JOURNAL OF BONE AND MINERAL RESEARCH
Volume 16, Number 3, 2001

Caspase-Dependent Cleavage of Cadherins and Catenins During Osteoblast Apoptosis

IRENE HUNTER,[1,2] DUNCAN McGREGOR,[1] and SIMON P. ROBINS[1]

ABSTRACT

As transmembrane, Ca^{2+}-dependent cell-cell adhesion molecules, cadherins play a central role in tissue morphogenesis and homeostasis. Stable adhesion is dependent on interactions of the cytoplasmic domain of the cadherins with a group of intracellular proteins, the catenins. In the present study, we have detected the expression of α-, β-, and γ-catenins in human osteoblasts, which assemble with cadherins to form two distinct complexes containing cadherin and α-catenin, with either β- or γ-catenin. In osteoblasts undergoing apoptosis, proteolytic cleavage of N-cadherin and β- and γ- catenins but not α-catenin was associated with the activation of caspase-3 and prevented by the caspase inhibitor Z-VAD-fmk. The pattern of cadherin/catenin cleavage detected in apoptotic osteoblasts was reproduced in vitro by recombinant caspase-3. The presence of a 90-kDa extracellular domain fragment of N-cadherin in conditioned medium from apoptotic cells indicates that additional extracellular or membrane-associated proteases also are activated. Disruption of N-cadherin–mediated cell-cell adhesion with function-blocking antibodies induced osteoblast apoptosis, activation of caspases, and cleavage of β-catenin. These findings provide compelling evidence that N-cadherin–mediated cell-cell adhesion promotes osteoblast survival and suggest that the underlying mechanism may involve activation of β-catenin signaling. (J Bone Miner Res 2001;16:466–477)

Key words: osteoblast, apoptosis, caspase, cadherin, catenin

INTRODUCTION

SKELETAL REMODELING, the process whereby old bone is replaced by new bone, continues throughout adult life, by the concerted action of bone-resorbing osteoclasts and bone-forming osteoblasts. On completion of their task, some osteoblasts become embedded within the bone matrix as osteocytes and others form flattened lining cells on the bone surface. However, as recent studies have shown, the fate of the majority of osteoblasts is to undergo apoptosis.[1] Apoptosis is a controlled mechanism of cell death, necessary to maintain tissue homeostasis and characterized by ultrastructural changes that include membrane blebbing, chromatin condensation, nuclear fragmentation, and formation of apoptotic bodies.[2]

Apoptosis is regulated by a complex set of environmental signals. In addition to the well-characterized growth factor signaling,[3] recent evidence suggests that adhesion-mediated signaling also may regulate the apoptotic process.[4–7] The disruption of cell-matrix and cell-cell adhesion is a feature of apoptosis in involuting tissues[8] and is an early event in cultured cells undergoing apoptosis.[9,10] In osteoblasts, cell rounding and detachment has been detected during phosphatase-induced[11] and Fas-mediated[12] apoptosis. In anchorage-dependent cells, attachment to the extracellular matrix has been shown to transmit signals that suppress apoptosis.[13,14] Recent data, linking cadherin-mediated adhesion to survival of ovarian granuloma cells[15,16] and oral squamous cell carcinoma,[17] suggest that cadherin-dependent adhesion also could transduce antiapoptotic sig-

[1]Matrix Biochemistry Group, Rowett Research Institute, Aberdeen, Scotland.
[2]Present address: Department of Biomedical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, Scotland.

nals. Previous studies have indicated that attachment to the extracellular matrix protein fibronectin promotes osteoblast survival,[18] but currently there are no reports on the role of cadherin-mediated cell-cell adhesion in the regulation of osteoblast apoptosis.

The cadherin family of Ca^{2+}-dependent transmembrane proteins play an essential role in the establishment and maintenance of cell-cell contacts.[19,20] The extracellular domain of the cadherins mediates homophilic interactions with cadherins on neighboring cells,[21] whereas the cytoplasmic domain interacts with a group of intracellular proteins, the catenins.[22,23] Both β- and γ-catenins can interact directly with cadherins, linking them to α-catenin, which in turn interacts with the actin cytoskeleton.[24–27] In addition, both β- and γ-catenins act as intracellular signaling proteins, which can interact with the lymphoid-enhancer binding factor/T cell factor (LEF/TCF) family of transcription factors, to regulate gene transcription.[28–32]

Osteoblasts have been reported to express a limited repertoire of cadherins,[33–35] which contribute to cell-cell adhesion and the development of the differentiated phenotype. In the present study, we have examined the expression and organization of cadherins and catenins in human osteoblasts and followed the fate of these proteins during both spontaneous and induced apoptosis. Our results indicate that both cadherins and catenins are targeted for cleavage during apoptosis. In addition, the induction of apoptosis by function-blocking N-cadherin antibodies suggests that N-cadherin–mediated cell-cell adhesion is a novel mechanism whereby osteoblasts can regulate apoptosis.

MATERIALS AND METHODS

Antibodies and reagents

Actinomycin D and staurosporine were from Sigma (Poole, Dorset, UK). Genistein was from Lancaster Synthesis, Ltd. (Morecombe, UK). The caspase-3 substrate N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) was purchased from Biomol (Plymouth Meeting, PA, USA). The caspase-1 substrate N-acetyl-Tyr-Val-Ala-Asp-p-nitroanilide (Ac-YVAD-pNA) and the caspase inhibitors N-acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) and benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-fmk) were from Calbiochem (Nottingham, UK). Recombinant human caspase-3 was from Pharmingen (San Diego, CA, USA).

Monoclonal antibodies to β-catenin, plakoglobin (γ-catenin), desmoglein, and caspase-3 (CPP32) were from Transduction Laboratories (Lexington, KY, USA). Polyclonal antibodies to cadherins (pan-cadherin) and β-catenin, a monoclonal antibody recognizing the extracellular domain of N-cadherin (anti-A-CAM; clone GC-4) and horseradish peroxidase (HRP)–conjugated and fluorescein isothiocyanate (FITC)–labeled secondary antibodies were purchased from Sigma. A monoclonal antibody (3B9) recognizing the cytoplasmic of N-cadherin was obtained from Zymed Laboratories (San Francisco, CA, USA). Tissue culture media and fetal bovine serum (FBS) were from Life Technologies Ltd (Paisley, UK). Tissue culture supplements and all other chemicals were from Sigma (Poole, Dorset, UK), unless otherwise indicated.

Cell culture

Primary osteoblast cultures were established from trabecular bone explants as described previously.[36] Briefly, trabecular bone particles, obtained at orthopedic operation from two donors with no known bone disease, were extracted sequentially with EDTA and trypsin and the cells released by 0.2% collagenase in phosphate-buffered saline (PBS) were maintained in α-modified essential medium (α-MEM) containing 10% FBS with 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2 humidified atmosphere. Cells prepared in this way were shown to exhibit an osteoblastic phenotype as judged by high levels of production of alkaline phosphatase, collagen type I, and osteocalcin.

The human osteoblastic cell lines MG-63, TE-85, and SaOS-2 were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C in 5% CO_2 humidified atmosphere.

Reverse transcription and polymerase chain reaction

Messenger RNA (mRNA) was isolated from approximately 10^7 cells for each cell line using the polyATract 1000 mRNA isolation system (Promega, Inc., Southampton, UK). First-strand complementary DNA (cDNA) synthesis was performed at 37°C for 1 h, with 1 μg of mRNA primed with 25 pmol oligo $(dT)_{15}$, in a 50-μl reaction volume containing 400 U Superscript II reverse transcriptase (Life Technologies Ltd., Paisley, UK), 10 mM dithiothreitol (DTT), 12.5 mM Tris-HCl (pH 8.3), 18.75 mM KCl, and 0.75 mM $MgCl_2$. Cadherin cDNA was polymerase chain reaction (PCR) amplified from 5 μl of first-strand cDNA reaction, using 25 pmol of each cadherin family degenerate primer (5′-ACNGCNCCNCCNTAYGA and 5′-TCNG-CNARYTTYTTRAA)[37] in 50-μl reaction volumes containing 2.5 U *Taq* DNA polymerase (Promega, Inc.), 100 μM deoxynucleoside triphosphate (dNTP), 120 μM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), and 0.1% Triton X-100. Amplification was carried out on a Techne Progene thermal cycler (Scotlab, Coatbridge, UK) for 30 cycles at 94°C for 1 minute, at 50°C for 1 minute, and at 72°C for 2 minutes. The 160-base pair (bp) reaction product was gel purified using a MERMAID DNA purification kit (BIO 101, Inc., Vista, CA, USA) according to the manufacturer's instructions and ligated overnight at 16°C into 50 ng of pGEM-T vector (Promega, Inc.) as described by the manufacturer. Precipitated DNA was electroporated into TG-1 *Escherichia coli* bacteria and plated onto LB-agar plates containing 100 μg/ml ampicillin, 1 mM isopropylthio-β-D-galactoside (IPTG), and 10 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and incubated overnight at 37°C. Plasmid DNA was isolated from insert containing colonies from 1.5 ml of overnight culture using a Wizard-SV kit (Promega, Inc.), and insert sequence was determined with

M13FOR primer using a BigDye terminator sequencing kit (PE-Applied Biosystems, Warrington, Cheshire, UK).

Immunofluorescence microscopy

Cells, grown on glass coverslips, were fixed in cold (−20°C) methanol/acetone (1:1, vol:vol) for 7 minutes, blocked with 1% bovine serum albumin (BSA) in PBS for 30 minutes, and incubated with primary antibodies for 1 h at room temperature (RT). After three washes with 1% BSA in PBS, cells were incubated with FITC-labeled secondary antibodies for 1 h at RT. Coverslips were mounted on glass slides in antifading reagent (Vectashield; Vector Laboratories, Inc., Burlinghame, CA, USA) and examined using a Zeiss Axioplan phase-epifluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Induction and detection of apoptosis

Apoptosis was induced by treating cells with actinomycin D (1 μg/ml) or staurosporine (1 μM) for 24 h or with genistein (100 μM) for 72 h at 37°C. Cells showing morphological features of apoptosis (cell shrinkage and membrane blebbing) were found floating in the culture medium, while the remaining adherent cells appeared normal. In some experiments, cells were transferred to serum-free medium and incubated with staurosporine (1 μM) for 24 h. After removal of nonadherent cells, the supernatants were concentrated 10-fold using Centriprep-30 concentrators (Amicon, Beverly, MA, USA). In experiments designed to evaluate the role of caspases, the tripeptide caspase inhibitor Z-VAD-fmk (100 μM) was added to cells 1 h before the induction of apoptosis.

Adherent and nonadherent cells were collected separately, washed with PBS, and analyzed for DNA fragmentation using the Suicide-Track DNA ladder isolation kit (Oncogene Research Products, Cambridge, MA, USA) according to the manufacturer's instructions. For the assessment of DNA fragmentation at the single cell level, osteoblasts were grown on glass coverslips and treated with apoptosis-inducing reagents as described previously. Nonadherent cells were collected by centrifugation and cytocentrifuged onto glass coverslips. Apoptosis was detected using a terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) assay (Promega, Madison, WI, USA) based on the incorporation of fluorescein-12-dUTP into 3′-OH ends of cleaved DNA. Cells were photographed using a Zeiss Axioplan phase-epifluorescence microscope.

Preparation of cell extracts, immunoprecipitation, and immunoblotting

Cells were lysed in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, and 20 μg/ml each leupeptin, pepstatin A, and aprotinin for 1 h on ice. Samples were centrifuged at 15,000*g* for 15 minutes at 4°C and the supernatants were retained. For immunoprecipitation, cell lysates were incubated overnight at 4°C with 2.5–5 μg of antibody. When monoclonal antibodies were used, rabbit anti-mouse immunoglobulin G (IgG) was added, before incubation with a 50% slurry of protein A–Sepharose. After constant mixing at 4°C for 1–2 h, samples were centrifuged and pellets were washed extensively with lysis buffer. For immunoblotting, cell lysates were mixed with an equal volume of 2× reducing sodium dodecyl sulfate (SDS) sample buffer[38] and incubated at 100°C for 5 minutes. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. After blocking with 5% milk powder/10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20, membranes were incubated for 1 h at RT with primary antibody. Specific antibody binding was detected with HRP-conjugated secondary antibodies and developed using enhanced chemiluminescence.

Measurement of caspase activity

Cells were lysed in caspase buffer (10 mM HEPES-KOH, pH 7.4, 10% sucrose, 2 mM EDTA, 0.1%, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) 5 mM DTT, 1 mM PMSF, and 20 μg/ml each pepstatin A, leupeptin, and aprotinin) for 15 minutes on ice. After centrifugation at 15,000*g* for 15 minutes at 4°C, supernatants containing 50 μg of protein were incubated with either 200 μM Ac-DEVD-pNA (caspase-3 substrate) or 200 μM Ac-YVAD-pNA (caspase-1 substrate). In some experiments, the caspase-3 inhibitor Ac-DEVD-CHO was included at a concentration of 0.1 μM. Caspase activity, measured by the enzymatic release of the chromophore, pNA from the labeled substrate, was monitored at 405 nm.

In vitro caspase cleavage assay

For direct cleavage of adherens junction proteins, β- and γ-catenins were immunoprecipitated from 500 μg aliquots of total TE-85 cell protein, washed with cleavage buffer (10 mM HEPES-KOH, pH 7.4, 10% sucrose, 2 mM EDTA, 0.1% CHAPS, and 5 mM DTT), and resuspended in 25 μl of the same buffer. Recombinant human caspase-3 (20 ng) was added and samples were incubated at 37°C for 0–60 minutes. The reaction was stopped by the addition of an equal volume of 2× reducing SDS sample buffer and the samples were incubated at 100°C for 5 minutes. Cleavage products were resolved by SDS-PAGE and detected by immunoblotting as described previously.

Antibody blocking experiments

To prevent cell attachment and spreading, tissue culture plates were coated with the nonadhesive polymer polyhydroxylethyl-methacrylate (poly-HEMA) as described previously[5] and washed extensively with PBS before use. Primary human osteoblasts (HOBs) were plated on poly-HEMA–coated 24-well plates, in the presence of anti-N-cadherin antibody (A-CAM, clone GC-4) or an isotype-matched control antibody at a concentration of 800 μg/ml or with 5 mM EDTA. After overnight incubation, cells were cytocentrifuged onto glass coverslips and assessed for apo-

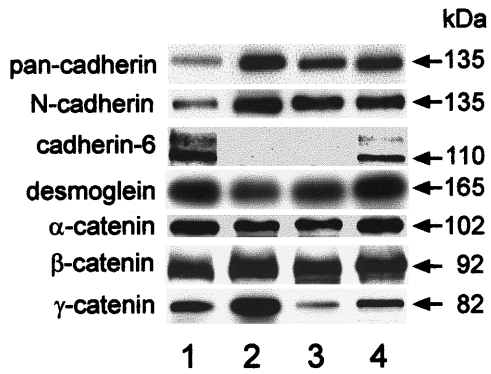

FIG. 1. Expression of cadherins and catenins in osteoblastic cells and HOBs. Extracts of the osteoblastic cell lines MG-63 (lane 1), TE-85 (lane 2), and SaOS-2 (lane 3) and primary human osteoblasts (HOBs; lane 4) were fractionated by SDS-PAGE and immunoblotted with antibodies against cadherins and catenins.

ptosis by TUNEL staining or extracted and analyzed by immunoblotting as described previously. Analysis of cells fixed before cytocentrifugation confirmed that this procedure did not induce cell damage.

RESULTS

Expression and distribution of cadherins in osteoblastic cells

We have detected the expression of two type I (N-cadherin and cadherin-4) and two type II (cadherin-6 and cadherin-11) classical cadherins in human osteoblasts. Using RT-PCR with specific primers, cadherin-11 and N-cadherin were identified in MG-63, TE-85, and SaOS-2 cells, confirming a recent report on the expression of these cadherins in osteoblastic cells.[34] Immunoblotting, using an antibody that recognizes a highly conserved sequence in the cytoplasmic domain of all classical cadherins, revealed that the major reactive species in both HOBs and osteoblastic cell lines had an apparent molecular mass of 135 kDa (Fig. 1, pan-cadherin), identical to that of N-cadherin (Fig. 1) but significantly higher than that reported for cadherin-11 (120 kDa).[33,34] These results suggest that the major classical cadherin expressed in human osteoblasts is likely to be N-cadherin and not cadherin-11 (120 kDa) as previously reported.[34] This conclusion also is consistent with the finding that a synthetic peptide containing the His-Ala-Val (HAV) adhesion motif, found in the extracellular domain of type I (e.g., N-cadherin) but not type II (e.g., cadherin-11) classical cadherins, was effective in inhibiting cell-cell adhesion in osteoprogenitor cells by >70%.[34]

Cadherin-4, present in HOBs, was reportedly absent from osteoblastic cell lines.[34] However, using cadherin-4–specific primers, we have obtained PCR products of the correct size in MG-63, SaOS-2, and TE-85 cells. In addition, sequence analysis of cDNA clones from TE-85 cells, generated with primers to a highly conserved region in the cytoplasmic domain of cadherins, confirmed the presence of cadherin-4 in these cells. HOBs, SaOS-2, and bone marrow stromal cells recently have been reported to lack cadherin-6[34] while cadherin-6 transcripts have been detected in human stromal cells, the MG-63, and TE-85 osteoblastic cell lines and in osteoclast-like cells.[39] Using a specific antibody, we have confirmed the expression of cadherin-6 in MG-63 cells but not in TE-85 or SaOS-2 cells (Fig. 1). It has been suggested that cadherin-6 may mediate heterotypic interactions between osteoblast and osteoclast precursors, important in the generation of osteoclasts.[39] In the present study, the finding that cadherin-6 also is expressed in HOBs (Fig. 1) indicates that it is likely to have specific functions in differentiated osteoblasts.

Desmogleins, members of the cadherin superfamily normally present in cell-cell desmosomal junctions,[40,41] also were expressed in osteoblasts (Fig. 1). RT-PCR analysis revealed that osteoblasts expressed desmoglein-2, a widely distributed desmoglein and the only isoform expressed in nonepithelial tissues.[42] The detection of an immunoreactive band of the correct size (165 kDa) in HOBs, as well as in osteoblastic cell lines, suggested that desmoglein expression was not an artifact of cultured osteosarcoma-derived cell lines. To our knowledge, neither desmosomes nor their constituent proteins have been detected previously in osteoblastic cells and the functional significance of this observation remains to be elucidated.

In TE-85 cells, which have an epithelial-like morphology, forming closely associated arrays of polygonal cells at high density, cadherins and catenins were localized to lateral cell borders at sites of cell-cell contact (Fig. 2). An identical distribution of cadherins/catenins also was detected in MG-63 and SaOS-2 cells (data not shown). In HOBs, which have a more spread morphology, cadherins and catenins were localized similarly to cell-cell contacts, both at lateral cell borders and where fingerlike processes contacted an adjacent cell (Fig. 2), as has recently been described.[34]

Organization of cadherin/catenin complexes in osteoblastic cells

The interaction of the cytoplasmic domain of the cadherins with a group of intercellular proteins, the catenins, and subsequent linkage to the actin cytoskeleton is essential for stable cell-cell adhesion.[24–27] Although it has been reported that β-catenin interacts with cadherins in bone marrow stromal cells[34] and with cadherin-6 in MG-63 cells,[39] neither the presence nor the organization of other catenins has been described in osteoblasts.

In the present study, we have detected the expression of α-, β-, and γ-catenins in both osteoblastic cells and HOBs, in which their colocalization with cadherins at sites of cell-cell contact (Fig. 2) is consistent with the assembly of these proteins into complexes. To define in detail the molecular organization of cadherins and catenins in osteoblasts, extracts of TE-85 cells were immunoprecipitated with β-catenin and the presence of additional proteins was determined by immunoblotting. The results clearly show both the effective precipitation of β-catenin (Fig. 3A, β-cat) and the coprecipitation of cadherins (Fig. 3A, pan-cad),

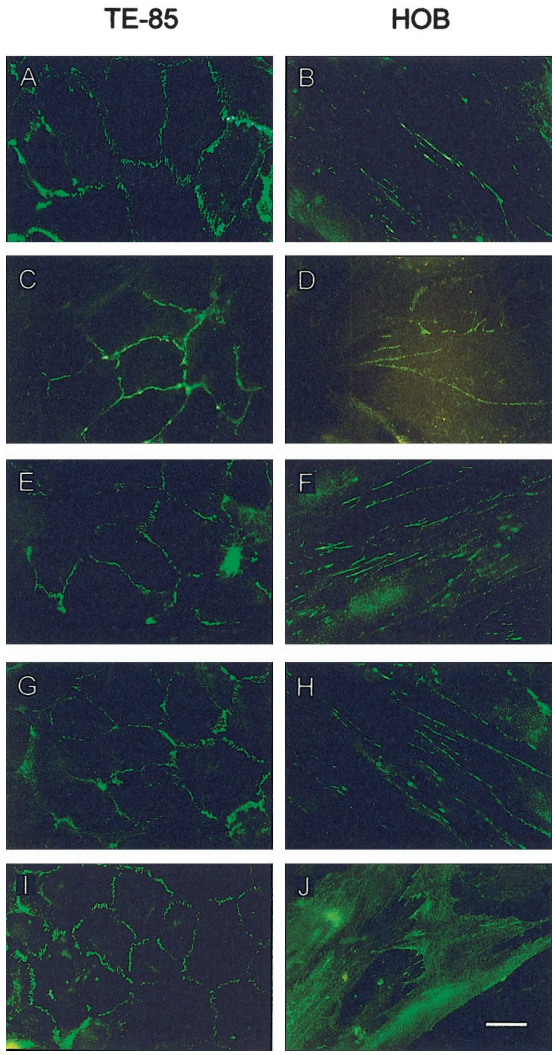

FIG. 2. Immunolocalization of cadherins and catenins in osteoblastic TE-85 cells and HOBs. Cells were grown to confluence on glass coverslips, fixed and incubated with antibodies recognizing (A and B) pan-cadherin, (C and D) N-cadherin, (E and F) α-catenin, (G and H) β-catenin, and (I and J) γ-catenin. For each cell type, antibody distribution was assessed in multiple cell monolayers, in at least three independent experiments. Bar = 25 μm.

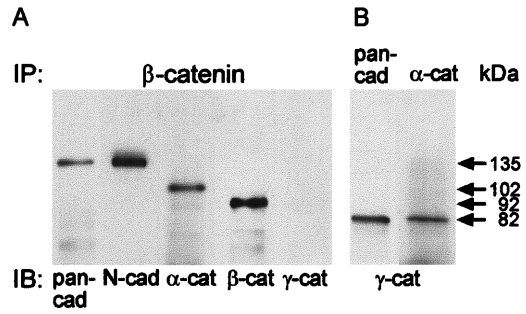

FIG. 3. The composition of cadherin/catenin complexes in osteoblastic cells. (A) Extracts of TE-85 cells were immunoprecipitated (IP) with antibodies against β-catenin, resolved by SDS-PAGE, and immunoblotted (IB) with antibodies recognizing pan-cadherin (pan-cad), N-cadherin (N-cad), α-catenin (α-cat), β-catenin (β-cat), and γ-catenin (γ-cat). Cadherin/catenin complexes of identical composition also were detected in MG-63 and SaOS-2 cells. (B) TE-85 cell extracts, immunoprecipitated with antibodies recognizing either pan-cadherin (pan-cad) or α-catenin (α-cat) were immunoblotted with anti–γ-catenin (γ-cat) antibodies.

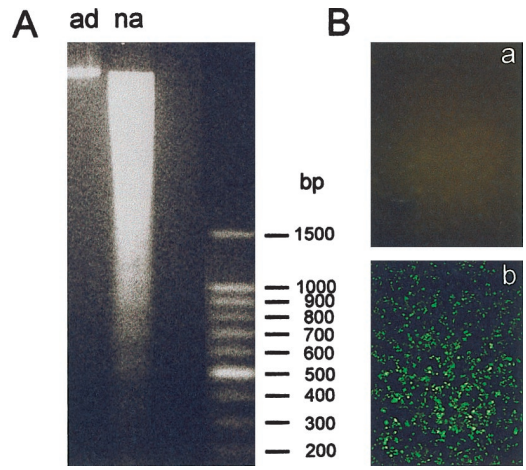

FIG. 4. SaOS-2 cells undergo spontaneous apoptosis. During routine culture, a small number of SaOS-2 cells are found as single cells, floating in the culture medium. (A) DNA isolated from adherent (ad) and nonadherent (na) SaOS-2 cells was electrophoresed through 2% agarose gels containing ethidium bromide and DNA laddering visualized with UV light. DNA molecular weight markers, expressed as base pairs (bp), are indicated to the right. Extensive cleavage of DNA indicated that nonadherent cells had undergone apoptosis. (B) Adherent (a) and nonadherent (b) SaOS-2 cells, were fixed and permeabilized and apoptosis was detected by positive TUNEL staining.

N-cadherin (Fig. 3A, N-cad), and α-catenin (Fig. 3A, α-cat). In contrast, γ-catenin was not coprecipitated with β-catenin from these cells (Fig. 3A, γ-cat) but was present in both pan-cadherin and α-catenin immunoprecipitates (Fig. 3B). These results show that at least two distinct complexes are present in osteoblasts, one containing cadherin/α-catenin/β-catenin and one containing cadherin/α-catenin/γ-catenin. Although γ-catenin is the only catenin reported to interact with both classical and desmosomal cadherins,[22,43] we have been unable to coprecipitate γ-catenin and desmogleins. Because the desmoglein and α-catenin binding sites on γ-catenin overlap,[44] it is possible that the predominant interaction of γ-catenin in these cells is with α-catenin.

Fate of cadherins/catenins during osteoblast apoptosis

During routine culture, we noticed that a small number of SaOS-2 cells are found as single cells in the culture medium. The detached cells were shrunken in appearance, with membrane blebbing and nuclear condensation, morphological features suggestive of apoptotic cell death. Agarose gel electrophoresis revealed a ladder of 180–200-bp multiple DNA fragments in nonadherent (Fig. 4A, na) but not in adherent (Fig. 4A, ad) cells. Laddering reflects internucleo-

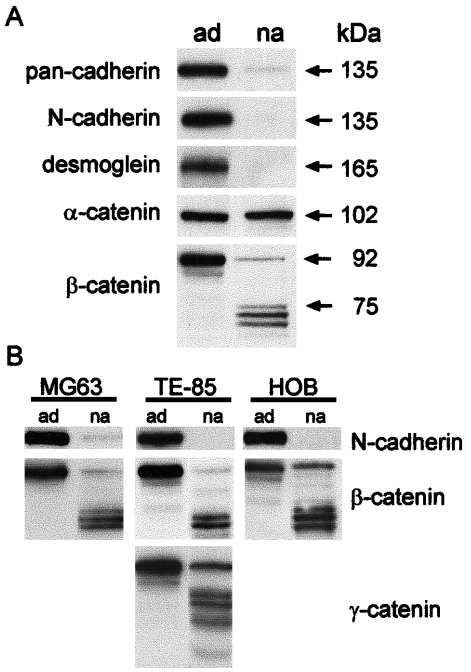

FIG. 5. Expression of cadherins and catenins in osteoblasts undergoing spontaneous and induced apoptosis. (A) Extracts of adherent (ad) and nonadherent (na) SaOS-2 cells were resolved by SDS-PAGE and immunoblotted with antibodies against cadherins and catenins. (B) Apoptosis was induced by treating MG-63 cells for 72 h with 100 μM genistein, TE-85 cells with 1 μM staurosporine for 24 h, and HOBs with 1 μg/ml actinomycin D for 24 h. Adherent (ad) and nonadherent (na) cell extracts were immunoblotted with antibodies raised against N-cadherin, β-catenin, and γ-catenin.

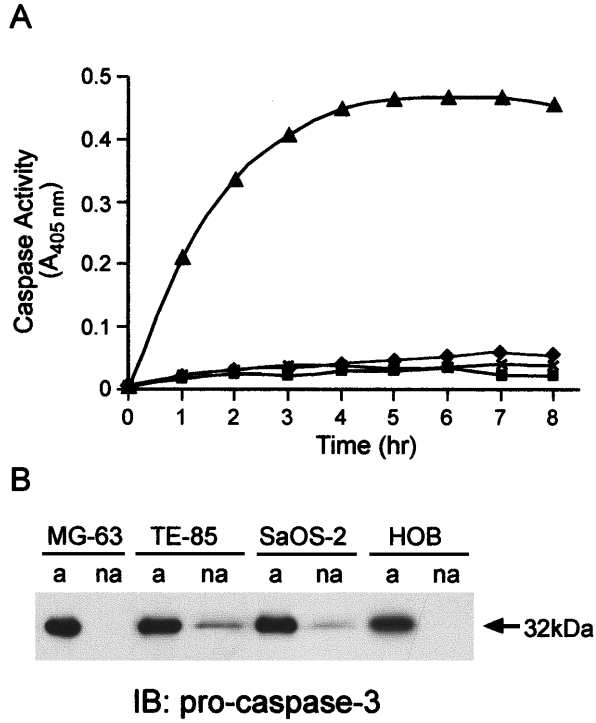

FIG. 6. Caspase-3 is activated in apoptotic osteoblasts. (A) Direct measurement of caspase-3 activity in extracts of adherent (ad) and nonadherent (na) SaOS-2 cells. Hydrolysis of the caspase-3 substrate Ac-DEVD-pNA was measured at 405 nm over a period of 8 h, in the absence and presence of the caspase-3 inhibitor Ac-DEVD-CHO at a concentration of 0.1 μM ($\times$, ad; ■, ad + inhib; ▲, na; ♦, na + inhib). (B) Osteoblast apoptosis was induced as described in .Fig. 5B. Extracts of adherent (ad) and nonadherent (na) cells were immunoblotted with an antibody recognizing the inactive proenzyme form of caspase-3. Activation of caspase-3 is measured by a decrease in the expression of the intact 32-kDa proenzyme.

somal DNA cleavage and is considered a hallmark of apoptosis.[2] Positive TUNEL staining of nonadherent (Fig. 4B, b) but not adherent (Fig. 4B, a) SaOS-2 cells confirmed that the detached cells had undergone apoptosis.

In apoptotic SaOS-2 cells, immunoblotting revealed that cellular cadherins, including N-cadherin, were significantly decreased in amount and that β-catenin was cleaved into three major polypeptides of 70- to 75-kDa and minor 85- to 90-kDa fragments (Fig. 5A). An antibody recognizing amino acids 768–781 of β-catenin failed to detect any of the cleavage products in apoptotic cells (data not shown), indicating that these fragments lack the COOH-terminal domain. Cleavage of γ-catenin also was apparent but was more clearly seen in TE-85 cells (Fig. 5B), which express higher levels of this protein. By contrast, there was no significant difference in the level of α-catenin in adherent and nonadherent cells (Fig. 5A). We also detected a dramatic reduction in the expression of desmogleins in nonadherent SaOS-2 cells (Fig. 5A). To determine if these effects were a common feature of osteoblast apoptosis, the expression of cadherins/catenins examined in osteoblasts was treated with genistein, staurosporine, or actinomycin D, all known inducers of apoptosis.[45–47] Consistent with the induction of apoptosis, osteoblasts detached from the culture dish by treatment with these reagents showed positive TUNEL staining (data not shown). Apoptosis in MG-63 and TE-85 cells and in HOBs resulted in the loss of N-cadherin and the cleavage of β-catenin into 70- to 75-kDa peptides (Fig. 5B), identical to those detected in apoptotic SaOS-2 cells. In TE-85 cells, which express high levels of γ-catenin (Fig. 1), major fragments of 75, 72, and 65 kDa and a minor fragment of about 55 kDa were detected in nonadherent but not adherent cells (Fig. 5B).

Caspase-dependent cleavage of cadherins/catenins in apoptotic osteoblasts

Because activation of the caspase family of cysteine proteases and their cleavage of key cellular substrates is a crucial step in the apoptotic process,[48] the presence of caspase-1 and caspase-3 activity in extracts of SaOS-2 cells was investigated by measuring the cleavage of their respective substrates Ac-YVAD-pNA and Ac-DEVD-pNA. Caspase-3 activity, barely detectable in adherent cells, was strongly activated in nonadherent, apoptotic cells (Fig. 6A), reaching a maximal activity 11-fold higher than basal levels. In the presence of the specific peptide inhibitor Ac-DEVD-CHO caspase-3 activity was reduced to basal levels (Fig.

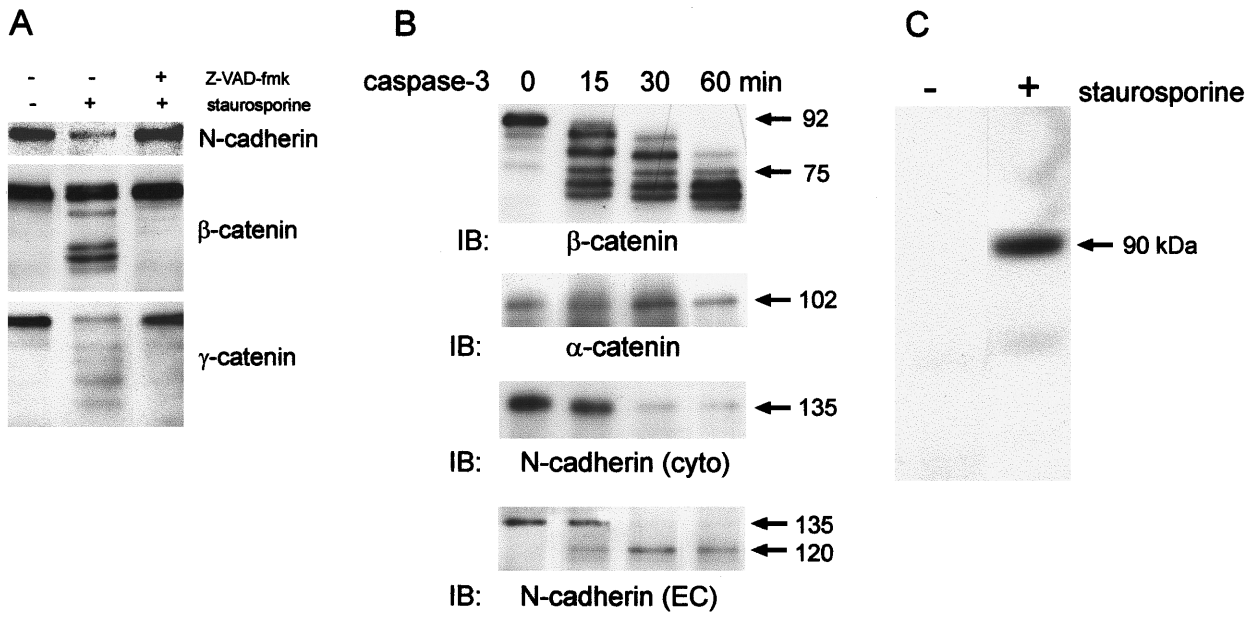

FIG. 7. N-cadherin and β- and γ-catenins but not α-catenin are cleaved by caspases. (A) TE-85 cells were grown without additions (−) or with (+) 1 μM staurosporine for 24 h to induce apoptosis, in the absence (−) or presence (+) of the caspase inhibitor Z-VAD-fmk at a concentration of 100 μM. Cell extracts from control and treated cells were resolved by SDS-PAGE and immunoblotted with antibodies against N-cadherin, β-catenin, and γ-catenin. (B) Extracts of untreated TE-85 cells were immunoprecipitated with an antibody to β-catenin and incubated with recombinant human caspase-3 at 37°C for 0–60 minutes. Cell extracts were fractionated by SDS-PAGE before immunoblotting with antibodies against β-catenin, α-catenin, and either the cytoplasmic (cyto) or the extracellular (EC) domains of N-cadherin. (C) Conditioned medium from TE-85 cells grown in the absence (−) or presence (+) of 1 μM staurosporine for 24 h was concentrated 10-fold and analyzed by immunoblotting with an antibody raised to the extracellular domain of N-cadherin.

6A). Caspase-1 was not activated in apoptotic osteoblasts (data not shown). Activation of caspase-3 in apoptotic cells was further confirmed by the disappearance of the inactive, 32-kDa proenzyme in nonadherent SaOS-2 cells undergoing spontaneous apoptosis (Fig. 6B) and in nonadherent MG-63 and TE-85 cells and in HOBs (Fig. 6B), induced to undergo apoptosis with staurosporine. The ability of the tripeptide caspase inhibitor Z-VAD-fmk to inhibit cleavage of both β- and γ-catenins and to abrogate the decrease in N-cadherin expression in staurosporine-treated TE-85 cells (Fig. 7A) strongly implicated activated caspases in the cleavage of these proteins.

To assess if cadherins/catenins were direct substrates for caspases, in the absence of endogenous cellular proteases, β-catenin was immunoprecipitated from untreated TE-85 cell extracts before incubation with recombinant human caspase-3. Initially, β-catenin was cleaved into several polypeptides ranging in size from 70 to 90 kDa (Fig. 7B). The higher molecular-mass species gradually disappeared with time and after 1 h, the predominant β-catenin fragments had a molecular mass of 70–75 kDa (Fig. 7B), identical to those found in apoptotic cells (Fig. 4). We have taken advantage of the fact that both N-cadherin and α-catenin coprecipitate with β-catenin (Fig. 3) to analyze the effect of caspase-3 on these proteins present in β-catenin immunoprecipitates. In agreement with observations in apoptotic cells (Fig. 4), α-catenin was not a substrate for caspase-3 in vitro (Fig. 7B), despite the presence of three potential caspase-3 cleavage sites. Using an antibody that recognizes the cytoplasmic domain of N-cadherin, caspase-3 incubation caused a dramatic decrease in the amount of intact N-cadherin (Fig. 7B), consistent with the pattern seen in apoptotic cells (Fig. 5). Immunoblotting with an antibody against the extracellular domain indicated that, in the presence of caspase-3, N-cadherin was reduced in apparent molecular mass from 135 to 120 kDa (Fig. 7B). A fragment of this size has been detected in some apoptotic cells, but its absence from others suggests that additional proteolysis may occur (data not shown). The recent demonstration that N-cadherin can be cleaved extracellularly during ovarian granuloma cell apoptosis[16] led us to examine whether a similar cleavage might occur during osteoblast apoptosis. Immunoblotting with an antibody against the extracellular domain of N-cadherin did indeed reveal the presence of a 90-kDa fragment in conditioned medium from staurosporine-treated but not control TE-85 cells, consistent with extracellular domain cleavage in apoptotic cells (Fig. 7C). Caspase-3 also was able to cleave immunoprecipitated γ-catenin, producing the 75-kDa and 65-kDa fragments, but the additional 72-kDa and 55-kDa fragments seen in apoptotic osteoblasts were not detected (data not shown).

Effect of caspase cleavage on cadherin/catenin interactions

In osteoblasts, we have shown the presence of two distinct complexes containing cadherin and α-catenin, together with either β- or γ-catenin (Fig. 3). Because the adhesive

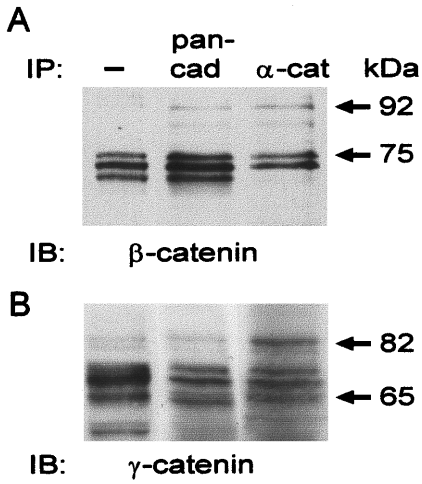

FIG. 8. Cadherin/catenin complex assembly in apoptotic osteoblasts. Extracts of apoptotic SaOS-2 cells were left untreated (−) or immunoprecipitated (IP) with antibodies recognizing pan-cadherin (pan-cad) or α-catenin (α-cat). After fractionation by SDS-PAGE, the composition of cadherin/catenin complexes was assessed by immunoblotting (IB) with antibodies against (A) β-catenin or (B) γ-catenin.

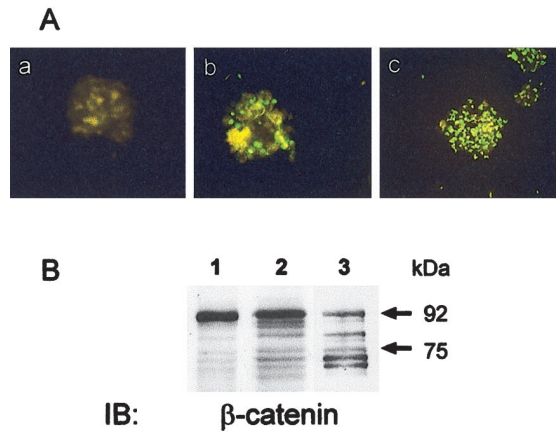

FIG. 9. Disruption of N-cadherin–mediated adhesion induces apoptosis. (A) HOBs were cultured on poly-HEMA–coated dishes in the presence of control Ig (a), a function-blocking antibody recognizing the extracellular domain of N-cadherin (b), or 5 mM EDTA (c) for 24 h. Cells were cytocentrifuged onto glass coverslips and subjected to TUNEL staining. (B) HOBs treated with control (lane 1), anti–N-cadherin (lane 2) or EDTA (lane 3) were extracted, resolved by SDS-PAGE, and immunoblotted with an antibody recognizing β-catenin.

function of cadherins is dependent on interactions with the intracellular catenins,[24–27,49] we have examined the effect of caspase cleavage on complex assembly. All three major β-catenin cleavage products present in apoptotic SaOS-2 cells retained the ability to interact with cadherins, whereas only the two higher-mass species were able to interact with α-catenin (Fig. 8A). Both cadherins and α-catenin interacted with the major cleavage products of γ-catenin, although they interacted poorly with the 55-kDa fragment (Fig. 8B). An increase in the relative amount of intact γ-catenin coprecipitated with α-catenin (Fig. 8B) may be indicative of a stronger interaction between the uncleaved forms of these proteins. Taken together, caspase-dependent cleavage of β- and γ-catenins appears to have little effect on their ability to form adhesion complexes, although the inability of the smallest β-catenin fragment to associate with α-catenin may lead to a decrease in adhesion.

Disruption of N-cadherin–mediated adhesion induces osteoblast apoptosis

During osteoblast apoptosis, the detachment of cells not only from each other but from the culture dish indicates that both cell-cell and cell-matrix interactions are disrupted. Because loss of cell-matrix adhesion has been shown to lead to apoptosis,[4] we have attempted to distinguish between these adhesive processes by culturing HOBs on dishes coated with poly-HEMA, a nonionic polymer, which prevents matrix deposition, cell attachment, and spreading.[4,50]

HOBs, grown on poly-HEMA–coated dishes in the presence of control antibody formed large, compact multicellular aggregates, with no detectable apoptosis as judged by the absence of TUNEL staining (Fig. 9A, a). In contrast, when cultured in the presence of a function-blocking N-cadherin antibody, HOBs formed aggregates that were only loosely associated and contained many apoptotic, TUNEL-positive cells (Fig. 9A, b). The inability of anti–N-cadherin to disrupt completely aggregate formation, strongly indicates that cadherins-4, -6, and -11, present in HOBs, also contribute to cell-cell adhesion. Cadherin-mediated cell adhesion is a Ca^{2+}-dependent process,[19] and in the presence of the calcium chelator, EDTA cells failed to form cell-cell contacts and showed extensive TUNEL-positive staining (Fig. 9A, c).

As a consequence of disrupting N-cadherin–mediated cell-cell adhesion, β-catenin was cleaved (Fig. 9B), giving rise to a number of lower molecular mass species, including the 70- to 75-kDa polypeptides characteristic of apoptotic osteoblasts (Fig. 5) and generated by caspase-3 activity in vitro (Fig. 7). In cells treated with EDTA, in which cell-cell adhesion was almost completely disrupted, β-catenin was cleaved more extensively to the 70- to 75-kDa fragments (Fig. 9B). Taken together, these results strongly suggest that disruption of N-cadherin–mediated cell-cell adhesion in osteoblasts leads to the activation of caspase-3, cleavage of β-catenin, and induction of apoptosis in human osteoblasts.

DISCUSSION

Although recent data[1] indicate that the majority of osteoblasts are destined to die by apoptosis, the molecular events underlying and regulating osteoblast-programmed cell death remain to be elucidated. In the present study, we have identified proteins associated with the intercellular adherens junctions as molecular targets during osteoblast apoptosis and provide evidence that cadherin-mediated cell-cell adhesion acts to inhibit the apoptotic process.

While studying the expression and organization of adherens junction proteins in osteoblasts, we detected a signifi-

cant reduction in the expression of classical cadherins, including N-cadherin, and the cleavage of β- and γ- but not α-catenin in MG-63, TE-85, and SaOS-2 osteoblastic cells as well as in primary osteoblasts (HOBs) undergoing apoptosis. The ability of the tripeptide caspase inhibitor Z-VAD-fmk to suppress cleavage of β- and γ-catenins and to abrogate the decrease in N-cadherin expression, suggested that the caspase family of cysteine proteases, activated during apoptosis,[48] may play an important role in the cleavage of these proteins. In support of this notion, caspase-3, a major effector caspase responsible for the cleavage of a number of key cellular proteins during apoptosis,[48] was activated in both apoptotic osteoblastic cell lines and HOBs. Cleavage of N-cadherin and β- and γ-catenins, but not α-catenin, by caspase-3 in vitro to generate fragments identical to those seen in apoptotic cells further confirmed the central role of caspase-3 in the cleavage of adherens junction proteins during osteoblast apoptosis.

Cleavage of β-catenin has been reported in apoptotic endothelial cells[10] and could be reproduced by the addition of caspase-3 to endothelial cell extracts but not to immunoprecipitated β-catenin. The authors' conclusion that complete cleavage of β-catenin is dependent on caspase-3–dependent activation of additional cellular proteases[10] contrasts sharply with our results, which clearly show that caspase-3 activity alone is sufficient to generate the β-catenin cleavage pattern found in apoptotic cells. In support of this conclusion, in vitro translated β-catenin has been shown to be cleaved efficiently by caspase-3 to give a pattern resembling that in apoptotic cells.[51]

Caspase-3–dependent cleavage of immunoprecipitated γ-catenin generated the major 75-kDa and 65-kDa polypeptides seen in apoptotic cells but failed to generate the 72-kDa and 55-kDa fragments. Caspase-3–dependent cleavage of either immunoprecipitated[10] or in vitro translated[52] γ-catenin also has failed to generate the pattern of fragments produced during apoptosis. Thus, although γ-catenin can clearly act as caspase-3 substrate, it remains to be shown whether additional proteases are involved in its cleavage in vivo.

In epithelial cells, both β- and γ-catenins interact directly with the cytoplasmic domain of the cadherins and with α-catenin to form a supramolecular complex that is regarded as the functional unit of cell adhesion.[24–27,49] In the present study, the identification in osteoblasts of two distinct complexes containing cadherin and α-catenin with either β- or γ-catenin allowed us to assess the impact of apoptosis on complex assembly. Cleavage of β- and γ-catenins during apoptosis had little effect on their ability to interact with the cadherins. The major cleavage products of γ-catenin retained the ability to interact with α-catenin as did the two higher molecular mass cleavage products of β-catenin. The 55-kDa fragment of γ-catenin showed reduced binding to α-catenin, and there was no detectable interaction between the 70-kDa fragment of β-catenin and α-catenin. Because the association of the cadherins with α-catenin and the actin cytoskeleton is essential for their adhesive function, a reduction in α-catenin binding by cleaved β- and γ-catenins may result in reduced cell-cell adhesion. However, taken together, our results indicate that protein-protein interactions required for adhesion complex assembly remain essentially intact in apoptotic osteoblasts.

In apoptotic osteoblasts, we have detected a decrease in the expression of cadherins, which we now report appears, at least in part, to be the result of caspase-dependent cleavage. N-cadherin was cleaved by caspase-3 in vitro, to give a polypeptide of 120 kDa, recognized by an antibody against the extracellular domain but not by an antibody against the cytoplasmic domain of the protein. Of the three caspase-3 consensus cleavage sites identified within the cytoplasmic domain of N-cadherin,[52] cleavage at the most COOH-terminal site (^{830}Asp-Ile-Gly-Asp^{833}) is inconsistent with the size of the cleaved fragment. However, cleavage at either of the two immediately adjacent, membrane proximal sites (^{760}Asp-Pro-Glu-Asp^{763} and ^{764}Asp-Val-Arg-Asp^{767}) would remove 138–142 amino acids and cause a reduction in the apparent molecular mass of N-cadherin (135 kDa) of about 15 kDa, consistent with the 120-kDa fragment detected after caspase-3 treatment. A fragment of this size has been detected in apoptotic osteoblasts, although its absence from some extracts may indicate subsequent cleavage by caspases or other proteases. Interestingly, by comparing the sequences of many different classical cadherins, we have found that one of the two membrane proximal sites (^{764}Asp-Val-Arg-Asp^{767}) is conserved in type I but not type II proteins, suggesting that it may define a function that differs between the two types of cadherins.

We are aware of only one previous report implicating caspases in the cleavage of cadherins. In that study,[53] a role for caspases was implied from the ability of caspase inhibitors to prevent cleavage of E- and P-cadherins in apoptotic cells, but direct cleavage of cadherins was not shown. The major cleavage product of P-cadherin in apoptotic cells had an apparent molecular mass of 104 kDa, consistent with cleavage at the site identified by us to be conserved in type I cadherins. In apoptotic cells, E-cadherin was cleaved to a 48-kDa product, which was suggested to be the result of caspase cleavage at amino acids 476–479.[53] However, this sequence, which forms part of a putative Ca^{2+}-binding site,[54] is located within the extracellular domain of E-cadherin, while the caspases are intracellular enzymes.

In addition to cleavage of the intracellular domain, the presence of a 90-kDa N-cadherin fragment in conditioned medium from apoptotic but not control cells indicated that N-cadherin also could be cleaved extracellularly. Although the nature of the proteases responsible for extracellular domain cleavage remain to be identified, surface-associated metalloproteinases have been implicated in the cleavage of N-cadherin during retinal development[55] and ovarian granuloma cell apoptosis[16] and in the release of a surface-associated fragment of VE-cadherin during endothelial apoptosis.[10]

Recent studies have provided evidence that cell-cell interactions may be one mechanism by which cells can regulate apoptosis.[6,7] In the present study, the induction of apoptosis in HOBs by a function-blocking N-cadherin antibody, but not by an isotype-matched control, indicated that N-cadherin–mediated cell-cell adhesion supports osteoblast survival. N-cadherin also has been linked recently to the

survival of ovarian granulosa cells,[15,16] and an association between E-cadherin–mediated aggregation and survival of squamous epithelial cells has been reported.[17]

The mechanisms whereby cadherin-mediated cell-cell adhesion generates cell survival signals remain poorly understood. N-cadherin–mediated adhesion of ovarian granulosa cells has been linked to tyrosine phosphorylation of the fibroblast growth factor (FGF) receptor,[56] while E-cadherin–dependent aggregation of epithelial cells results in activation of the retinoblastoma protein and G1 cell cycle arrest.[57] In the present study, apoptosis induced by the disruption of N-cadherin–mediated intercellular adhesion resulted in the cleavage of β-catenin to give a pattern indicative of caspase-3 activation. Because caspase-dependent cleavage of β-catenin has a limited effect on its ability to interact with its cellular binding partners, we think it unlikely that cleavage of this molecule plays a major role in the direct dismantling of cell-cell contacts during osteoblast apoptosis. However, in addition to a structural role in adherens junctions, β-catenin is an important transducer of cellular signals and can translocate to the nucleus, where it interacts with the LEF/TCF family of transcription factors to regulate gene expression.[28–32] We suggest, therefore, that β-catenin is involved in antiapoptotic signaling and that this is disrupted by caspase-dependent cleavage during osteoblast apoptosis. The ability of the cleavage products of β-catenin to interact with cadherins indicates that the LEF/TCF binding site is also intact, because these transcription factors share a large, overlapping binding domain with the cadherins in the center of β-catenin.[58] Within the LEF/TCF–β-catenin complex, the LEF/TCF transcription factors provide a DNA binding domain, while the transactivation function resides within the COOH-terminal domain of β-catenin.[32] In apoptotic osteoblasts, cleavage of β-catenin resulted in the loss of the COOH-terminal domain, as judged by the inability of an antibody to amino acids 768–781 of β-catenin to recognize the cleaved protein. Taken together, our results indicate that although cleaved β-catenin would retain the ability to interact with LEF/TCF, transcriptional activation and thus antiapoptotic signaling would be compromised. Although additional studies will be required to validate this mechanism, the recent demonstration that deletion constructs of β-catenin, resembling the proteolytic fragments seen in apoptotic cells, have strongly reduced transcriptional activity[59] is consistent with our hypothesis.

The factors responsible for disruption of N-cadherin–mediated adhesion during bone remodeling remain to be elucidated. However, the cytokines tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1), present in the bone microenvironment and recognized as powerful stimulants of bone resorption and inhibitors of bone formation,[60] recently have been shown to down-regulate the expression of N-cadherin in the mouse osteoblastic cell line MC3T3-E1.[61] Thus, in conditions where the levels of these cytokines are increased, such as during postmenopausal osteoporosis,[60] disruption of N-cadherin–mediated cell-cell adhesion and induction of osteoblast apoptosis may be one mechanism whereby access of osteoclasts to the bone surface and bone resorption is facilitated. That cadherin-mediated adhesion may play a role in promoting bone formation is further suggested by the observation that parathyroid hormone (PTH), which increases bone formation by preventing osteoblast apoptosis,[62] also increases the expression of cadherins in osteoblasts and osteoprogenitor cells.[63,64]

In summary, we have identified N-cadherin–mediated cell-cell adhesion as a molecular mechanism for suppressing apoptosis in human osteoblasts, which we suggest is mediated by a β-catenin signaling pathway.

ACKNOWLEDGMENTS

We are indebted to the Ministry of Agriculture, Fisheries and Food and to the Scottish Executive Rural Affairs Department for support.

REFERENCES

1. Jilka RL, Weinstein RS, Bellido T, Parfitt AM, Manolagas SC 1998 Osteoblast programmed cell death (apoptosis): Modulation by growth factors and cytokines. J Bone Miner Res **13:**793–802.
2. Wyllie AH, Kerr JF, Currie AR 1980 Cell death: The significance of apoptosis. Int Rev Cytol **68:**251–306.
3. Collins MK, Lopez Rivas A 1993 The control of apoptosis in mammalian cells. Trends Biochem Sci **18:**307–309.
4. Frisch SM, Francis H 1994 Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol **124:**619–626.
5. Re F, Zanetti A, Sironi M, Polentarutti N, Lanfrancone L, Dejana E, Colotta F 1994 Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. J Cell Biol **127:**537–546.
6. Bates RC, Buret A, van Helden DF, Horton MA, Burns GF 1994 Apoptosis induced by inhibition of intercellular contact. J Cell Biol **125:**403–415.
7. Hermiston ML, Gordon JI 1995 In vivo analysis of cadherin function in the mouse intestinal epithelium: Essential roles in adhesion, maintenance of differentiation, and regulation of programmed cell death. J Cell Biol **129:**489–506.
8. Potter SW, Gaza G, Morris JE 1996 Estradiol induces E-cadherin degradation in mouse uterine epithelium during the estrous cycle and early pregnancy. J Cell Physiol **169:**1–14.
9. Brancolini C, Lazarevic D, Rodriguez J, Schneider C 1997 Dismantling cell-cell contacts during apoptosis is coupled to a caspase-dependent proteolytic cleavage of beta-catenin. J Cell Biol **139:**759–771.
10. Herren B, Levkau B, Raines EW, Ross R 1998 Cleavage of beta-catenin and plakoglobin and shedding of VE-cadherin during endothelial apoptosis: Evidence for a role for caspases and metalloproteinases. Mol Biol Cell **9:**1589–1601.
11. Morimoto Y, Ohba T, Kobayashi S, Haneji T 1997 The protein phosphatase inhibitors okadaic acid and calyculin A induce apoptosis in human osteoblastic cells. Exp Cell Res **230:**181–186.
12. Kawakami A, Eguchi K, Matsuoka N, Tsuboi M, Koji T, Urayama S, Fujiyama K, Kiriyama T, Nakashima T, Nakane PK, Nagataki S 1997 Fas and Fas ligand interaction is necessary for human osteoblast apoptosis. J Bone Miner Res **12:** 1637–1646.
13. Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY 1996 Control of adhesion-dependent cell survival by focal adhesion kinase. J Cell Biol **134:**793–799.
14. Hungerford JE, Compton MT, Matter ML, Hoffstrom BG, Otey CA 1996 Inhibition of pp125FAK in cultured fibroblasts results in apoptosis. J Cell Biol **135:**1383–1390.

15. Peluso JJ, Pappalardo A, Trolice MP 1996 N-cadherin–mediated cell contact inhibits granulosa cell apoptosis in a progesterone-independent manner. Endocrinology **137:**1196–1203.
16. Makrigiannakis A, Coukos G, Christofidou-Solomidou M, Gour BJ, Radice GL, Blaschuk O, Coutifaris C 1999 N-cadherin–mediated human granulosa cell adhesion prevents apoptosis: A role in follicular atresia and luteolysis? Am J Pathol **154:**1391–1406.
17. Kantak SS, Kramer RH 1998 E-cadherin regulates anchorage-independent growth and survival in oral squamous cell carcinoma cells. J Biol Chem **273:**16953–16961.
18. Globus RK, Doty SB, Lull JC, Holmuhamedov E, Humphries MJ, Damsky CH 1998 Fibronectin is a survival factor for differentiated osteoblasts. J Cell Sci **111:**1385–1393.
19. Takeichi M 1991 Cadherin cell adhesion receptors as a morphogenetic regulator. Science **251:**1451–1455.
20. Kemler R 1993 From cadherins to catenins: Cytoplasmic protein interactions and regulation of cell adhesion. Trends Genet **9:**317–321.
21. Nose A, Tsuji K, Takeichi M 1990 Localization of specificity determining sites in cadherin cell adhesion molecules. Cell **61:**147–155.
22. Ozawa M, Baribault H, Kemler R 1989 The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. EMBO J **8:**1711–1717.
23. Ozawa M, Kemler R 1992 Molecular organization of the uvomorulin-catenin complex. J Cell Biol **116:**989–996.
24. Aberle H, Butz S, Stappert J, Weissig H, Kemler R, Hoschuetzky H 1994 Assembly of the cadherin-catenin complex in vitro with recombinant proteins. J Cell Sci **107:**3655–3663.
25. Hinck L, Nathke IS, Papkoff J, Nelson WJ 1994 Dynamics of cadherin/catenin complex formation: Novel protein interactions and pathways of complex assembly. J Cell Biol **125:**1327–40.
26. Jou TS, Stewart DB, Stappert J, Nelson WJ, Marrs JA 1995 Genetic and biochemical dissection of protein linkages in the cadherin-catenin complex. Proc Natl Acad Sci USA **92:**5067–5071.
27. Rimm DL, Koslov ER, Kebriaei P, Cianci CD, Morrow JS 1995 Alpha 1(E)-catenin is an actin-binding and -bundling protein mediating the attachment of F-actin to the membrane adhesion complex. Proc Natl Acad Sci USA **92:**8813–8817.
28. Molenaar M, van de Wetering M, Oosterwegel M, Peterson-Maduro J, Godsave S, Korinek V, Roose J, Destree O, Clevers H 1996 XTcf-3 transcription factor mediates beta-catenin–induced axis formation in *Xenopus* embryos. Cell **86:**391–399.
29. Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, Grosschedl R, Birchmeier W 1996 Functional interaction of beta-catenin with the transcription factor LEF-1. Nature **382:**638–42.
30. Berens ME, Rief MD, Shapiro JR, Haskett D, Giese A, Joy A, Coons SW 1996 Proliferation and motility responses of primary and recurrent gliomas related to changes in epidermal growth factor receptor expression. J Neurooncol **27:**11–22.
31. Huber O, Korn R, McLaughlin J, Ohsugi M, Herrmann BG, Kemler R 1996 Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. Mech Dev **59:**3–10.
32. van de Wetering M, Cavallo R, Dooijes D, van Beest M, van Es J, Loureiro J, Ypma A, Hursh D, Jones T, Bejsovec A, Peifer M, Mortin M, Clevers H 1997 Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. Cell **88:**789–799.
33. Okazaki M, Takeshita S, Kawai S, Kikuno R, Tsujimura A, Kudo A, Amann E 2098 1994 Molecular cloning and characterization of OB-cadherin, a new member of cadherin family expressed in osteoblasts. J Biol Chem **269:**12092–12098.
34. Cheng SL, Lecanda F, Davidson MK, Warlow PM, Zhang SF, Zhang L, Suzuki S, St John T, Civitelli R 1998 Human osteoblasts express a repertoire of cadherins, which are critical for BMP-2-induced osteogenic differentiation. J Bone Miner Res **13:**633–644.
35. Ferrari SL, Traianedes K, Thorne M, Lafage-Proust MH, Genever P, Cecchini MG, Behar V, Bisello A, Chorev M, Rosenblatt M, Suva LJ 2000 A role for N-cadherin in the development of the differentiated osteoblastic phenotype. J Bone Miner Res **15:**198–208.
36. Scheven BA, van der Veen MJ, Damen CA, Lafeber FP, Van Rijn HJ, Bijlsma JW, Duursma SA 1995 Effects of methotrexate on human osteoblasts in vitro: Modulation by 1,25-dihydroxyvitamin D3. J Bone Miner Res **10:**874–80.
37. Pytela R, Suzuki S, Breuss J, Erle DJ, Sheppard D 1994 Polymerase chain reaction cloning with degenerate primers: Homology-based identification of adhesion molecules. Methods Enzymol **245:**420–451.
38. Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
39. Mbalaviele G, Nishimura R, Myoi A, Niewolna M, Reddy SV, Chen D, Feng J, Roodman D, Mundy GR, Yoneda T 1998 Cadherin-6 mediates the heterotypic interactions between the hemopoietic osteoclast cell lineage and stromal cells in a murine model of osteoclast differentiation. J Cell Biol **141:**1467–1476.
40. Koch PJ, Franke WW 1994 Desmosomal cadherins: Another growing multigene family of adhesion molecules. Curr Opin Cell Biol **6:**682–687.
41. Garrod D, Chidgey M, North A 1996 Desmosomes: Differentiation, development, dynamics and disease. Curr Opin Cell Biol **8:**670–678.
42. Schafer S, Koch PJ, Franke WW 1994 Identification of the ubiquitous human desmoglein, Dsg2, and the expression catalogue of the desmoglein subfamily of desmosomal cadherins. Exp Cell Res **211:**391–399.
43. Mathur M, Goodwin L, Cowin P 1994 Interactions of the cytoplasmic domain of the desmosomal cadherin Dsg1 with plakoglobin. J Biol Chem **269:**14075–14080.
44. Witcher LL, Collins R, Puttagunta S, Mechanic SE, Munson M, Gumbiner B, Cowin P 1996 Desmosomal cadherin binding domains of plakoglobin. J Biol Chem **271:**10904–10909.
45. Naora H, Nishida T, Shindo Y, Adachi M, Naora H 1996 Constitutively enhanced nbl expression is associated with the induction of internucleosomal DNA cleavage by actinomycin D. Biochem Biophys Res Commun **224:**258–264.
46. Janicke RU, Sprengart ML, Wati MR, Porter AG 1998 Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J Biol Chem **273:**9357–9360.
47. Li Y, Upadhyay S, Bhuiyan M, Sarkar FH 1999 Induction of apoptosis in breast cancer cells MDA-MB-231 by genistein. Oncogene **18:**3166–3172.
48. Thornberry NA, Lazebnik Y 1998 Caspases: Enemies within. Science **281:**1312–1316.
49. Hinck L, Nathke IS, Papkoff J, Nelson WJ 1994 Beta-catenin: A common target for the regulation of cell adhesion by Wnt-1 and Src signaling pathways. Trends Biochem Sci **19:**538–542.
50. Folkman J, Moscona A 1978 Role of cell shape in growth control. Nature **273:**345–349.
51. Brancolini C, Sgorbissa A, Schneider C 1998 Proteolytic processing of the adherens junction components beta-catenin and gamma-catenin/plakoglobin during apoptosis. Cell Death Differ **5:**1042–1050.
52. Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT, Nicholson DW 1997 A combinatorial approach defines specificities of members of the

caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem **272:** 17907–17911.
53. Schmeiser K, Grand RJ 1999 The fate of E- and P-cadherin during the early stages of apoptosis. Cell Death Differ **6:**377–386.
54. Berndorff D, Gessner R, Kreft B, Schnoy N, Lajous-Petter AM, Loch N, Reutter W, Hortsch M, Tauber R 1994 Liver-intestine cadherin: Molecular cloning and characterization of a novel Ca(2+)-dependent cell adhesion molecule expressed in liver and intestine. J Cell Biol **125:**1353–1369.
55. Paradies NE, Grunwald GB 1993 Purification and characterization of NCAD90, a soluble endogenous form of N-cadherin, which is generated by proteolysis during retinal development and retains adhesive and neurite-promoting function. J Neurosci Res **36:**33–45.
56. Trolice MP, Pappalardo A, Peluso JJ 1997 Basic fibroblast growth factor and N-cadherin maintain rat granulosa cell and ovarian surface epithelial cell viability by stimulating the tyrosine phosphorylation of the fibroblast growth factor receptors. Endocrinology **138:**107–113.
57. Day ML, Zhao X, Vallorosi CJ, Putzi M, Powell CT, Lin C, Day KC 1999 E-cadherin mediates aggregation-dependent survival of prostate and mammary epithelial cells through the retinoblastoma cell cycle control pathway. J Biol Chem **274:** 9656–9664.
58. Huber O, Bierkamp C, Kemler R 1996 Cadherins and catenins in development. Curr Opin Cell Biol **8:**685–691.
59. Steinhusen U, Badock V, Bauer A, Beherens J, Wittman-Leibold B, Dorken B, Bommer K 2000 Apoptosis-induced cleavage of β-catenin by caspase-3 results in proteolytic fragments with reduced transactivation potential. J Biol Chem **275:**16345–16353.
60. Pacifici R 1996 Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis. J Bone Miner Res **11:**1043–1051.
61. Tsutsumimoto T, Kawasaki S, Ebara S, Takaoka K 1999 TNF-(alpha) and IL-1(beta) suppress N-cadherin expression in MC3T3–E1 cells. J Bone Miner Res **14:**1751–1760.
62. Jilka RL, Weinstein RS, Bellido T, Robertson P, Parfitt AM, Manolagas SC 1999 Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone. J Clin Invest **104:**439–446.
63. Babich M, Foti LR 1994 E-cadherins identified in osteoblastic cells: Effects of PTH and extracellular calcium on localization. Life Sci **54:**PL201–PL208.
64. Liu P, Oyajobi BO, Croucher P, Stringer B, Russell RGG, Scutt A 1997 Regulation of cadherin expression in human osteoprogenitor cells by osteotropic hormones and growth factors. Bone **20:**73S (abstract).

Address reprint requests to:
Irene Hunter
Department of Biomedical Sciences
Institute of Medical Sciences
University of Aberdeen
Foresterhill
Aberdeen AB25 2ZD, Scotland

Received in original form June 8, 2000; in revised form September 21, 2000; accepted October 30, 2000.