

FIBRONECTIN IS PRODUCED BY BLOOD  
VESSELS IN RESPONSE TO INJURY

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In culture, fibronectin, a 440,000-dalton glycoprotein produced by fibroblasts (1-3), monocytes (4, 5), and endothelial cells (6-8) and/or derived from serum (9), can bind to substratum and simultaneously to cell surfaces; and thereby promote attachment, spreading, and growth of these cells (10, 11). In vivo fibronectin is concentrated in areas of intense cellular activity during embryogenesis (12, 13), delayed-type hypersensitivity (14), and wound repair (15-19), suggesting that fibronectin in vivo may also provide a provisional substratum essential for cell migration and proliferation. Nevertheless, little is known of the source of in vivo fibronectin.

During the first 2 d after intradermal antigen challenge in sensitized guinea pigs (14) or excisional skin wounds in guinea pigs (18, 19), a large quantity of fibronectin (14, 18, 19) appeared as fibrillar strands and minute nodular foci in the extravascular space both within and adjacent to the damaged tissue as demonstrated by immunofluorescence. By radioisotopic studies, this interstitial accumulation of fibronectin was plasma derived (14). In contrast, fibronectin accumulation within blood vessels, as determined by immunofluorescence, began at 3 d after tissue injury (14, 19), when interstitial deposition of plasma fibronectin and fibrin had ceased and endothelial cells within blood vessels in these tissue sites had begun to proliferate. From these data, we postulated that intravascular fibronectin was produced *in situ* by endothelial cells. Such a hypothesis is quite tenable, in that endothelial cells have been shown to synthesize fibronectin in vitro (6-8) and deposit the fibronectin between themselves and the tissue culture dish. In this report we give direct evidence that the blood vessel fibronectin is produced *in situ* and is not plasma derived.

### Materials and Methods

*Experimental System.* (A/J × C47/B16)F<sub>1</sub> hybrid mice (The Jackson Laboratory, Bar Harbor, ME), were thymectomized and, 2-3 wk later, injected intraperitoneally with rabbit anti-mouse thymocyte serum (Microbiological Associates, Walkersville, MD) in doses of 0.25 ml before grafting, on the day of grafting, and at 2 and 4 d after graft placement. CD rat (Charles River Breeding Laboratories, Inc., Wilmington, MA) ear skin obtained from the pinna was prepared and transplanted as described previously (20, 21). By 2 wk after grafting, the sites were well-healed, allowing excisional wounds to be made with a 3-mm punch in the center of each graft. At various time intervals thereafter, ranging from 2 to 10 d, the wounded graft sites were harvested and bisected for immunofluorescence and 1- $\mu$ m sections.

*Autoradiography of 1- $\mu$ m Sections.* Mice carrying transplanted rat ear skin on their flanks for various numbers of days (2-10) were injected intraperitoneally with 25  $\mu$ Ci of [<sup>3</sup>H]thymidine 2

h before killing. Tissue was processed for 1- $\mu$ m sections as previously described (14). In addition, the sections were coated with a silver emulsion and incubated for 2 wk before Giemsa staining and microscopic examination. Autoradiography of [<sup>3</sup>H]thymidine incorporation into cell nuclei was used to quantitate endothelial cell proliferation. Labeled endothelial cells had >5 grains of precipitated silver superimposed on their nuclei. The percentage of labeled endothelial cells was determined within a 0.5-mm swath along the wound edge from the epidermis to the panniculus carnosus and within the adjacent swath 0.5–1.0 mm from the wound edge.

*Reciprocal Species-specific Anti-Fibronectin Antibodies.* Rat and mouse fibronectin was isolated from normal rat- and mouse-citrated plasma using gelatin-affinity chromatography (22). Fibronectin from each animal was mixed with complete Freund's adjuvant and injected into the footpads of reciprocal animals. This procedure produced species-specific antibodies. Antisera from each species were purified by adsorption on insolubilized fibronectin-depleted plasma of that species. Antibodies were isolated by passage of the antisera over a protein A affinity column (23) and then conjugated with either fluorescein or rhodamine by the dialysis method (24). Immunofluorescence was performed as previously described (14).

## Results

To test whether blood vessel fibronectin was produced *in situ*, we used rat ear skin transplanted onto the flanks of immunosuppressed mice and reciprocal species-specific anti-fibronectin antibodies. Previous studies have shown that donor (rat) blood vessels persisted in the xenograft for at least 4 wk after transplantation (20, 21); therefore, within this time period mouse anti-rat fibronectin antisera would identify fibronectin produced *in situ* by donor (rat) blood vessel cells, while rat anti-mouse fibronectin antisera would identify plasma derived fibronectin deposited in the vessels from the recipient (mouse) circulation. Because the graft sites were healed within 2 wk after surgery, it was possible to extirpate a 3-mm punch biopsy from the center of each graft site at that time.

2 d after excisional wounding, the endothelial cells of the microvasculature along the wound edge appeared markedly activated as characterized by swollen nuclei with an open chromatin pattern, swollen cytoplasm, and bulging of the entire cell into the vessel lumen. Some endothelial cells in blood vessels within 0.5 mm of the wound edge demonstrated mitotic figures by day 4. These cytologic findings correlated with the greatly increased numbers of [<sup>3</sup>H]thymidine-labeled endothelial cells near the wound edge demonstrated with 1- $\mu$ m section autoradiography at 2 and 4 d after injury (Fig. 1).

When the microvasculature near the wound was examined with species-specific, fluorescein-labeled, anti-rat fibronectin antibodies, an increased fluorescence of fibronectin was noted in the walls of blood vessels that coursed within 0.5 mm of the wound edge the second day after injury (Fig 2. A and B) compared with the staining of fibronectin in walls of rat blood vessels within the graft before injury (Fig 2. D). When the rat vessels near the wound edge were stained with anti-mouse fibronectin no staining was apparent (Fig 2. C). The lack of vessel fluorescence with anti-mouse fibronectin was not secondary to a failure of the anti-mouse fibronectin antibodies to stain mouse fibronectin in tissue sections, because the microvasculature of normal mouse skin fluoresced with the same anti-mouse antibodies (Fig 2. E) but not with anti-rat fibronectin antibodies (Fig 2. F). Specificity of the antisera was confirmed by the demonstration that anti-rat, but not anti-mouse, fibronectin antibody-staining activity could be abolished by passage over a rat-fibronectin Sepharose 4B affinity column; conversely, anti-mouse, but not anti-rat, fibronectin staining could be abrogated by absorption with insolubilized mouse fibronectin.

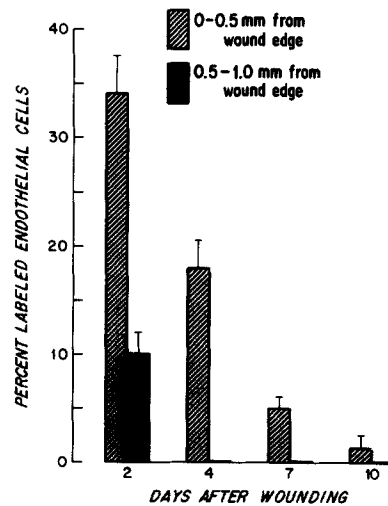


FIG. 1. Histogram demonstrates marked endothelial cell proliferation 0-0.5 mm from the wound edge within the graft at 2 and 4 d after extirpation. Each bar and bracket represents the mean  $\pm$  standard error of the mean for three mice at each time interval.

At the interface between transplanted skin and host skin, the microvasculature from the two animals might intermingle; therefore, we examined the graft/host interface with our reciprocal anti-rat and anti-mouse fibronectin fluorescent antibody probes. At this location, at 2 wk after transplantation, the blood vessels stained with either anti-rat fibronectin (Fig. 3 A) or anti-mouse fibronectin (Fig. 3 B) but not with both antibody preparations (Fig. 3 A and B). Thus, these data further support the hypothesis that fibronectin is synthesized *in situ* in blood vessels and that fibronectin production is stimulated during microvasculature proliferation in response to injury.

### Discussion

These studies provide the first clear indication that blood vessels synthesize fibronectin *in vivo* in association with endothelial proliferation. Whereas plasma fibronectin can deposit in other sites and bathes the endothelium, we could detect no significant recruitment of circulating fibronectin into the matrix of these proliferating vessels, as has been shown in fibroblast matrices *in vitro* (25). This suggests there might be a direct relationship between the observed endothelial proliferation and the increased production of fibronectin by cells in the vessel wall. Perhaps the increase in vessel wall fibronectin is an essential part of the reparative process of blood vessel basement membrane during wound healing or neovascularization.

### Summary

During the time of tissue repair that ensues subsequent to tissue injury, blood vessel wall fibronectin increases concomitantly with endothelial proliferation and angiogenesis. However, the source of this blood vessel fibronectin had not been delineated. In this report we have demonstrated that microvascular fibronectin is produced *in situ* by the proliferating vessels surrounding excisional wounds. This finding was established by extirpating 3 mm of skin from the center of a well-healed rat xenograph on the flanks of immunosuppressed mice, harvesting the injured skin sites at various

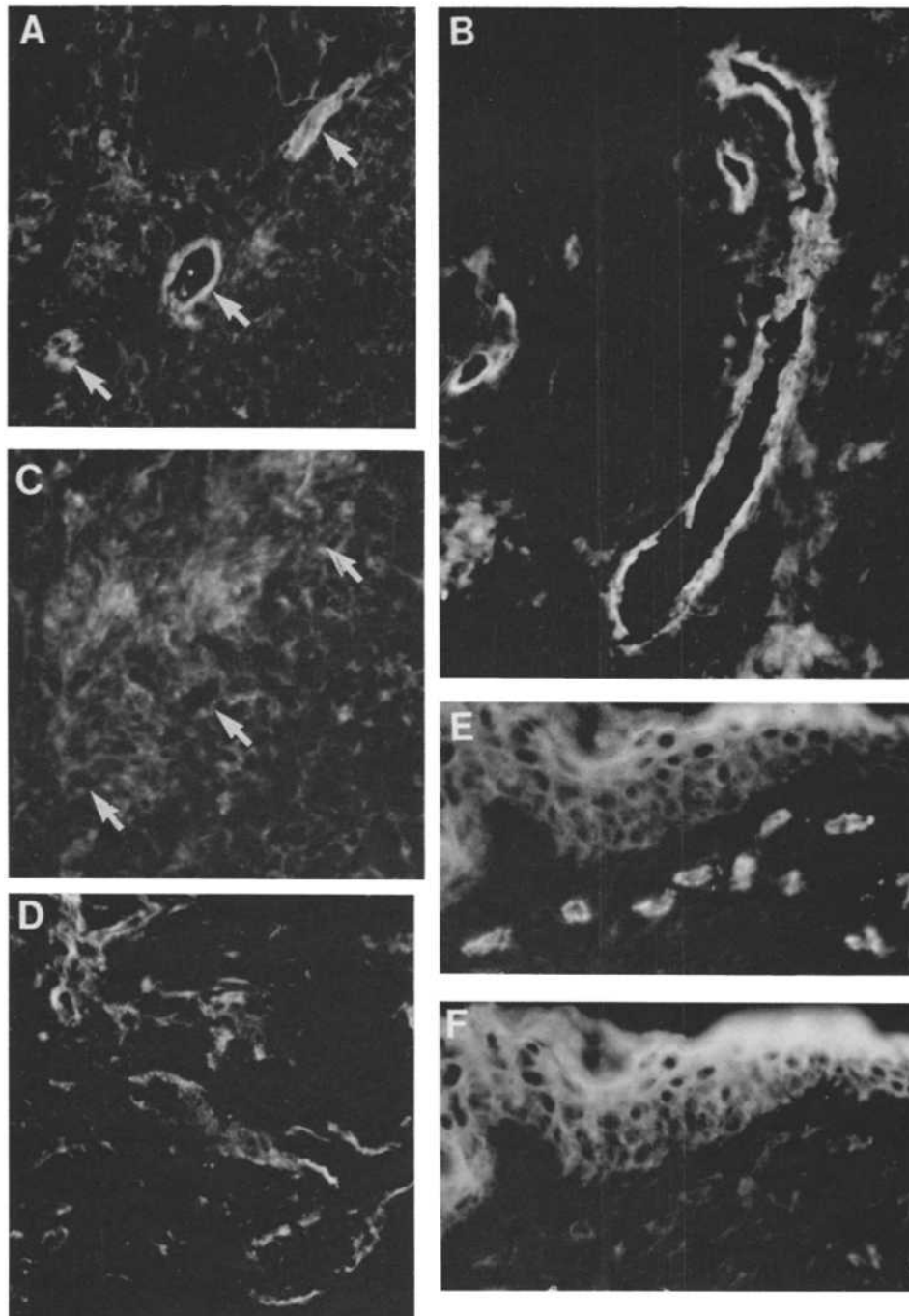


FIG. 2. Immunofluorescence studies of small blood vessels: (A) and (B) within 0.5 mm of an excisional wound 2 d after extirpation using fluorescein-conjugated anti-rat fibronectin; (C) same as (A), using rhodamine-conjugated anti-mouse fibronectin; (D) in a nonwounded rat graft 2 wk after transplantation using fluorescein-conjugated anti-rat fibronectin; (E) in normal mouse skin stained with rhodamine-conjugated anti-mouse fibronectin; (F) same, with fluorescein-conjugated anti-rat fibronectin. 800 X.

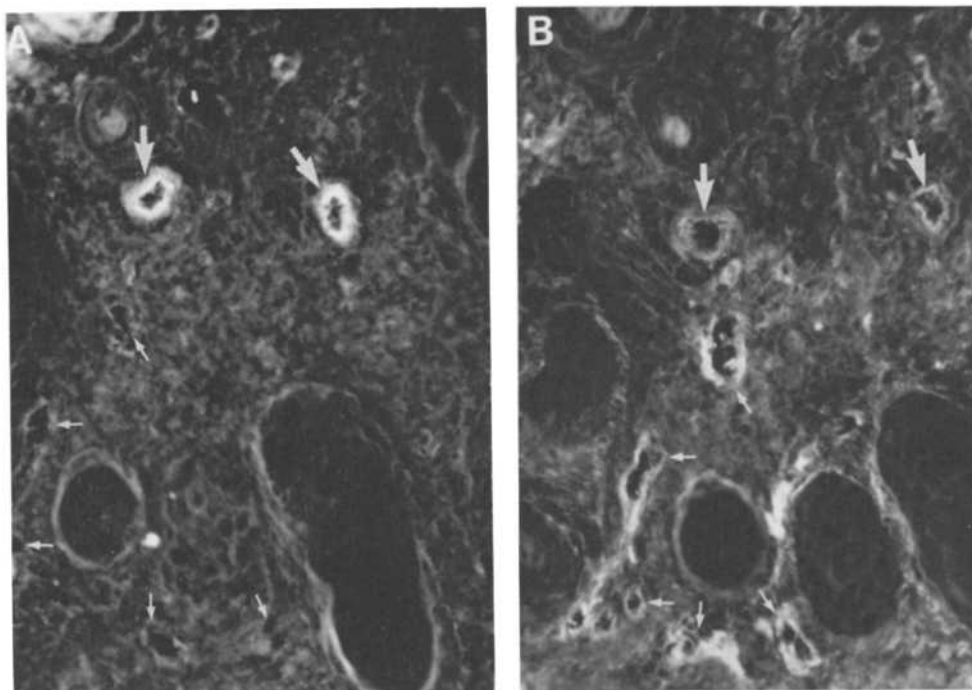


FIG. 3. Immunofluorescence studies of the rat graft/mouse skin interface at 2 wk after transplantation with (A) fluorescein-conjugated anti-rat fibronectin or (B) fluorescein-conjugated anti-mouse fibronectin. Adjacent tissue specimens are shown in which the rat vessels are indicated by large arrows and the mouse vessels with small arrows. 800  $\times$ .

stages during the healing process, and staining the specimens with reciprocal species-specific anti-fibronectin. The proliferating donor vessels that surrounded the wounded graft had increased fluorescence staining with FITC conjugated mouse anti-rat fibronectin and no staining with rat anti-mouse fibronectin. This finding was taken as direct evidence that the fibronectin was produced *in situ* by the rat vessels and not derived from circulating mouse plasma.

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