibition of endothelial cell migration by HA hexamer. Confluent monolayers of bovine aortic endothelial cells were wounded by scratching with a smoothened pasteur pipette and then incubated for 8 h in the presence (B) or absence (A) of 500 µg/ml of HA hexamer (see Materials and Methods). The curved arrows in the margins indicate the approximate positions of the cut edges immediately after wounding. The hexamer caused complete inhibition of emigration at this concentration. Bar, 60 µm.

as mAb IVd4 but does not recognize HABP (Banerjee and Toole, 1991), had no effect on migration of the endothelial cells (Table I).

In addition to the above experiments, we added mAb IVd4 to wounded cultures 8 h subsequent to wounding to test its effect on the continuing migration of cells that had already begun to emigrate from the cut edge of the monolayer. The antibody inhibited further migration of the cells (data not shown), thus illustrating that its effect was not only on initial emergence of the cells.

Effect of HA Hexamer on Endothelial Cell Migration

The results above suggest that HA-HABP interaction is important for endothelial cell migration. If this is the case, other reagents that disrupt HA-HABP interaction, in addition to antibody to HABP, would also be expected to affect migration. HA oligomers competitively inhibit interaction of polymeric HA with cell surface HABP (Underhill and Toole, 1979; Underhill et al., 1983) and so we tested their effect. We used HA hexamer since it does not compete for interaction of HA with link protein or proteoglycans (Hascall and Hascall, 1981; Tengblad, 1981; Yamagata et al., 1986) but does inhibit interaction with cell surface HA receptors (Underhill and Toole, 1979; Nemeč et al., 1987).

The hexamer was found to be inhibitory to migration in

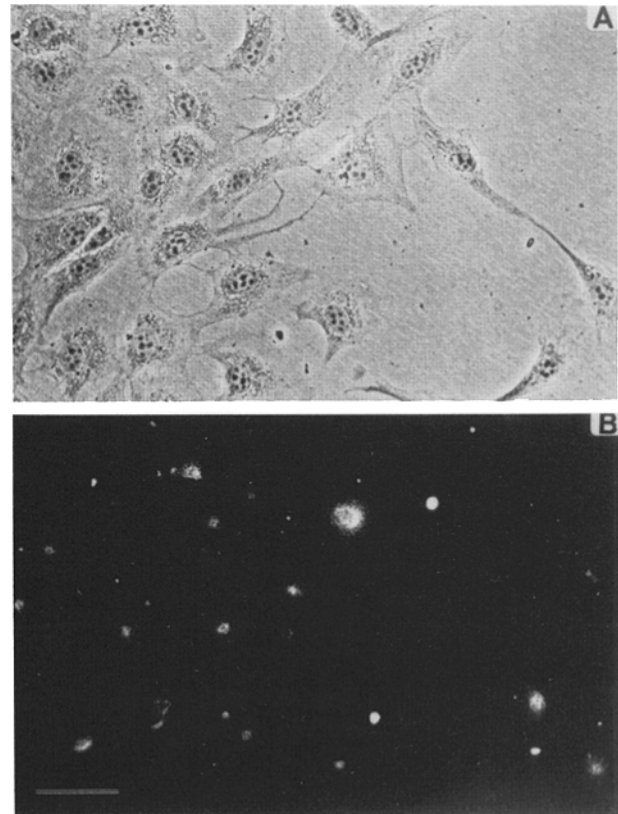


Figure 7. Loss of mAb IVd4 immunoreactivity in motile cells after exposure to HA hexamer. A confluent monolayer of pulmonary artery endothelial cells was wounded and incubated in the absence of hexamer for 8 h, and the cells emigrated from the wound edges in similar fashion to the cultures shown in Figs. 3 A, 5 A, and 6 A. The culture was then incubated for a further 17 h with 500 µg/ml HA hexamer, following which the culture was washed thoroughly with hexamer-free medium, photographed, and processed for immunocytochemistry. (A) Phase contrast; (B) Immunoreactivity with mAb IVd4. Exposure to the hexamer inhibited further emigration of the cells and caused loss of mAb IVd4 immunoreactivity in the lamellipodia and other areas of the membrane (compare with Fig. 3). However, reactivity persisted in the subcellular patches (see Fig. 1 B). Bar, 40 µm.

the wounding assay (Fig. 6). In a dose response, no apparent inhibition was obtained with 25 µg/ml of hexamer, partial inhibition with 50–125 µg/ml, almost complete inhibition with 300 µg/ml, and complete inhibition with 500 µg/ml. Table I shows that, in a series of experiments performed with both bovine aortic and bovine pulmonary arterial endothelium, 75 µg/ml hexamer gave rise to an average of 66% inhibition and 500 µg/ml totally inhibited emigration. Inhibition was not obtained with 500 µg/ml of HA tetrasaccharide, Na uronate, *N*-acetylglucosamine, or a mixture of 500 µg/ml each of Na uronate and *N*-acetyl glucosamine.

We also examined the effect of hexamer on mAb IVd4 immunoreactivity. To do this we treated cells with 250 and 500 µg/ml of hexamer 8 h after wounding of a confluent monolayer. At this time many endothelial cells had emigrated from the edges of the wound but they ceased to migrate further on exposure to the hexamer (Fig. 7 A). After a 24-h exposure to the hexamer the cells were washed thoroughly with hexamer-free medium, fixed, and examined for immunoreactivity with

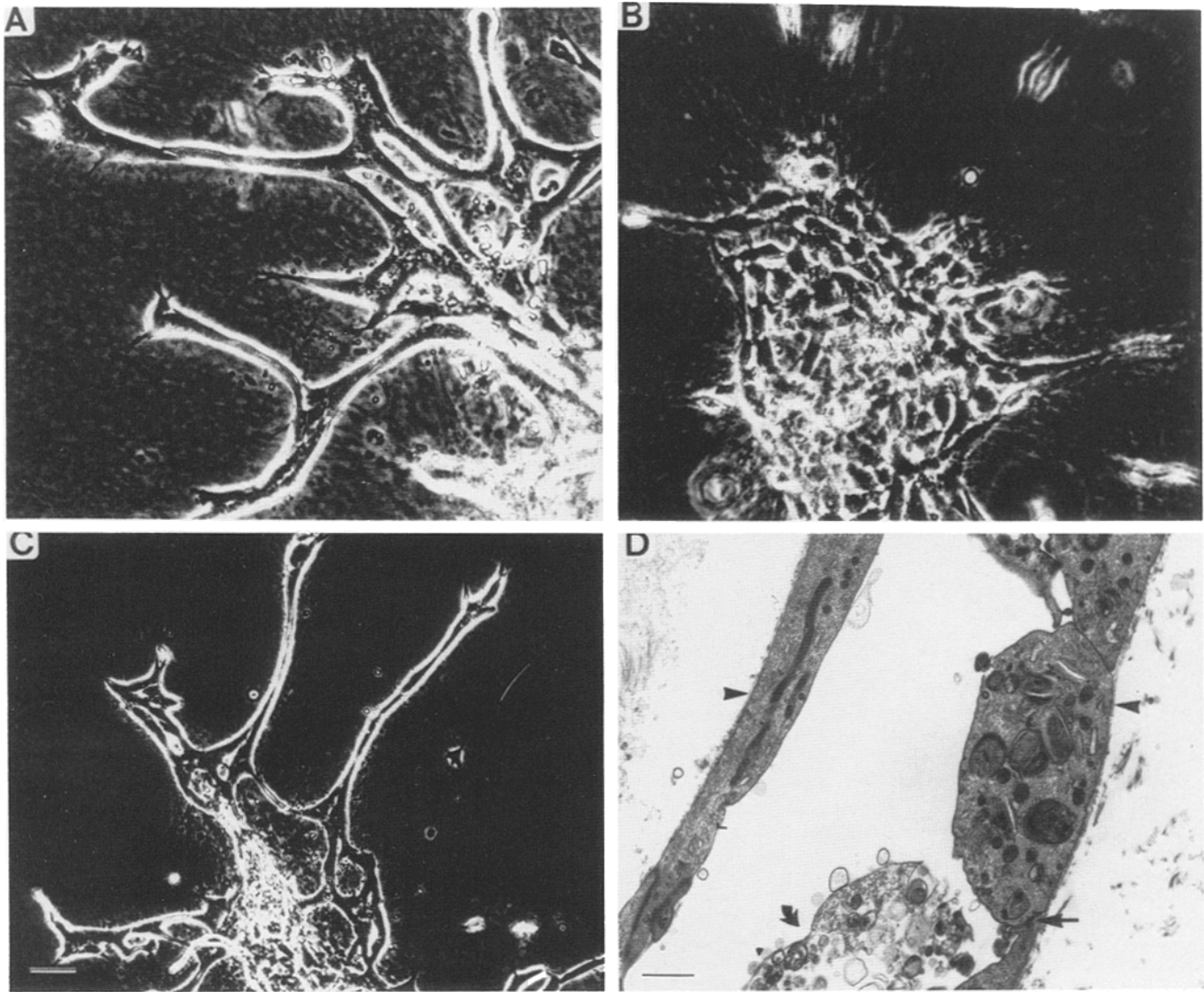


Figure 8. Inhibition of capillary-like tubule formation by mAb IVd4. Pulmonary artery endothelial cells were cultured for 48 h within gels containing type I collagen and basement membrane components as described in the Materials and Methods. (A) Control culture showing the capillary-like tubules that form in these cultures. (B) Inhibition of tubule formation by addition of 280 $\mu\text{g/ml}$ of mAb IVd4 to a similar culture. (C) Recovery of tubule formation after treatment with 280 $\mu\text{g/ml}$ mAb IVd4 as in B, followed by washing of the gel with medium and reincubation for 48 h in the absence of antibody. (D) Electron micrograph of one of the capillary-like tubules within a culture similar to that in A. A continuous basal lamina (arrowheads) surrounding the tubule and tight junctions (straight arrow) between the cells are visible. The lumen of the tubule is clearly apparent between the apical aspects of the endothelial cells. The curved arrow indicates a degenerating endothelial cell in the lumen that did not become incorporated into the tubule wall. Bars: (A, B, and C) 60 μm ; (D) 1 μm .

mAb IVd4. Reactivity was virtually absent in the cell membrane of the treated cells but persisted in the subcellular patches (Fig. 7 B). These results suggest that the HA hexamer may inhibit endothelial cell migration by causing loss of HAP from the plasma membrane rather than by competition for HA polymer-HAP interaction.

Effect of mAb IVd4 and HA Hexamer on Capillary-like Tubule Formation

We have developed a culture system, based on previous observations from other laboratories (Montesano et al., 1983; Madri et al., 1988; Kubota et al., 1988), where endothelial cells grown to confluence in monolayer are transferred into a gel composed of a mixture of type I collagen and basement membrane components. In this system, the confluent en-

dothelial cells rearrange into capillary-like tubular networks in 24–48 h (Fig. 8 A). The endothelial cells first form cords in which a partial lumen appears in many locations; these cylindrical regions subsequently fuse to form a longer lumen (e.g., Fig. 8 D), in similar fashion to that described previously by Folkman and Haudenschild (1980). Addition of 250 $\mu\text{g/ml}$ mAb IVd4 into the gel inhibited tubule formation (Fig. 8 B). On removal of the antibody, the capillary-like tubules formed in similar manner to the controls (Fig. 8 C).

HA hexamer also inhibited tubule formation; 75 $\mu\text{g/ml}$ gave rise to partial inhibition (Fig. 9 B) and 500 $\mu\text{g/ml}$ caused complete inhibition (Fig. 9 C). Tubules formed readily after removal of the hexamer (Fig. 9 D).

Bovine aortic and pulmonary arterial endothelial cells responded in similar fashion in the above experiments.

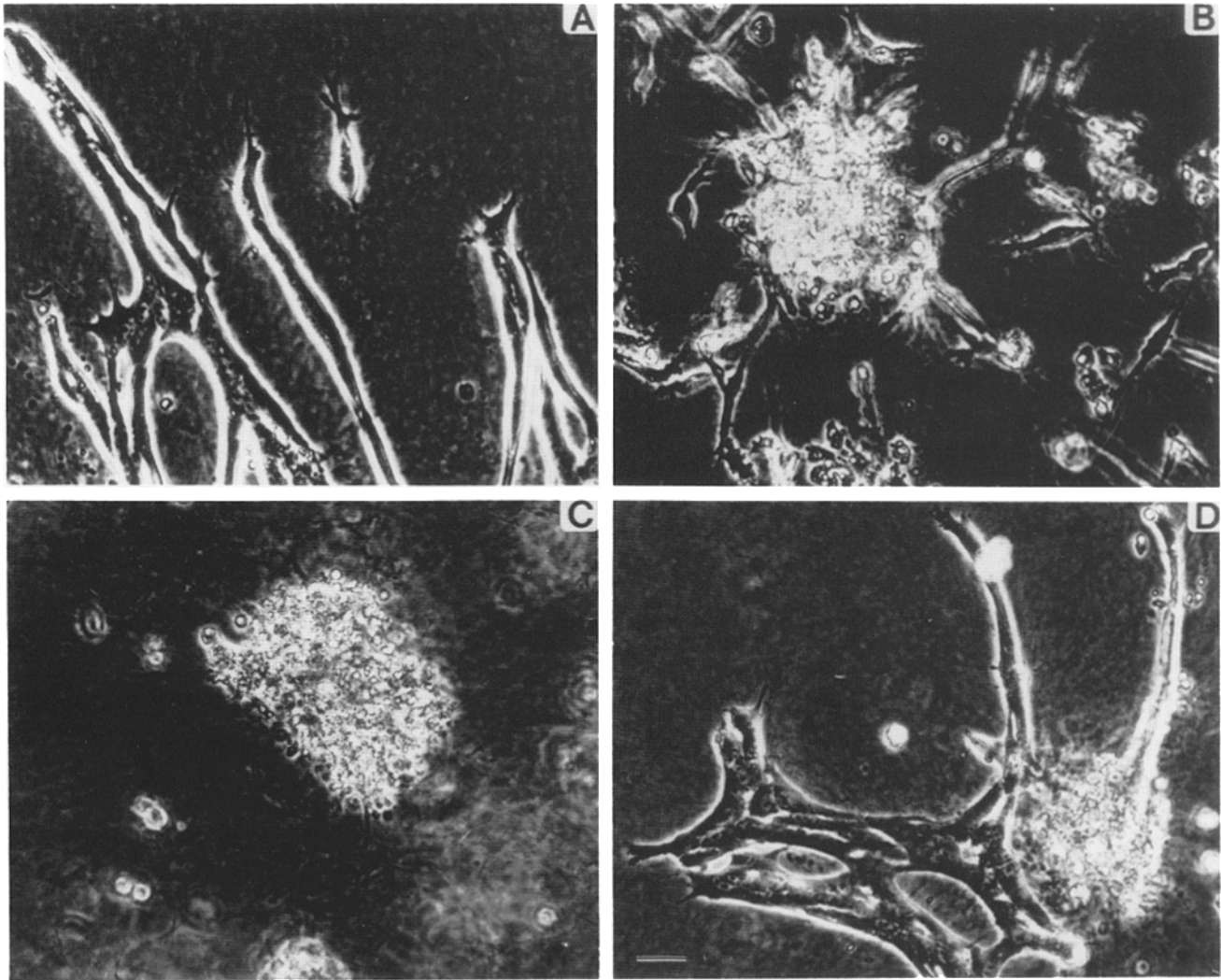


Figure 9. Inhibition of capillary-like tubule formation by HA hexamer. Pulmonary artery endothelial cells were cultured as in Fig. 8. (A) Control culture. (B) Partial inhibition of tubule formation by addition of 75 $\mu\text{g/ml}$ HA hexamer. (C) Complete inhibition of tubule formation by 500 $\mu\text{g/ml}$ hexamer. (D) Recovery of tubule formation after treatment with 500 $\mu\text{g/ml}$ hexamer as in C, followed by washing of the gel with medium and reincubation for 48 h in the absence of hexamer. Bar, 60 μm .

Discussion

In the present study we have examined the function of a hyaluronan receptor in endothelial cell behavior, specifically migration and formation of capillary-like tubules *in vitro*. HA oligomers and mAb to HABP both inhibit these processes; also, the appearance of HABP in the cell membrane of motile cells, including within many of their lamellipodia, is consistent with its association with endothelial cell movement. These results implicate HA-HABP interactions in endothelial cell morphogenesis.

The mechanism whereby mAb IVd4 inhibits endothelial cell migration and capillary-like tubule formation is presumably due to its specific binding to HABP which in turn would block interaction of endogenous HA with the HABP. Our previous studies have shown that mAb IVd4 blocks binding of exogenous HA to soluble or membrane-bound HABP (Banerjee and Toole, 1991) and formation of pericellular matrices which are dependent on endogenous HA-HABP interaction (Yu et al., 1992). Several types of cells exhibit pericel-

lular matrices that are dependent on both HA and HABP, and assembly of these matrices is inhibited by mAb IVd4 and HA oligomers (Knudson and Knudson, 1991; Yu et al., 1992). Although endothelial cells do not normally produce large amounts of HA in culture (Amanuma and Mitsui, 1991), they do produce small pericellular matrices that are dependent on HA and HABP (Yu et al., 1992). It is likely that these pericellular matrices play an important role in morphogenetic processes such as those involved in angiogenesis and vasculogenesis.

The inhibitory mode of action of HA oligomer on endothelial cell migration and capillary-like tubule formation is not yet certain. Our initial rationale for use of HA oligomers was their ability to block HA polymer-HABP interactions competitively (Underhill and Toole, 1979; Underhill et al., 1983). However, our results strongly suggest that treatment of motile endothelial cells with HA hexamer leads to reduction in the level of membrane-associated HABP and consequently to loss of HA-HABP interactions. The observation upon which this is based is that high concen-

trations of HA hexamer cause disappearance of mAb IVd4 immunoreactivity in lamellipodia and other areas of the cell membrane of motile cells. It might be argued that this absence of immunoreactivity results from occupation of HABP by the added HA oligomer, thus blocking access of the antibody to the HABP. There are at least two reasons why this would not be the case. First, HA oligomers have a low affinity of binding to HABP (Underhill and Toole, 1979) and thus are readily removed in the process of washing before reaction with antibody. Second, concomitant with loss of immunoreactivity in the cell membrane, immunoreactivity persists in the subcellular matrix (Fig. 7). Therefore, we conclude that treatment with HA hexamer leads to loss of HABP from the endothelial cell membrane and that this loss results in inhibition of migration. Whether this would also explain the inhibitory action of HA hexamer on capillary-like tubule formation is not yet clear.

The effect of HA hexamer discussed above suggests the possibility that membrane-bound HABP is subject to "down-regulation" in similar fashion to hormone receptors in the presence of excess ligand. Our finding that the subcellular immunoreactivity observed in endothelial cultures is largely attached to the substratum (Fig. 2) would explain why it is not lost in similar fashion to the membrane HABP when exposed to HA hexamer, since this extracellular HABP would not be subject to the membrane events involved in down-regulation. We are currently attempting to determine more definitively whether the mechanism underlying the apparent loss of HABP from the membrane is indeed down-regulation.

The above findings strongly suggest that HA-HABP interaction is required for endothelial cell morphogenesis. However, the molecular nature of the HABP involved is not yet known. In previous studies, we have shown that mAb IVd4 recognizes three proteins, of mol wt 93, 90, and 69 kD, in Western blots of partially purified HABP preparations from chick embryo brain (Banerjee and Toole, 1991), and an additional HABP of ~50 kD from several other chick and rat embryo tissues (unpublished data). We have found here that extracts of endothelial cells contain mAb IVd4-reactive proteins of 95, 77, and 50 kD. The interrelationship of these protein variants is not yet known, nor is their relationship to the widely distributed HA receptor, CD44 (Culty et al., 1990). Another HABP, isolated from normal and transformed fibroblasts, is of particular interest since interaction of HA with this protein promotes cell movement (Turley et al., 1985, 1991; Boudreau et al., 1991) and since it is preferentially located in lamellipodia of motile cells (Turley and Torrance, 1984; Turley and Auersperg, 1989). This 52/58 kD HABP is part of a cell membrane complex of proteins (Turley, 1989) and has recently been cloned (Hardwick et al., 1992). It is not related to CD44 but its relationship to the HABP recognized by mAb IVd4 is not yet known.

Previous studies have shown that HA oligomers are angiogenic (West et al., 1985), and that this angiogenic effect may derive from their stimulatory effect on endothelial cell proliferation (West and Kumar, 1989). However, HA oligomers are only stimulatory at low concentrations, i.e., <10 $\mu\text{g}/\text{ml}$, and high concentrations appear to be somewhat inhibitory (West and Kumar, 1989). In the present study we have found that higher concentrations of HA oligomer inhibit, rather than stimulate, endothelial cell migration and assembly into

capillary-like tubules. The stimulatory effect of low concentrations of HA oligomer on proliferation is unlikely to be due to competitive inhibition of the interaction of endogenous polymeric HA with HABP since, in other systems, oligomers have been shown to have a much lower affinity for cell surface HA receptors than polymer (Underhill and Toole, 1979; Laurent et al., 1986). Possibly the interaction of HA oligomer with unoccupied cell surface HABP, albeit with low affinity, may stimulate second messenger-generating assemblies within the cell; the inhibitory effects at high concentration would result from loss of the receptor by down-regulation, as discussed above. There is considerable evidence that the intracellular domain of HA receptors, or receptor complexes, of several cell types is linked to the cytoskeleton (Lacy and Underhill, 1987; Turley et al., 1990; Lokeshwar and Bourguignon, 1991; Camp et al., 1991) and can be phosphorylated (Carter and Wayner, 1988; Kalomiris and Bourguignon, 1989; Turley, 1989; Culty et al., 1990; Camp et al., 1991), suggesting that it may indeed be involved in signal transduction. However, as discussed above, there is no detailed information yet available regarding the nature of the endothelial HA receptor or its involvement in transmembrane signaling.

We conclude that HA-HABP interactions at the endothelial cell surface are most likely essential to endothelial cell morphogenesis. However, blood vessels fail to form in HA-enriched tissues in vivo (Feinberg and Beebe, 1983). This latter phenomenon is presumably due to inhibition of the proliferative phase of angiogenesis since high concentrations of polymeric HA, greater than 100 $\mu\text{g}/\text{ml}$, inhibit endothelial cell proliferation in culture; low concentrations do not (West and Kumar, 1989). Thus the concentration of HA in the extracellular matrix may be in part responsible for specifying the sites of angiogenesis or vasculogenesis during embryonic development. Low concentrations of HA would be essential whereas high concentrations would be inhibitory.

Modulation of angiogenesis is also of importance therapeutically. For example, angiogenic inhibitors may be beneficial in treating cancer since solid tumor growth requires new blood vessel formation (Folkman, 1986). HA oligomers in particular may be useful in this regard.

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