Annexin V/β5 Integrin Interactions Regulate Apoptosis of Growth Plate Chondrocytes*

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Apoptosis of terminally differentiated chondrocytes allows the replacement of growth plate cartilage by bone. Despite its importance, little is known about the regulation of chondrocyte apoptosis. We show that overexpression of annexin V, which binds to the cytoplasmic domain of β5 integrin and protein kinase C α (PKCα), stimulates apoptotic events in hypertrophic growth plate chondrocytes. To determine whether the balance between the interactions of annexin V/β5 integrin and annexin V/active PKCα play a role in the regulation of terminally differentiated growth plate chondrocyte apoptosis, a peptide mimic of annexin V (Penetratin (Pen)-VVISYSMPD) that binds to β5 integrin but not to PKCα was used. This peptide stimulated apoptotic events in growth plate chondrocytes. Suppression of annexin V expression using small interfering ribonucleic acid (siRNA) and by increasing the apoptosis rate of terminally differentiated growth plate chondrocytes occurs in chondrocytes at the chondro-osseous junction in chicken growth plate and sternal cartilage (1, 5). Furthermore, mice with targeted disruptions of both alleles for the antiapoptotic protein bcl-2 have short limbs and accelerated ossification of their growth plates (6). In addition, two chondrodysplastic conditions (parathyroid hormone-related peptide knock-out mice and activating mutations of the fibroblast growth factor receptor-3 (FGFR-3)) are associated with increased apoptosis of the growth plate chondrocytes (7, 8).

The various differentiation events of growth plate chondrocytes, including apoptosis, are precisely regulated to allow coordinated longitudinal bone growth. A disturbance in the regulation of these events leads to growth retardation. For example, glucocorticoid treatment results in growth retardation by decreasing the proliferation rate of growth plate chondrocytes and by increasing the apoptosis rate of terminally differentiated growth plate chondrocytes (9, 10). Therefore, the understanding of the mechanisms regulating the various differentiation events is highly relevant. However, very little is known about the regulation of apoptosis of growth plate chondrocytes. Recent studies have shown that annexin V, a cytosolic protein that binds to membranes in the presence of calcium, binds to the cytoplasmic domain of β5 integrin and to active protein kinase C (PKCα)2 and that these interactions play a key role in the regulation of apoptosis of endothelial cells (11, 12). Interestingly, annexin V and β5 integrin are expressed in hypertrophic and terminally differentiated growth plate chondrocytes (13, 14). Therefore, we hypothesized that the interactions among annexin V, β5 integrin, and PKCα play a role in the regulation of apoptosis of growth plate chondrocytes. To address this hypothesis, we used a peptide mimic of annexin V, which has been shown to bind to β5 integrin and induce apoptosis in endothelial cells (11), overexpression of annexin V using a retroviral expression vector, and suppression of annexin V using small interfering RNA (siRNA), and determined cell viability, bcl-2 and bax expression, and caspase-3 activity.

During endochondral ossification, the bone structures are first cartilaginous. Chondrocytes in these growth plate cartilages undergo a series of differentiation events, including proliferation, hypertrophy, and terminal differentiation, eventually leading to the replacement of mineralized cartilage by bone. Evolving in vitro and in vivo evidence shows that the final fate of terminally differentiated growth plate chondrocytes is apoptosis (programmed cell death) (1, 2). We have demonstrated that retinoic acid treatment of growth plate chondrocytes stimulates terminal differentiation events and eventually leads to apoptosis of these cells (3, 4). In addition, several in vivo studies have also revealed that apoptosis is the final fate of terminally differentiated growth plate chondrocytes. For example, we and others have demonstrated that apoptosis of growth plate chondrocytes occurs in chondrocytes at the chondro-osseous junction in chicken growth plate and sternal cartilage (1, 5).

5 Integrin Interactions Regulate Apoptosis

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Annexin V/β5 Integrin and Chondrocyte Apoptosis

EXPERIMENTAL PROCEDURES

Reagents—The preparation and specificity of antibodies specific for annexin V were described previously (13). Antibodies specific for β5 integrin subunit were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Penetratin (Pen) peptides (Pen-SDNYRIGSW and Pen-VIVISMPD) were purchased from Global Peptide Services (Fort Collins, CO). Calphostin C, myristoylated (Myr)-PKCa/β, and phorbol 12-myristate 13-acetate (PMA) were purchased from EMD Biosciences/Calbiochem. Myr-PKCζ was purchased from BIOSOURCE (Camarillo, CA).

Chondrocyte Culture—Chondrocytes were isolated from the hypertrophic zone of day 19 embryonic chick tibia growth plate cartilage as described previously (15). Cells were grown in monolayer cultures in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 5% fetal calf serum (HyClone, Logan, UT), 2 mM l-glutamine (Invitrogen), and 50 units/ml penicillin and streptomycin (Invitrogen) (complete medium). After 3 days, cells were incubated with high titer retroviral stocks of replication-competent, non-transforming Rous sarcoma virus-based expression vector (RCAS-BP) or RCAS-BP containing full-length annexin V cDNA in a small volume (5 × 10⁶ colony-forming units/10⁶ cells in less than 1 ml of medium) for 4 h. Thereafter, cells were cultured in complete medium until ~90% of chondrocytes were infected. The degree of overexpression was detected by immunoblotting using antibodies specific for annexin V (16). For siRNA experiments, growth plate chondrocytes were transfected with 200 nM siRNA specific for annexin V using Lipofectamine 2000 transfection reagent according to the manufacturer’s protocol (Invitrogen) (16). After transfection, cells were cultured in complete medium in the absence or presence of peptides and/or calphostin C (10 nM), PMA (3 μM), Myr-PKCα/β (100 μM), or Myr-PKCζ (100 μM).

Construction and Production of Chicken Retrovirus RCAS-BP—Full-length annexin V cDNA was first cloned into an adaptor vector SLAX-myc, which contained a 10-amino acid epitope of human c-Myc tag fused to the carboxyl-terminal end of the recombinant protein and then subcloned into RCAS-BP (17). To obtain viral stocks, the plasmid constructs and RCAS-BP containing no insert were used to transfect chicken embryonic fibroblasts using the Lipofectamine 2000 transfection reagent as described previously (16).

Construction of siRNA to Silence Annexin V Expression in Growth Plate Chondrocytes—We used the Silencer siRNA construction kit from Ambion, Inc. (Austin, TX) to synthesize siRNA. Four pairs of oligonucleotides encoding the desired sense and antisense siRNA strands were designed according to the chicken annexin V sequence by using a computer program (Ambion Inc.) Oligonucleotides were designed to include an 8-base sequence complementary to the 5’ end of T7 promoter primer included in the kit. The procedure was performed as described previously (16). The different siRNAs were tested for the efficiency to suppress annexin V protein expression in 10-day embryonic chicken dorsal fibroblasts to select the most effective siRNAs for transfection of growth plate chondrocytes. The sequences of the most efficient oligonucleotides were: anti-sense, 5’-AAGCATGCAATCAAGGGAGCACCTGTCTC-3’, and sense, 5’-AATGCTCCCTTGTATTGATCGACCTGTCTC-3’. Double Immunofluorescence and Peptide Internalization and Visualization—Cells were incubated with 10 μg/ml biotinylated peptides. After 2 h, the cultures were washed with phosphate-buffered saline, fixed, permeabilized with ethanol/acetic acid (9:1, v/v) for 5 min at −20 °C, blocked for 30 min with goat serum, and incubated with fluorescein isothiocyanate-conjugated streptavidin for 1 h. After washing, cells were analyzed by fluorescence microscopy (Nikon). Cell nuclei were counter-stained with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI). For double immunostaining for β5 integrin and annexin V, cells were washed, fixed with ethanol, and incubated with primary mouse monoclonal antibodies specific for β5 integrin and rabbit polyclonal antibodies specific for annexin V. After washing, cells were incubated with Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies (Invitrogen/Molecular Probes) and analyzed by inverted fluorescence microscopy (Nikon). For double immunostaining of β5 integrin and 5 integrin subunit was expressed in hypertrophic zone of growth plate cartilage (Fig. 1). Double immuno-
Annexin V/β5 Integrin and Chondrocyte Apoptosis

A

β5 Integrin  Type X  Merged  Annexin V

Prolif.

Hyp.

B

beta5  AnV  Merged  Phase

FIGURE 1. Immunostaining of sections from day 19 embryonic chicken growth plate cartilage with antibodies specific for type X collagen, β5 integrin, and annexin V and growth plate chondrocytes in culture with antibodies specific for annexin V (AnV) and β5 (beta5) integrin. A, sections of day 19 embryonic chicken growth plate cartilage were double-stained with mouse anti-β5 integrin and rabbit anti-type X collagen immunoglobulin G and then by Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies. Sections were also stained with antibodies specific for annexin V. Note that positive immunostaining for β5 integrin (green) and type X collagen (red) was co-detected in the hypertrophic (Hyp.) but not in the proliferative (Prolif.) zone of growth plate cartilage. Annexin V was also detected in the hypertrophic zone but not in the proliferative zone. B, growth plate chondrocytes isolated from the hypertrophic zone of day 19 embryonic chicken growth plate cartilage were double-stained with rabbit anti-annexin V and mouse anti-β5 integrin and then by Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies. Note the co-localization of annexin V (green) and β5 integrin (red) in growth plate chondrocytes (Merged). Bar, 100 μm.

cartilage showed no staining for type X collagen and β5 integrin (Fig. 1A). Type X collagen staining was localized in the extracellular matrix, whereas β5 integrin staining was cellular. Annexin V is also expressed in the hypertrophic region of growth plate cartilage (Fig. 1A) (see also Ref. 13). Thus, annexin V, β5 integrin, and type X collagen are highly expressed in the hypertrophic region of growth plate cartilage. A previous study has shown that annexin V binds to the cytosolic domain of the β5 integrin subunit (11). Double immunostaining with antibodies specific for β5 integrin and annexin V revealed a co-localization of both proteins in hypertrophic growth plate chondrocytes in culture (Fig. 1B), confirming previous findings showing an interaction between annexin V and the β5 integrin subunit (11).

To determine the role of annexin V and its interactions with β5 integrin, we overexpressed annexin V in hypertrophic growth plate chondrocytes using the retroviral expression vector RCAS-BP. Infection of growth plate chondrocytes with RCAS-BP containing full-length annexin V cDNA (Fig. 2A, AnV/RCAS) resulted in a notable increase (2–3-fold) of annexin V protein expression when compared with the expression levels of uninfected or RCAS-BP-infected (Fig. 2A, Uninfected, RCAS) growth plate chondrocytes. Overexpression of full-length annexin V led to a decrease of bcl-2 expression and increases of bax expression and caspase-3 activity when compared with the levels of RCAS-infected cells (Fig. 3, A and B, AnV/RCAS, RCAS). To determine whether annexin V and β5 integrin interactions are involved in mediating apoptotic events in growth plate chondrocytes, we used a peptide mimic of annexin V (VVI-SYSMPD) that has been shown to bind to β5 integrin and trigger cell death of endothelial cells (11). We synthesized an internalizing version of the peptide by using the Pen system for intracellular delivery. Pen, a peptide containing 16 amino acids that are part of the third helix of the Antennapedia protein homeodomain, has translocating properties and therefore is capable of carrying hydrophilic compounds across the plasma membrane and delivering them to the cytoplasmic space without degradation (18). We fused the peptide mimic of annexin V (VVISYSMPD) or a control unrelated peptide (SDNRYIGSW) to Pen and added a biotin moiety to visualize internalization. Both Pen-VVISYSMPD peptide and the control peptide were internalized and uniformly distributed in the cytoplasm (Fig. 2B, Pen-SDNRYIGSW, Pen-PVVISYSMPD). Cells not incubated with these peptides showed no cytoplasmic fluorescence staining (Fig. 2B, Control). Treatment of growth plate chondrocytes with 12 μM Pen-PVVISYSMPD for 6 h resulted in a decreased expression of the anti-apoptotic factor bcl-2 and an increased expression of the apoptotic factor bax when compared with the expression levels of untreated cells (Fig. 3A, Pen-PVVISYSMPD, Control). Cells treated with the control peptide showed similar expression levels of bcl-2 and bax as untreated cells (Fig. 3A, Pen-SDNRYIGSW, Control). Treatment of growth plate chondrocytes with 12 μM Pen-PVVISYSMPD for 24 h led to a notable increase of caspase-3 activity when compared with untreated cells or cells treated with Pen-SDNRYIGSW (Fig. 3B, Pen-PVVISYSMPD, Pen-SDNRYIGSW, Control).

Next we determined whether Pen-PVVISYSMPD and β5 integrin are sufficient to induce apoptotic events in growth plate chondrocytes or whether full-length annexin V is also required. Therefore, we suppressed annexin V expression in Pen-PVVISYSMPD-treated growth plate chondrocytes using siRNA
technology and measured cell viability and caspase-3 activity. Using annexin V-specific siRNA, annexin V expression was notably suppressed in hypertrophic growth plate chondrocytes (annexin V expression was suppressed by ~70%; Fig. 4, siAnV). Pen-VVISYSMPD treatment reduced the cell viability to ~40% (Fig. 5A, Pen-VVISYSMPD). The control peptide Pen-SDNRYIGSW did not affect cell viability (Fig. 5A, Pen-SDNRYIGSW). Transfecting cells with siRNA specific for annexin V or treatment with annexin V-mimicking peptide (Pen-VVISYSMPD) resulted in up-regulation of gene expression of bax and caspase-3 activity and down-regulation of bcl-2 gene expression when compared with the levels in untreated (Pen-SDNRYIGSW, Pen-VVISYSMPD). Transfection of growth plate chondrocytes with annexin V-specific siRNA, treatment with Pen-SDNRYIGSW, or treatment with Pen-SDNRYIGSW (control peptide) and transfection with annexin V-specific siRNA had no effect on caspase-3 activity (Fig. 5B, Pen-SDNRYIGSW, Pen-SDNRYIGSW/siAnV).

Annexin V has been shown not only to bind to the cytoplasmic domain of the β5 integrin subunit but also to bind to active PKCα (11, 12). Binding of annexin V to active PKCα inhibits PKCα activity (12, 19). Since active PKCα has been shown to be involved in mediating cell survival of chondrocytes (20), we asked whether inhibition of PKCα through annexin V and/or annexin V/β5 integrin interactions may play a role in mediating apoptosis of growth plate chondrocytes using an activator of PKC (PMA) or generic inhibitors of PKC (calphostin) or subtype-specific PKC inhibitors (Myr-PKCa/β, Myr-PKζ). Treatment of growth plate chondrocytes with PMA, calphostin, or Pen-SDNRYIGSW did not alter cell viability or caspase-3 activity when compared with cell viability and caspase-3 activity of untreated cells (Fig. 6, A and B, PMA, Calphostin, Pen-SDNRYIGSW, Control). Co-treatment with Pen-SDNRYIGSW and PMA or calphostin also did not alter cell viability or caspase-3 activity (Fig. 6, A and B, Pen-SDNRYIGSW/PMA, Pen-SDNRYIGSW/ Cal.). PMA further reduced cell viability of Pen-VVISYSMPD-treated cells (from ~40% for Pen-VVISYSMPD-treated cells to ~25% for Pen-VVISYSMPD- and PMA-treated cells) (Fig. 6A, Pen-VVISYSMPD, Pen-VVISYSMPD/PMA), whereas calphostin increased cell viability of Pen-VVISYSMPD-treated cells (~80% viability in Pen-VVISYSMPD and calphostin-treated cells when compared with ~40% viability in Pen-VVISYSMPD-treated cells) (Fig. 6A, Pen-VVISYSMPD, Pen-VVISYSMPD/Cal.). Similar results were obtained for caspase-3 activity. Pen-VVISYSMPD and PMA treatment (Fig. 6B, Pen-VVISYSMPD/PMA) further increased caspase-3 activity when compared...
with the activity in Pen-VVISYSMPD-treated cells. On the other hand, co-treatment with Pen-VVISYSMPD and calphos-tin (Fig. 6B, Pen-VVISYSMPD/Ca+) decreased caspase-3 activity to levels similar to those of untreated (Fig. 6B, Control) cells.

Next, we tested whether PKC regulation by annexin V/β5 integrin interactions is specific for PKCa. We treated growth plate chondrocytes with Pen-VVISYSMPD and Myr-PKCa/β (specific for PKCa) or Myr-PKCζ (specific for PKCζ). In the absence of Pen-VVISYSMPD, Myr-PKCa/β did not change cell morphology and viability when compared with the morphology and viability of untreated cultures (Fig. 7A, Untreated, Myr-PKCa/β, and B, white bars, Untreated, Myr-PKCa/β). However, Myr-PKCζ treatment in the absence of Pen-VVISYSMPD resulted in a notable decrease of growth plate chondrocyte viability (Fig. 7B, white bars, Myr-PKCζ). As already shown above, treatment of growth plate chondrocytes with Pen-VVISYSMPD resulted in a morphological appearance of dead cells (Fig. 7A, Pen-VVISYSMPD) and a marked decrease of growth plate chondrocyte viability when compared with untreated cells (Fig. 7B). Pen-SDNRYIGSW-treated cells showed a similar morphology as untreated cells (Fig. 7A, Untreated, Pen-SDNRYIGSW). Myr-PKCa/β and Pen-VVISYSMPD-treated growth plate chondrocytes had a morphological appearance similar to untreated cells (Fig. 7A, Untreated, Pen-VVISYSMPD/Myr-PKCa/β). In addition, Myr-PKCa/β led to an increase of cell viability of Pen-VVISYSMPD-treated growth plate chondrocytes (Fig. 7B, black bars, Myr-PKCa/β). Myr-PKCζ resulted in a further decrease of cell viability of Pen-VVISYSMPD-treated growth plate chondrocytes (Fig. 7B, black bars, Myr-PKCζ).

**DISCUSSION**

In this study, we provide evidence that the balance between annexin V/β5 integrin and annexin V/PKCα interactions plays a role in the regulation of growth plate chondrocyte apoptosis. Apoptosis is the final fate of terminally differentiated growth plate chondrocytes and is required for normal endochondral bone formation (2, 21). Disturbance of apoptosis in growth plate cartilage results in abnormal bone development. For example, bcl-2 knock-out mice show accelerated chondrocyte differentiation and apoptosis, resulting in accelerated endochondral bone formation and short stature of these mice (6). Our results show that a peptide mimic of annexin V (Pen-VVISYSMPD) that binds to β5 integrin but not to PKCa (11) stimulates apoptotic events in hypertrophic growth plate chondrocytes. Similarly, overexpression of annexin V in hypertrophic growth plate chondrocytes increased caspase-3 activity and the proapoptotic bax gene expression and decreased expression of the antiapoptotic bcl-2 gene, suggesting that high expression of annexin V in growth plate chondrocytes results in apoptosis of these cells. On the other hand, suppression of annexin V in hypertrophic growth plate chondrocytes using siRNA resulted in an increase of cell viability and a decrease of caspase-3 activity in Pen-VVISYSMPD-treated growth plate chondrocytes, further confirming our model that both the interactions between β5 integrin and annexin V (or its peptide mimic) and the interactions between annexin V and PKCa are required for the regulation of growth plate chondrocyte apoptosis.

Annexin V and β5 integrin are expressed by hypertrophic and terminally differentiated chondrocytes in growth plate cartilage (13, 14). Furthermore, we have previously shown that retinoic acid-induced terminal differentiation and apoptotic events in growth plate chondro-
Annexin V/β5 Integrin and Chondrocyte Apoptosis

cytes are accompanied by stimulation of annexin V, annexin II, and annexin VI expression (3, 4). How does annexin V regulate terminal differentiation events and apoptosis of growth plate chondrocytes? Apoptotic events are regulated by the interactions of a variety of pathways, including alterations of cytosolic Ca²⁺ homeostasis. Annexins II, V, and VI form Ca²⁺ channels in the plasma membrane of terminally differentiated growth plate chondrocytes, leading to the influx of extracellular Ca²⁺ into these cells. These increases in cytoplasmic Ca²⁺ stimulate a whole series of events, including stimulation of expression of terminal differentiation and mineralization-related marker genes, release of mineralization-competent matrix vesicles, and apoptotic-related events (4). The present study shows that annexin V regulates apoptosis not only by forming Ca²⁺ channels but also through its interactions with β5 integrin and PKCα. Both events together are required for effective regulation of apoptosis (Fig. 8). The inhibition of one pathway results in an only partial inhibition of cell death (Figs. 5–7) (see also Ref. 4). Although the peptide mimic of annexin V only stimulates the β5 integrin/annexin V/PKCα pathway, overexpression of annexin V stimulates both the cytosolic Ca²⁺ and the β5 integrin/annexin V/PKCα pathways. An inhibitor of PKC (calphostin) decreased caspase-3 activity and increased cell viability of growth plate chondrocytes treated with Pen-VVISYMPD, whereas treatment of cells with Pen-VVISYMPD and an activator of PKC (PMA) further increased caspase-3 activity and decreased cell viability when compared with the levels of Pen-VVISYMPD-treated cells. This effect was specific for PKCα in that a specific inhibitor of PKCα (Myr-PKCo/β) increased viability of Pen-VVISYMPD-treated growth plate chondrocytes. A previous study showed that Pen-VVISYMPD binds to β5 integrin but not to PKCα. In contrast, modest amounts of annexin V, annexin V-specific inhibition of PKCα and subsequent cell death can be mimicked by Pen-VVISYMPD, which binds to β5 integrin but not PKCα. The interaction of Pen-VVISYMPD with β5 integrin releases annexin V from β5 integrin binding, allowing annexin V to bind to active PKCα (Fig. 8B). The presence of an activator of PKCα (PMA) further decreased cell viability of Pen-VVISYMPD-treated growth plate chondrocytes because PMA has been shown to facilitate binding of annexin V to active PKCα (Fig. 8C) (see also Ref. 11). On the other hand, inhibitors of PKCα prevent binding of annexin V to PKCα in the presence of high amounts of annexin V or Pen-VVISYMPD, thereby hindering annexin V-specific inhibition of PKCα and subsequent cell death (Fig. 8D).

Interestingly, active PKCα has been implicated in mediating cell survival in a variety of cell types (23, 24). Our findings that suppression of annexin V expression in growth plate chondrocytes increased cell viability and decreased caspase-3 activity in Pen-VVISYMPD-treated cells suggest that active PKCα also mediates cell survival in chondrocytes and that inhibition of PKCα by annexin V and/or the interactions among annexin V, β5 integrin, and PKCα are required for the stimulation of apoptotic events in growth plate chondrocytes. Inactivation of PKCα has been shown to play an important role in modulating hepatic apoptosis during sepsis. Apoptosis of hepatocytes after sepsis is associated with a decrease in bcl-2 expression and an increase in bax expression, which is similar to the findings of our study in which PKCα in growth plate chondrocytes was inhibited by annexin V, resulting in decreased expression of bcl-2 and increased expression of bax (23). Other findings have shown that PKCα inhibition in NIH3T3 cells reduced proliferation and induced apoptosis and that these effects were also

![FIGURE 6. Cell viability and caspase-3 activity of untreated (Control), Pen-SDNYIGSW-treated (Pen-SDNYIGSW), Pen-VVISYMPD-treated (Pen-VVISYMPD), PMA-treated (PMA), calphostin-treated (Calphostin), Pen-SDNYIGSW- and PMA-treated (Pen-SDNYIGSW/PMA), Pen-VVISYMPD- and PMA-treated (Pen-VVISYMPD/PMA), Pen-SDNYIGSW- and calphostin-treated (Pen-SDNYIGSW/Cal), and Pen-VVISYMPD- and calphostin-treated (Pen-VVISYMPD/Cal) growth plate chondrocytes. PMA further decreased cell viability (A) and further increased caspase-3 activity (B) in Pen-VVISYMPD-treated cells. Data were obtained from three different cultures and expressed as mean ± S.D. (Pen-VVISYMPD or Pen-VVISYMPD/PMA versus control; *, p ≤ 0.01).](image-url)
Annexin V/β5 Integrin and Chondrocyte Apoptosis

mediated by alterations of bcl-2 and bax expression (25). Similar to the findings of our study, a recent study showed that Pen-VVISYSMPD mediates apoptosis of endothelial cells by a similar PKCα/β5 integrin/annexin V-mediated mechanism (11). Therefore, it is plausible to assume that PKCα/annexin V/β5 integrin interactions play a role not only in the regulation of growth plate chondrocyte apoptosis but also in the regulation of apoptosis of other cell types.

PKCs have been shown to play important roles in cell differentiation and to act as either proapoptotic or antiapoptotic depending on the cell type, extracellular stimuli, and specific isoform that is activated or inhibited. Our findings showing that the interactions among annexin V, β5 integrin, and active PKCα play a proapoptotic role in growth plate chondrocytes suggest that not only activation or inhibition of PKC but also the mechanism of PKC activation or inhibition plays a role in whether PKCs act pro- or antiapoptotic. Although the interactions among annexin V, β5 integrin, and active PKCα were able to affect cell survival of growth plate chondrocytes, a specific inhibitor of PKCζ (Myr-PKCζ) decreased cell survival of untreated and Pen-VVISYSMPD-treated growth plate chondrocytes, suggesting that PKCζ plays an additional role in cell survival of hypertrophic growth plate chondrocytes independent of the role of β5 integrin/annexin V/PKCα interactions. These findings are consistent with previous findings showing that inhibition of PKCζ is required for nitric oxide-induced apoptosis of articular chondrocytes and that inhibition of PKCζ activity is required for the apoptosis of various cell types (20, 26, 27). Therefore, it is possible that the regulation of the activities of various PKCs by different mechanisms plays a crucial role in the regulation of growth plate and articular chondrocyte apoptosis.

Thus, within the growth plate, the ratio of Bcl-2 to bax progressively decreases in chondrocytes in favor of Bax (6). This change in the bcl-2:bax ratio in favor of bax results in the apoptotic death of terminally differentiated chondrocytes (1, 2, 5). Our study findings show that annexin V/β5 integrin/PKCα interactions result in the alteration of bcl-2 and bax expression in favor of bax, suggesting that annexin V/β5 integrin/PKCα interactions may affect apoptosis of growth plate chondrocytes by altering the bcl-2:bax ratio in favor of bax. We and others have shown that articular chondrocytes in osteoarthritic cartilage undergo differentiation events similar to those of growth plate chondrocytes, resulting in terminal differentiation of

FIGURE 7. A, light microscopical analysis revealed a morphology of dead cells in Pen-VVISYSMPD-treated growth plate chondrocytes, whereas Myr-PKCα/β resulted in a morphology of Pen-VVISYSMPD-treated growth plate chondrocytes similar to untreated, Pen-SDNYIGSW, or Myr-PKCα/β-treated growth plate chondrocytes. B, quantification of cell viability of control, Myr-PKCα/β-treated, or Myr-PKCζ-treated growth plate chondrocytes in the absence (white bars; −Pen-VVISYSMPD) or presence (black bars; +Pen-VVISYSMPD) of Pen-VVISYSMPD. Cell viability was measured using the MTT assay after 24 h of treatment. Data were obtained from three different cultures and expressed as mean ± S.D. (control + Pen-VVISYSMPD versus control − Pen-VVISYSMPD; Myr-PKCζ − Pen-VVISYSMPD versus control − Pen-VVISYSMPD; Myr/PKCζ + Pen-VVISYSMPD versus control − Pen-VVISYSMPD; *, p = 0.01).
osteoarthritic chondrocytes (13, 28, 29). Interestingly, these cells also express annexin V and β5 integrin (13, 30). Furthermore, several studies have shown that chondrocytes in osteoarthritic or damaged cartilage undergo apoptotic changes (13, 31–34). Therefore, it is possible that annexin V, β5 integrin, and PKCα are involved in the regulation of apoptosis of articular chondrocytes in osteoarthritis by a mechanism similar to that described in the present study for growth plate chondrocytes. Interestingly, previously it has been reported that nitric oxide-induced apoptosis of articular chondrocytes requires the inhibition of PKCα and -ζ consistent with the findings of our study (20).

β5 integrin is expressed by various skeletal cell types. A previous study detected high expression of β5 integrin in developing cartilages and suggested that β5 integrin is a key integrin involved in bone formation (35). However, mice lacking β5 integrin show no obvious skeletal phenotype (36). It is possible that other integrins, such as β3 integrin, might compensate for the loss of β5 integrin. Sometimes a lack of phenotype in mice can also result from other reasons, including the location of the neomycin cassette and/or the genetic background of the mice. For example, recently two different lines of annexin VII−/− mice were generated; one mouse line shows a severe phenotype, whereas the other mouse line has only a mild phenotype (37, 38).

In conclusion, we present evidence that the link among annexin V, β5 integrin, and PKCα interactions mediates regulation of apoptosis of growth plate chondrocytes. Our findings reveal that a certain amount of annexin V is required to interact with β5 integrin and PKCα and that the interactions of annexin V with both β5 integrin and PKCα are required for the induction of apoptosis of growth plate chondrocytes. Increasing the amounts of annexin V present in growth plate chondrocytes to interact with β5 integrin and/or PKCα either by overexpression of annexin V or by adding the annexin V-mimicking peptide (Pen-VVISYMPD) stimulated apoptotic events in growth plate chondrocytes, whereas suppressing annexin V expression prevented apoptotic events in growth plate chondrocytes even in the presence of the annexin V peptide mimic Pen-VVISYMPD. Endothelial cells seem to undergo apoptosis by a similar mechanism involving annexin V/β5 integrin and PKC (11). Considering that annexin V and β5 integrin are also expressed in osteoarthritic articular cartilage (13, 30, 39) and that inhibition of PKCα seems to be involved in articular chondrocyte apoptosis (20), it is possible that a similar mechanism leads to cell death of osteoarthritic chondrocytes.

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Annexin V/β5 Integrin and Chondrocyte Apoptosis

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