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Fibronectin is a survival factor for differentiated osteoblasts

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SUMMARY

The skeletal extracellular matrix produced by osteoblasts contains the glycoprotein fibronectin, which regulates the adhesion, differentiation and function of various adherent cells. Interactions with fibronectin are required for osteoblast differentiation in vitro, since fibronectin antagonists added to cultures of immature fetal calvarial osteoblasts inhibit their progressive differentiation. To determine if fibronectin plays a unique role in fully differentiated osteoblasts, cultures that had already formed mineralized nodules in vitro were treated with fibronectin antagonists. Fibronectin antibodies caused >95% of the cells in the mature cultures to display characteristic features of apoptosis (nuclear condensation, apoptotic body formation, DNA laddering) within 24 hours. Cells appeared to acquire sensitivity to fibronectin antibody-induced apoptosis as a consequence of differentiation, since antibodies failed to kill immature cells and the first cells killed were those associated with mature nodules. Intact plasma fibronectin, as well as fragments corresponding to the amino-terminal, cell-binding, and carboxy-terminal domains of fibronectin, independently induced apoptosis of mature (day-13), but not immature (day-4), osteoblasts. Finally, transforming growth factor-β1 partially protected cells from the apoptotic effects of fibronectin antagonists. Thus, in the course of maturation cultured osteoblasts switch from depending on fibronectin for differentiation to depending on fibronectin for survival. These data suggest that fibronectin, together with transforming growth factor-β1, may affect bone formation, in part by regulating the survival of osteoblasts.

Key words: Fibronectin, Osteoblast, Transforming growth factor-β1, Apoptosis, Differentiation

INTRODUCTION

Skeletal tissue undergoes modeling and remodeling of its mineralized extracellular matrix throughout life in response to changing mechanical and systemic demands. Osteoblasts, the cells responsible for the formation of new bone, first differentiate from precursors adjacent to bone surfaces. Once osteoblasts have matured to produce an extracellular matrix (ECM) that mineralizes, they subsequently become either flattened, inactive bone lining cells or osteocytes, entrapped within the mineralized matrix. However, the majority of mature osteoblasts in adult remodeling bone fail to differentiate further into either lining cells or osteocytes, and presumably undergo apoptosis (Parfitt, 1990). ECM components are likely to be important in regulating the survival of osteoblasts (Meredith and Schwartz, 1997), although at this time there is no mechanistic insight into the factors that determine whether mature osteoblasts further differentiate or die.

The ECM produced by osteoblasts is complex and consists of several different classes of molecules that may regulate the modeling and remodeling of bone. The ECM contains structural components such as type I collagen and fibronectin (FN), as well as proteases that degrade the matrix (Nordahl et al., 1995; Winnard et al., 1995; Robey, 1996). The ECM also serves as a reservoir for growth factors, including members of the transforming growth factor-β (TGF-β) and fibroblast growth factor (FGF) superfamilies (Canalis et al., 1993; Erlebacher et al., 1995). Acting either alone or together, these components of the ECM, which are produced by osteoblasts, may subsequently affect differentiation and survival, thus acting via autocrine feedback mechanisms to regulate the rate of bone formation.

FN is a heterodimeric ECM glycoprotein which has been shown to regulate adhesion, migration and differentiation of various mesenchymal cells (Yamada et al., 1992). FN interacts with other matrix components as well as with cell surface adhesion receptors via well-defined domains. The amino-terminal 70-kDa domain of FN binds to collagen and includes a region important for *in vitro* matrix assembly, while the central cell-binding and carboxy-terminal heparin-binding domains include sites for interacting with cell surface receptors, including several integrins and transmembrane proteoglycan receptors (Damsky and Werb, 1992; Hynes, 1992). FN has been shown to induce apoptosis of monocytes through interactions with α5β1 (Terui et al., 1996), the integrin heterodimer that recognizes FN exclusively. In contrast to its

role in myeloid cells, FN may function to promote the survival of some attached cells. Specific subpopulations of migrating neural crest cells and endodermal cells from α5 subunit-deficient embryonic mice undergo apoptosis (Goh et al., 1997). In addition, immortalized CHO cells that express α5β1 are able to survive serum withdrawal when plated on dishes coated with FN, suggesting that α5β1 interactions with FN can suppress apoptosis (Zhang et al., 1995). In fact, the ECM produced by normal cells functions as a survival factor for many cell types (Frisch and Francis, 1994; Frisch and Ruoslahti, 1997; Meredith and Schwartz, 1997). However, existing studies have not identified component(s) of the complex ECM produced by normal cells responsible for an autocrine survival-promoting activity, which is the subject of this report.

Cells isolated from fetal rat calvaria and grown in culture provide a useful model of osteogenesis since they have been shown to faithfully recapitulate the stepwise sequence of differentiation and formation of a mineralized ECM that is characteristic of osteoblasts *in vivo* (Bellows et al., 1986; Bhargava et al., 1988; Owen et al., 1990; Liu et al., 1994). We have demonstrated previously that interactions between cultured fetal calvarial osteoblasts and FN are required for differentiation, since the addition to immature cells of function-perturbing antibodies against FN or FN integrin receptors, as well as FN fragments that include the central cell-binding domain of FN, suppresses the formation of mineralized nodules and expression of genes characteristic of mature osteoblasts (Moursi et al., 1996, 1997). In this study, we asked whether FN continues to play an important, yet distinct, role in mature osteoblasts. Therefore, function-perturbing FN antibodies and fragments corresponding to discrete domains of FN were added to cultures that had already formed mineralized nodules. Our results identify FN as a component of the ECM that is required for the survival of osteoblasts once they have matured in culture. We report as well that TGF-β1 partially protects cells from the apoptotic effects of FN function-perturbing reagents, suggesting that TGF-β and FN together protect osteoblasts from apoptosis in vitro.

MATERIALS AND METHODS

Cell culture

Cells were isolated from 21-day-old fetal rat calvariae by sequential collagenase digestion as previously described (Moursi et al., 1996). Briefly, cells were plated in culture dishes overnight, released using 0.25% trypsin and 0.53 mM EDTA in Hanks' buffered salt solution (Gibco/BRL), and then plated at a density of 3.6×10^4 cells/cm^2 either in gelatin-coated 35-mm dishes (Corning) or onto chamber slides (Permanox, Nunc Inc.) that were pre-coated with 0.2% gelatin cross-linked with cyanamide as previously described (Globus et al., 1989). Cells were grown in α-Minimum Essential Medium supplemented with 10% heat-inactivated fetal calf serum (growth medium, Gibco/BRL). After confluence (3 days), the medium was further supplemented with ascorbic acid (50 μg/ml) and β-glycerophosphate (3 mM) to induce differentiation, and was refreshed every 2-3 days.

ECM ligands and antibodies

Polyclonal rabbit anti-rat plasma FN antiserum (FNAb) was purchased from Chemicon Intl. Inc (Temecula, CA.) and purified by protein A-Sepharose chromatography (Pharmacia Biotech Inc.) as previously described (Moursi et al., 1996). FNAb from the two different sources had similar effects on cell survival; the data shown were obtained using the FNAb from Chemicon. Osteocalcin antiserum was a kind gift from Dr K. Nishimoto (University of Tennessee, Memphis, TN). Amino-terminal fragments of FN included a 70-kDa fragment from human plasma prepared by Cathepsin D digestion, as well as the 30-kDa (matrix assembly) and 45-kDa (gelatin binding) fragments prepared by trypsin digestion of the 70 kDa fragment (Sigma). The 120-kDa fragment of FN (FN120) (consisting of approximately FN type III repeats 3-11) and soluble rat fibronectin, were purchased from Gibco/BRL. A central cell-binding FN fragment containing FN type III repeats 6-10 (FN6-10) was a gift from S. Aota (National Institute for Dental Research; Aota et al., 1994). The carboxy-terminal heparin binding domain fragment consisted of type III repeats 12-15 with a deletion of the IIICS domain (H0; Mould et al., 1994). A 40-kDa carboxy-terminal domain of FN prepared by digestion of human plasma fibronectin with α-chymotrypsin that includes the IIICS domain was purchased from Sigma and yielded similar results to the H0 fragment. GRGDSPK and control GRADSP peptides were purchased from Gibco/BRL.

Apoptosis assays

To test for the effects of various treatments on apoptosis, cells were grown for the times indicated in the figure legends. Medium was then refreshed and supplemented with a single addition of FNAb, soluble FN, FN fragments or RGD peptides, and cells were incubated for 24 hours. The concentrations of the FN function-perturbing reagents used were those doses that caused maximum apoptosis, as follows: FNAb (30 μg/ml), soluble FN (0.2 μM), 70-kDa fragment (3 μM), 30-kDa fragment (2.7 μM), 45-kDa fragment (4.5 μM), 120-kDa fragment (1 μM), carboxy-terminal heparin-binding fragment (2 μM), GRGDSPK peptide and control GRADSP peptides (100 μg/ml). To assess the ability of growth factors to affect FN antagonist-induced apoptosis, TGF-β1 (Gibco/BRL), Bone Morphogenetic Protein-2 (30 ng/ml; BMP-2, a gift from Genetics Institute) or FGF (50 ng/ml; bovine pituitary gland, Sigma) was added to the cultures at the same time as the antagonist. To assess nuclear morphology, cultures were fixed for 10 minutes in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, stained in 4′,6-diamidino-2-phenylindole (DAPI, 2.5 μg/ml) for 5 minutes, and washed once in water, after which coverslips were placed with Aquamount (Polysciences Inc.). Photographs were taken using a Nikon camera mounted on a Nikon Diaphot 300 microscope and UV illumination.

To quantify the number of surviving cells, images of DAPI-stained nuclei illuminated by UV light were captured using a COHO series 500 TV camera linked to a MacIntosh Quadra computer. Images were digitized, then enhanced using NIH Image 1.54 (National Institutes of Health, public domain). The numbers of surviving cells were quantified by manually counting intact nuclei from three randomly selected fields within a given sample, such that the total number of nuclei counted in fully viable control samples was >200. This technique was reproducible between investigators and triplicate wells and yielded values for intact nuclei in control samples that were consistent with values for cells that excluded Trypan Blue, counted using a hemocytometer. The data shown are mean ± s.d. and are representative of at least three separate experiments.

To assess DNA degradation, DNA was purified by standard phenol/chloroform extraction methods, then resolved on a 1.2% agarose gel containing 0.5 μg/ml ethidium bromide. DNA was visualized on a transilluminator and then photographed with a Polaroid camera.

Electron microscopy

Cells prepared for electron microscopy were fixed in 2% paraformaldehyde and 0.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, for 12-18 hours at 4°C. Samples were further processed for microscopy as previously described in detail (Moursi et al., 1996).

Immunocytochemistry

Cells were grown for the indicated times in culture, fixed as for DAPI staining, then incubated first with rabbit anti-osteocalcin antiserum (1/200) (a kind gift from Dr K. Nishimoto, University of Tennessee, Memphis, TN) then with Texas Red-conjugated donkey anti-rabbit IgG, as previously described (Moursi et al., 1996).

RESULTS

As described previously (Moursi et al., 1996), fetal rat calvarial osteoblasts grown in medium supplemented with ascorbic acid and β-glycerophosphate form mineralized nodules after 6-9 days of culture, depending on the particular cell preparation. The addition of polyclonal FNAb to immature cultures inhibited differentiation and morphogenesis without affecting survival (Moursi et al., 1996). In contrast, the addition of FNAb to mature 13-day cultures containing nodules caused cells to shrink. DAPI staining revealed that nuclei became condensed and fragmented within 24 hours of treatment (Fig. 1). Both nodular and internodular cells displayed these morphological changes, which are characteristic of apoptosis (Fig. 1F-J). FNAb induced changes in morphology characteristic of apoptosis whether the cells were plated on gelatin-coated dishes (Fig. 1) or on substrata coated with plasma FN (data not shown).

Transmission electron microscopy of day-13 osteoblast cultures after 12 hours of FNAb treatment revealed membrane blebbing, condensation of chromatin at the periphery of nuclei, and extensive cytoplasmic vacuolization (Fig. 2). In addition nuclear condensation, assessed by DNA staining, was evident as early as 10 hours after the addition of FNAb, whereas release of lactate dehydrogenase into spent medium, a marker of compromised surface membrane integrity, was not detected until 16 hours after treatment (data not shown). Thus, nuclear fragmentation preceded changes in membrane permeability in cells treated with FNAb, a characteristic feature of apoptosis as opposed to necrosis. Furthermore, DNA purified from cells treated with FNAb demonstrated a laddering pattern (Fig. 3), characteristic of internucleosomal degradation (Compton, 1992).

In contrast to the effects of FNAb on 13-day cultures, treatment with protein A-purified non-immune IgG did not cause apoptosis (Fig. 1A-E). Furthermore, FNAb did not induce apoptosis when added to immature (3- to 6-day) primary osteoblast cultures prior to nodule formation (Fig. 4). Finally, FNAb did not affect survival of immortalized osteoblast-like cell lines (MG63, MC3T3-E1, ROS 17/2.8) (data not shown), indicating that the ability of FNAb to induce apoptosis may be restricted to normal, mature osteoblasts.

The data presented thus far suggest that cultured osteoblasts become dependent on FN for survival only after they have matured. To test this idea further, the responses of cells to FNAb at different stages of growth and differentiation were determined (Fig. 4). After 24 hours of treatment with FNAb, cells were stained with DAPI and remaining viable cells quantified by

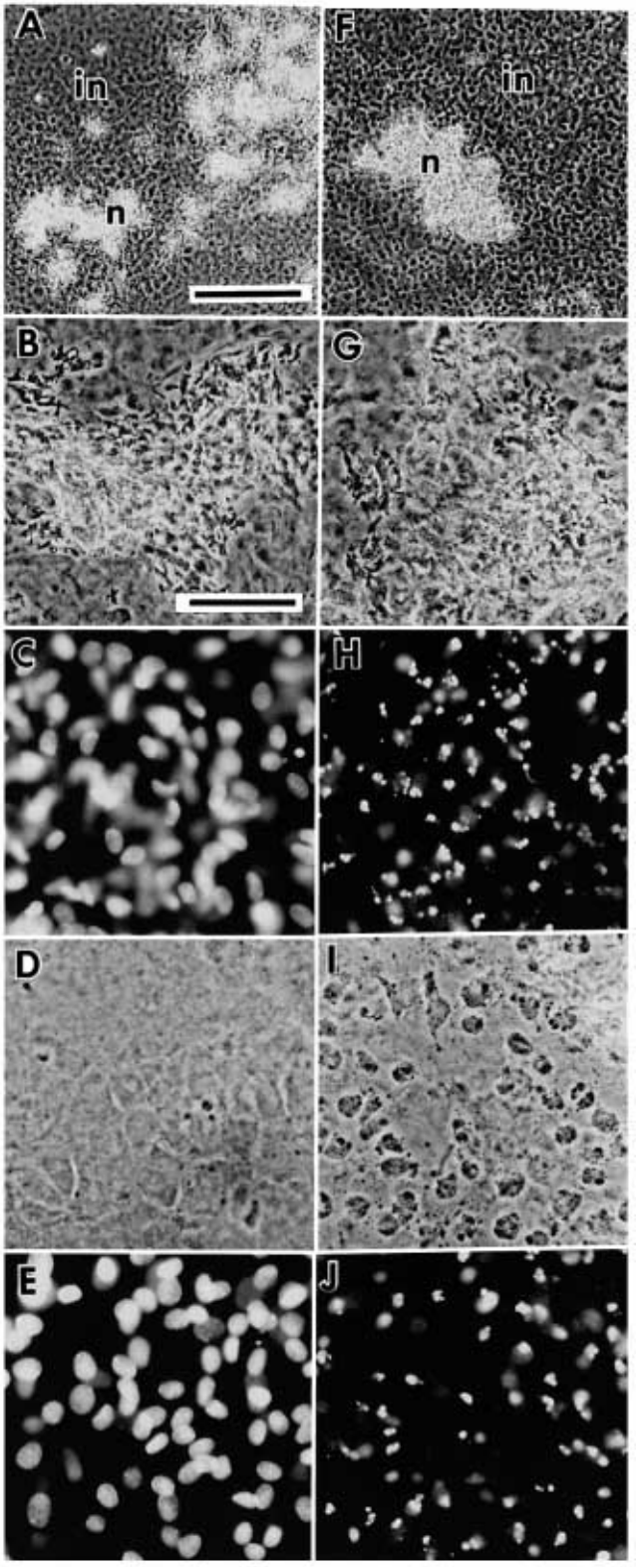

Fig. 1. Treatment of mature osteoblasts with FNAb causes morphological changes characteristic of apoptosis. The medium of mature 13-day cultures was supplemented for 24 hours with either non-immune IgG (A-E) or with anti-FNAb (F-J). Cultures were fixed, stained with DAPI to visualize nuclear morphology, then photographed. Images shown in B-E and G-J include phase contrast images followed by corresponding UV images of nodular (n) or internodular (in) regions of the culture as depicted at lower magnification in A and B. B, C, G and H, nodular regions; D, E, I and J, internodular regions. Bars, A,F, 200 μm; B-E, G-J, 50 μm.

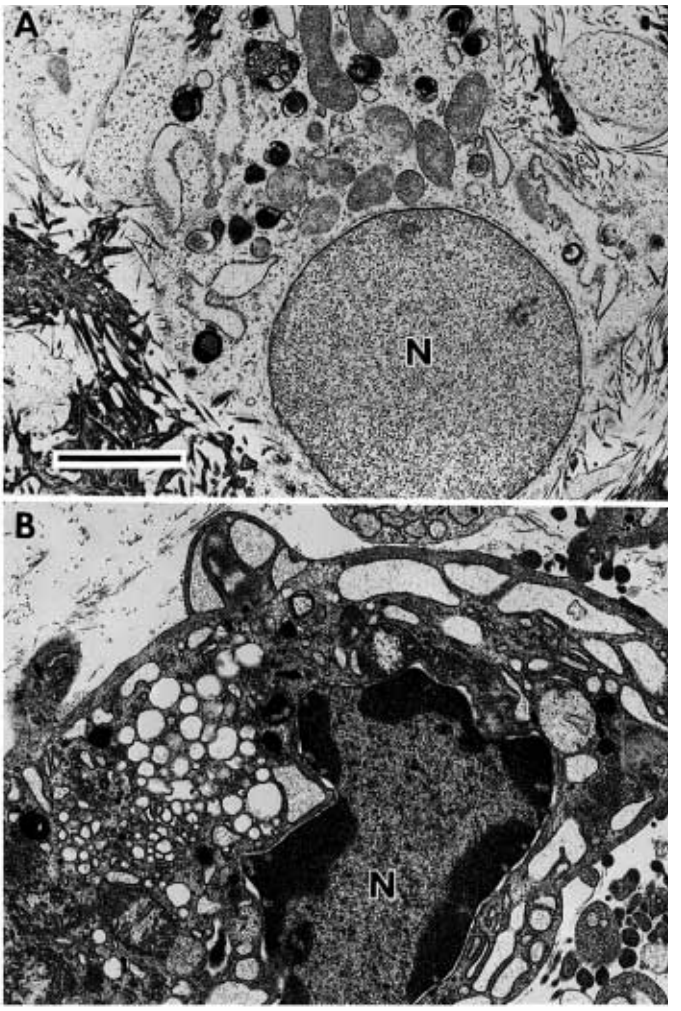

Fig. 2. Treatment of mature osteoblasts with FNAb causes ultrastructural changes characteristic of apoptosis. The medium of mature 13-day cultures was supplemented for 12 hours with either non-immune IgG (A) or with FNAb (B). Cultures were then processed for transmission electron microscopy. N, nucleus. Bar, 2 μm.

image analysis of intact nuclei. In cultures treated with ascorbic acid to promote differentiation, FNAb did not induce apoptosis if the cells were treated at early times prior to the formation of mature nodules (6 days). However, when they were treated after 9 days in culture, >95% of the cells underwent apoptosis in response to FNAb. In mature cultures, cellular sensitivity to FNAb-induced apoptosis continued unabated for as long as 18 days in culture, which was the latest time tested (data not shown). In contrast, cells that were grown in the absence of ascorbate, and therefore failed to form nodules, demonstrated minimal cell death in response to the addition of FNAb (Fig. 4).

To determine which cells are the first to undergo apoptosis in response to FNAb, maturing 8-day cultures were treated for 24 hours with FNAb and then stained with DAPI (Fig. 5). FNAb caused cells within maturing nodules to undergo apoptosis (Fig. 5C,D), whereas internodular cells located at some distance from nodules did not (Fig. 5E,F). If FNAb treatment was initiated on day 8, the majority of internodular cells did not undergo apoptosis despite prolonged exposure to

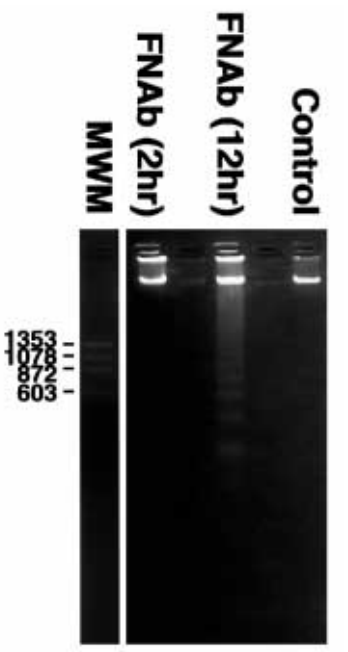

Fig. 3. Treatment of osteoblasts with FNAb causes DNA degradation into oligonucleosome-sized fragments. The medium of mature 13-day cultures was supplemented with either non-immune IgG (Control) or with FNAb for the indicated times. DNA was purified, resolved on an agarose gel, and stained with ethidium bromide. MWM, molecular mass markers.

FNAb (4 days) (data not shown). Thus, internodular cells were resistant to FNAb-induced apoptosis at this stage in culture. Furthermore, only cells located within maturing nodules of 8-day cultures stained positively for osteocalcin, a late marker of the differentiated osteoblast phenotype (Fig. 5G). In contrast, the majority (approx. 80%) of cells in 13-day cultures stained positively for osteocalcin, including those located in

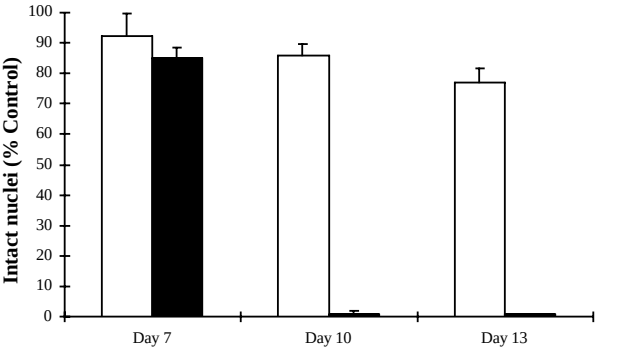

Fig. 4. The apoptotic response of osteoblasts to FNAb is related to maturation. Cells were grown in the absence (open bars) or presence (closed bars) of ascorbate and β-glycerophosphate, which trigger differentiation and nodule formation. Cultures were treated with a single addition of either non-immune IgG (Control) or FNAb for 24 hours, then stained with DAPI at the indicated times in culture (day 7, 10 or 13). Intact nuclei were quantified by imaging techniques to assess cell viability. IgG (control) values ranged from 2300-2700 intact nuclei per mm^2. Data shown are mean ± s.d., expressed as a percentage of IgG (Control).

internodular regions (Fig. 5H). Since mature osteoblasts display a heterogeneous phenotype and express only a subset of differentiation-associated genes (Liu et al., 1994, 1997), these data indicate that the proportion of cells in 13-day cultures that are mature osteoblasts exceeds the 80% or so that stain positively for osteocalcin. This stage of maturation corresponds to maximal sensitivity (>95%) of both nodular and internodular cells to the apoptotic effects of FNAb (Figs 1I,J, 4). Together, these results suggest that osteoblasts acquire a dependence on FN for survival as they mature.

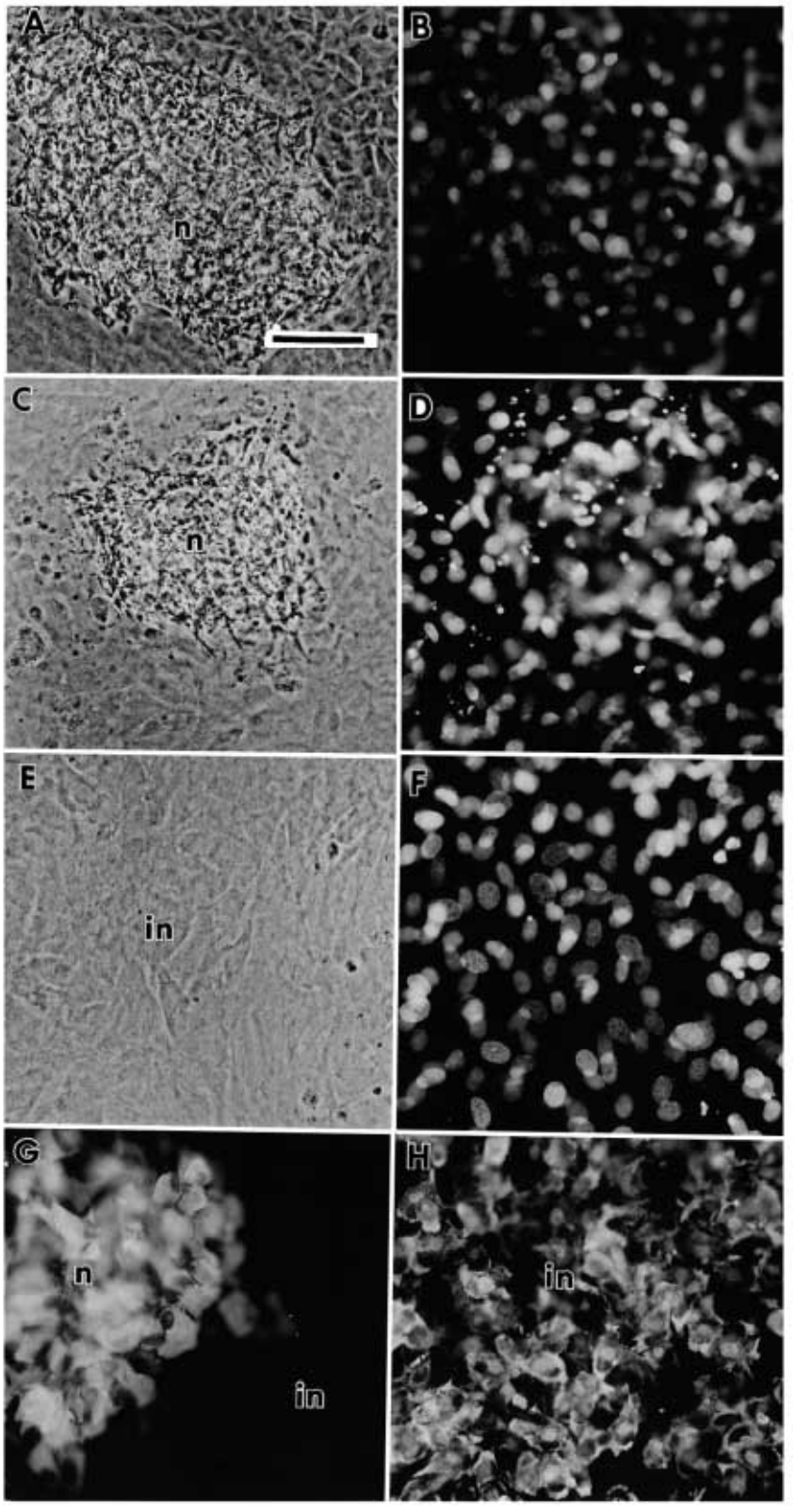

Fig. 5. Nodule-associated cells are the first to become sensitive to the apoptotic effects of FNAb. (A-F) The medium of cultures at intermediate stages of differentiation (8 days) was supplemented with either non-immune IgG (A,B) or FNAb (C,D and E,F) for 24 hours. Cultures were fixed, stained with DAPI to visualize nuclear morphology, then photographed. Images shown in A-F include phase contrast images (A,C,E) followed by corresponding UV images (B,D,F) of nodular (n, A-D) or internodular (in, E,F) regions of the cultures. (G,H) Cells were grown for 8 days (G) or 13 days (H), then fixed and stained for osteocalcin expression. Bar, 50 μm.

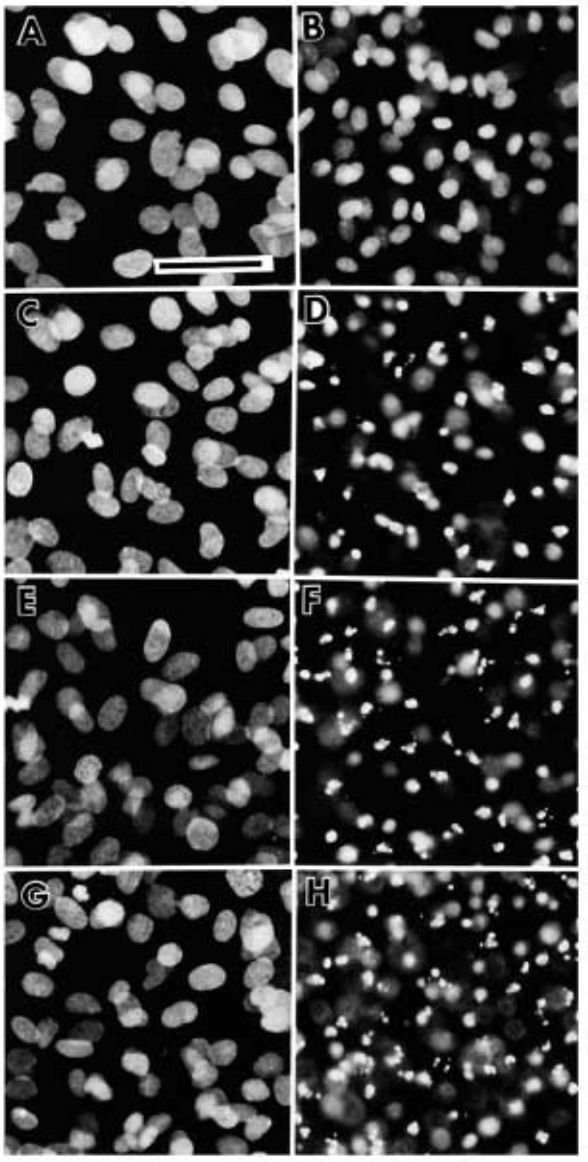

Fig. 6. FN fragments induce apoptosis of mature but not immature osteoblasts. Cells were grown for 4 days (A,C,E,G) or 13 days (B,D,F,H), then the media were supplemented for 24 hours with either bovine serum albumin as a control (A,B), an amino-terminal FN fragment, FN70 (C,D), a fragment that encompassed the central cell binding domain, FN120 (E,F), or a carboxy-terminal fragment, H0 (G,H). Cultures were fixed, stained with DAPI to visualize nuclear morphology, then photographed. Bar, 50 μm.

To identify the domains of FN important for regulating osteoblast survival, immature (4-day) and mature (13-day) cultures were treated with soluble FN or with FN fragments that correspond to the amino-terminal domain, central cell-binding domain, or carboxy-terminal heparin-binding domain (Figs 6, 7), or with an RGD-containing peptide, GRGDSPK (Fig. 7). Although the nuclei in 13-day control cultures were generally smaller than in 4-day control cultures (Fig. 6A,B), these differences are apparently typical of healthy osteoblasts since >95% of cells from 13-day cultures did not display characteristic features of apoptosis (cell shrinkage, nuclear fragmentation, DNA laddering) (Figs 1-3). Mature cells (13 days), treated for 24 hours with either intact soluble FN or FN fragments, underwent extensive apoptosis, whereas treatment with GRGDSPK had no effect. In contrast, none of the fragments induced apoptosis of immature cells (4 days) (Figs 6, 7). Thus, fragments of FN have a similar effect to FN Ab, in causing apoptosis of mature but not immature osteoblasts.

Table 1. TGF-β1 partially inhibits the apoptotic effects of FN fragments

	Intact nuclei (% untreated controls)	
Treatment	FN antagonist	FN antagonist +TGF-β1
FNAb	2	30
70FN amino-terminal	0	45
120FN central cell binding	2	51
H0 carboxy-terminal	0	80

The medium of mature 13-day cultures was supplemented for 24 hours with FNAb or FN fragments in the absence or presence of TGF-β1 (100 ng/ml) as indicated. Cultures were stained with DAPI, then intact nuclei were quantified by imaging techniques to assess cell viability. Data shown are expressed as a percentage of untreated controls.

Previous studies have shown that growth factors and structural ECM components can act synergistically to regulate cell growth and differentiation (Damsky and Werb, 1992). Furthermore, growth factors recently shown to regulate cell survival are also sequestered in bone (Canalis et al., 1993; Prehn et al., 1993; Sachsenmeier et al., 1996). Therefore, we tested the ability of several important skeletal growth factors to promote osteoblast survival when challenged with FN antagonists. When added together with FNAb to mature 13-day cultures, TGF-β1 partially inhibited apoptosis whereas BMP-2 and FGF had no detectable effects on cell survival (Fig. 8). TGF-β1 inhibited FNAb-induced apoptosis in a dose-dependent manner, while the addition of TGF-β1 alone had no effect (Fig. 9). TGF-β1 consistently protected cells from apoptosis induced by FNAb, although the extent of the protection varied between experiments, depending upon the particular cell preparation. We next examined the ability of TGF-β1 to protect osteoblasts from apoptosis induced by FN fragments that correspond to the amino-terminal (70FN), the central cell binding (120FN), or carboxy-terminal domain of FN (H0) (Table 1). TGF-β1 partially inhibited apoptosis induced by each of the fragments although it appeared to be most effective at inhibiting apoptosis induced by carboxy-terminal fragments of FN.

DISCUSSION

Our results support the hypothesis that FN functions as a survival factor for mature osteoblasts. Function-perturbing antibodies, as well as soluble FN and FN fragments, caused extensive cell death throughout mature osteoblast cultures within 24 hours of treatment. The cell death induced by FNAb displayed characteristic features of apoptosis based both on morphological criteria, including chromosomal condensation and nuclear fragmentation, and on the appearance of approx. 200 bp DNA fragments that are characteristic of internucleosomal degradation (laddering) (Compton, 1992). In contrast, non-immune IgG added to mature cells, and FNAb added to cells at early stages of culture, did not cause cell

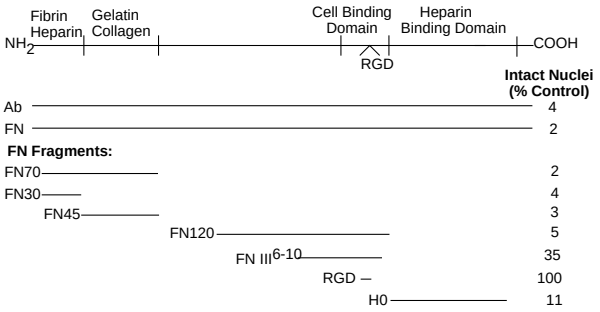

Fig. 7. Fragments from different domains of FN independently trigger apoptosis of mature osteoblasts. The medium of mature 13-day cultures was supplemented with FNAb, soluble FN, fragments corresponding to the indicated regions of FN, or the peptide GRGDSPK (RGD), for 24 hours. Intact nuclei were quantified by imaging techniques to assess cell viability, and the results were expressed as a percentage of untreated controls. Control values ranged from 2300-2700 intact nuclei per mm^2. H0, carboxy-terminal heparin-binding fragment of FN.

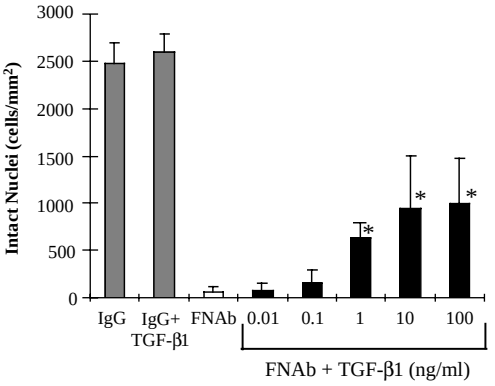

Fig. 9. TGF-β1 partially inhibits the apoptotic effects of FNAb. The medium of mature 13-day cultures was supplemented for 24 hours as shown with non-immune IgG, FNAb and TGF-β1. Cultures were stained with DAPI then quantified by imaging techniques to assess cell viability. Data shown are mean ± s.d. from three separate experiments. * indicates $P<0.05$ (ANOVA) relative to cultures treated with FNAb alone.

death. Thus FNAb selectively induces death of mature osteoblasts by an apoptotic mechanism.

The response of mature cells to disrupting interactions with FN contrasts sharply with that of immature cells. The continuous addition of FNAb and FN fragments containing the central cell-binding domain (Moursi et al., 1996) to immature cultures inhibits morphological differentiation and suppresses expression of genes characteristic of mature osteoblasts, while the cells remain viable. The addition to immature cultures of fragments that correspond to the amino-terminal and carboxy-terminal domains also inhibits nodule formation (data not shown). In contrast, the addition of FNAb to cultures that had already formed nodules caused extensive apoptosis, suggesting that cells switch from differentiation-dependent to survival-dependent interactions with FN as they mature. This proposal is supported by several lines of evidence. When cultures were deprived of ascorbate to prevent differentiation, they failed to undergo extensive apoptosis in response to FNAb. In addition, the cells that first became sensitive to the apoptotic effects of FN function-perturbing agents were those cells that were associated with maturing nodules. Whereas the addition of ascorbate was required for FNAb to induce apoptosis, the further addition of β-glycerophosphate, which is required for mineralization, was not (data not shown). Therefore, cellular differentiation and the associated formation of an osteoblast-derived ECM appear to confer on osteoblasts a dependence on FN for survival that does not require mineralization of that ECM. In an analogous situation involving mammary epithelial cells, a basement membrane ECM both induces differentiation and suppresses apoptosis (Boudreau et al., 1996), although the specific component(s) of the basement membrane responsible for supporting survival have not been identified.

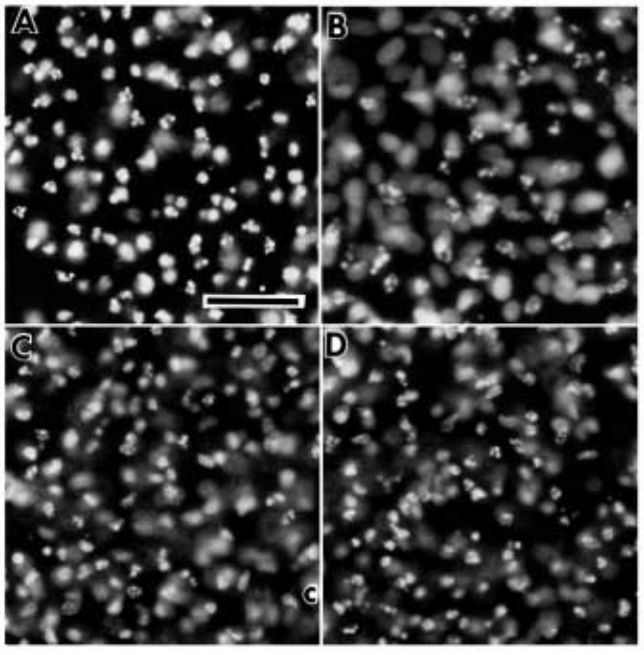

Fig. 8. TGF-β, but not BMP-2 or FGF, partially inhibits the apoptotic effects of FNAb. The medium of mature 13-day cultures was supplemented for 24 hours with FNAb (A-D) together with TGF-β1 (B), BMP-2 (C) or FGF (D). Cultures were fixed, stained with DAPI to visualize nuclear morphology, then photographed. Bar, 50 μm.

FN may support the survival of mature osteoblasts by transmitting a survival signal via adhesion receptors such as integrins, or by maintaining the overall structural integrity of the ECM, or both. The structural integrity of the ECM is essential for cell survival, perhaps because adhesive ligands must be positioned geometrically within an intact ECM to maintain adherent cells in a spread configuration (Chen et al., 1997). We found that fragments from all the major FN regions caused apoptosis of mature osteoblasts. These function-perturbing reagents may all act through a common mechanism, or through distinct pathways. The amino-terminal collagen-binding and matrix-assembly FN fragments may act to perturb matrix organization, whereas fragments that include the central cell-binding domain may act as antagonists for integrin receptor signals. Scott et al. (1997) have shown recently that plating melanocytes on FN suppresses apoptosis by a β1-

integrin-dependent mechanism. However, small RGD-containing peptides, previously shown to inhibit nodule formation by immature osteoblasts (Moursi et al., 1996), did not induce apoptosis of mature cells in this study, suggesting that interfering with RGD-dependent integrin/ECM interactions alone may not be sufficient for inducing apoptosis. The mammary epithelial system provides evidence to support the possibility that both integrin signaling and ECM architecture can participate in maintaining cell viability. Apoptosis of mammary epithelial cells is induced by anti-β1 and anti-α3 antibodies (Boudreau et al., 1995; Howlett et al., 1995), showing that integrins provide a survival signal for these cells. In addition, soluble basement membrane components, or culture conditions that do not lead to the formation of a three-dimensional alveolar structure, do not support survival, leading to the proposal that suppression of apoptosis by the basement membrane requires a three-dimensional tissue organization (Boudreau et al., 1996). Although the precise mechanisms whereby the FN fragments perturb osteoblast function are not known, taken together our data suggest that FN plays an essential role in the mature osteoblast-derived ECM to sustain osteoblast survival, perhaps in part by maintaining the integrity of the ECM.

TGF-β is produced by osteoblasts, is stored in abundant amounts in bone, and affects the activity of osteoblasts and their precursors (Canalis et al., 1993). Since TGF-β can function as a survival factor for neural cells (Prehn et al., 1993) and keratinocytes (Sachsenmeier et al., 1996), we tested whether it may regulate apoptosis of osteoblasts induced by FN antagonists. When added in combination with FN antagonists, TGF-β1 partially inhibited their effects. TGF-β1 was most effective in inhibiting apoptosis induced by fragments from the carboxy-terminal domain of FN. Thus, distinct functional domains of FN may induce apoptosis by different cellular mechanisms. In contrast, we found that two other growth factors known to be sequestered in bone matrix, BMP-2 and FGF, do not protect the cells from FNAb-induced apoptosis. The ability of TGF-β1 to protect mature osteoblasts from apoptosis may contribute to its anabolic properties in vivo, a possibility that warrants further study.

As primary osteoblasts continue to age for as long as 30 days in culture (after having formed mature nodules), a gradual increase in the number of apoptotic cells within the nodules occurs (R. K. G., unpublished observations; McCabe et al., 1995). Whether the apoptosis observed in aging cultures is related to the loss of functional interactions with FN needs to be determined. Recent studies report that triiodothyroinine increases the number of apoptotic cells in cultures of MC3T3-E1 osteoblast-like cells while the fas/fasL pathway stimulates apoptosis of cultured human osteoblasts (Kawakami et al., 1997; Fratzl-Zelman et al., 1997). Therefore endocrine, as well as paracrine and autocrine factors such as FN and TGF-β1, may regulate the survival of osteoblasts (Hill et al., 1997).

In conclusion, although the factors that determine whether mature osteoblasts undergo apoptosis or further differentiate into osteocytes or lining cells *in vivo* have not yet been identified, results from this study demonstrate that FN and TGF-β1 cooperate to regulate the survival of mature osteoblasts in vitro. Furthermore, the ability of various fragments of FN to induce widespread apoptosis of mature osteoblasts suggests that this process may contribute to the loss of skeletal integrity in those pathologies that generate fragments of FN within the local milieu of bone, such as osteoarthritis (Xio et al., 1985; Werb et al., 1989).

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