Fibronectin regulates calvarial osteoblast differentiation

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SUMMARY

The secretion of fibronectin by differentiating osteoblasts and its accumulation at sites of osteogenesis suggest that fibronectin participates in bone formation. To test this directly, we determined whether fibronectin-cell interactions regulate progressive differentiation of cultured fetal rat calvarial osteoblasts. Spatial distributions of α5 integrin subunit, fibronectin, osteopontin (bone sialoprotein I) and osteocalcin (bone Gla-protein) were similar in fetal rat calvaria and mineralized, bone-like nodules formed by cultured osteoblasts. Addition of anti-fibronectin antibodies to cultures at confluence reduced subsequent formation of nodules to less than 10% of control values, showing that fibronectin is required for normal nodule morphogenesis. Anti-fibronectin antibodies selectively inhibited steady-state expression of mRNA for genes associated with osteoblast differentiation; mRNA levels for alkaline phosphatase and osteocalcin were suppressed, whereas fibronectin, type I collagen and osteopontin were unaffected. To identify functionally relevant domains of fibronectin, we treated cells with soluble fibronectin fragments and peptides. Cell-binding fibronectin fragments (type III repeats 6-10) containing the Arg-Gly-Asp (RGD) sequence blocked both nodule initiation and maturation, whether or not they contained a functional synergy site. In contrast, addition of the RGD-containing peptide GRGDSPK alone did not inhibit nodule initiation, although it did block nodule maturation. Thus, in addition to the RGD sequence, other features of the large cell-binding fragments contribute to the full osteogenic effects of fibronectin. Nodule formation and osteoblast differentiation resumed after anti-fibronectin antibodies or GRGDSPK peptides were omitted from the media, showing that the inhibition was reversible and the treatments were not cytotoxic. Outside the central cell-binding domain, peptides from the IIICS region and antibodies to the N terminus did not inhibit nodule formation. We conclude that osteoblasts interact with the central cell-binding domain of endogenously produced fibronectin during early stages of differentiation, and that these interactions regulate both normal morphogenesis and gene expression.

Key words: Fibronectin, Osteoblast differentiation, RGD sequence, Fetal rat calvarial osteoblast

INTRODUCTION

Osteogenesis involves the recruitment of mesenchymal cells to the osteoblast lineage and their progressive differentiation to produce a mineralized extracellular matrix (ECM). Structural ECM proteins, cytokines sequestered in the ECM, and ECM-degrading proteases and their inhibitors are all potential regulators of osteoblast differentiation and function. The observation that the production and composition of ECM are carefully modulated during osteoblastic differentiation (Stein et al., 1990) suggests an important regulatory role for osteoblast-ECM interactions. The functional significance of these interactions, however, is poorly understood.

One ECM protein that may provide information to osteoblasts during their differentiation is fibronectin (FN). FN is a heterodimeric ECM glycoprotein that has several cell- and matrix-binding domains (Fig. 1) (Hynes, 1990). Sequences in at least three different regions of FN bind to the cell surface via two classes of transmembrane receptors: integrins and cell-surface proteoglycans (Damsky and Werb, 1992; Hynes, 1992). The arginine-glycine-aspartic acid (RGD) sequence, present in FN type III repeat 10 (FNIII10) of the central cell-binding domain, recognizes several members of the integrin family. These include the specific integrin FN receptor α5β1 as well as several αv-containing integrins, which also interact with other RGD-containing ECM components besides FN. A second sequence in FNIII9, referred to as the synergy sequence, has been found to selectively enhance binding of the FN RGD sequence to α5β1 (Bowditch et al., 1994; Aota et al., 1994). The C-terminal heparin-binding region of FN (FNIII11-13)
contains several sequences that bind to transmembrane cell-surface proteoglycans, including CD44 and members of the syndecan family (Iida et al., 1992; Jalkanen and Jalkanen, 1992). This region also has low-affinity binding activity for the α4β1 integrin (Mould and Humphries, 1991). Finally, the variably spliced IIICS connecting sequence contains the CS-1 high-affinity binding sequence for α4β1 (Komoriya et al., 1991). Distinct functions for several of these cell-binding FN domains have been demonstrated. For example, the central cell-binding and heparin-binding regions of FN cooperate to promote the formation of focal contact sites in cultured fibroblasts (Woods et al., 1993). The central cell-binding region and the CS-1 sequence cooperate to establish a low basal level of collagenase expression in synovial fibroblasts (Huhtala et al., 1995). These and other studies in many systems have shown that cell interactions with intact FN, or specific FN fragments, can initiate signals that affect cytoskeletal organization, cell motility, tissue-specific gene expression, and matrix remodeling (Adams and Watt, 1993; Damsky and Werb, 1992; Homandberg et al., 1993; Huhtala et al., 1995; Juliano and Haskill, 1993; Werb et al., 1989).

The distribution of FN in areas of skeletogenesis suggests that it may be involved in early stages of bone formation. FN is detected in the periosteum of cultured rat calvaria (Gronowicz et al., 1991), where it could participate in regulation of osteoblast recruitment and commitment to terminal differentiation. FN is also localized to the non-mineralized, dense ECM of osteoid surrounding implants (Weiss and Reddi, 1980), where it could help to regulate matrix assembly. A role in matrix organization is also suggested by ultrastructural immunolocalization studies which demonstrate an association of FN with individual type I collagen fibrils in the matrix of mature bone tissue during endochondral ossification in young rats (Nordahl et al., 1995). In culture, FN is synthesized by chicken osteoblasts and accumulates in the ECM during cell proliferation and early differentiation; synthesis is sharply reduced as cells mature (Winnard et al., 1995). In addition, the expression of FN, as well as of type I collagen, increases during the early stages of differentiation in rat calvarial osteoblasts (Stein et al., 1990). Furthermore, factors known to regulate osteoblast differentiation, such as parathyroid hormone, estrogen, glucocorticoids, 1, 25-dihydroxy vitamin D₃, and transforming growth factor beta (TGF-β), affect both FN expression and osteoblast attachment to FN (Breen et al., 1994; Eielson et al., 1994; Franceschi et al., 1987; Gronowicz et al., 1991; Gronowicz and McCarthy, 1995; Shalhoub et al., 1992).

Integrins are likely to transduce the signals generated by FN. Although there are conflicting data, osteoblasts in normal human bone have been reported to express integrin receptors for FN; α3β1 (Clover et al., 1992), α4β1 (Grzesik and Robey, 1994), α5β1 (Hughes et al., 1993; Grzesik and Robey, 1994) and αvβ3 (Grzesik and Robey, 1994). Similarly, normal human osteoblasts in culture express α3β1 (Clover et al., 1992; Saito et al., 1994), α4β1 (Grzesik and Robey, 1994), α5β1 (Grzesik and Robey, 1994; Saito et al., 1994) and αvβ3 (Grzesik and Robey, 1994). In addition, αvβ5 has been identified in the osteoblast cultures (Saito et al., 1994) but was not localized to osteoblasts in human tissue (Grzesik and Robey, 1994). In rat, the FN receptors αvβ3 and αvβ5 were localized to normal bone tissue (Hultenby et al., 1993) and α5β1 was identified in normal osteoblast cultures (Brighton and Albeida, 1992).

These correlative data suggest the hypothesis that interaction of osteoblasts with FN generates signals that are required for normal morphogenesis and gene regulation during osteogenesis and bone remodeling. To test this hypothesis directly and to study the role of FN in osteoblast differentiation, we used a well-characterized rat calvarial osteoblast model (Bellows et al., 1986; Stein et al., 1990) in which mineralized, bone-like nodules form. We disrupted FN-osteoblast interactions in these cultured osteoblasts by using anti-FN antibodies and fragments of FN. Here we report that perturbing interactions between osteoblasts and the central cell-binding region of endogenously produced FN inhibited mineralized nodule morphogenesis and gene expression in vitro. These findings provide novel evidence that FN plays a role in regulating osteoblast differentiation.

**MATERIALS AND METHODS**

**Cells**

Osteoblasts were isolated from 21-day-old fetal rat calvariae as described by Bellows et al. (1986), with several modifications. Following an initial treatment of calvariae for 10 minutes at 37°C with 570 U/ml collagenase ( Worthington Biochemical Corp., Freehold, NJ), the cells released from calvariae by two 10 minute, and two 20 minute sequential collagenase digestions were pooled, and then filtered through both 100 μm and 37 μm Nitex filters. Cells were grown overnight at 25,000 cells/cm² on plastic dishes (Corning, Corning, NY). For the differentiation experiments, cells were plated at a density of 36,000 cells/cm² in 35 mm dishes or 24-well plates coated with 0.2% gelatin (bovine skin, type B, Sigma Chemical Co., St Louis, MO). For some experiments, cells were plated on 8-well chamber slides, 0.81 cm² per well (Permanox, Nunc Inc., Naperville, IL) that had been coated with 0.25% gelatin cross-linked with cyanamide (Sigma) (Macklis et al., 1985) as previously described (Globus et al., 1989). The cells were grown in alpha-Minimum Essential Medium supplemented with 10% heat-inactivated fetal calf serum (Gibco). After confluence (3 days) the medium was further supplemented with freshly prepared ascorbic acid (50 μg/ml) and β-glycerophosphate (3 mM) (Sigma) to trigger differentiation. Addition of this level of β-glycerophosphate, which is lower than that used in most previous studies, promotes both high cell viability over the 2-4-week culture period and an orderly deposition of mineral extracellularly, in association with fibrillar collagen (see Fig. 2E). Unless otherwise noted, antibodies, fragments, or small peptides were added with every medium change (every 2-3 days) until termination of the experiment. Multiple concentrations of each reagent were tested and the minimum dose providing maximal inhibition was used throughout.

**ECM ligands and antibodies**

**GRGDS** and **GRADSP** peptides, the 120 kDa central cell-binding fragment of FN (120 FN) consisting of approximately FN type III repeats 3-11, and rabbit anti-rat FN antisera were purchased from Gibco/BRL (Gaithersburg, MD). An additional rabbit anti-rat FN antisera was purchased from Chemicon International Inc. (Temecula, CA). The IgG fractions of the anti-FN antiserum were purified by binding to Protein A-Sepharose (Pharmacia Biotech Inc., Piscataway, NJ) and eluting at pH 3.0. Shorter central cell-binding FN fragments containing FN type III repeats 6-10 (FNIII6-10) with, and without, a 16-amino-acid substitution at the synergy site were prepared as described previously (Aota et al., 1994). Rabbit anti-rat osteocalcin antisera was kindly provided by Dr K. Nishimoto (University of Tennessee, Memphis, TN). Monoclonal mouse anti-
osteopontin antibody (MP11B10) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Monoclonal mouse anti-rat FN antibody (BR5.4) was kindly provided by Dr Paul Johnson (University of California, San Francisco). Rabbit anti-mouse α5 antibody was obtained from Chemicon International Inc. The CS-1 peptide (DELPLQVTLPHPNLHPGEILDVPSST) and two control peptides, CS-1C (DELPQLTLPNLPNLDPEILDVPSST) and CS-1S (LTEHTHQ1PLPVPDGLSDDPPPL), were synthesized at the Howard Hughes Medical Institute (University of California, San Francisco). They were conjugated to ovalbumin as described previously (Huhtala et al., 1995). Rabbit anti-human antibody to the 70 kDa amino-terminal region of FN was provided by Dr Deane Mosher (University of Wisconsin, Madison, WI).

**Histology**

Calvaria from 21-day-old rat fetuses were imbedded in Tissue Tek OCT (Miles, Inc., Elkhart, IN) and cryosectioned at 8 μm. Tissue sections were then fixed in 4% paraformaldehyde in PBS for 30 minutes. Cells prepared for immunocytochemistry were grown on plastic chamber slides, fixed with 4% formaldehyde, permeabilized with cold methanol, and then rinsed three times with PBS. Samples were incubated with 5% sucrose in PBS and then carefully removed as an intact sheet, embedded in Tissue Tek OCT, frozen in isopentane in a liquid nitrogen bath, and cryosectioned at 8 μm. Cells prepared for toluidine blue staining and electron microscopy were fixed with a solution of 2% paraformaldehyde, 0.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, for 12-18 hours at 4°C. The cells and their associated matrix were removed from the dishes/slides and placed into 2% aqueous osmium tetroxide for 1 hour, dehydrated in a graded series of alcohols, and embedded in Spurr’s or Araldite resin. Sections 1 μm thick were obtained from each block and stained with 1% toluidine blue containing sodium borate at 55°C. For electron microscopy, sections approximately 70-80 nm thick were collected on water containing bromothymol blue with pH adjusted to 8.0 or above. This prevented loss of mineral from the thin section into the collecting water. These sections were counter-stained with lead citrate and uranyl acetate and examined in a Philips CM-12 electron microscope.

**Immunocytochemistry**

Cryostat sections of fixed calvarial tissue and osteoblast cultures were washed with PBS, incubated with 0.1 M glycine (Sigma) in PBS, and then incubated for 1 hour in a blocking solution containing 0.5% casein (Sigma), 5.0% BSA (Sigma), 0.1% Tween-20 (Fisher Scientific, Fair Lawn, NJ) and 2.0% donkey serum (Jackson Immunoresearch, West Grove, PA). Slides were incubated in primary antibody diluted in the blocking solution (anti-α5 anti-serum, 1:100; mouse anti-rat FN ascites, 1:200; anti-osteopontin hybridoma supernatant 1:1; anti-osteocalcin anti-serum 1:100) overnight at 4°C. Next, slides were washed in PBS for 30 minutes, incubated in blocking solution for 1 hour, and then incubated for 30 minutes at room temperature with rhodamine-conjugated goat anti-mouse or donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch) diluted 1:200 in the blocking solution. Finally, slides were washed with PBS for 15 minutes and coverslips were placed with Aqua Polymount (Polysciences Inc., Warrington, PA).

**Quantification of nodule formation**

To calculate the surface area of the osteoblast cultures occupied by nodules, a photographic image of the central 80% of the entire well was captured with a ScanMaker 1850S scanner (Microtek, Taiwan, Rep. of China) linked to a Macintosh Ici computer (Apple Computer Inc., Cupertino, CA) and then analyzed with NIH Image version 1.57 (National Institutes of Health, public domain). Since the nodules were more phase dense than the surrounding inter-nodular areas we were able to determine nodule surface area by measuring regions in the culture that displayed a density greater than a set threshold. This threshold value was determined and verified by comparing thresholds derived surface areas to manually-circumscribed surface areas. This technique was reproducible between investigators and duplicate wells. Surface areas were calculated from the average of three to four independent experiments containing duplicate dishes or wells ± s.e.m. P values were determined by Student’s t-test.

**Northern analysis**

Total RNA (10 μg/lane) was fractionated on 1% agarose/formaldehyde gels, blotted to a nylon membrane (Hybond N, Amersham, Arlington Heights, IL), and then incubated at 68°C with an [α-32P]dCTP-labeled cDNA probe (see below) in hybridization solution (QuikHyb, Stratagene, La Jolla, CA) for 1 hour. The final wash of the filters was at high stringency in 0.3 M sodium chloride, 30 mM sodium citrate, 0.1% sodium dodecyl sulfate, pH 7.0 at 60°C for 30 minutes. The filters were exposed to film (X-OMAT AR, Eastman Kodak Co., Rochester, NY) for 1-9 days with enhancer screens. Uniformity of sample loading was confirmed by densitometric scanning of the 28 S ribosomal RNA bands stained on the gel with acridine orange. Densitometric analysis of the steady-state expression of mRNA was performed by capturing the video image of the autoradiograph and then analyzing the bands using NIH Image version 1.57 on a Macintosh Ici computer. The data shown are the means of two to three experiments ± s.e.m.

The following cDNA fragments were used for probing the northern blots. A 363-bp cDNA encoding a fragment of rat osteocalcin (bone γ-carboxyglutamic acid protein) (Pan and Price, 1985) was a gift from P. Price (University of California, San Diego, CA). A 520 bp cDNA (pRAP-1) for rat alkaline phosphatase (Noda et al., 1987) was a gift from M. Noda (Tokyo Medical and Dental University, Tokyo) and G. Rodan (Merck Research Labs, West Point, PA). A 1,600 bp cDNA (pRAP-1) encoding a fragment of the α(1) chain of rat procollagen (Genovese et al., 1984) was provided by B. Kream (University of Connecticut, CT). A 350 bp cDNA for rat osteopontin cDNA (Ridall et al., 1995) was a gift from A. Ridall (University of Texas Health Science Center, Houston, TX). A 100 bp cDNA for rat FN (Peters et al., 1995) was provided by P. Johnson (University of California, San Francisco).

**Cell number**

Cell number was determined on day 10. Cells were washed with Ca2+- and Mg2+-free PBS and treated with 1 mg/ml collagenase for 1 hour at 37°C. An equal volume of 0.05% trypsin was then added to the collagenase, and the cells were incubated in this mixture for an additional 30 minutes. Detached cells were then aggregated to obtain a cell suspension. Exclusion of 0.1% trypan blue was used to determine cell viability during counting. Cells from duplicate or triplicate wells of three independent experiments were counted using a hemocytometer. Data are reported as mean ± s.e.m.

**RESULTS**

A fetal rat calvarial osteoblast culture model of osteogenesis preserves normal spatial regulation of ECM components, including FN

When osteoblasts isolated from fetal rat calvariae are plated at near confluent density, they proliferate and differentiate in culture over a 14-16 day period to form multilayered sheets of cells that include prominent mineralized nodules (Bellows et al., 1986; Stein et al., 1990). Cells isolated as described in Materials and Methods formed confluent monolayers within 3 days (Fig. 2A), at which time ascorbic acid and β-glycerophosphate were added. By days 6-8, discrete clusters of cells first appeared that possessed a markedly rounder shape than surrounding cells (nodule initiation) (Fig. 2B).
clusters of round cells subsequently became increasingly phase dense over the next week as observed by phase microscopy (nodule maturation), and mineral deposition was evident by day 14 (Fig. 2C), as shown by von Kossa staining (Fig. 2D). Fully mature nodules (assessed at days 20-29) shared important features of normal woven bone, as judged by electron microscopy. The osteoblast surface membrane was surrounded by an ECM rich in banded type I collagen. Areas of mineralization were associated with collagen fibrils (Fig. 2E, arrows), as is found in normal bone tissue.

To determine whether the spatially regulated distribution of ECM components characteristic of bone in vivo was preserved in this in vitro model, cross-sections of 21 day-old fetal rat calvaria and osteoblast cultures were stained with antibodies against the \( \alpha 5 \) integrin subunit of the FN receptor \( \alpha 5 \beta 1 \) and ECM components characteristic of early and late stages of osteogenesis. In the calvaria the strongest staining for \( \alpha 5 \) was observed in the peristomial and osteoblast layer adjacent to mineralized matrix. Less intense staining was detected among cells within the mineralized matrix (Fig. 3A). Similarly, in day 8 and day 14 osteoblast cultures (similar to cultures depicted en face in Fig. 2B,C) \( \alpha 5 \) staining was most intense in the interstitial areas and the periphery of nodules with some intra-nodular cells also staining (Fig. 3C,E). In the case of FN, the predominant area of tissue staining was the peristomial surface adjacent to bone. Little staining was evident in the mineralized matrix (Fig. 3B). This pattern was similar to that demonstrated by osteoblast cultures where FN was localized to interstitial areas and at the edges of nodules, but not within the nodule core (Fig. 3D,F), where mature osteocyte-like cells surrounded by mineralized matrix were present. In contrast, staining for both osteopontin (Fig. 3G) and osteocalcin (Fig. 3H), which are characteristic of mature, mineralized bone matrix, was restricted largely to the nodular region. This spatially regulated distribution of ECM components serves both to validate the culture system as a suitable in vitro model in which to study the role of FN-osteoblast interactions, and to reinforce the hypothesis that FN is likely to play a role in relatively early stages of osteogenesis.

**Osteoblast interactions with FN are required for nodule formation in vitro**

To determine whether there is a functional role for FN-osteoblast interactions in the progressive differentiation of osteoblasts, we added a polyclonal anti-FN antibody (100 \( \mu \)g/ml) or control rabbit IgG (100 \( \mu \)g/ml) to cultures of confluent day 3 osteoblasts. Ascorbate and \( \beta \)-glycerophosphate were also added to the medium at this time to induce differentiation and mineralization. Antibody was replenished every other day at each medium change. Samples were photographed every other day and harvested at several time points to evaluate proliferation, extent of nodule formation, and the expression of genes associated with osteoblast differentiation.

In control, IgG-treated cultures, nodule initiation was evident by phase or Hoffman optics microscopy by day 8 (Fig. 4A). These nodules appeared increasingly phase-dense by phase contrast microscopy and mineralization could be observed in the maturing nodules by day 14 (Fig. 4C) without staining. In cultures treated with anti-FN antibody, nodule formation was inhibited to less than 10% of control values at both early (day 10) and late (day 20) stages (Fig. 5). Anti-rat FN antibodies from two different commercial sources produced similar results. To determine whether soluble FN present in fetal bovine serum affected the function of the anti-FN antibodies, we repeated the experiments using serum depleted FN on a gelatin-Sepharose column. The results were similar (data not shown), suggesting that the antibody is interacting primarily with the FN produced by the rat osteoblasts, as opposed to the soluble FN in the fetal bovine serum added to the medium.

To determine whether the inhibition of nodule formation by antibody treatment was reversible, cell cultures treated with anti-FN antibody from day 3 to day 14 were allowed to recover for an additional 6-9 days in medium that did not include antibody. Nodule formation recovered significantly by day 20 (Fig. 4H), when compared both with anti-FN antibody-treated day 14 cultures (Fig. 4D) and with parallel cultures exposed to anti-FN antibody continuously through day 20 (Fig. 4F).

**The effect of FN on nodule formation is a property of the central cell-binding domain**

The data above documented an important role for osteoblast-FN interactions in regulating nodule morphogenesis. To determine which domain(s) in FN are required for normal nodule formation, we first added large fragments derived from the RGD-containing central cell-binding region of FN to the medium of confluent osteoblast cultures (day 3). Fragment was replenished every other day at each medium change. Addition of a 120 kDa fragment (120 FN), encompassing approximately FNIII\( ^{1-11} \) (see Figs 1 and 9), at 100 \( \mu \)g/ml (0.83 \( \mu \)M) reduced nodule formation to 10% of control values as assessed on day 10 and day 20 (Fig. 5). Nodule formation resumed after the cultures were provided with medium without the fragment, although the extent of recovery was less consistent than that
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observed following treatment with anti-FN antibody (Fig. 5). The RGD sequence present in FNIII$^{10}$ is the major cell-recognition motif in the cell-binding domain, although additional sequences present in FNIII$^{9}$, such as the synergy site, have been shown to influence integrin receptor specificity and binding affinity (Bowditch et al., 1994; Aota et al., 1994). Addition of the smaller cell-binding fragments FNIII$^{6-10}$ at 200 μg/ml (4.0 μM) inhibited nodule formation to 15% of control.
whether or not they contained a functional synergy site. These soluble fragments are likely to function by interfering with interactions between osteoblasts and the cell-binding domain of intact FN present in their pericellular ECM. Therefore, these data suggest an important role for the central cell-binding domain of FN in regulating osteoblast differentiation.

To determine effects on osteoblast differentiation of the RGD sequence alone, we began addition of the peptide GRGDSPK (100 μg/ml) to osteoblast cultures on either day 3 or day 8. Peptide was replenished every other day at each medium change. GRGDSPK perturbed nodule formation in this assay, when compared with a control peptide, GRADSP (100 μg/ml). However, the morphological effects were distinct from those produced by treatment with polyclonal anti-FN antibody (Fig. 6). When added at day 3, GRGDSPK did not block nodule initiation as detected by the formation of clusters of rounded cells (Fig. 6B); however, the nodules that formed by day 8 were no longer evident by day 10. This inhibition of nodule maturation was also observed when GRGDSPK was added at day 8, after nodule initiation had already occurred (not shown). In addition, the number of nodules detected by Hoffman optics and phase microscopy (without staining) was reduced to less than 5% of control values (Figs 5 and 6C,D). The peptide was omitted from the media of selected cultures at day 14 and the cultures were allowed to continue for an additional 6-9 days. Nodule formation recovered significantly by day 20, when compared both with day 10 cultures and with parallel cultures exposed to RGD continuously through day 20 (Fig. 6E-H). These data suggest that GRGDSPK acts after the initiation of nodule formation, at a critical step in which integrin-mediated interactions are required for stabilization and

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**Fig. 3.** Extracellular matrix components have distinct distribution patterns in fetal rat calvaria and differentiated osteoblast cultures. Cryostat cross-sections of fetal 21 day-old calvaria were incubated with antibodies against the α5 integrin subunit (A) and FN (B). α5 staining was strongest in the periosteal surface adjacent to bone (P). FN staining was strongest in the periosteal surface adjacent to bone, with little localization in the mineralized tissue (MN). 8 day osteoblast cultures, corresponding to nodule initiation, were incubated with antibodies against the α5 integrin subunit (C) and FN (D). 14-day osteoblast cultures were incubated with antibodies against α5 (E), FN (F), osteopontin (G) and osteocalcin (H). In cultured osteoblasts α5 staining was most intense in the internodular (IN) regions and the periphery of the nodules (N). Some cells within the nodule (N) also stained for α5. FN was detected in the internodular region (IN) and around the periphery of the nodule (N), but not within the nodule itself. Staining for osteopontin and osteocalcin was confined largely to the core of the nodule (N). All samples were incubated with the appropriate secondary antibodies conjugated to rhodamine. MS, marrow space. Bars, 40 μm.
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continued maturation of nodules. By contrast, the polyclonal anti-FN antibody blocks both initiation and maturation of nodules.

To evaluate at higher resolution the effects of the RGD peptide treatment on osteoblast differentiation and ECM organization, we examined treated and control cultures histologically in toluidine blue-stained sections. Both control and GRGDSPK-treated cultures formed multilayered sheets of cells (Fig. 7A,B). GRGDSPK-treated cultures had very few nodules, and those that formed were small and poorly developed (Fig. 7B). In contrast, only the control cultures formed numerous nodules containing large depositions of fibrillar collagen (Fig. 7A, arrow), which on ultrastructural observation were shown to have a typical banded pattern (Fig. 2E, arrows).

To determine whether cell number was affected by the anti-FN antibody or GRGDSPK peptide, we treated cultures on day 3 then harvested them on day 10, when nodules were well-formed in control cultures and nodule inhibition was evident in treated cultures. The numbers of cells in treated and control cultures were not significantly different, indicating that anti-FN antibody and GRGDSPK were not toxic and that their inhibitory effects on nodule formation did not reflect large changes in cell proliferation or cell detachment.

To evaluate potential roles in osteoblast differentiation for FN domains outside the cell-binding region, we first used an antibody against the N-terminal 70 kDa region of FN (200 µg/ml). This region includes the N-terminal heparin/fibrin-binding and matrix assembly region as well as the adjacent collagen-binding domain of FN. The antibody against this region blocks FN matrix assembly in fibroblasts (Mosher et al., 1991). However, we found that this antibody had no effect on the formation of nodules by osteoblasts (not shown), suggesting that the N-terminal 70 kDa region of FN is not required for this process. Similarly, addition of the CS-1 peptide (200 µg/ml) from the variably spliced IIICS domain that interacts with α4β1 integrin also did not affect nodule formation. This peptide is active, however, in regulating the expression of col-

Fig. 4. Anti-FN antibody inhibits nodule formation. Cultures were incubated with control rabbit IgG (A,C,E,G) or rabbit anti-rat FN antibody (B,D,F,H) starting on day 3. Cultures were photographed on days 8 (A,B) using Hoffman optics, 10×. Day 14 (C,D) 4×; and day 20 (E-H) 1.5×. Cultures were photographed using phase optics, without staining. In control cultures, nodules were evident by day 8 and became increasingly phase dense, whereas in experimental cultures anti-FN antibody suppressed nodule formation. (G,H) D20-R, cultures were treated with control or anti-FN antibody from day 3 to day 14, then allowed to recover by omitting the antibody from the medium during days 14–20. Dense nodules were able to form following removal of anti-FN antibody. Bars, 400 µm.
lagerase in response to FN in synovial fibroblasts (Huhtala et al., 1995). Taken together, these results indicate that the central cell-binding domain has a preeminent role in regulating nodule morphogenesis.

The disruption of osteoblast-FN interactions reduces expression of genes characteristic of differentiating osteoblasts

Our results thus far indicated that perturbing osteoblast-FN interactions interfere with the morphogenesis of mineralized nodules. To determine whether interfering with FN- and RGD-dependent interactions also alters the pattern of gene expression characteristic of osteoblasts, we used northern blotting to assess the steady-state expression of mRNA for genes characteristic of early (alkaline phosphatase, FN and type I collagen) or late (osteopontin, osteocalcin) stages of differentiation. Data are presented as the values for treated cultures as a percentage of control at the indicated time points (Fig. 8A,C). Alkaline phosphatase (AP) levels in control cultures were elevated by day 3 (not shown) and remained high at day 10. When assessed on day 10, AP levels in cultures treated with anti-FN antibody (100 μg/ml) were 25% of control levels (Fig. 8A), indicating that anti-FN activity suppressed AP expression. Consistent with the results of others (Stein et al., 1990), osteocalcin (OC) expression was low in control cultures through day 10 and then rose at later time points. When assessed on days 10 and 14, treatment with anti-FN antibody suppressed the rise in expression of osteocalcin mRNA, to 25% and 20%, of IgG-treated controls, respectively (Fig. 8A,B). However, anti-FN treatment had no significant effect on the expression of mRNA for type I collagen, osteopontin or FN itself (not shown). When anti-FN antibody was removed at day 14 and the cultures were allowed to recover, the mRNA levels for AP and OC increased to control levels by day 20, showing that the inhibitory effects of the antibody were reversible (Fig. 8A,B). Collectively, these results indicate that interfering with osteoblast-FN interactions disrupts morphogenetic processes involved in early nodule formation and selectively suppresses the expression of specific genes associated with the osteoblast phenotype, while leaving others unaffected.

In contrast to the inhibitory effects of anti-FN antibody, GRGDSPK (100 μg/ml) treatment had only a modest inhibitory effect on the expression of AP and OC after 20 days in culture (Fig. 8C). As with anti-FN antibody treatment, GRGDSPK treatment did not markedly inhibit expression of FN, type I collagen or osteopontin. These differences between the effects of anti-FN antibody and GRGDSPK on gene expression during the early stages of nodule formation (day 10) are consistent with the finding that they exert distinct effects on nodule morphogenesis.

DISCUSSION

The data presented in this study strongly support our hypothesis that FN plays a crucial role in the progressive differentiation of osteoblasts. In order to inhibit interactions between FN and osteoblasts, we first used a polyclonal anti-FN antibody that is expected to bind to multiple sites on FN. This antibody interfered with both the formation of mineralized nodules (nodule morphogenesis) and the expression of genes characteristic of the osteoblast phenotype. Since the cultures resumed nodule morphogenesis once the antibody was removed, the inhibition was reversible and not cytotoxic. Furthermore, addition of a function-perturbing antibody (Young et al., 1994) to tenascin-c, an ECM component localized to developing bone and produced by osteoblasts (Mackie et al., 1992), did not inhibit nodule formation, though after day 14 some wells showed slight detachment of the entire multi-layered cell sheet from the surface. Nodules in these detached cultures appeared normal (data not shown). Thus, inhibition of nodule formation by anti-FN antibody was not simply a generic response to perturbation of cell-ECM interactions; instead, we conclude that blocking cell-FN interactions in osteoblast cultures inhibits one or more steps required for differentiation to progress.

As an alternative approach to perturbing osteoblast interactions with FN, and to identify functionally relevant domains, we then used fragments of FN from the central cell-binding region (120FN). This region contains the RGD sequence involved in early nodule formation and selectively suppresses the expression of specific genes associated with the osteoblast phenotype. Since the cultures resumed nodule morphogenesis once the antibody was removed, the inhibition was reversible and not cytotoxic. Furthermore, addition of a function-perturbing antibody (Young et al., 1994) to tenascin-c, an ECM component localized to developing bone and produced by osteoblasts (Mackie et al., 1992), did not inhibit nodule formation, though after day 14 some wells showed slight detachment of the entire multi-layered cell sheet from the surface. Nodules in these detached cultures appeared normal (data not shown). Thus, inhibition of nodule formation by anti-FN antibody was not simply a generic response to perturbation of cell-ECM interactions; instead, we conclude that blocking cell-FN interactions in osteoblast cultures inhibits one or more steps required for differentiation to progress.

As an alternative approach to perturbing osteoblast interactions with FN, and to identify functionally relevant domains, we then used fragments of FN from the central cell-binding region (120FN). This region contains the RGD sequence in FNIII10, as well as sequences in FNIII11, reported to be important for optimal interaction of the central cell-binding region with specific integrin receptors (Bowditch et al., 1994), including α5β1 (Aota et al., 1994). Addition of either 120FN (corresponding approximately to FNIII11), or a smaller fragment containing FNIII6-10, to osteoblast cultures blocked nodule formation (summarized in Fig. 9), indicating that the central cell-binding domain of FN plays an important role in regulating normal nodule morphogenesis. In contrast, addition of either an antibody against the N-terminal 70 kDa matrix assembly/collagen-binding region or the CS-1 peptide, which interacts with the α5β1 integrin, did not affect nodule formation (Fig. 9), suggesting that these domains of FN are not required for nodule morphogenesis. Not all FN domains were...
tested in this assay, so we cannot rule out important contributions to nodule morphogenesis from regions outside the cell-binding domain, such as the C-terminal heparin-binding domain. For example, it has been reported that human osteoblasts attach to the heparin-binding domain as well as to the cell-binding domain of FN (Dalton et al., 1995). However,
cultures were allowed to recover for 6 days with antibody or peptide demonstrating osteocalcin expression. 28 S ribosomal mRNA was analyzed by northern blotting followed by densitometry. Results are the average of at least two independent experiments ± s.e.m. AP, alkaline phosphatase; OC, osteocalcin.

Results are depicted as a percentage of the control values (GRADSP- FN antibody-treated (A) and GRGDSPK-treated cultures (C). Total RNA was analyzed by northern blotting followed by densitometry. Results are the average of at least two independent experiments ± s.e.m. AP, alkaline phosphatase; OC, osteocalcin.

Fig. 8. Expression of mRNA for osteoblast-associated genes in anti-FN antibody-treated (A) and GRGDSPK-treated cultures (C). Total RNA was analyzed by northern blotting followed by densitometry. Results are depicted as a percentage of the control values (GRADSP- and IgG-antibody-treated) obtained from cultures at the time points indicated. Results are the average of at least two independent experiments ± s.e.m. AP, alkaline phosphatase; OC, osteocalcin.

Besides blocking nodule initiation and morphogenesis, treatment with anti-FN antibody suppressed steady-state mRNA levels of alkaline phosphatase and osteocalcin, two genes characteristic of the osteoblast phenotype. Alkaline phosphatase expression was actively suppressed, since its mRNA level was lower at day 10 in cultures treated with anti-FN antibody than it was at day 3 in control cultures. Anti-FN treatment delayed the rise in expression of osteocalcin, a late osteoblast marker. In contrast, anti-FN antibody had no sig-
significant effect on the expression of mRNA for FN, type I collagen or osteopontin, important ECM components that are characteristic of the osteoblastic phenotype. Thus, the effects of anti-FN antibody on osteoblast gene expression are selective. Taken together, these data suggest that addition of anti-FN antibody initiates a shift in the pattern of gene expression toward a less mature osteoblast.

Surprisingly, continuous treatment with GRGDSPK had no effect on expression of mRNAs for any of the genes tested prior to day 20, despite the fact that nodules were not detected beyond day 10. These data suggest that, in contrast to nodule morphogenesis, the program of osteoblast gene expression is relatively stable in the continued presence of GRGDSPK. Furthermore, these results indicate that morphogenesis and biochemical differentiation of osteoblasts can be uncoupled by factors that perturb those integrin-ECM interactions that are RGD-dependent.

Our results suggest a functional role for integrins as transducers of the signals generated by FN in this culture model, since the central cell-binding domain of FN was critical for inhibition of osteoblast differentiation and since an RGD peptide also suppressed nodule morphogenesis. We found that the distribution of the specific FN receptor α5β1 was similar to that reported by others in both human (Hughes et al., 1993; Grzesik and Robey, 1994; Saito et al., 1994) and rat (Brighton and Albelda, 1992) osteoblasts. Another FN receptor identified in human bone and cultured osteoblasts, α4β1 (Grzesik and Robey, 1994), was not detected in either rat calvarial tissue or osteoblasts in culture. Future studies will be aimed at using function-perturbing antibodies that recognize β1 and αα-associated integrin subunits in this normal rat osteoblast system. Using this approach in MG-63 human osteosarcoma cells, the α5β1 FN receptor was reported to promote osteoblastic differentiation (Dedhar, 1989; Dedhar et al., 1987, 1989). In those studies, amplification of α5β1 expression was associated with increased mineralization in MG-63 cultures. In addition, anti-α5 and anti-β1 antibodies blocked induction of alkaline phosphatase in response to IL-1 in MG-63 cells. Thus, the α5β1 integrin is a likely candidate for signaling at least some of the responses of rat calvarial osteoblasts to FN.

Evidence from these studies indicates that FN acts as an autocrine factor that provides either permissive or instructive cues required for osteoblast differentiation, although our results do not preclude a regulatory role for other bone matrix components. Since FN expression appears to be highly localized to the bone surfaces in vivo and at the periphery of nodules in vitro, it is possible that FN promotes a step in the recruitment or migration of osteoblast precursors. FN may also promote the synthesis and/or organization of an ECM by osteoblasts that is permissive for signaling by growth factors, such as members of the insulin growth factor, TGF-β or fibroblast growth factor families, which are known to reside in the ECM of bone (Erlebacher et al., 1995). In addition, it is possible that contact of osteoblasts with FN via integrins increases expression of TGF-β/bone morphogenetic protein family members which in turn enhance osteoblast differentiation. Such cooperative signaling by ECM and growth/differentiation factors has been demonstrated elegantly in the case of the mammary gland (Streuli et al., 1991). In that system, the expression of milk proteins in pregnant mammary epithelium requires both basement membrane and lactogenic hormones.

In summary, this study demonstrates that osteoblast-FN interactions are required for both morphogenesis and gene expression in this model of osteoblast differentiation. There appear to be critical periods in the differentiation program of osteoblasts during which specific adhesion receptor-FN interactions provide important regulatory signals required for normal morphogenesis and gene expression. Further investigation of the role of osteoblast-FN interactions and how they may collaborate with growth/differentiation factors should provide important new insights into the regulation of tissuespecific differentiation in osteoblasts.

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Fig. 9. Summary of the effects of anti-FN antibodies and FN fragments and peptides on nodule formation. Solid bar represents FN molecule. Striped bars represent regions of FN recognized by the antibodies listed. Open bars represent FN fragments and peptides. Anti-FN Ab, anti-fibronectin antibody; anti-70 kDa Ab, antibody to the N-terminal 70 kDa region of fibronectin; 120 FN, 120 kDa fragment of fibronectin; FN III5-10, type III repeats 6-10 of FN; Sy, ‘synergy’ sequence; RGD, arginine-glycine-aspartic acid; CS-1, connecting segment 1. See text for more information.

Heparin/Collagen
Inhibits Nodule
Initiation
Antibodies
FN
Anti-FN
Heparn/Collagen Binding
FN
Anti-70 kDa
FN III
Heparn/Collagen Binding
FN III
GRGDSPK
CS-1
Inhibits Nodule Maturation
FN
Anti-70 kDa
FN III
GRGDSPK
CS-1
Inhibits Nodule Maturation

1 Anti-FN
3 Anti-70 kDa
FN III
GRGDSPK
CS-1

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