

# Appearance of Fibronectin during the Differentiation of Cartilage, Bone, and Bone Marrow

ROY E. WEISS and A. H. REDDI

*Laboratory of Biological Structure, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205. Dr. Weiss's present address is the Department of Orthopedics, University of Southern California Medical School, Los Angeles, California 90007.*

**ABSTRACT** Fibronectin has been localized by indirect immunofluorescence during the various phases of endochondral bone formation in response to subcutaneously implanted demineralized bone matrix. Its histologic appearance has been correlated with results of biosynthetic experiments. (a) The implanted collagenous bone matrix was coated with fibronectin before and during mesenchymal cell proliferation. (b) During proliferation of mesenchymal precursor cells, the newly synthesized extracellular matrix exhibited a fibrillar network of fibronectin. (c) During cartilage differentiation, the fibronectin in the extracellular matrix was apparently masked by proteoglycans, as judged by hyaluronidase treatment. (d) Differentiating chondrocytes exhibited a uniform distribution of fibronectin. (e) Fibronectin was present in a cottony array around osteoblasts during osteogenesis. (f) The developing hematopoietic colonies revealed fibronectin associated with them. Therefore, it appears that fibronectin is ubiquitous throughout the development of endochondral bone and bone marrow.

Fibronectin is a cell surface glycoprotein involved in cell-substratum (usually collagen) interaction (7, 9, 11, 16, 22, 27). A structurally related and immunologically cross-reactive protein, plasma fibronectin, is present in the blood plasma and was previously known as cold insoluble globulin (26). Although many studies have investigated the involvement of fibronectin in cell attachment *in vitro* (for review see reference 27), important questions regarding its *in vivo* function remain, as yet, unanswered. In particular, the role that fibronectin plays in the development of various connective and skeletal tissues is unknown. We have previously shown that fibronectin is synthesized throughout the development of endochondral bone and that its synthesis is maximal during mesenchymal precursor cell proliferation and hematopoiesis (24). However, the localization of fibronectin in the developing bone and bone marrow is not known. To study this problem, we have used an *in vivo* matrix-induced endochondral bone-forming system (18–20) and extended our earlier observations (24). In response to subcutaneous implantation of demineralized collagenous bone matrix, a cascade of events ensues starting with proliferation of mesenchymal precursor cells at 3 d after implantation, chondrogenesis on day 7, followed by chondrolysis with calcification

of cartilage and culminating in osteogenesis on day 10, and hematopoiesis 21 d after implantation. Therefore, by the use of this *in vivo* system, the sequential development of bone from mesenchymal precursor cells can be studied. This study describes the localization of fibronectin by indirect immunofluorescence during differentiation of cartilage, bone, and bone marrow.

## MATERIALS AND METHODS

### *Matrix and Implantation*

Demineralized bone matrix prepared from rat diaphyses (18–20) was implanted subcutaneously into 28–31-d-old (120–140 g) rats of Long-Evans strain. The day of implantation was designated as day 0. Animals were killed by CO<sub>2</sub> asphyxiation and tissues were removed at various days during the development of the bone plaque.

### *Protein Labeling and Extraction*

Plaques at different stages of development were excised and minced into pieces not <1 mm<sup>3</sup> and incubated in 4 ml of Dulbecco's Modified Eagle's Medium containing 25 mM HEPES, 4.0 mM L-glutamine, 5.6 mM glucose, and 1.0 mM sodium pyruvate without methionine (Grand Island Biological Co., Grand Island, New York) for 15 min. The medium was replaced with fresh

medium containing 100  $\mu\text{Ci/ml}$  of L-[ $^{35}\text{S}$ ]methionine, and the pieces were incubated for 3 h at 37° C. This method ensured that there would be no possible adsorption of any labeled fibronectin from circulating plasma.

After 3 h of incubation, the tissue was removed from the medium and extracted (24). Plaque tissues were homogenized in 5 vol of 50 mM Tris-HCl, 20 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.4, containing 0.25% Triton X-100 and a mixture of protease inhibitors (50 mM EDTA, 5 mM benzamide, 0.1 M 6-aminohexanoic acid, 0.5 mM phenylmethylsulfonylfluoride (PMSF)). The homogenate was then stirred at 4°C for 4 h and centrifuged at 20,000 g for 20 min. The precipitate was reextracted three times in the above medium. The supernates were pooled and dialyzed against deionized  $\text{H}_2\text{O}$  at 4°C. The precipitate was then extracted in 4.0 M guanidine-HCl, 50 mM Tris-HCl, 20 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.4, containing 0.25% Triton X-100 with the protease inhibitor mixture described above. Subsequently, a final extraction was carried out at 4°C in 4.0 M guanidine-HCl, 50 mM  $\text{Na}_2\text{CO}_3$ , protease inhibitor mixture, pH 11.0, with 0.25% Triton X-100. More than 90% of the total radioactivity is recovered by this sequential method of extraction (24). The extracts were then dialyzed against  $\text{H}_2\text{O}$  and lyophilized.

## Gel Electrophoresis and Fluorography

Gel electrophoresis (5% polyacrylamide with 0.1% SDS) was performed using vertical slab gels (1.5-mm thick) (12). Samples were heated in a boiling water bath for 5 min with 0.1 M  $\beta$ -mercaptoethanol, 0.1% SDS, and glycine/Tris electrode buffer, pH 8.8. A sample containing 50–100  $\mu\text{g}$  of protein with 200,000 cpm of each sample was applied to each well. Fluorography was performed according to a modified procedure of Laskey and Mills (13). Gels were fixed in TCA 10% (wt/vol), glacial acetic acid 10% (vol/vol), methanol 30% (vol/vol), and impregnated with EN $^3$ HANCER (New England Nuclear, Boston, Mass.).

Fibronectin was identified in the gels by its (a) comigration with rat and human plasma fibronectin, (b) cross reactivity with purified antibody to rat plasma fibronectin, (c) affinity for gelatin, and (d) insensitivity to collagenase digestion (24).

## Preparation of Antibody to Rat Plasma Fibronectin

**ISOLATION OF RAT PLASMA FIBRONECTIN:** Swine skin gelatin (Sigma Chemical Co., St. Louis, Mo.) was coupled to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N. J.) at 56°C in 1.0 M NaCl, 0.5 M  $\text{H}_3\text{BO}_3$ , pH 9.0, to prepare the gelatin-Sepharose affinity column (6). The Sepharose 4B was activated by pretreating with CNBr (3). Freshly obtained rat plasma, with EDTA as an anticoagulant, was chilled on ice immediately after exsanguination and then applied to the gelatin-Sepharose column (4  $\times$  25 cm; 75 ml of plasma). The column was washed with 5 vol of 1.0 M urea, 0.05 M Tris-HCl, 0.02 M  $\text{NaH}_2\text{PO}_4$ , 0.15 M NaCl, 0.5 mM PMSF. Fibronectin was eluted with two-column volumes of 4.0 M urea, 0.02 M  $\text{NaH}_2\text{PO}_4$ , 0.15 M NaCl, 0.05 M Tris-HCl, 0.5 mM PMSF. All chromatography was performed at 4°C. The 4.0 M urea wash of the gelatin-Sepharose affinity column was dialyzed against distilled water at 4°C and then lyophilized.

**PRODUCTION OF THE ANTISERUM:** The lyophilized preparation above was treated for 5 min in a boiling water bath in the following sample buffer: 0.1 M  $\beta$ -mercaptoethanol, 0.1% SDS, 1.0% glycerol, glycine/Tris-HCl electrode buffer, pH 8.5, and then subjected to preparative 5% polyacrylamide gel electrophoresis with 0.1% SDS. Gel slices containing fibronectin were then homogenized with incomplete Freund's adjuvant and injected into male New Zealand rabbits. Booster injections were given 2 and 4 wk later.

**ISOLATION OF PURIFIED ANTIFIBRONECTIN ANTIBODIES:** The lyophilized 4.0 M urea eluate, containing the fibronectin, was further purified by preparative electrophoresis. This purified fibronectin, from the gelatin-Sepharose column, was coupled to CNBr-activated Sepharose 4B at 4°C in 0.5 M  $\text{H}_3\text{BO}_3$ , 1.0 M NaCl for 15 h with gentle agitation. The antiserum (20 ml) was applied to the fibronectin-Sepharose affinity column (1.5  $\times$  12 cm) at 4°C. The column was washed with buffer containing 0.002% sodium azide, 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5. The eluent was monitored for protein by absorbance at 280 nm. The fibronectin-specific antibody was eluted with 3.0 M KSCN, 0.02 M  $\text{NaH}_2\text{PO}_4$ , pH 6.0. The antibody was then immediately dialyzed against 0.02 M  $\text{NaH}_2\text{PO}_4$ , 0.15 M NaCl (phosphate-buffered saline [PBS]) at 4°C. The fibronectin monospecific antibodies did not cross react with types I and II collagens, albumin, or fibrin, as determined by immunodiffusions. There was weak cross reactivity with plasma fibronectins of human, chicken, and horse.

## Enzyme-linked Immunoabsorbant Assay [ELISA]

Fibronectin was quantitated in tissue extracts and tissue culture medium by an ELISA assay. The procedure used was similar to that described by Rennard

et al. (21) and Vuento and Vaheri (23). Immulon micro-ELISA plates (Dynatech Corp., Alexandria, Va.) were coated with 100–200 ng of purified rat plasma fibronectin/well. The tissue extract was incubated with the purified rabbit antibody to rat plasma fibronectin for 60 min in PBS with 0.05% Tween 20. The soluble antigen-antibody mixture was then placed in the micro-ELISA wells and incubated for 60 min. After washing, peroxidase-conjugated anti-rabbit IgG made in goat (Miles Laboratories, Inc., Elkhart, Ind.) was placed in the wells for 60 min. After washing, a substrate solution containing  $\text{H}_2\text{O}_2$  and *o*-phenylenediamine was added. The reaction was stopped with 8.0 M  $\text{H}_2\text{SO}_4$  and the optical density read at 480 nm. A standard curve was prepared at the time of each assay. The amount of fibronectin was reported per unit protein by the method of Lowry et al. (14).

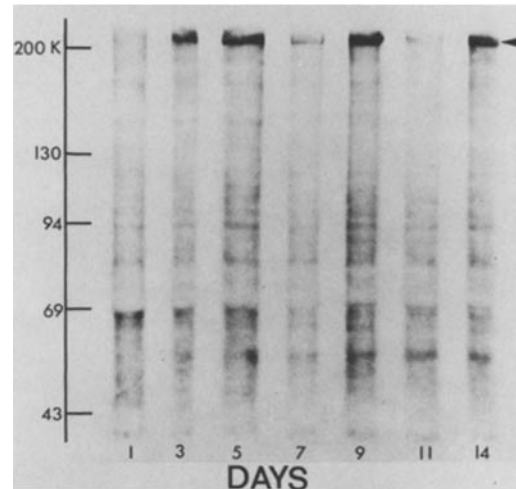


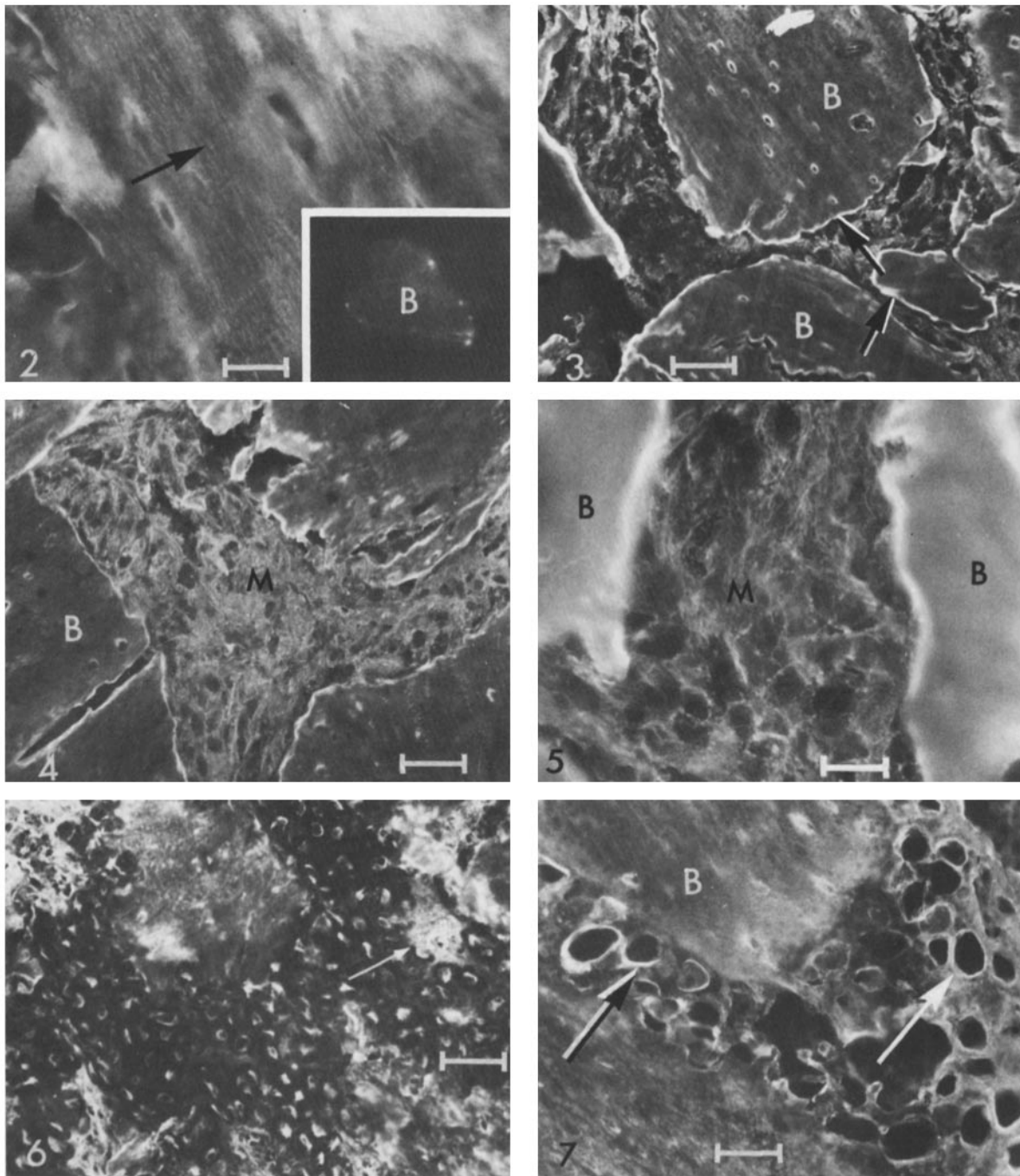
FIGURE 1 Fluorograph of 5% SDS polyacrylamide slab gel electrophoretogram under reducing conditions. Samples were extracted with 20 mM  $\text{Na}_2\text{HPO}_4$ , 50 mM Tris-HCl, 0.25% Triton X-100, pH 7.4, containing a mixture of protease inhibitors (cf. Materials and Methods). The arrow on the upper right indicates where purified rat fibronectin migrates in this gel system. Note the absence of labeled fibronectin on day 1. The amount of fibronectin synthesized on days 7 and 11 appears diminished.

TABLE I  
ELISA Measurement of Accumulated Fibronectin during Endochondral Bone Formation

Day	Stages	Fibronectin $\mu\text{g}/\text{mg}$ protein	
		Tris/ $\text{PO}_4$ extract	Guanidine, 4.0 M extract
3	Mesenchymal proliferation	4.57	8.58
7	Chondrogenesis	20.91	12.47
12	Osteogenesis	4.00	2.18

TABLE II  
Major Phases of Matrix-induced Endochondral Bone and Bone Marrow Development

Day of development	Stage of development and cell phenotype
1	Cell attachment
3	Mesenchymal cell proliferation
5	Chondrogenesis
7	Cartilage formation
9	Calcified cartilage, Vascular invasion
10–12	Bone formation
12–18	Bone remodeling
21	Bone marrow formation

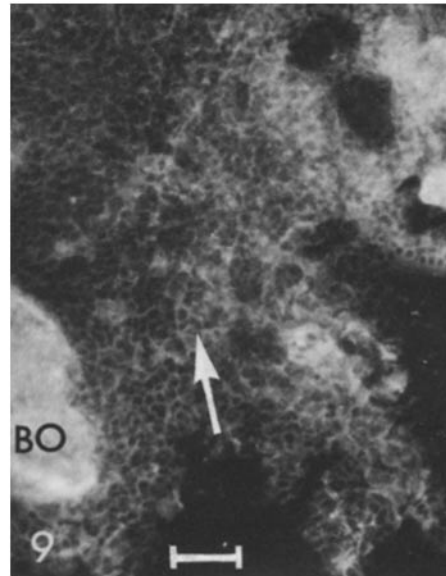
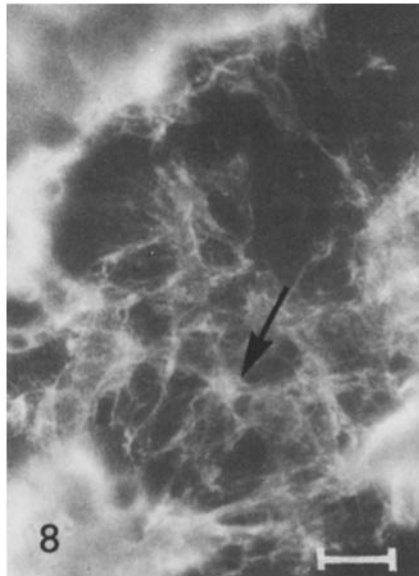


FIGURES 2-9 Frozen sections of unfixed developing plaques stained with affinity-purified rabbit anti-rat plasma fibronectin and FITC-conjugated goat anti-rabbit IgG. *B*, Demineralized bone matrix that was implanted on day 0; *M*, extracellular matrix. Negligible background fluorescence was observed in control sections stained with preimmune normal rabbit serum. Fig. 2 shows a demineralized bone matrix 3 d after implantation. Note the appearance of fibronectin in a fibrillar orientation on the matrix pieces (arrow). *Inset*, day 0, matrix particle before implantation reveals no fibronectin associated with it. Fig. 3 shows day 1, 24 h after implantation. Note the fibronectin coating the matrix particles (arrows). Fig. 4 shows day 3, most of this mesenchymal cell matrix is reactive for fibronectin. Fig. 5 shows day 5, the intensity of the staining has decreased in the extracellular matrix. Fig. 6 shows day 7, this typical cartilaginous matrix reveals fibronectin associated with the chondrocytes but absent from the extracellular matrix. Areas that are undergoing chondrolysis reveal fibronectin in the immediate vicinity (arrow). Fig. 7 shows day 9, extensive chondrolysis has occurred and endosteal surfaces are seen; fibronectin is localized around the periphery of the hypertrophic chondrocytes (arrows). Fig. 8 shows day 14, osteogenesis is in progress and osteoblasts (arrow), which line the endosteal surface, have a characteristic cottony fibrillar distribution of fibronectin. Fig. 9 shows day 21, hematopoiesis is evident and the bone marrow is reactive for fibronectin (arrow); *BO*, newly formed bone. Bar, 25  $\mu\text{m}$ .  $\times 400$ .

### Immunofluorescence Staining

Tissues were immediately placed on dry ice ( $-70^{\circ}\text{C}$ ) and then frozen, unfixed, in O.C.T. embedding medium (Miles Laboratories, Inc.). Frozen sections (6- $\mu\text{m}$

thick) were cut in a cryostat at  $-20^{\circ}\text{C}$  within 3 h of autopsy. Sections of unfixed matrix were incubated for 30 min at  $22^{\circ}\text{C}$  with the affinity-purified antifibronectin. The protein concentration of the antibody solution was 20  $\mu\text{g}/\text{ml}$ , as determined by the method of Lowry et al. (14). After incubation, the sections were



washed three times for 5 min each in PBS and then air dried. The sections were incubated for 30 min at 22°C with fluorescein isothiocyanate (FITC)-conjugated goat antibodies to rabbit IgG (diluted 1:20 with PBS; Miles Laboratories, Inc.). A control was observed for each day of a sampling by using preimmune rabbit sera as the first incubating solution. Another control consisted of preabsorbing the antibody with purified antigen which yielded no fluorescence.

### Enzymatic and EDTA Treatment of Sections

Frozen sections were treated for 30 min at 22°C with a 1 mg enzyme/ml PBS, pH 7.5, solution of the following: testicular hyaluronidase (Sigma Chemical Co.), leech hyaluronidase (Biotrics, Inc., Boston, Mass.), streptomyces hyaluronidase (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.), and pronase (Calbiochem-Behring Corp.). The slides were then washed three times for 5 min each in PBS, air dried, and then stained for fibronectin as described above. After each digestion, a control section was observed by using preimmune rabbit sera as the first incubating solution. There were no qualitative differences in the fibronectin staining between the different hyaluronidases. Enzymatic treatment did not result in autofluorescence. Sections of tissues from day 9 through day 18 (during osteogenesis) were demineralized with 0.5 M EDTA, 0.05 M Tris-HCl, pH 7.4, for 1.5 h at 22°C. Demineralized sections were then stained for fibronectin and revealed no increase or diminution of staining.

### RESULTS

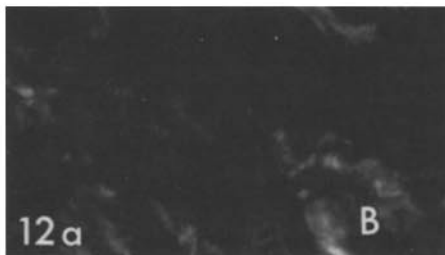
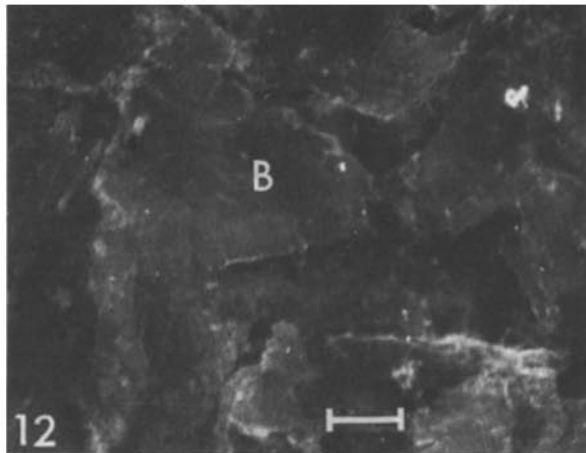
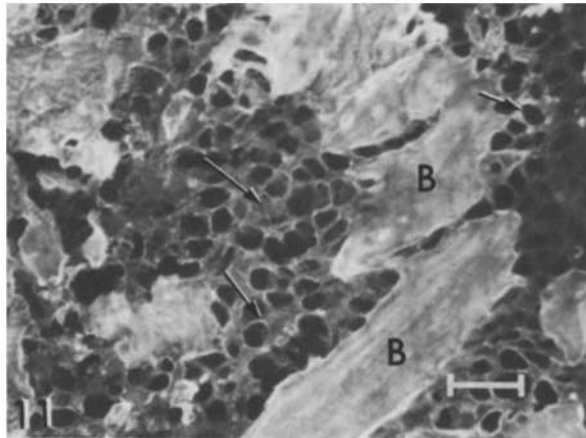
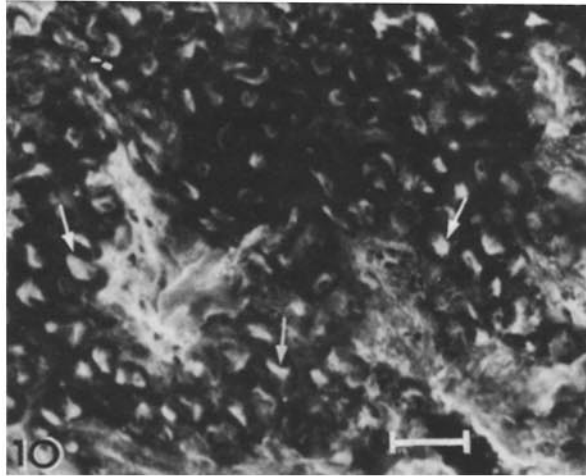
A fluorograph of the electrophoretic fractionation of proteins extracted at various stages during endochondral bone formation in Tris/PO<sub>4</sub>/Triton buffer, pH 7.4, (>70% of the radioactivity was extracted) is shown in Fig. 1. The radiolabeled fibronectin was present on all days except day 1 (24 h after implantation). It should be noted that a Coomassie Blue-stained gel (not shown) revealed that fibronectin was present on day 1, but the fluorograph indicated that it was not synthesized during the pulse. The fibronectin band was faint on days 7 and 11. This band, as seen on days 3–14, was collagenase insensitive, comigrated with rat and human plasma fibronectins, and stained with purified antibody to rat plasma fibronectin and bound to gelatin. Therefore, fibronectin was synthesized throughout the development of the plaque. 10–18% of the newly synthesized fibronectin was extracted in the 4.0 M guanidine pH 7.4 buffer. It is likely that this might represent a second pool of tissue fibronectin.

To quantify total fibronectin accumulating in the implants, we conducted ELISA assays on extracts of tissues at various days of development (Table I). The data obtained from this analysis do not directly indicate the amount synthesized but

rather the total amount accumulated in the tissue. For both extractions shown in Table I, day-7 tissue (i.e., cartilage) accumulated the most fibronectin and accounted for 2% of the total protein extracted in Tris/PO<sub>4</sub>. The decrease in fibronectin per milligram of protein seen in day-12 plaques (osteogenesis) may be indicative of decreased fibronectin synthesis relative to other proteins or increased degradation occurring during chondrolysis.

The major phases of matrix-induced endochondral bone and bone marrow development is summarized in Table II. Immunofluorescence staining of the bone matrix before implantation showed no reaction for the presence of fibronectin (Fig. 2, insert). It was previously shown (24) that no detectable fibronectin could be released from the matrix after treatment with 8.0 M urea. Within 24 h of implantation, the matrix was strongly reactive for fibronectin—especially around the periphery of the matrix particle (Fig. 3, arrow). The source of the fibronectin in day-1 tissue is the circulating form, based on the fact that it was not synthesized *in situ* and that matrix particles can bind fibronectin *in vitro* (24). 3 d after implantation, mesenchymal precursor cells proliferated and the extracellular matrix was highly reactive for fibronectin (Fig. 4). Observation of the implanted, demineralized matrix revealed a fibrillar orientation of the fibronectin on the matrix particles (Fig. 2, arrow). On day 5, chondroblasts appeared and the matrix remained positive for fibronectin, but the staining was diminished in intensity (Fig. 5). By day 7, a typical cartilaginous matrix was evident. Differentiating chondrocytes stained for fibronectin but the extracellular matrix was devoid of fluorescence (Fig. 6). On day 9, chondrolysis occurred with the onset of cartilage calcification. In contrast to differentiating chondrocytes, which have fibronectin uniformly over their surface, hypertrophic chondrocytes have fibronectin localized around their periphery (Fig. 7). In areas of chondrolysis, where endogenous hyaluronidases and/or proteases were presumably active, fluorescence was seen, suggesting the apparent unmasking of fibronectin (Fig. 5, arrow). By day 11, further chondrolysis was evident and fibronectin was observed coating endosteal surfaces. On day 14, osteoblasts in apposition to the implanted matrix were reactive for fibronectin (Fig. 8, arrow). On day 21, although bone showed slight reactivity, the hematopoietic colonies indicated the presence of fibronectin (Fig. 9).

To determine whether the failure to visualize fibronectin in the cartilaginous matrix on day 7 was a result of possible masking of the fibronectin by proteoglycans or whether, in fact, fibronectin was absent, we treated sections with leech,



fungus, and bovine testicular hyaluronidases. As shown in Fig. 10, the fibronectin in a day-7 plaque was associated with the chondrocytes but absent from the matrix. However, upon treatment of the section with hyaluronidase, the fibronectin in the matrix was stained (Fig. 11). A similar pattern was observed irrespective of the source of the hyaluronidase. It is also noted that upon treatment with hyaluronidase the cell-associated fluorescence was lost, possibly because of contaminating proteases in the hyaluronidase preparations. The loss of fibronectin staining was not caused by cell loss because chondrocytes were still present in the matrix, as observed with toluidine blue staining. Treatment of the sections with pronase eliminated all detectable fibronectin, supporting the sensitivity of cell-associated fibronectin to proteases (Fig. 12). No staining of cartilage was observed when monospecific antifibronectin was preabsorbed on a fibronectin affinity column (Fig. 12 a).

Because we found that hyaluronidase treatment unmasked cartilage matrix fibronectin, the localization of fibronectin was attempted in articular cartilage of young rats. Antibody staining showed high reactivity for fibronectin in the perichondrium and proliferating chondrocytes (Fig. 13). The extracellular matrix was not reactive but, after hyaluronidase pretreatment, fluorescence was present (Fig. 14). Treatment of articular cartilage sections with pronase completely abolished fluorescence as a result of fibronectin (Fig. 15).

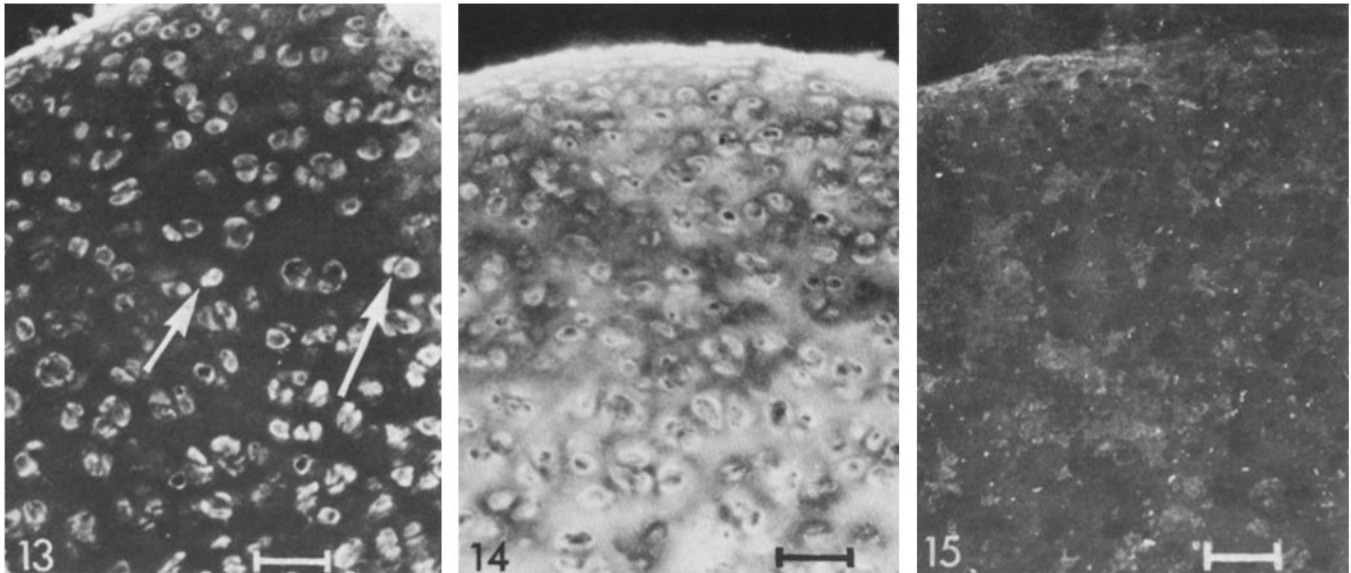
In contrast to the development of endochondral bone by the matrix-induced system, in the epiphyseal growth plate the events of endochondral osteogenesis occur in a continuum. A section of the epiphyseal growth plate treated with hyaluronidase and stained for fibronectin is shown in Fig. 16. The same patterns of fibronectin distribution are observed as were described for the matrix-induced bone forming system. Hyaluronidase unmasked the cartilaginous matrix fibronectin. Hypertrophic chondrocytes accumulated fibronectin in the cell periphery. The transverse septa of the hypertrophic zone were positive for fibronectin. In the endosteal surfaces of the spongy bone of an untreated section, fibronectin was seen in a typical lacy network (Fig. 17).

## DISCUSSION

Fibronectin was synthesized during the various stages of matrix-induced endochondral bone formation. Fibronectin was also localized by immunofluorescence and was ubiquitous throughout the development of bone. Particularly significant was the apparent masking of fibronectin by cartilage proteoglycans. By day 5, when chondroblasts were beginning to differ-

---

FIGURES 10-12 Effect of hyaluronidase and pronase treatment of frozen sections of day 7 plaques stained for fibronectin. B, implanted bone matrix. Fig. 10 shows the untreated control. The fibronectin is localized within the chondrocytes (arrows) but absent in the cartilaginous matrix. Fig. 11 shows the section treated with 25 µl of 1 mg/ml of testicular hyaluronidase in PBS, pH 7.4, at 22°C for 30 min. Note the appearance of fibronectin in the extracellular matrix and the accumulation of fibronectin at the periphery of the chondrocytes (arrows). Fig. 12 shows the section treated with 25 µl of 1 mg/ml of pronase in PBS, pH 7.4, at 22°C for 30 min. There is no evidence of fibronectin remaining after treatment. Fig. 12 a shows the section treated with absorbed antifibronectin revealed no staining. The exposure times for these micrographs were identical. Appropriate controls stained with preimmune rabbit serum after digestion revealed negligible fluorescence. Bar, 25 µm. × 400.



FIGURES 13–15 Effect of hyaluronidase and pronase treatment on frozen sections of articular cartilage from 21-d-old rats stained for fibronectin. Fig. 13 shows the untreated control. The fibronectin is localized with the chondrocytes (arrows) but absent in the cartilaginous matrix. The perichondrium is highly reactive for fibronectin. Fig. 14 shows the section treated with 25  $\mu$ l of 1 mg/ml testicular hyaluronidase in PBS, pH 7.4, at 22°C for 30 min. Note the unmasking of the fibronectin in the cartilaginous matrix with this treatment. Fig. 15 shows the section treated with 25  $\mu$ l of 1 mg/ml pronase in PBS, pH 7.4, at 22°C for 30 min. There is no evidence of fibronectin remaining after pronase treatment. The exposure times for these micrographs were identical. Appropriate controls stained with preimmune rabbit serum after digestion revealed no background fluorescence. Bar, 25  $\mu$ m.  $\times$  384.

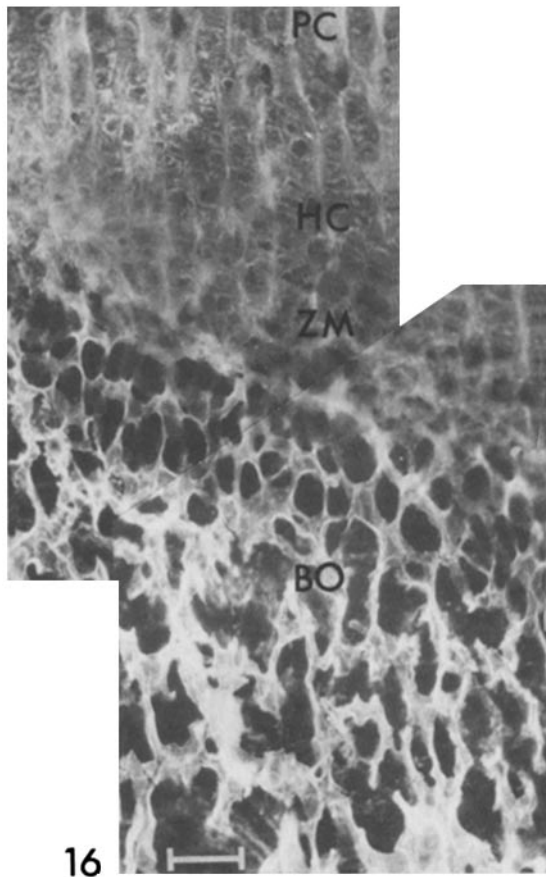


FIGURE 16 Frozen section of epiphyseal growth plate stained for fibronectin after hyaluronidase digestion. PC, proliferating chondrocytes; HC, hypertrophic chondrocytes; ZM, zone of provisional mineralization; BO, bone. Bar, 25  $\mu$ m.  $\times$  400.

entiate (independently assessed by  $^{35}\text{SO}_4$  incorporation [18, 19]), there was already a significant decrease in fluorescence, and by day 7, only chondrocytes exhibited fluorescence. Dessau et al. (4) have shown that dispersed chondrocytes *in vitro* synthesize fibronectin. The interaction of fibronectin and proteoglycans has been demonstrated *in vitro* (10, 17). Data presented here also indicate the close relationship between fibronectin and proteoglycans *in situ*. Dessau et al. (4, 5) reported that fibronectin was absent in sternal cartilage and embryonic chick limb bud cartilage. The matrix-induced cartilage system described in this report appears to be different from the system described by Dessau et al. (4) in this regard when observed with hyaluronidase pretreatment, possibly caused by the fact that, in the present model, cartilage is replaced by bone, reminiscent of embryonic endochondral bone development. Alternatively, the difference may be caused by fixation of tissues in the earlier study (4).

There has been much speculation about the *in vivo* role of fibronectin (7, 9, 11, 16, 22, 27). This report describes the appearance of fibronectin during the *in vivo* development of bone. Based on the evidence in this report, a possible scheme for the role of fibronectin is outlined as follows. Initially, plasma fibronectin binds to the implanted collagenous demineralized bone matrix. This may allow for initial cell attachment and subsequent recognition of the implanted matrix. It is also possible that fibronectin may be chemotactic (H. Kleinman, personal communication); therefore, it may also play a role in cell recruitment to implant site. When the mesenchymal precursor cells proliferate, fibronectin is present in a cottony fibrillar array, described in various cell cultures (2, 8, 25). When these cells differentiate into chondrocytes, they still have their cell-associated fibronectin. During chondrolysis and the removal of proteoglycans, the fibronectin in the extracellular matrix is unmasked. This "exposed" fibronectin may support



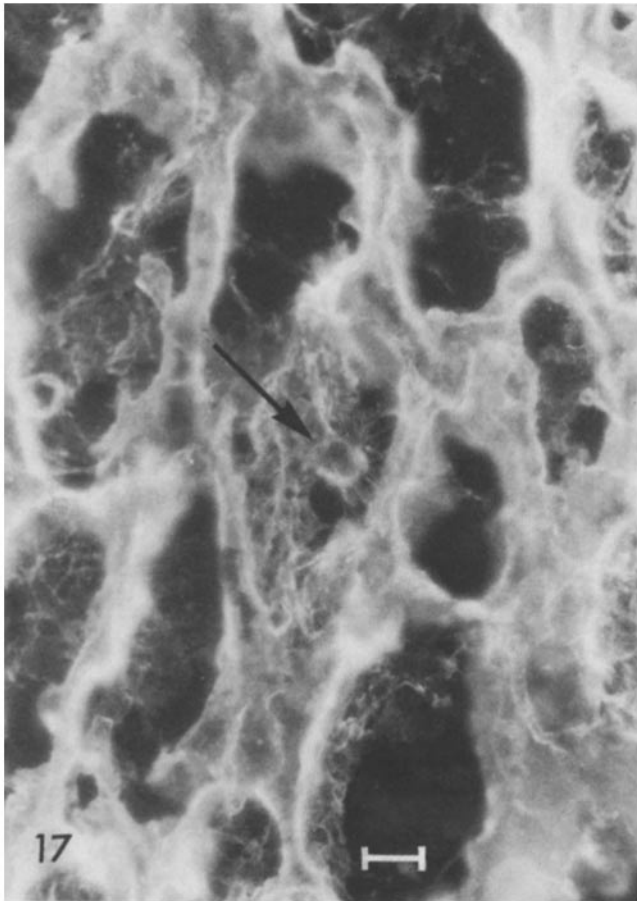


FIGURE 17 Frozen section of spongy bone (untreated) stained for fibronectin. Note the appearance of osteoblasts with typical cottony array of fluorescence (arrow). This localization was not seen in the spongy bone of Fig. 16 which was digested with hyaluronidase. Bar, 1.25  $\mu\text{m}$ .  $\times 800$ .

recruitment and attachment of mesenchymal osteoprogenitor cells that will soon infiltrate the area and differentiate into osteoblasts.

Endothelial cells are known to synthesize fibronectin (1, 15) and it is clear from this study that endosteal surfaces, which have numerous blood vessels associated with them, are rich in fibronectin. Fibronectin was also observed in the osteoblasts and bone, as seen by immunofluorescence localization. Fluorographs confirmed the biosynthesis of fibronectin during osteogenesis. It is of interest to note, in contrast to extracellular matrix fibronectin, that the chondrocyte and osteoblast cell surface fibronectins were susceptible to the hyaluronidase treatment. Furthermore, the observation that some fibronectin is extracted in low salt and some in high salt (e.g., 4.0 M guanidine-HCl) may lend additional support to the occurrence of different pools or classes of tissue fibronectins based on their extractability and susceptibility to enzymes. Finally, fibronectin in the bone marrow may be a critical element of the hematopoietic microenvironment.

In summary, fibronectin was present in developing bone and was synthesized by mesenchymal cells, chondrocytes, and osteoblasts. Fibronectin appears to be a ubiquitous component of developing skeletal matrix.

We thank Dr. Waltraud Dessau of Max Planck Institute of Biochemistry, Munich, West Germany, for sharing with us the unpublished findings. A. Reddi thanks Dr. K. von der Mark for introducing him to the intricacies of immunofluorescence techniques.

R. Weiss was a National Institutes of Health (NIH) postdoctoral fellow.

Reprint requests should be addressed to A. H. Reddi at NIH.

Received for publication 18 June 1980, and in revised form 17 November 1980.

#### REFERENCES

- Birdwell, C. R., D. Gospodarowicz, and G. L. Nicholson. 1978. Identification, localization, and role of fibronectin in cultured bovine endothelial cells. *Proc. Natl. Acad. Sci. U. S. A.* 75:3273-3277.
- Chen, B., A. Murray, R. A. Segal, A. Bushnell, and M. L. Walsh. 1978. Studies on LETS glycoprotein matrices. *Cell* 14:377-391.
- Cuatrecasas, P. 1970. Protein purification by affinity chromatography: derivatizations of agarose and polyacrylamide beads. *J. Biol. Chem.* 245:3059-3065.
- Dessau, W., J. Sasse, R. Timpl, F. Jilek, and K. von der Mark. 1978. Synthesis and extracellular deposition of fibronectin in chondrocyte cultures. *J. Cell Biol.* 79:342-355.
- Dessau, W., H. von der Mark, K. von der Mark, and S. Fischer. 1980. Changes in the patterns of collagens and fibronectin during limb bud chondrogenesis. *J. Embryol. Exp. Morph.* 57:51-60.
- Engvall, E., and E. Ruoslahti. 1977. Binding of soluble forms of fibroblast surface protein, fibronectin to collagen. *Int. J. Cancer.* 20:1-5.
- Grinnell, F., D. G. Hays, and D. Minter. 1977. Cell adhesion and spreading factor: partial purification and properties. *Exp. Cell Res.* 110:175-190.
- Hedman, K., A. Vaheri, and J. Wartiovaara. 1978. External fibronectin of cultured human fibroblasts is predominately a matrix protein. *J. Cell Biol.* 76:748-760.
- Hynes, R. O. 1976. Cell surface proteins and malignant transformation. *Biochim. Biophys. Acta.* 459:73-107.
- Jilek, F., and H. Hörmann. 1979. Fibronectin (cold insoluble globulin). VI. Influence of heparin and hyaluronic acid on the binding of native collagen. *Hoppe-Seyler's Z. Physiol. Chem.* 360:597-603.
- Klebe, R. J. 1974. Isolation of a collagen dependent cell attachment factor. *Nature (Lond.)* 250:248-251.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Laskey, R. A., and A. B. Mills. 1975. Quantitative film detection of  $^3\text{H}$  and  $^{14}\text{C}$  in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 53:335-341.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Macarak, E. J., E. Kirby, T. Kirk, and N. Kefalides. 1978. Synthesis of cold-insoluble globulin by cultured calf endothelial cells. *Proc. Natl. Acad. Sci. U. S. A.* 75:2621-2625.
- Pearlstein, E., L. Gold, and A. Garcia-Pardo. 1980. Fibronectin: a review of its structure and biological activity. *Mol. Cell Biochem.* 29:103-123.
- Perkins, M. E., T. H. Ji, and R. O. Hynes. 1979. Cross-linking of fibronectin to sulphated proteoglycans at the cell surface. *Cell.* 16:941-952.
- Reddi, A. H. 1976. Collagen and cell differentiation. In *Biochemistry of Collagen*. G. N. Ramachandran and A. H. Reddi, editors. Plenum Publishing Co., New York. 449-478.
- Reddi, A. H., and C. B. Huggins. 1972. Biochemical sequences in the transformation of normal fibroblasts in adolescent rats. *Proc. Natl. Acad. Sci. U. S. A.* 69:1601-1605.
- Reddi, A. H., and C. B. Huggins. 1975. Formation of bone marrow in fibroblast-transformation ossicles. *Proc. Natl. Acad. Sci. U. S. A.* 72:2212-2216.
- Rennard, S. I., R. Berg, G. R. Martin, J. M. Foidart, and P. Gehron Robey. 1980. Enzyme-linked immuno-assay (ELISA) for connective tissue components. *Anal. Biochem.* 104:205-214.
- Vaheri, I., E. Ruoslahti, and D. F. Mosher. 1978. Fibroblast surface proteins. *Ann. N. Y. Acad. Sci.* 312:456.
- Vuento, M., and A. Vaheri. 1978. Dissociation of fibronectin from gelatin-agarose by amino compounds. *Biochem. J.* 175:333-336.
- Weiss, R. E., and A. H. Reddi. 1980. Synthesis and localization of fibronectin during collagenous matrix-mesenchymal cell interaction and differentiation of cartilage and bone. *Proc. Natl. Acad. Sci. U. S. A.* 77:2074-2078.
- Yamada, K. M. 1978. Immunological characterization of a major transformation-sensitive fibroblast cell surface glycoprotein. *J. Cell Biol.* 78:520-541.
- Yamada, K. M., and D. W. Kennedy. 1979. Fibroblast cellular and plasma fibronectins are similar but not identical. *J. Cell Biol.* 80:492-498.
- Yamada, K. M., and K. Olden. 1979. Fibronectins-adhesive glycoproteins of cell surface and blood. *Nature (Lond.)* 275:179-184.