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Wound Fluid Angiogenesis Factor Stimulates the Directed Migration of Capillary Endothelial Cells

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During wound healing, new capillaries grow into the wound site. An angiogenesis factor isolated from wound fluid stimulates the movement of capillary endothelial cells in a filter migration assay. Experiments were carried out to determine whether the movement seen in the assay was chemokinetic or chemotactic. Capillary endothelial cells were plated onto a collagen-coated coverslip and inverted over a visualization apparatus. Cells exposed to a constant concentration of wound fluid angiogenesis factor (WAF) were more mobile than cells not exposed to WAF, and this movement was chemokinetic. When exposed to a gradient of WAF, the cells translocated toward the higher concentration; this directional movement was chemotactic. Cells in a gradient of WAF morphologically aligned with the gradient. These data support the idea that wound healing angiogenesis is regulated by the chemotaxis of capillary endothelial cells.

Key words: angiogenesis, capillary endothelial cells, chemotaxis, wound fluid

Angiogenesis is a feature of pathologic conditions such as the growth of tumors [1–4] and diabetic retinopathy [5]. It is also a feature of normal tissue growth [6], inflammation [7–10], and wound repair [11–13]. Because angiogenesis is a complex process involving the directed growth of blood vessels into tissue not previously vascularized, it cannot be adequately characterized by a single event at the cellular level. Recent data suggest that capillary endothelial cells respond to an angiogenic stimulus by synthesizing and secreting basement membrane- and connective tissue-degrading proteinases [14], by replicating in response to a specific mitogen or mitogens [15,16], and by moving in the direction of the angiogenic stimulus [12,17–21]. That these responses are due to the recognition of a single angiogenic factor or are the result of several components acting in concert are not necessarily mutually exclusive possibilities.

An angiogenesis factor has been isolated from experimentally induced rabbit wound fluid [12]. Wound fluid also contains endothelial cell mitogens [11]. Although

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wound fluid angiogenesis factor (WAF) is not a capillary endothelial cell mitogen, it does stimulate capillary endothelial cell movement [12]. In this study the nature of that movement was examined to determine if capillary endothelial cells respond to WAF by chemokinesis or by chemotaxis and if these cells can orient in a gradient of WAF.

MATERIALS AND METHODS

Capillary Endothelial Cells

Primary rabbit brain capillary endothelial cells were isolated and cultured on collagen-coated surfaces (Vitrogen, Collagen Corp., Palo Alto, CA) as previously described [12]. The endothelial nature of these cells was verified morphologically and by the presence of factor VIII antigen. The cells used in this study were from the second and third passages only.

Wound Fluid Angiogenesis Factor

A nonmitogenic wound fluid angiogenesis factor was isolated from experimentally induced rabbit wound fluid as previously described [12]. This factor stimulates angiogenesis in the rabbit corneal implant assay [12].

Filter Migration Assay

Cellular migration through porous filters was carried out by a modification [18,22] of the method of Boyden [23] as previously described [12]. Polycarbonate filters (Nucleopore) containing pores 10 μm in diameter were used. Assay times were typically 5 hr.

Visualization Assay

Cellular orientation and migration were also examined by means of a 7.5 cm $\times$ 2.5 cm $\times$ 0.5 cm Plexiglas slide that had milled into it two 1-mm deep by 4-mm wide wells separated by a 1-mm "bridge" [24] (Fig. 1). Approximately 3.3×10^4 capillary endothelial cells were plated onto a collagen-coated 22 mm $\times$ 40 mm coverslip for migration studies, and 1×10^5 cells were used for orientation studies. The culture medium used in all of the studies was serum-free HB 101 (Hana Media, Inc., Berkeley, CA) with 20 mM HEPES (UCSF Cell Culture Facility). The coverslip was inverted over the apparatus and the wells were filled with culture medium alone or culture medium with 10 ng/ml of WAF. When one of the wells contained WAF, a gradient was created across the bridge by capillary action and diffusion. As soon as

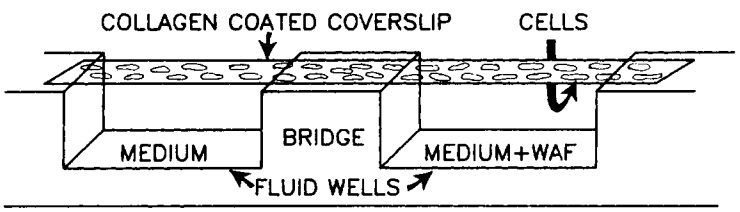

Fig. 1. Visualization apparatus.

the wells were filled (time zero), the cells were photographed over each well and over the bridge, and the apparatus was then placed in an incubator. The areas photographed were chosen at random and were considered acceptable if there was sufficient space between each cell to ensure that it could be unambiguously followed throughout the course of the experiment. Small marks had been etched onto the surface of the bridge so that each field could subsequently be located. At each hour for the next 5 hr the cells were again photographed, the medium in the wells was replenished, and the apparatus was returned to the incubator.

For analysis of cell movement, the positions of the capillary cell nuclei were traced from successive photographs and the tracings were superimposed. Orientation of capillary endothelial cells with a gradient of WAF was determined by measuring the angles made by all of the cellular projections from every cell in the field. No attempt was made to distinguish between leading and trailing projections. Therefore, all angles were normalized to a 0–90° measurement (0° = 0°, 90° = 90°, 180° = 0°, 270° = 90°, >270° = <90°). A cellular projection parallel to the direction of the gradient would make an angle of 0°.

RESULTS

Previously we demonstrated that WAF is not an endothelial cell mitogen [12], and we presented preliminary data that WAF stimulates endothelial cell migration. To determine the optimal concentration for the stimulation of capillary endothelial cell migration by WAF, we carried out a dose-response experiment using the filter migration assay. In a range of 0.01–100 ng/ml of WAF the optimal concentration was 10 ng/ml (Fig. 2). The shape of the dose-response curve indicated that 100 ng/ml WAF was only as effective as 1 ng/ml.

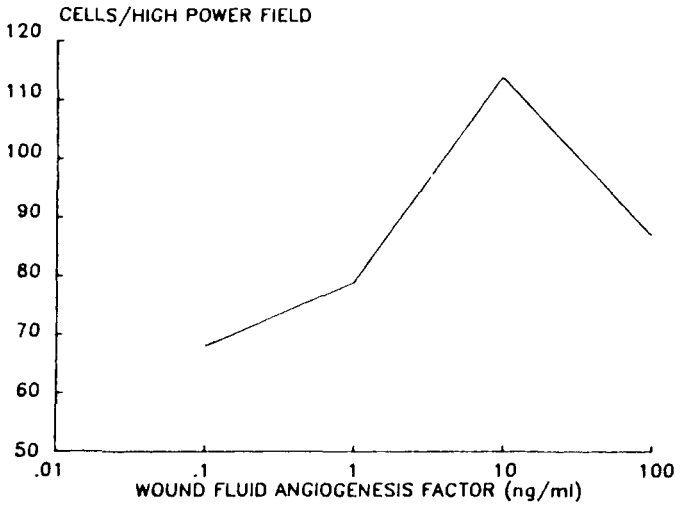

Fig. 2. Dose response of capillary endothelial cell migration to WAF in the filter migration assay. Cells were allowed to migrate for 5 hr toward various concentrations of WAF. Each concentration was tested on the same day with the same preparation of capillary endothelial cells. The experiment was conducted three times with different preparations of WAF.

Capillary endothelial cells in the filter migration assay changed their morphology while migrating in a gradient of WAF. Normally these cells appeared to have a well-spread cytoplasm and a polygonal morphology. While migrating through a 10-ng/ml gradient of WAF, they appeared elongated and spindle shaped (Fig. 3). When the cells reached the bottom of the filter, they once again took on their typical spread and polygonal morphology. Because the bottom compartment of the chambers contained WAF, the morphologic change was apparently induced by the cells' sensing a gradient of WAF and not merely by the presence of WAF.

To determine whether the stimulated movement of endothelial cells was chemokinetic or chemotactic, we placed optimal concentrations of WAF (10 ng/ml) in the upper compartment as well as in the lower compartment. There was no net increase in migration of capillary endothelial cells over that of cells in chambers containing only medium. This suggests that the movement of capillary endothelial cells stimulated by WAF in the previous experiment was chemotactic. However, because these assays were carried out over 5 hr, it was possible that a stable gradient of WAF was not maintained. In the absence of a stable gradient the conclusion that the movement stimulated by WAF was chemotactic would not be warranted.

A series of experiments was therefore performed by using a visualization apparatus. With this apparatus, stable gradients can be maintained for several hours and the cells can be observed and photographed throughout the course of the experiment. In these studies WAF, when present, was always placed in the right-hand well, and all of the figures are shown with this orientation. Because 10 ng/ml of WAF gave optimal results in the filter migration assay (Fig. 2), the same concentration was used in the visualization assay. Because capillary endothelial cells may randomly align with or against an experimental gradient when the cells are initially plated onto collagen-coated coverslips, the cells were exposed to 2 μg/ml of cytochalasin B immediately before establishment of a gradient. This caused the cells to become spherical and would abolish any alignment or morphologic bias that might result from the initial plating on the collagen-coated coverslip. Once the cells became spherical, they were washed free of the drug and inverted over the visualization apparatus.

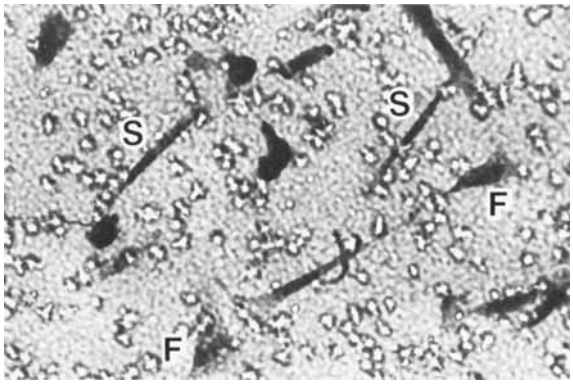

Fig. 3. Morphology of capillary endothelial cells in the filter migration assay. The plane of focus is at the bottom of a 10-μm pore diameter polycarbonate filter. Cells were allowed to migrate for 5 hr toward 10 ng/ml of WAF. The spindle-shaped cells (S) are in the process of migrating, while the flat cells (F) have finished migrating and have resumed their normal morphology. ×670.

Although this precaution was taken in all of the experiments described here, similar results were obtained without the pretreatment with cytochalasin B.

The movement of cells inverted over the left well, which contained only medium, was compared to that of cells inverted over the right well, which contained medium with 10 ng/ml of WAF (Fig. 4). The cells exposed to medium alone showed very little movement, and that movement occurred in several different directions (Fig. 5). In contrast, the cells exposed to 10 ng/ml of WAF in the absence of a gradient showed accelerated movement, also in several different directions. It is likely that this was a chemokinetic response to WAF.

The analysis of the capillary endothelial cells inverted over the 1-mm bridge betweeen the two fluid-filled wells was more complex. When both the left and right wells were filled only with medium, the cells inverted over the bridge were dendritic, with highly branched projections (Fig. 6a), in contrast to cells plated over the well containing medium (Fig. 4). Cells inverted over the bridge with 10 ng/ml of WAF on both sides were also dendritic, but the projections were less branched (Fig. 6b). This dendritic morphology was similar to that of capillary endothelial cells cultured in a collagen gel [25]. The bridge may have approximated the conditions of culturing in a collagen gel because the cells were plated onto a collagen-coated coverslip and then inverted over the bridge, with only about 10 μm between the top of the bridge and the bottom of the coverslip.

When the right well contained 10 ng/ml of WAF, the cells on the 1-mm bridge were in a gradient of 0–10 ng/ml/mm of WAF (Fig. 6c). These cells had fewer and less branched projections and were slightly more elongated than control cells (Fig. 6a) or cells in a constant 10 ng/ml of WAF (Fig. 6b). This morphologic change was, therefore, due to the cells' detecting a concentration gradient of WAF, not merely the presence of WAF. Tracings of the nuclei of cells on the bridge between two medium-filled wells (Fig. 7) showed slightly more movement than tracings of cells over the medium-filled well (Fig. 5). Tracings of the nuclei of cells on the bridge between two chambers, each containing 10 ng/ml of WAF, showed more random movement than cells on the bridge between medium-filled wells (data not shown). Tracings of the endothelial cell nuclei in a gradient of WAF showed increased movement that was not random but was directed up an increasing concentration of WAF (Fig. 7). Similar gradients of either rabbit serum or bovine serum albumin did not stimulate increased

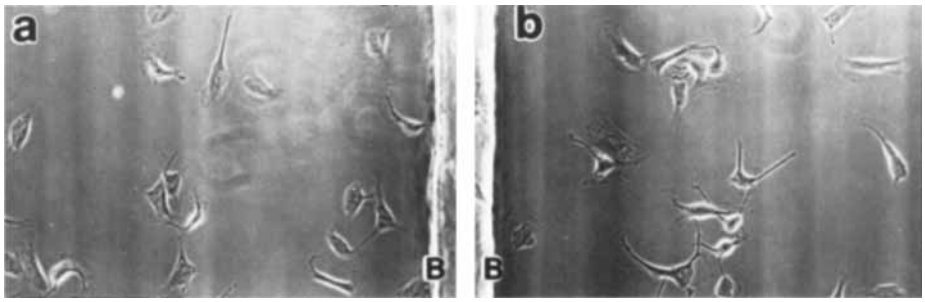

Fig. 4. Morphology of capillary endothelial cells over the wells of the visualization apparatus after the 1-hr time point. The fields are oriented as if they were in the apparatus with the bridge separating the two wells. The bright line (B) indicates the edge of the bridge. The panels show cells over wells filled with medium alone (a) or medium with 10 ng/ml of WAF (b). ×340.

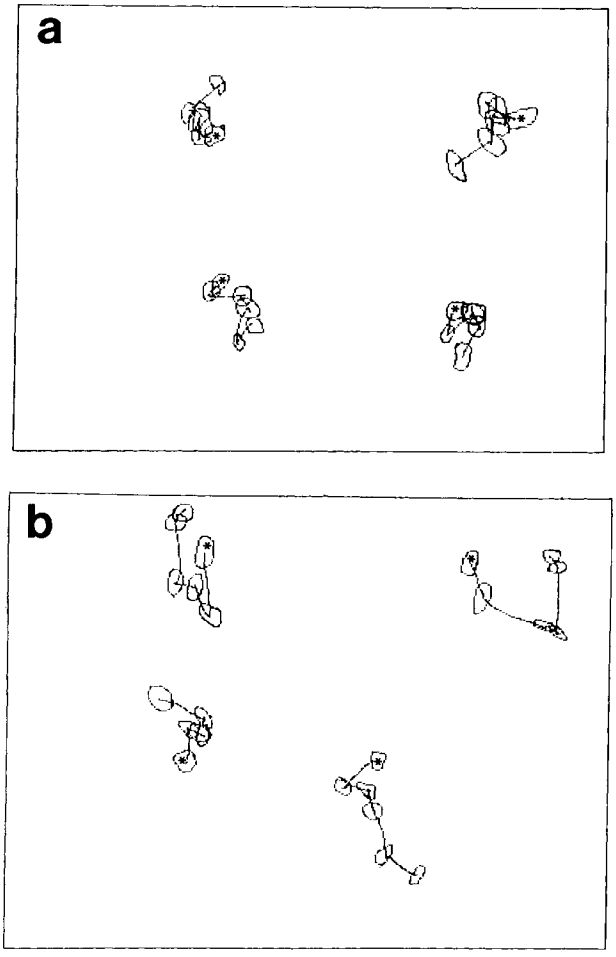

Fig. 5. Chemokinetic movement of capillary endothelial cells. The positions of the nuclei of cells shown in Figure 4 were traced at each hourly time point for a total of 6 hr. Asterisks indicate time zero. The lines connect consecutive positions. a) Movement resulting from exposure to medium alone. b) Movement after exposure to WAF in the absence of a gradient.

random or directed movement. These data suggest that capillary endothelial cells translocate by chemotaxis up an increasing concentration gradient of WAF.

Alignment of capillary endothelial cells parallel to the direction of the gradient of WAF may contribute to the directionality of the movement seen in Figure 7. The analysis of capillary endothelial cells over the bridge was based on cells near the edge of the bridge closest to the 10 ng/ml of WAF (in the right well). To determine if alignment was occurring, we densely plated capillary endothelial cells onto a collagen-coated coverslip. At this plating density it was impossible to identify the position of each individual cell from one hourly time point to the next. However, when a portion of the entire width of the bridge was photographed, the morphology of the capillary endothelial cells varied with position in the gradient (Fig. 8). The cells in the lowest concentration of WAF had the morphologic characteristics of cells over the bridge in

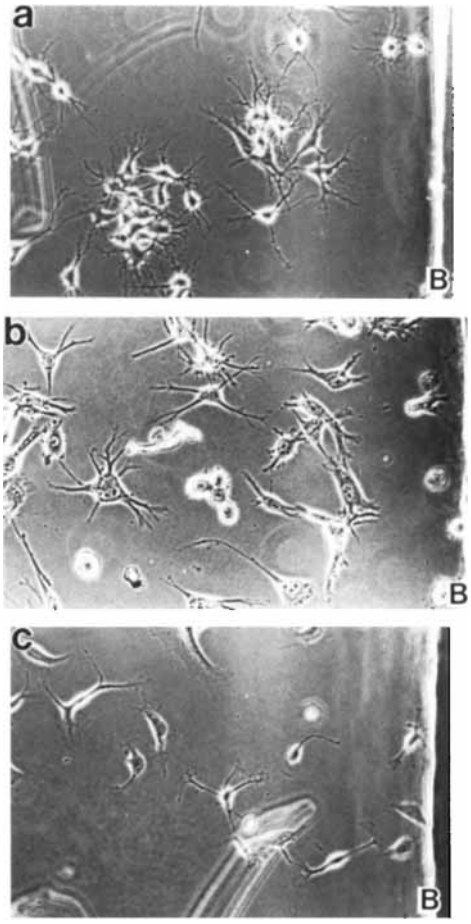

Fig. 6. Morphology of capillary endothelial cells on the bridge after the 1-hr time point. a) Dendritic morphology of cells on a bridge between two wells filled with medium alone. b) Less branched dendritic morphology of cells on the bridge between two wells, each filled with 10 ng/ml of WAF. c) Cells on a bridge in a gradient of 10 ng/ml/mm WAF entering at the highest concentration from the right. Note the reduced number of cellular projections. In each panel the right edge of the bridge is indicated (B). ×340.

the absence of a gradient of WAF, whereas cells in the higher concentration portion of the gradient showed fewer projections that were more elongated and less branched, as were the cells in Figure 6c. The cells in this portion of the gradient appeared to aggregate, and the cell bodies were more rounded and refractile. The cellular projections of the cells in the lower concentration of WAF, on the left side of the bridge, appeared to make more random angles with respect to the line of the gradient, whereas those on the high concentration portion of the gradient appeared to make shallow angles with respect to the gradient. When the angles made by the cellular projections were measured, there was an increased relative frequency of low-degree angles in the high concentration portion of the gradient (Fig. 9). These data suggest that capillary endothelial cells align with a gradient of WAF and that they are more

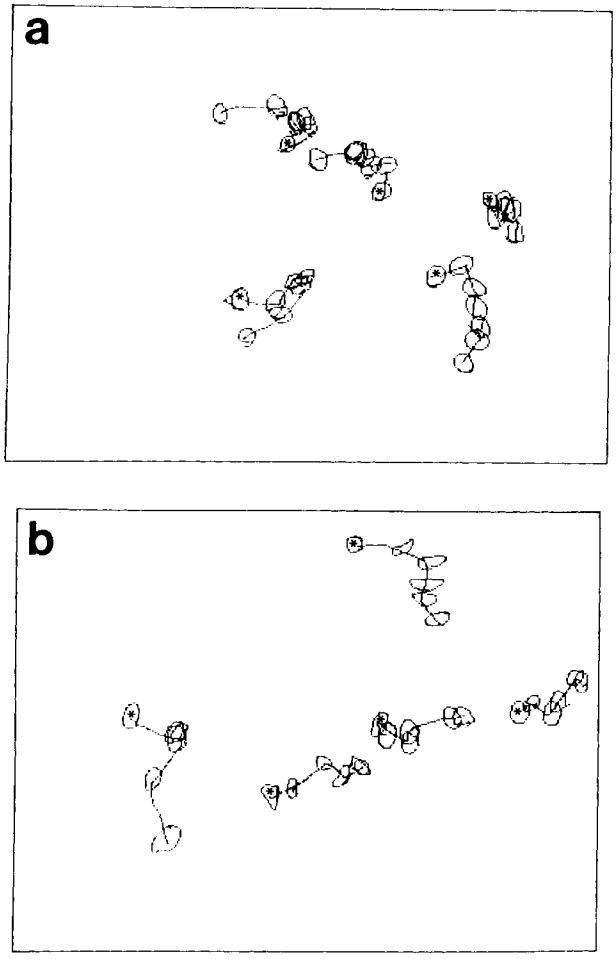

Fig. 7. Chemotactic movement of capillary endothelial cells. The positions of the nuclei of cells shown in Figure 6 were traced at each hourly time point for a total of 6 hr. Asterisks indicate time zero. The lines connect consecutive positions. a) Movement of cells on the bridge that were exposed to medium alone (see Fig. 6a). b) Movement of cells in a gradient of 10 ng/ml/mm WAF entering at the highest concentration from the right (see Fig. 6b).

aligned in the higher concentration portion of the gradient than in the lower concentration portion of the gradient.

DISCUSSION

The data presented here establish that when capillary endothelial cells are exposed to a constant concentration of WAF they increase their random mobility and when placed in a gradient of WAF they translocate up the concentration gradient. This directionality is characteristic of the capillary growth during angiogenesis. When capillary endothelial cells, which are usually stationary cells, are stimulated to move they do so slowly in comparison to the more mobile inflammatory cells. In vivo, the

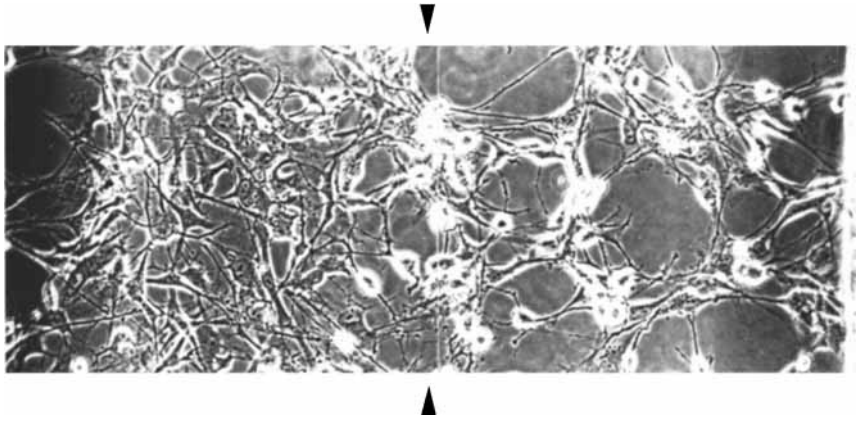

Fig. 8. Morphologic orientation in the visualization assay. Capillary endothelial cells were plated at an increased density ($1.1 \times 10^4/cm^2$) and exposed for 5 hr to a gradient of 10 ng/ml/mm WAF entering at the highest concentration from the right. The field shown spans the entire bridge. The arrowheads indicate the middle of the bridge. ×370.

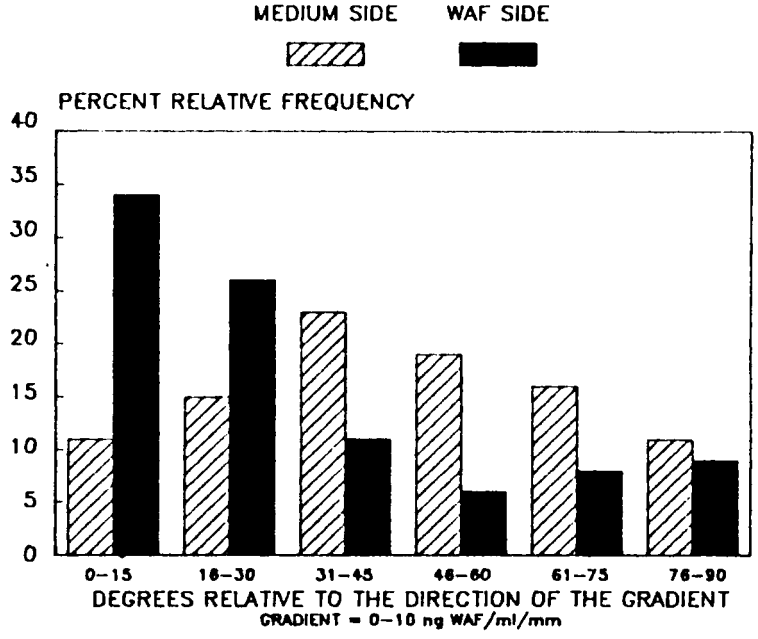

Fig. 9. Alignment of capillary endothelial cells with a gradient of WAF. The angle, relative to the direction of the gradient, made by all of the cytoplasmic projections of each cell shown in Figure 8 was measured and normalized to 0–90°. The relative frequency of the angles made by the projections on the side of the bridge toward the medium (left of the arrow in Fig. 8) was compared to the relative frequency of those made on the side of the bridge toward the WAF (right of the arrow in Fig. 8).

stimulation of capillary endothelial cell migration would constitute an unusual event for these cells and under normal circumstances may be limited to an angiogenic response.

Because capillary endothelial cells move slowly, traditional migration studies may be difficult to interpret. In the filter migration assay described here, 3 to 5 hr were necessary to detect significant movement, whereas studies on polymorphonuclear leukocytes have been completed in 45 min [26]. The gradient in a 3 to 5 hr filter migration assay may be stable for only 1 hr [24,27]. To determine unambiguously whether slow-moving cells are translocating up an increasing concentration gradient of stimulant, the stability of the gradient must be maintained throughout the course of the experiment. The visualization apparatus can maintain a stable gradient for several hours, especially if the contents of the wells are replenished during the experiment [24,27].

Our data suggest that the morphologic changes associated with directed migration are the result of the capillary endothelial cells' detecting a concentration gradient of WAF rather than a constant concentration of WAF. The morphologic changes that were induced in capillary endothelial cells by a gradient of WAF indicated that the cells aligned parallel with the direction of the gradient. This behavior was not uniform across the full width of the bridge, suggesting that the extent of the response was concentration dependent. In another system, bovine adrenal capillary endothelial cells have been shown to change their morphology from spread to spindle shaped in a concentration-dependent manner in response to retina-derived growth factor [21].

Studies of angiogenesis in vivo have established that there is a role for nonmitogenic movement of capillary endothelial cells during capillary growth. Transmission electron micrographic studies of growing capillaries showed cell division occurring behind a group of nondividing, migrating capillary endothelial cells [28]. Scanning electron micrographic analysis of angiogenesis in the rat cornea showed that an initial response of individual microvascular endothelial cells to an angiogenic stimulus was the production of cellular projections in the direction of the angiogenic response; this appeared to take place in the absence of cell division [29]. A study with irradiated animals established that limited angiogenesis can proceed from a capillary bed incapable of a mitogenic response; however, the extent of capillary growth was greater in animals that could support cell division [30]. Microvascular endothelial cell movement may, therefore, be sufficient to establish the initial capillary bed during angiogenesis.

Some angiogenesis factors may provide both mitogenic and chemotactic signals [15,21]. It has not yet been established whether the capillary endothelial cells first divide or first migrate in response to such a factor. The data presented here suggest that a component of wound fluid can stimulate angiogenesis by initiating the directed movement of capillary endothelial cells in the absence of a mitogenic response by these cells. These data further imply that during angiogenesis the mitogenic response need not be delivered in the form of a gradient as long as the chemotactic gradient is maintained.

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REFERENCES

1. Gimbrone MA Jr, Cotran RS, Leapman SB, Folkman J: JNCI 52:413, 1974.
2. Folkman J, Cotran R: Int Rev Exp Pathol 16:207, 1976.
3. Weiss JB, Brown RA, Kumar S, Phillips P: Br J Cancer 40:493, 1979.
4. Fenselau A, Watt S, Mello RJ: J Biol Chem 256:9605, 1981.
5. D'Amore PA, Glaser BM, Brunson SK, Fenselau AH: Proc Natl Acad Sci USA 78:3068, 1981.
6. Castellot JJ Jr, Karnovsky MJ, Spiegelman BM: Proc Natl Acad Sci USA 79:5597, 1982.
7. Sidky YA, Auerbach R: J Exp Med 141:1084, 1975.
8. Polverini PJ, Cotran RS, Gimbrone MA Jr, Unanue ER: Nature 269:804, 1977.
9. Polverini PJ, Leibovich SJ: Lab Invest 51:635, 1984.
10. Polverini PJ, Leibovich SJ: J Leuk Biol 37:279, 1985.
11. Greenburg GB, Hunt TK: J Cell Physiol 97:353, 1978.
12. Banda M, Knighton DR, Hunt TK, Werb Z: Proc Natl Acad Sci USA 79:7773, 1982.
13. Knighton DR, Hunt TK, Scheuenstuhl H, Halliday BJ, Werb Z, Banda MJ: Science 221:1283, 1983.
14. Gross JL, Moscatelli D, Rifkin DB: Proc Natl Acad Sci USA 80:2623, 1983.
15. Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsbrun M: Science 223:1296, 1984.
16. D'Amore PA, Klagsbrun M: J Cell Biol 99:1545, 1984.
17. Azizkhan RG, Azizkhan JC, Zetter BR, Folkman J: J Exp Med 152:931, 1980.
18. Glaser BM, D'Amore PA, Seppa H, Seppa S, Schiffmann E: Nature 288:483, 1980.
19. Hannan GN, McAuslan BR: J Cell Physiol 111:207, 1982.
20. Zetter BR: Nature 285:41, 1980.
21. Herman IM, D'Amore PA: J Musc Res Cell Motil 5:697, 1984.
22. Snyderman R, Shin HS, Hausman MH: Proc Soc Exp Biol Med 138:387, 1971.
23. Boyden S: J Exp Med 115:453, 1962.
24. Zigmond SH: J Cell Biol 75:606, 1977.
25. Stopak D, Werb Z, Banda MJ: J Cell Biol 97:452a, 1983.
26. Postlethwaite AE, Kang AH: J Exp Med 143:1299, 1976.
27. Zigmond SH: In van Furth R (ed): "Mononuclear Phagocytes, Functional Aspects," Pt. I. The Hague: Martinus Nijhoff, 1980, p 461.
28. Ausprunk DH, Folkman J: Microvasc Res 14:53, 1977.
29. Burger PC, Chandler DB, Klintworth GK: Lab Invest 48:169, 1983.
30. Sholley MM, Ferguson GP, Seibel HR, Montour JL, Wilson JD: Lab Invest 51:624, 1984.