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Synthesis and localization of fibronectin during collagenous matrix–mesenchymal cell interaction and differentiation of cartilage and bone *in vivo*

(anchorage-dependence/cell attachment/cell differentiation/hematopoiesis)

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ABSTRACT **The biosynthesis of fibronectin during the *in vivo* development of matrix-induced endochondral bone was investigated by using [^{35}S]methionine in rats. The demineralized bone matrix that was implanted subcutaneously to induce local bone formation bound circulating fibronectin. This may be an important initial requirement for cell attachment to the matrix. Fibronectin was present throughout the development of bone but accounted for the largest percentage of total protein synthesized during mesenchymal cell proliferation and hematopoiesis. Fibronectin was identified in tissue extracts by its (*i*) comigration on electrophoretic NaDodSO$_4$/polyacrylamide gels with human and rat plasma fibronectin, (*ii*) affinity for denatured collagen, (*iii*) crossreactivity with purified antibody of rat plasma fibronectin, and (*iv*) insensitivity to collagenase digestion. Fibronectin was localized by immunofluorescence in the extracellular matrix during the period of mesenchymal cell proliferation. During chondrogenesis, fibronectin was demonstrated in the differentiating chondrocytes. Fibronectin was detectable in the cartilage matrix only after hyaluronidase treatment. During vascular invasion, prior to osteogenesis, fibronectin was localized in association with endothelial cells. These observations demonstrate a possible role of fibronectin in collagenous matrix–mesenchymal cell interaction *in vivo*.**

It has been well documented (1, 2) that anchorage to a substratum is a fundamental condition for *in vitro* growth of normal fibroblasts. After the initial contact, cellular extensions attach to the surrounding surface (3) before the cells proliferate. It has been shown for many cell types that, without the addition of serum to the culture medium, cell attachment does not occur and subsequent growth does not ensue (4, 5). Fibronectin is a cell surface glycoprotein that is also present in serum and has been shown to function *in vitro* as an adhesive component for cell–substratum (usually collagen) or cell–cell interaction (for a review see ref. 6). The relevance of these observations to the *in vivo* situation has not been established. Extrapolating from the *in vitro* data to the *in vivo* situation, similar conditions for growth and differentiation may be required—i.e., anchorage of cells to a suitable substratum prior to cell proliferation. The aim of this study was to investigate changes in tissue fibronectin during mesenchymal cell proliferation and differentiation of cartilage and bone elicited in response to implanted collagenous matrix.

In order to study changes in tissue fibronectin during growth and development, we investigated matrix-induced endochondral bone formation in rats (7–10). After subcutaneous implantation of demineralized collagenous bone matrix, an invariant sequence of events ensues culminating in *de novo* cartilage, bone, and red marrow formation. In brief, 1 day after implantation of matrix, a transient inflammatory reaction is observed. Three days later mesenchymal cell proliferation occurs with the subsequent differentiation into chondroblasts on day 5. On day 7, a typical metachromatic matrix is evident. This is followed on days 8–9 by vascularization, chondrolysis, and calcification of cartilage. Concurrently, mesenchymal precursor cells proliferate and differentiate into osteoblasts on day 10. Starting on day 11, bone develops and concomitant remodeling occurs on days 12–18 with the formation of hematopoietic bone marrow by day 21. Each of the above events can be characterized histologically and quantitated with specific biochemical markers (7–10). The present paper describes the biosynthesis and localization of fibronectin at the different developmental stages of collagenous matrix-induced endochondral bone formation.

MATERIALS AND METHODS

Preparation of Matrix and Implantation. Demineralized bone matrix prepared from rat diaphyses was implanted subcutaneously into 28- to 31-day-old (120–140 g) Long–Evans rats. The day of implantation was designated as day 0, with all surgical procedures and autopsies performed between 10 and 11 a.m. The present study investigated protein biosynthesis of various stages of matrix-induced bone formation that have been previously defined histologically and biochemically (7–10).

Labeling of Proteins. Two methods were used to evaluate protein biosynthesis. In the first, implanted rats were injected intravenously with L-[^{35}S]methionine (specific activity, 546.7 Ci/mmol; New England Nuclear) with a dose of 1 μCi/g of body weight (1 Ci = 3.7×10^{10} becquerels). Two hours after injection, the rats with plaques at different stages of development were killed, plaque tissue was removed, and proteins were extracted as described below. In the second method, the plaques at different stages of development were removed and minced into particles not greater than 1 mm^3 and incubated *in vitro*. The tissues were placed in 4 ml of Dulbecco's modified Eagle's medium containing 25 mM Hepes, 4.0 mM L-glutamine, 5.6 mM glucose, 1.0 mM sodium pyruvate, 0.1 mM unlabeled methionine, and 100 μCi of L-[^{35}S]methionine per ml for 3 hr at 37°C. Essentially the same results were obtained by using both methods. Unless otherwise mentioned, the second method was used in order to detect fibronectin synthesized *in situ* by the developing plaque. This ensured there would be no possible adsorption of any labeled fibronectin from circulating plasma.

Extraction of Proteins. Plaques (taken either directly from the animal or after a 3-hr *in vitro* incubation) were initially extracted by homogenization in 5 vol of 50 mM Tris·HCl/20 mM Na_2HPO_4 at pH 7.4, which contained a mixture of protease inhibitors (50 mM EDTA/5 mM benzamidine/0.1 M 6-aminohexanoic acid/0.5 mM phenylmethylsulfonyl fluoride). The homogenate was then extracted at 4°C for 4 hr and centrifuged

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at 20,000 × g for 20 min. The precipitate was reextracted three times in the above medium. The supernatants were pooled and dialyzed against deionized H_2O at 4°C. The precipitate was then extracted in 50 mM Tris·HCl/20 mM Na_2HPO_4 at pH 7.4, containing 0.25% Triton X-100 and the protease inhibitor mixture described above. Subsequently, extractions were carried out at 4°C in 4.0 M guanidine·HCl/50 mM Tris/protease inhibitor mixture/0.25% Triton X-100 at pH 7.4. This was followed by extraction in 4.0 M guanidine·HCl/50 mM Na_2CO_3/protease inhibitor mixture, adjusted to pH 11 with NaOH and then in the same buffer with 0.25% Triton X-100. The remaining insoluble material was extracted with 4.0 M guanidine/0.5 M Na_4EDTA/50 mM Tris·HCl, pH 7.4, for 16 hr in order to extract any mineral-bound radioactive proteins. The remaining precipitate was dissolved in 0.15% NaDodSO$_4$/0.1 M 2-mercaptoethanol/50 mM Tris·HCl, pH 7.4. Aliquots of each dialyzed extract were assayed for radioactivity and protein was determined according to Lowry *et al.* (11). The efficiency of this extraction method was assessed by using tissues of mesenchymal cell proliferation (day 3), chondrogenesis (day 7), and osteogenesis (day 12). Five to 8% of the total activity was released into the medium. Approximately 35% of the radioactivity was extracted in Tris·HCl/PO_4 buffer, pH 7.4. Addition of 0.25% Triton X-100 released a further 35%. Twelve to 18% of the radioactivity was extracted in the presence of 4.0 M guanidine·HCl in Tris·HCl, pH 7.4. The remaining 5–10% radioactivity was extracted in 4.0 M guanidine·HCl, (pH 11.0). Based on the above information, a simplified extraction procedure was initiated by using Tris·HCl/PO_4 buffer (pH 7.4) followed by 4.0 M guanidine/50 mM Tris·HCl, pH 7.4, and 4.0 M guanidine/50 mM Na_2CO_3, pH 11.0, all with 0.25% Triton X-100 and the protease inhibitor solution described above. The trends of extractability as described above were also seen.

Determination of Radioactive Components Binding to Gelatin in Plaque Extracts. Gelatin-Sepharose affinity chromatography was performed on extracts from the developing plaques at different stages. The columns were prepared according to the procedure of Engvall and Ruoslahti (12) except for the use of salt-soluble lathrytic rat skin collagen (13) coupled to CNBr-activated Sepharose 4B at 56°C for 3 hr. An aliquot of the extract containing 200,000 cpm was applied to 5 ml of plain Sepharose 4B to remove any entity binding to the Sepharose 4B alone. It was determined that less than 5% of the total counts bound to the plain Sepharose 4B. The equilibrating and eluting buffer was 20 mM Na_2HPO_4/0.15 M NaCl/0.5 mM phenylmethylsulfonyl fluoride at pH 7.4. The eluate from the plain Sepharose 4B was then passed over a 2-ml bed volume of the gelatin-Sepharose. The columns were eluted with 10 vol each of 1, 4, and 8 M urea/50 mM Tris·HCl, pH 7.4/0.5 mM phenylmethylsulfonyl fluoride. The eluents were collected and assayed for radioactivity. Results are expressed as percentage of total activity that eluted with a particular concentration of urea. The recovery of radioactivity was greater than 95%.

Gel Electrophoresis and Fluorography. Gel electrophoresis (5% polyacrylamide/0.1% NaDodSO$_4$) was performed with vertical slab gels (1.5 mm thick) as described by Laemmli (14). Samples were heated in a boiling water bath for 5 min with 0.1 M 2-mercaptoethanol/0.1% NaDodSO$_4$ in glycine/Tris electrode buffer, pH 8.8. Protein (50–100 μg) from each sample was applied to each gel and stained for protein with Coomassie blue. Certain samples were heated with purified bacterial collagenase (15). Fluorography was performed according to the procedure of Laskey *et al.* (16). Gels were treated with 25% 2,5-diphenyloxazole in dimethyl sulfoxide, dried, and exposed to Kodak X-omatic X-ray film by using a fluorescent image intensifying screen. For fluorography an aliquot containing 200,000 cpm was applied for each sample.

Immunohistological Localization of Fibronectin in Frozen Tissue Sections and Electrophoretic Gels. (*i*) *Antibody production.* Rat plasma fibronectin was purified by affinity chromatography of freshly obtained rat plasma with EDTA as an anticoagulant over a gelatin-Sepharose column, followed by preparative gel electrophoresis in 5% polyacrylamide. The slices of polyacrylamide gel containing the fibronectin were homogenized with incomplete Freund's adjuvant and injected into rabbits. Booster injections were given 2 and 4 weeks later. Immune rabbit serum was collected 2 weeks after the last booster and antifibronectin antibodies were purified by fibronectin-Sepharose affinity chromatography. The isolation and characterization of rat fibronectin and antibody of rat fibronectin will be reported in detail elsewhere.

(*ii*) *Immunofluorescent localization.* Frozen sections of plaque tissues were freshly prepared and incubated for 30 min at 22°C with the affinity-purified antifibronectin antibody (27 μg/ml). After incubation the sections were washed three times for 5 min each with 0.15 M NaCl/20 mM Na_2HPO_4, pH 7.5 and then dried. The sections were then incubated for 30 min at 22°C with fluorescein isothiocyanate conjugated goat antibodies to rabbit IgG (diluted 1:20 with 0.15 M NaCl/20 mM Na_2HPO_4). A control was tested by using preimmune normal rabbit serum as the first incubating solution in one set of sections. These control slides did not exhibit any fluorescence. Some sections were pretreated with testicular hyaluronidase (1 mg/ml of 0.15 M NaCl/20 mM Na_2HPO_4) for 30 min prior to immunofluorescent staining.

(*iii*) *Gel staining.* Fibronectin was localized in protein profiles of tissue extracts on electrophoretic slab gels according to the method of Olden and Yamada (17). Gels were fixed in 50% trichloroacetic acid for 2 hr, washed in 7% acetic acid for 1 hr, and then washed in 0.5% Triton X-100/7% acetic acid for 16 hr. Gels were then incubated with purified rabbit antifibronectin antibody and treated with peroxidase-linked anti-rabbit IgG. The fibronectin bands in the gel were visualized by the reaction product of the diaminobenzidine reaction for localization of peroxidase.

RESULTS

Gel Electrophoresis and Fluorography. Electrophoretic fractionation of proteins extracted at various stages during endochondral bone formation in Tris·HCl/PO_4 buffer (pH 7.4) is shown in Fig. 1. A band at about M_r 220,000 is present on all days. This band was collagenase insensitive (data not shown), comigrated with human and rat plasma fibronectin, and stained with purified antibody to rat plasma fibronectin. Therefore, this 220,000 M_r band was designated as fibronectin. Fluorographs of the gels indicated fibronectin was present on all days except day 1.

The 4.0 M guanidine buffer (pH 7.4) extract (after the Tris·HCl/PO_4 extraction) of a day 3 plaque is shown in Fig. 2*A*. The fibronectin band appears as a doublet at 220,000 on day 3 and is collagenase insensitive (Fig. 2*A*, lane 3). The purified collagenase preparation was shown to degrade purified type I collagen under the same conditions. A similar doublet pattern was seen on days 7 and 12 as well. A fluorograph of 4.0 M guanidine buffer (pH 7.4) extracts of plaques is shown in Fig. 3. It is noteworthy that there are labeled bands at high molecular weights (greater than 220,000), which may represent complexes of fibronectin with collagen extractable only in 4.0 M guanidine or incompletely reduced dimers and multimers of fibronectin.

The gel stained for protein revealed fibronectin on day 1 (Fig. 1, day 1); however, a fluorograph indicated that it was not synthesized during the labeling pulse. Therefore, we tested to

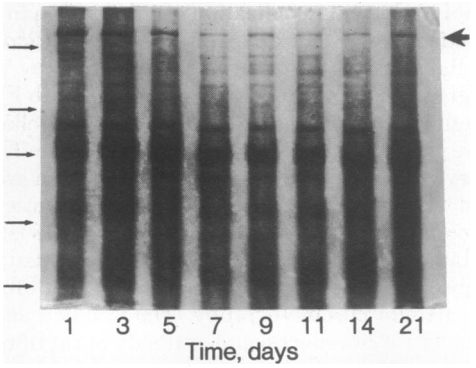

FIG. 1. NaDodSO$_4$/5% polyacrylamide slab gel electrophoresis under reducing conditions. Samples were soluble components extracted with 20 mM Na_2HPO_4/50 mM Tris·HCl/0.25% Triton X-100 at pH 7.4, containing a mixture of protease inhibitors. The arrows on the left indicate known molecular weight standards starting at the top: 200,000 (myosin); 130,000 (β galactosidase); 94,000 (phosphorylase *b*); 69,000 (bovine serum albumin); 43,000 (ovalbumin). The thick arrow indicates where purified circulating human and rat fibronectin migrated in the same gel system.

determine if the demineralized bone matrix that was implanted could, by itself, bind plasma fibronectin. A column was prepared of demineralized bone matrix and was washed with 8.0 M urea to remove any urea-soluble materials. Fibronectin was not detectable in this wash. Fresh rat plasma was then passed over the column, which was washed with buffer until no further protein eluted. The column was then eluted with 4.0 M urea. Gel electrophoresis demonstrated that fibronectin bound to the matrix (Fig. 2*B*), suggesting that circulating (plasma) fibronectin *in vivo* could bind to implanted collagenous bone matrix. It should also be noted that, in gels overloaded with respect to protein, a band with an apparent M_r of 70,000 was observed.

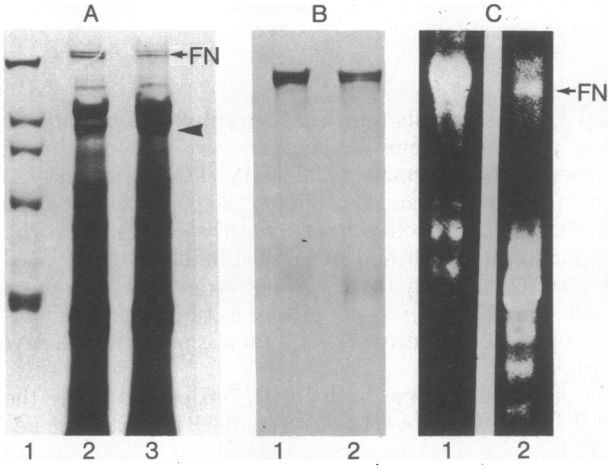

FIG. 2. (*A*) NaDodSO$_4$/5% polyacrylamide slab gel electrophoresis under reducing conditions stained for protein. Samples were soluble components extracted with 50 mM Tris·HCl/4.0 M guanidine·HCl/0.25% Triton X-100 at pH 7.4, containing a mixture of protease inhibitors. Lanes: 1, molecular weight standards (200,000; 130,000; 94,000; 69,000; and 43,000); 2, day 3; 3, day 3 extract digested with purified bacterial collagenase. Arrow head, purified bacterial collagenase. FN, fibronectin. (*B*) Lane 1, protein profile of material eluted with 4.0 M urea from a column of demineralized bone matrix. Initially, fresh rat plasma was passed over the column. Lane 2, protein eluted with 4.0 M urea from a gelatin-Sepharose column. The major protein in both lanes is the doublet fibronetcin. (*C*) Tris·HCl/PO$_4$ buffer (pH 7.4) tissue extracts from day 7 plaques were passed through gelatin-Sepharose columns. A fluorograph of material eluted with 4.0 M urea (lane 1) and 8.0 M urea (lane 2) is shown. FN, fibronectin.

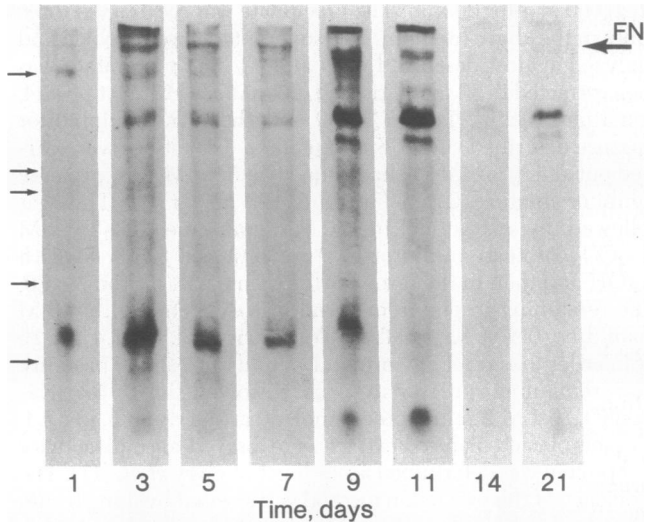

FIG. 3. Fluorograph of proteins extracted in 50 mM Tris·HCl/4.0 M guanidine·HCl/0.25% Triton X-100 at pH 7.4, containing a mixture of protease inhibitors. There are protein bands with an apparent M_r of 150,000 that are synthesized at the onset of mineralization (day 9), and there is an increased synthesis of proteins of about M_r 170,000, whereas a protein of an apparent M_r of 50,000 is not synthesized. Arrows on the left indicate molecular weight markers. FN, rat plasma fibronectin.

Determination of Gelatin-Binding Activity. Because fibronectin binds well to denatured collagen and gelatin-affinity columns are useful in its purification, we determined the amount of fibronectin present in the various extracts by measuring the percentage of total radioactivity bound to gelatin-Sepharose. Plaques were incubated for 3 hr in tissue culture medium with L-[^{35}S]methionine prior to extraction in Tris·HCl/PO$_4$ buffer. The percentage of total radioactivity washed off the column with 1.0 M urea for plaques from day 1 through day 21 ranged from 50 to 59%. A significant amount of radioactivity was eluted with 4.0 M urea. Days 3 and 21 had greater activities compared to days 1, 7, 9, 11 and 14, whereas day 1 had the least (Fig. 4). Another radioactively labeled gelatin-binding component was eluted with 8.0 M urea and was an average of 5% of the total activity applied to the column. Fluorographs of

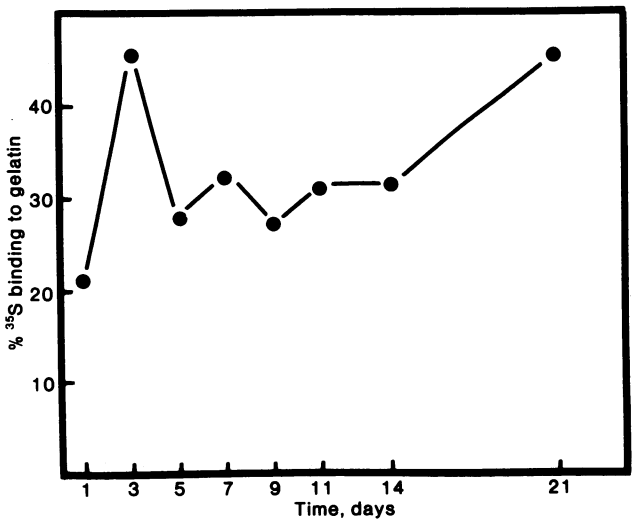

FIG. 4. Determination of radiolabeled gelatin-binding components. Ordinate, percentage of radioactivity (amount of fibronectin) passed over a gelatin-Sepharose column that eluted with 4.0 M urea; abscissa, days after implantation.

the electrophoresed 4.0 and 8.0 M urea eluents from day 7 plaques showed that the 4.0 M eluent was primarily fibronectin, whereas the 8.0 M urea extract was primarily a 70,000 M_r protein (Fig. 2C). There clearly are other lower molecular weight proteins in addition to fibronectin evident in the 4.0 M urea wash. Therefore, there may be more than one gelatin-binding protein in these tissue extracts. Alternatively, the lower molecular weight protein may represent breakdown products of fibronectin, even in the presence of the protease inhibitor phenylmethylsulfonyl fluoride.

Immunofluorescent Localization of Fibronectin. Indirect immunofluorescent localization of fibronectin in frozen sections of developing plaques by use of affinity purified antibody against rat fibronectin is shown in Fig. 5. Negligible autofluorescence was detected when preimmune rabbit serum was used. On day 3 (Fig. 5A) the extracellular matrix was reactive for fibronectin, especially in the area adjacent to the implanted matrix. On day 7 (Fig. 5B) in the differentiating cartilage, fibronectin was not detectable in the extracellular cartilage matrix whereas the chondrocytes were positive for fibronectin. Pretreatment of the sections with hyaluronidase revealed a moderate reaction in the matrix (unpublished results). In day 7 plaques, the area of undifferentiated mesenchymal tissue was also positive for fibronectin. During vascular invasion and prior to osteogenesis, fibronectin was localized in association with invading endothelial cells.

DISCUSSION

The biosynthesis of fibronectin has been demonstrated during the *in vivo* development of matrix-induced endochondral bone. Although fibronectin was present in the plaque throughout its development, an increase in fibronectin synthesis was greatest on day 3 after implantation when mesenchymal precursor cells (destined to be chondrocytes) proliferated and on day 21 during hematopoiesis. Fibronectin was identified by its (*i*) comigration on electrophoretic $NaDodSO_4$ gels with human and rat plasma fibronectin, (*ii*) affinity for denatured collagen, (*iii*) insensitivity to collagenase digestion, and (*iv*) crossreactivity with purified antibody to rat fibronectin. Fibronectin extracted from developing plaque tissues in Tris·HCl/PO_4 buffer (pH 7.4) migrated as a single band on $NaDodSO_4$/5% polyacrylamide gels, whereas that extracted with 4.0 M guanidine (pH 7.4) consistently migrated as a double band. It is not clear whether this represents two different pools of tissue fibronectin. Fibronectin was also localized by immunofluorescence in the extracellular matrix surrounding implanted demineralized bone matrix particles. The appearance of fibronectin during cell proliferation *in vitro* has been well documented (cf. ref. 6). Evidence presented here indicates this to be true *in vivo* as well.

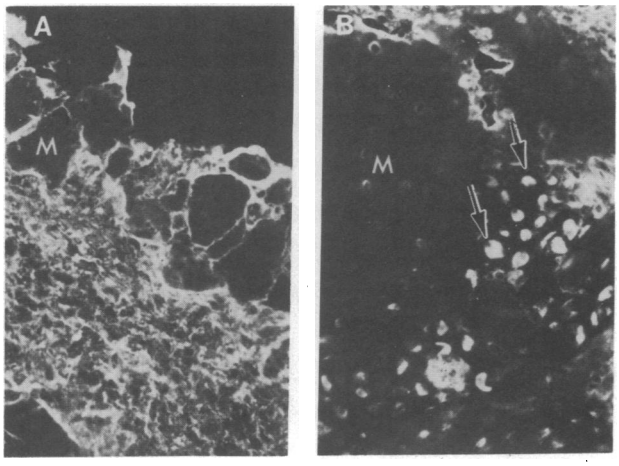

FIG. 5. Frozen sections of developing plaque stained with affinity-purified rabbit anti-rat plasma fibronectin and goat anti-rabbit IgG conjugated with fluoresceinisothiocyanate. M, demineralized bone matrix that was implanted on day 0. Negligible background fluorescence was observed in control sections stained with preimmune normal rabbit serum. (*A*). Day 3. Mesenchymal cells proliferate; the extracellular matrix is highly reactive for fibronectin. (*B*) Day 7. In areas of cartilage differentiation there is no fibronectin associated with the cartilage matrix, but chondrocytes appear to be fluorescent (arrow). In undifferentiated areas there is evidence for extracellular fibronectin accumulation.

Although there was significant fibronectin detectable in the 24-hr implant, there was no indication of local synthesis of the protein. It was previously demonstrated that an early response to the implanted matrix is local fibrin accumulation (9). Mosher (18) has shown that fibronectin can be crosslinked with fibrin. However the implanted bone matrix, by itself, has the potential for binding circulating fibronectin (Fig. 2B). This property of the matrix may account for the binding of preformed unlabeled fibronectin on day 1 plaques. Therefore, it is suggested that the circulating plasma fibronectin binds to the implanted collagenous matrix prior to or concurrent with mesenchymal cell attachment. Whether this interaction is a necessary prerequisite for mesenchymal cell proliferation is currently under investigation. Previous studies (19, 20) have reported that type III collagen was localized and found to be synthesized in the day 3 plaque, which coincides with the appearance of the fibronectin. Engvall *et al.* (21) and Jilek and Hörman (22) have shown that fibronectin binds most avidly to native type III collagen compared to native types I and II. Therefore, the visualization of fibronectin in the extracellular matrix of day 3 plaques may be attributed to the attachment of fibronectin to the type III collagen present.

Seven days after implantation the plaque is characterized histologically by a cartilage-like metachromatic matrix as well as by its ability to incorporate $^{35}SO_4$ into proteoglycans (7–10). We have shown that fibronectin is synthesized in day 7 plaques, although to a much lesser extent than days 3 and 21. Immunohistological localization revealed that the fibronectin in day 7 plaques was in the area of the plaque not yet fully developed into cartilage and in differentiating chondrocytes. Linder *et al.* (23) and Dessau *et al.* (24) showed fibronectin to be absent from differentiated cartilage matrix. Yamada and Olden (6) and Pennypacker *et al.* (25) suggested that losses of fibronectin may be a prerequisite for differentiation of certain cells. These observations by earlier workers were on *in vitro* systems and on cartilage that does not subsequently differentiate into bone, in contrast to the endochondral bone formation studies here. Our data suggest that differentiating chondrocytes may have fibronectin associated with them. Preliminary observations based on hyaluronidase pretreatment indicate that proteoglycans appeared to mask the localization of fibronectin in the cartilage matrix. The appearance of a significant amount of fibronectin in day 21 plaques can be correlated with the onset of hematopoiesis and the presence of endothelial elements in the sinusoids (8, 26).

In summary, maximal biosynthesis of fibronectin was observed during the early phase of *in vivo* collagenous matrix–mesenchymal cell interaction. Continued synthesis of fibronectin was noted during chondrogenesis and osteogenesis. During hematopoiesis considerable fibronectin synthesis was detected in the plaques. Fibronectin was present in early differentiating chondrocytes and could be localized in the extracellular cartilage matrix only after hyaluronidase pretreatment.

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