Synthesis and localization of fibronectin during collagenous matrix-mesenchymal cell interaction and differentiation of cartilage and bone \textit{in vivo}

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

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ABSTRACT The biosynthesis of fibronectin during the \textit{in vivo} development of matrix-induced endochondral bone was investigated by using \textsuperscript{35}Smethionine 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\textsubscript{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 \textit{in vivo}.

It has been well documented (1, 2) that anchorage to a substratum is a fundamental condition for \textit{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, with 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 \textit{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 \textit{in vivo} situation has not been established. Extrapolating from the \textit{in vitro} situation to the \textit{in vitro} 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 \textit{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 quantitatively 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-[\textsuperscript{35}S]methionine (specific activity, 546.7 Ci/mmol; New England Nuclear) with a dose of 1 \textmu Ci/g of body weight (1 Ci = 3.7 \times 10\textsuperscript{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\textsuperscript{3} and incubated \textit{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 \mu Ci of L-[\textsuperscript{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 \textit{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 \textit{in vitro} incubation) were initially extracted by homogenization in 5 vol of 50 mM Tris-HCl/20 mM NaH\textsubscript{2}PO\textsubscript{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.
Cell Biology: Weiss and Reddi

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. Sections of frozen sections of 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 Na2HPO4, 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 Na2HPO4). 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 Na2HPO4) 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/PO4 buffer (pH 7.4) is shown in Fig. 1. A band at about M, 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, 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/PO4 extraction) of a day 3 plaque is shown in Fig. 2A. The fibronectin band appears as a doublet at 220,000 on day 3 and is collagenase insensitive (Fig. 2A, 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. NaDodSO4/5% polyacrylamide slab gel electrophoresis under reducing conditions. Samples were soluble components extracted with 20 mM Na2HPO4/50 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. 2B), 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 Mr of 70,000 was observed.

FIG. 2. (A) NaDodSO4/5% 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 fibronectin. (C) Tris-HCl/PO4 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.

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-[35S]methionine prior to extraction in Tris-HCl/PO4 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 5 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. 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 Mr of 150,000 that are synthesized at the onset of mineralization (day 9), and there is an increased synthesis of proteins of about Mr, 170,000, whereas a protein of an apparent Mr of 50,000 is not synthesized. Arrows on the left indicate molecular weight markers. FN, rat plasma fibronectin.

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.
Fibronectin accumulation. Areas
In (arrow). 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 vitro 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 NaDodSO4 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/PO4 buffer (pH 7.4) migrated as a single band on NaDodSO4/5% 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 vitro as well.

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 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 35S 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 vitro 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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