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Laboratory Investigations

Monoclonal Antibodies against Osteonectin Show Conservation of Epitopes across Species

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Summary. Several monoclonal antibodies were produced to bovine osteonectin, a major noncollagenous protein in the extracellular matrix of bone, and four were characterized. These antibodies showed different reactivities in Western immunoblots, immunoprecipitation, and indirect immunofluorescence, indicating that they recognize different epitopes on the protein. The data indicate that an epitope recognized by one of the antibodies is masked in interactions of osteonectin within cells and in the extracellular matrix. The high degree of cross-species immunoreactivity observed against bone osteonectin with these monoclonal antibodies indicates that these common epitopes have been conserved during evolution.

Key words: Osteonectin — Monoclonal antibodies — Epitope conservation.

Osteonectin (M_r = 32,000) is the major noncollagenous protein in the extracellular matrix of developing bone. Though the function of this protein is not known, its location in newly deposited osteoid, its high affinity for calcium, hydroxyapatite, and collagen, and its reduction in bone from some animals and humans with osteogenesis imperfecta point up its importance to bone metabolism [1–5].

Although bone is a particularly rich source of osteonectin, recent studies indicate that osteonectin, or a homologous protein, (SPARC), an anagram for secreted protein, acidic and rich in cysteine, is produced by mouse parietal endoderm [6, 7], Reichert's membrane [6], endothelial cells [7–9], and fibroblasts [10] in culture and is found in human platelets [11]. Furthermore, this protein and/or its mRNA are detected in several nonbone tissues [8, 12]. However, only bone matrix accumulates osteonectin in appreciable quantity [1, 10].

A monoclonal antibody was previously reported to recognize bovine and human osteonectin [13]. In this study, we report the isolation of four hybridomas which secrete monoclonal antibodies to osteonectin. The specificity of these antibodies was investigated in several systems and data is presented indicating that the epitope recognized by one of them may be involved in interactions of osteonectin in cells and in the extracellular matrix.

Materials and Methods

Protein and Tissue Preparation

Bovine osteonectin was prepared as described earlier [2]. Additionally, guanidine HCl-EDTA extracts of bone were made [2] from young or fetal animals of a variety of species including human, bovine, monkey, pig, dog, sheep, rabbit, rat, and chick.

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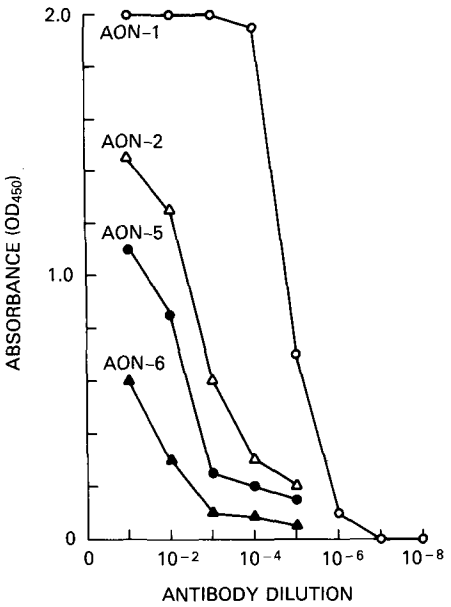

Fig. 1. Titers of ascites fluid from mice injected with hybridomas producing anti-osteonectin antibodies. Titers were evaluated by direct ELISA after purification of the monoclonal antibody on osteonectin affinity columns. The plateau of 2.0 absorbance units for AON-1 was off-scale when analyzed on the Flow Multiscan, possibly indicating a very high affinity for this antibody when compared with other antibodies.

These were desalted by molecular sieve column chromatography, lyophilized, and stored at room temperature prior to analysis.

Preparation of Monoclonal Antibodies

BALB/C mice from the Jackson Laboratory (Bar Harbor, ME) were injected intraperitoneally with 100 mg each of purified bovine osteonectin dissolved in PBS (phosphate-buffered saline) without adjuvant. The mice were boosted with 50 mg of antigen 7 and 14 days after the first injection. Three days after the third injection, the mice were sacrificed and their spleens were removed under sterile conditions. Hybridomas were prepared by the procedure described by Prabhakar et al. [14]. Fusion was with X63-AG8.653 myeloma cells in the presence of 50% polyethylene glycol (M_r = 10^3).

The medium from the hybridoma colonies as assayed for osteonectin antibody by enzyme-linked immunoassay (ELISA) using purified osteonectin (described below). Medium from wells showing a single colony were screened against an extract of bovine bone and the positive colonies were again subcloned. The hybridomas producing osteonectin antibodies were successfully converted to ascites tumors by injecting approximately 2 × 10^6 cells into the peritoneal cavity of mice primed with Pristane. Mice showed evidence of tumor growth in approximately 7–10 days. Ascites fluid was obtained under sterile conditions on the tenth day.

Immunoglobulin from ascites fluid was purified by affinity chromatography on a column of osteonectin coupled to cellulose (Biorad, Rockville Center, NY). Bound immunoglobins were eluted with 3.5 M sodium thiocyanate. Eluted antibody was dialyzed against two changes of PBS and evaluated by direct ELISA for antibody activity.

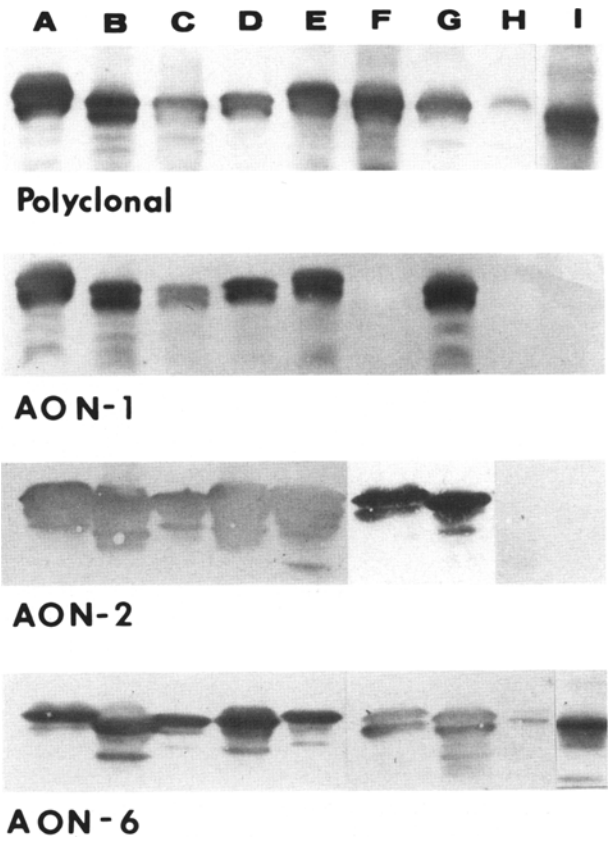

Fig. 2. Recognition of osteonectin in total bone extracts from different species evaluated by Western immunoblots. Monoclonal antibodies AON- 1, AON-2, and AON-6 were compared with polyclonal anti-osteonectin antibody. Species include (A) human, (B) bovine, (C) monkey, (D) pig, (E) dog, (F) sheep, (G) rabbit, (H) rat, and (I) chick. All samples were reduced prior to electrophoresis. The polyclonal anti-osteonectin has been shown previously to be specific in recognizing osteonectin [1, 2, 4].

ELISA and Ouchterlony Assays

The presence of osteonectin antibody in medium from primary and subcloned cultures was tested by direct ELISA against either purified osteonectin or total bone extracts [15]. Wells in plastic microtiter plates (Dynatech Laboratories, Alexandria, VA.) were coated overnight with 100 ng of osteonectin suspended in PBS. Subsequently, 3,3′, 5,5′- trimethylbenzidine (Miles Scientific, Naperville, FL.) was added to the wells and the color was developed according to the manufacturer's instructions. The reaction product was measured at 450 nm with a Titertek Multiscan (Flow Labs, McLean, VA). Fresh culture medium incubated with second antibody in coated microtiter wells served as controls.

The isotype of the anti-osteonectin monoclonal antibodies was determined by double immunodiffusion in 3 mm agarose gels using specific mouse anti-isotype immunoglobulin [16] obtained from Cappel Labs (West Chester, PA.).

Table 1. Summary of species recognition of osteonectin determined by Western immunoblot and direct ELISA

	Human	Bovine	Monkey	Pig	Dog	Sheep	Rabbit	Rat	Chick
Polyclonal	+ + +	+ +	+ +	+ +	+ +	+ +	+ +	+	+
AON-1	+ + +	+ + +	+ +	+ +	+ +	−	+ +	−	−
AON-2	+ +	+ + +	+ +	+ + +	+ +	+ +	+ +	−	−
AON-5[a]	+ +	+ + +	+ +	+ +	+ +	+ +	−	−	−
AON-6	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+	+

[a] Data for AON-5 were obtained by direct ELISA only. The relative reactivity for each species is indicated as determined from optical density readings of ELISA plates and scanned immunoblots (− indicates no reaction; + indicates weak but positive reaction; + + indicates intermediate reaction; + + + indicates strong reaction)

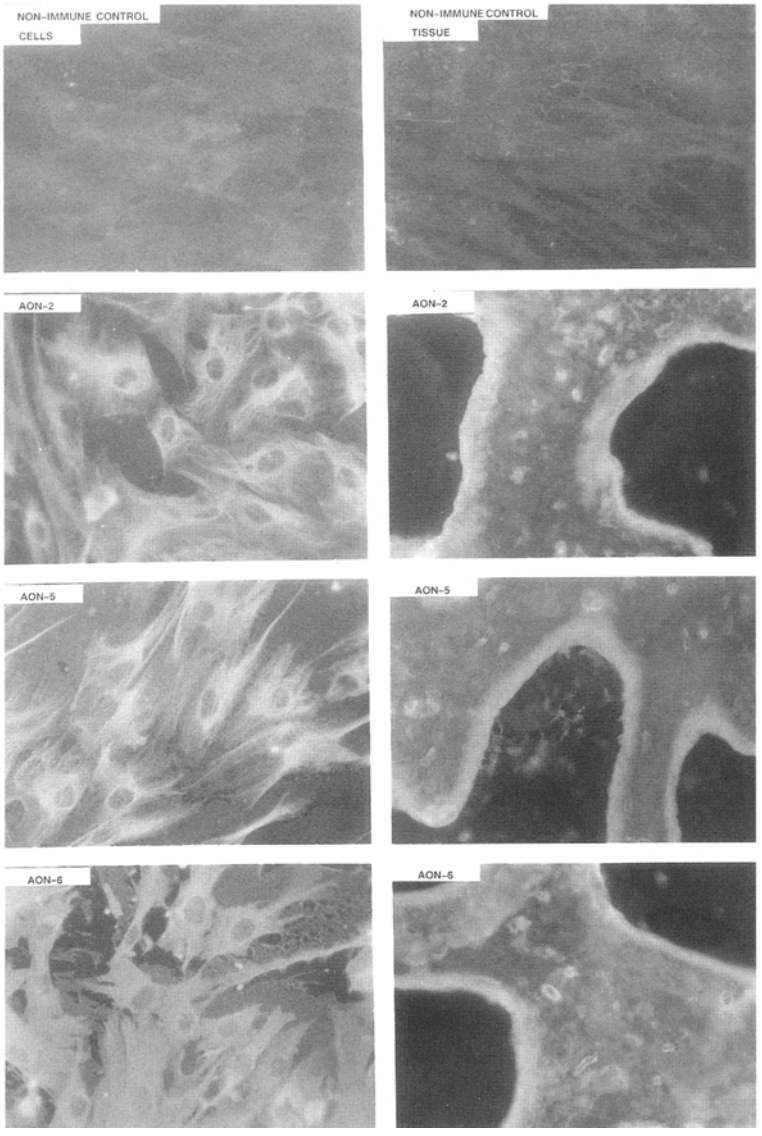

Fig. 3. Indirect immunofluorescent localization of osteonectin in subperiosteal fetal calf bone and cultured human bone-forming cells. In cultures of human bone cells (left panels) a positive reaction for osteonectin is seen in cells, and points of contact between cells and the dish. When undemineralized subperiosteal fetal calf bone is stained with antibodies against osteonectin, the osteoid seam fluoresces, but neither the interstitial mesenchyme nor the mineralized bone trabeculae fluoresce significantly.

SDS Gel Electrophoresis and Western Immunoblot Analysis

Electrophoresis was performed with gradient (4–20%) polyacrylamide gels, prepared as described previously [17]. Bone proteins extracted with guanidine HCl-EDTA were dissolved in sample buffer with mercaptoethanol prior to loading on the gels. For subsequent immunostaining, the proteins in the gel were transferred by electrophoresis to nitrocellulose sheets [18]. Nitrocellulose sheets were blocked with 3% bovine serum albumin in PBS/0.025% Tween 20, incubated with antibodies, and reacted with chromogens. Purified bovine osteonectin was electropho-

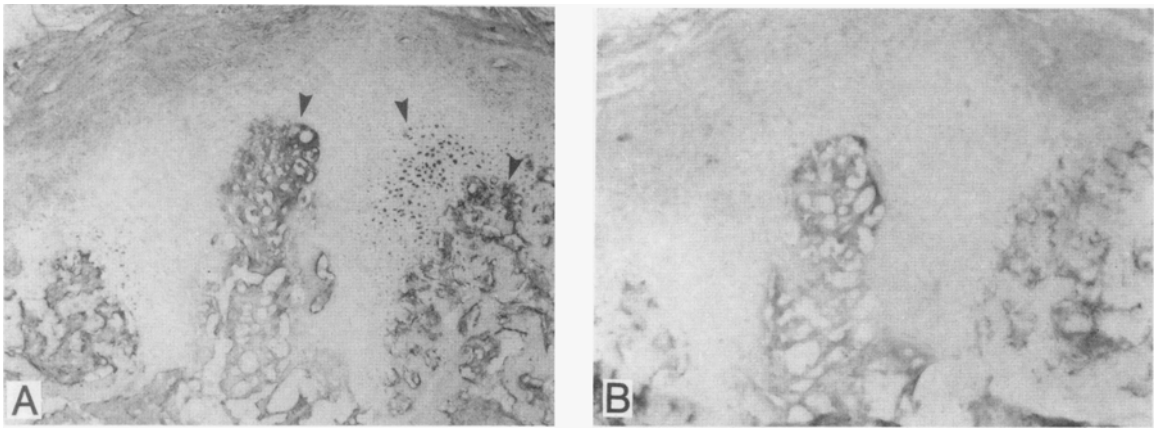

Fig. 4. Immunoperoxidase staining of the callus in a rat femur 16 days after fracture with the AON-6 antibody **(A)**. There is staining of newly formed endochondral bone and of a subpopulation of chondrocytes (arrows). There is no staining in control sections incubated without the AON-6 antibody **(B)**.

resed as an internal standard. Several controls were performed, including immunoblots without primary antibody and immunoblots against bone extracts from Texas variant osteogenesis imperfecta cattle, which are known not to incorporate osteonectin in their bones [4].

Immunoprecipitation Assays

Immunoprecipitation of osteonectin was performed according to the method of Bumol and Reisfeld [19] with minor modifications. Cells isolated from human and bovine bone and adapted to culture [20] were labeled with [^{14}C]leucine for 6 hours. One milliliter samples of culture medium were incubated with rabbit anti-Mouse IgG bound to agarose resin (Cappel Labs) to remove proteins that bind to these reagents. The samples were then incubated with the rabbit anti-mouse IgG agarose to which normal mouse serum had been added in order to remove proteins bound by normal serum IgG's (nonimmune precipitate). Finally, the samples were incubated with rabbit anti-mouse conjugates to which monoclonal antibodies had been bound (immunoprecipitate). Both immune and nonimmune precipitates were solubilized in SDS gel sample buffer containing dithiothreitol to reduce disulfide bonds and electrophoresed on 4–20% polyacrylamide gradient gels.

Immunostaining of Fetal Tissue, Bone Cell Cultures, and Fracture Callus

Slices of subperiosteal bone were shaved from the cortices of fetal bovine bones and frozen in liquid nitrogen. Rat fracture calluses harvested 16 days after fracture were frozen in liquid nitrogen (G. G. Nemeth et al., unpublished data). Frozen sections were cut with a steel knife on a CTF microtome-cryostat (International Equipment Co., Needham Hts., MA) at −15°C. Serial sections of subperiosteal bone or fracture callus were picked up on albumin-coated glass slides, allowed to air dry for 30 minutes, and stored at −20°C. Human bone cells were grown on glass cover slips in culture [20]. The cells on the slides were fixed in 80% methanol at −20°C for 30 minutes, rinsed in PBS-BSA (1%), and stored at −20°C. Slides of fetal tissue, cultured bone cells, and fracture callus were exposed to various dilutions of each monoclonal anti-osteonectin antibody. Pre-immune mouse serum was used as a control. After 30 minute incubation at room temperature in a moist chamber, the slides were washed five times in PBS-BSA (1%), followed by a second 30 minute incubation in either fluorescein-labeled goat anti-mouse antibody (Cappel Labs) diluted 1:16 in PBS-BSA (1%) or a second incubation with a biotin-labeled goat anti-mouse antibody (Vector Labs, Burlington, CA). After additional rinses in PBS-BSA (1%), slides with fluorescein label were mounted in 90% glycerol-10% PBS and photographed using a Leitz ultraviolet microscope. Slides with biotin-labeled second antibody were incubated with horseradish peroxidase-strepavadin complex according to the manufacturer's instruction and photographed. Control samples were photographed for identical lengths of time to allow accurate comparisons.

Results

Six antibody-producing hybridoma lines were obtained, two of which were of the IgM class and not further characterized. The other four lines were isolated, subcloned at least twice, and adapted to ascites culture. Based on ELISA assay, hybridoma AON-1 had the highest titer of the four lines against bovine osteonectin (Fig. 1). The antibodies from three of the lines, AON-1, AON-2, and AON-6, also reacted with bovine bone osteonectin in Western immunoblots when the protein was electrophoresed either with reduction (Fig. 2, column A) or without reduction (data not shown). The other line, AON-5, did not react in Western immunoblots under either condition. The positive hybridomas showed two main bands of staining (Mr = 43,000 and 42,000). Purified osteonectin also showed these two bands on staining with coomassie blue. Negative controls showed no reaction on Western immunoblots.

The monoclonal antibodies were evaluated for

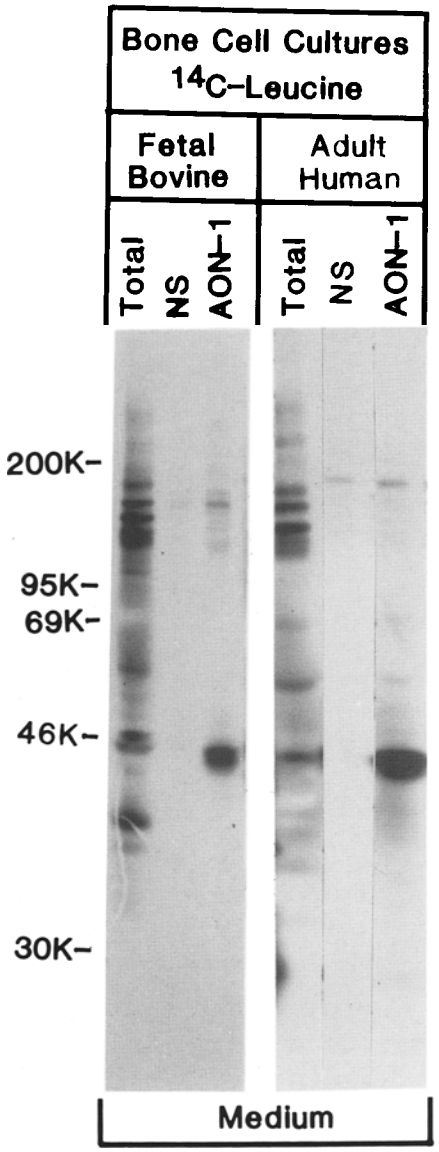

Fig. 5. Immunoprecipitation of osteonectin by monoclonal antibody AON-1 from [^{14}C]leucine conditioned medium of cultured bovine and human bone-forming cells. Total: labeled medium prior to immunoprecipitation; NS: precipitate obtained from normal mouse serum (non-immune); AON-1: precipitate obtained with AON-1 ascites fluid. All samples were heated in the presence of dithiothreitol to reduce disulfide bonds prior to electrophoresis.

their ability to react with bone osteonectins from other species (Fig. 2 and Table 1). One of these (AON-6) seemed to recognize an epitope that was conserved for all species studied. The four monoclonal antibodies were tested for ability to localize osteonectin in fetal bovine bone tissue and in cultures of human bone-forming cells by indirect immunofluorescence. Three, AON-2, AON-5, and AON-6, showed a reaction with bone tissue and with cells by this procedure. Immunofluorescence was observed in nondemineralized subperiosteal fetal bovine bone, particularly in osteoid and in pericellular regions (Fig. 3), a pattern similar to that reported earlier with polyclonal antibodies against this protein [1]. These same three antibodies also reacted with osteonectin in cultures of human bone cells. At low cell density, even when there was little extracellular matrix production, the monoclonal antibodies stained cells, filamentous structures on the cells surfaces, and points of contact between the cells and their substrata (Fig. 3). At high cell density, where considerable matrix had accumulated, extracellular immunofluorescence was very intense and obscured cellular structures (data not shown). Monoclonal antibody AON-1 did not stain bovine tissues or cells, despite its high ELISA titer. Immunohistochemical staining of fracture callus with the AON-6 antibody localized this protein to osteoblasts, osteoid, and a subpopulation of chondrocytes (Fig. 4). As suggested by Western blotting, the other monoclonal antibodies did not stain rat tissues.

The monoclonal antibodies were also tested for ability to immunoprecipitate osteonectin from conditioned medium. Monoclonal AON-1 precipitated osteonectin from radiolabeled conditioned medium of both bovine and human bone cell cultures (Fig. 5). The immunoprecipitates contained small amounts of pro α1(I) collagen. Osteonectin has been shown to bind type I collagen [1], and this association is not completely disrupted with detergent, even at the high levels used in this study (Gehron, Robey and Young, unpublished results). The other monoclonal antibodies did not precipitate osteonectin when tested individually or when the three antibodies were mixed together. The isotype and some other characteristics of these four antibodies were determined (Table 2). Monoclonals AON-1, AON-2, and AON-6 were of the IgG_3 class, whereas AON-5 was of the IgG_1 class.

Discussion

Four new monoclonal cell lines producing antibodies against osteonectin were isolated and tested in a number of systems. Bone extracts were used to evaluate the ability of these four antibodies to cross-react with osteonectin from bone of human and other animal species. Each antibody showed a different species recognition pattern suggesting that they reacted with different epitopes on the protein. Osteonectin is a complex molecule with distinct structural domains as deduced from sequence data [21]. The high degree of cross-reactivity observed

Table 2. Characteristics of monoclonal antibodies to osteonectin

Antibody	Isotype	Immunoprecipitation	Indirect immunofluorescence	
			Bovine tissue	Human cells
Polyclonal		+[a]	+	+
AON-1	IgG_3	+	−	−
AON-2	IgG_3	−[b]	+	+
AON-5	IgG_1	−	+	+
AON-6	IgG_3	−	+	+

[a] Positive reaction; [b]no reaction

against osteonectins from the developing bone of different species suggests considerable conservation of at least some of these domains during evolution.

Although antibodies AON-2, AON-5, and AON-6 appeared unable to react with osteonectin in immunoprecipitation assays, they did react on indirect immunofluorescence with cultures of bone-forming cells and with cells in fetal bone. Conversely, we found a lack of cytochemical reaction between AON-1 and osteonectin in cultures of bone-forming cells or in fetal bone tissue, despite a high antibody titer and strong reaction in immunoprecipitation assays. This suggests that the epitope recognized by AON-1 may be masked by intermolecular interactions within cells and in the extracellular matrix, thus rendering it unavailable for interaction with antibody. These observations raise the possibility that the epitope recognized by AON-1 has a functional role within developing bone.

Diseases of bone may be accompanied by changes in the biosynthetic capacity of osteoblasts, and abnormally low levels of osteonectin have been observed in osteogenesis imperfecta bone [4, 5]. The monoclonal antibodies described above may prove useful in studying the structure of osteonectin in normal and diseased tissues and in evaluating potential abnormalities associated with the protein. Further, they may be useful in distinguishing what differences, if any, exist between bone osteonectin and its homologues in other tissues. Finally, these monoclonal antibodies should be excellent tools to elucidate the role that osteonectin may play in maintaining the functional integrity of bone matrix, and should facilitate the quantitation of osteonectin levels in different disease states.

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