Chondrocyte-Matrix Attachment Complexes Mediate Survival and Differentiation

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ABSTRACT Integrin mediated cell-extracellular matrix interactions are required for survival and differentiation of many cell types. In this review, the cell-matrix attachment complex (CMAX) is described for chondrocytes. The evidence that integrin-mediated signal transduction is necessary for normal chondrocyte differentiation and survival in various culture conditions and in vivo are reviewed. The possible signal transduction pathways stimulated by the extracellular matrix components are discussed with a review of current data from chondrocyte experiments. In addition, the influence of parathyroid hormone and transforming growth factor β on chondrocyte survival has been included as they may function in concert with integrin mediated signal transduction. Finally, specific changes in gene expression preceding apoptosis are discussed. The current understanding of how integrin-mediated signals prevent apoptosis and implications of anchorage-dependent survival for development and differentiation of the chondrocyte phenotype are discussed. *Microsc. Res. Tech. 43:111–122, 1998.* © 1998 Wiley-Liss, Inc.

INTRODUCTION

It is established that many cell types require integrin-mediated interactions with extracellular matrix to differentiate, migrate, proliferate, and even survive. Some epithelial and endothelial cells actually require specific matrix molecules for survival (see Meredith and Schwartz 1997, for recent review). Cells that require cell-matrix interactions to survive undergo a specific sequence of degradation events termed programmed cell death or apoptosis (Kerr et al., 1972) in the absence of integrin-mediated attachment. This phenomenon was first described for vascular endothelial cells (Meredith et al., 1993), followed shortly by a report that MDCK cells, an epithelial kidney cell line, died when placed in suspension culture (Frisch and Francis, 1994).

Recently, chondrocytes were added to the list of cells that depend on integrin-mediated attachment to survive (Hirsch et al., 1997). Although previous experiments had demonstrated anti integrin antibodies blocked attachment to purified matrix components (type I and II collagen or FN) (Durr et al., 1993; Enomoto et al., 1993; Loeser et al., 1995), these studies did not include apoptosis analysis, whereas in the whole chick sterna model, blocking cell-matrix interactions with specific integrin subunit antibodies changed cell and nuclear shape and increased apoptosis. The actin cytoskeleton was disrupted in chondrocytes from integrin antibody treated tissue, indicating that the integrin-mediated signal transduction pathways were altered (Hirsch et al., 1997). A recent analysis of chondrogenic apoptosis from transgenic mice lacking type II collagen is also strong evidence that chondrocytes require a specific matrix for survival (Yang et al., 1997). In this review, the characterization of the *c*ell *m*atrix *a*ttachment comple*x* (CMAX) will include a discussion of the component proteins (actin-associated and signal transduction pathways) followed by the role of this complex in chondrocyte differentiation and survival. Some of the hormones and growth factors that also have a role in differentiation and survival will be discussed. Although some discussion of integrin subunits expressed by chondrocytes is included, the detailed description of integrins and other extracellular receptor molecules will be covered in another review in this issue (Kosher, 1998).

CHARACTERIZATION OF CELL MATRIX ATTACHMENT COMPLEXES

Proliferating chondrocytes synthesize and secrete collagen types II, IX, and XI (Mendler et al., 1989). Prior to bone deposition, chondrocytes terminally differentiate into hypertrophic chondrocytes and change their collagen gene expression to type X collagen (Linsenmayer et al., 1991; Schmid and Linsenmayer, 1985). Hypertrophy has been classified as the terminal stage of chondrocyte differentiation and a marker for maturation (LuValle et al., 1992). Hypertrophy is characterized by an increased cell volume of individual chondrocytes, matrix accommodation for the enlarged chondrocytes, production of type X collagen, and a concomitant decrease in the synthesis of collagen types II, IX, and XI. The fate of hypertrophic chondrocytes has been controversial (Gibson et al., 1995; Roach, 1997). In culture, chondrocytes from hypertrophic regions of chick sternum have a shorter survival rate than cells from proliferative regions (Gibson et al., 1997). In contrast, a recent detailed histological study of growth plates from

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9- to 20-day embryonic chick long bones determined that hypertrophic chondrocytes at regions of vascular invasion undergo apoptosis, whereas other cells appear to re-enter the cell cycle (Roach, 1997; Gibson, 1998).

Following hypertrophy, these regions calcify, blood vessels invade, and bone marrow forms. This differentiation process involves the interaction of integrin receptors with collagen molecules extracellularly and the cytoskeleton intracellularly via signal transduction pathways. Chondrocytes express a variety of integrin receptors including: $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_v\beta_3$ (Durr et al., 1993; Enomoto et al., 1993; Hirsch and Svoboda, 1994; Hirsch et al., 1996; Shakibaei et al., 1995).

Attachment assays have demonstrated that freshly isolated (Durr et al., 1993) and cultured chondrocytes (Enomoto et al., 1993) bind to types I and II collagen via β_1 (Durr et al., 1993; Enomoto et al., 1993) and α_2 integrin (Durr et al., 1993). The combination of anti-α_1 and anti-α_2 acted synergistically to decrease binding to types I and II collagen further (Durr et al., 1993). Chondrocyte binding to collagen substrates was also found to be Mg^{++} dependent (Durr et al., 1993; Enomoto et al., 1993) but Ca^{++} and RGD sequence independent (Durr et al., 1993). Articular chondrocytes also require the β_1 integrin subunit for attachment to FN, matrix Gla protein, and type II collagen, whereas the β_3 subunit was required for attachment to bone sialoprotein II and osteopontin (Loeser, 1993). In recent studies using monolayer cultures, chondrocyte adhesion was also shown to be β_1 integrin dependent (Shakibaei et al., 1997). In other experiments, RGD peptides or antibodies against $\alpha_5\beta_1$ blocked chondrocyte spreading and proliferation (Enomoto-Iwamoto et al., 1997), indicating that these receptors were necessary for attachment, spreading, and proliferation. However, none of these studies examined the fate of the unattached cells, probably because chondrocytes differentiate in suspension cultures.

Two growth factors, insulin growth factor (IGF-1) and transforming growth factor β (TGFβ), were shown to stimulate articular chondrocyte cell surface expression of the α_3/α_5 integrin subunits and adhesion to fibronectin and type II collagen. The two growth factors have opposite effects on expression of $\alpha_1\beta_1$. IGF-1 increased and TGF β decreased cell surface levels of $\alpha_1\beta_1$. TGF β-treated cells also had decreased adhesion to type VI collagen. These opposing effects of IGF-1 and TGFβ on chondrocyte $\alpha_1\beta_1$ expression and adhesion suggested that $\alpha_1\beta_1$ mediated chondrocyte adhesion to type VI collagen. An antibody to the α_1 integrin subunit also blocked chondrocyte adhesion to type VI collagen (Loeser, 1997). Apoptosis rates were not reported.

The localization of integrin subunits on cartilage sections with immunofluorescence has been problematic. The α_2 integrin subunit was not detected in human fetal cartilage (clone P1E6) although the antibody blocked attachment to types I and II collagen by 40–50% (Durr et al., 1993). The same antibody did not block articular chondrocyte attachment to type II collagen (Loeser et al., 1995) or reduce collagen gel contraction by dermal fibroblasts at 40 μg/ml (Langholz et al., 1995). However, clone P1E6 immuno-detected α_2 on chick chondrocytes in situ (Fig. 1C,D) (Hirsch et al., 1996; Wu et al., 1995) and decreased sternal growth and differentiation (Hirsch et al., 1997). We have shown with immunohistochemistry that embryonic chick hyaline chondrocytes form cell matrix attachment complexes (CMAX) that include integrin molecules (β_1, α_2, and α_3) (Fig. 1) and cytoskeletal proteins (F-actin, vinculin, focal adhesion kinase [FAK], α-actinin, zyxin, and paxillin) (Fig. 2) (Hirsch et al., 1996; Wu et al., 1995). These morphological observations have led to the hypothesis that changes in chondrocyte cell size and gene expression may be regulated by integrin receptor signaling. Inhibition of cell-matrix interactions with blocking antibodies that recognize extracellular domain epitopes of β_1, α_2, and α_3 integrin subunits decreased growth, altered the actin cytoskeleton, and prevented terminal differentiation as measured by the deposition of type X collagen into the matrix (Hirsch et al., 1997).

IDENTIFICATION OF ACTIN ASSOCIATED PROTEINS IN CHONDROCYTES

In freshly isolated whole hyaline cartilage (embryonic chick sternum), confocal images demonstrated that chondrocytes organize actin bundles near cell surfaces (Fig. 2A,D). In optical sections through the central regions of cells, actin staining was closely associated with plasma membranes. The actin-associated protein, vinculin, decorated the ends of the actin filaments in chondrocytes from whole mount cartilage preparations (Fig. 2D) (Hirsch et al., 1996). Additional actin-associated proteins that are known to interact

Abbreviations:

Bax	bcl-associated x protein that accelerates cell death
Bcl-2	B cell lymphoma protein that blocks cell death
BMPR	bone morphogenetic protein receptors, BMP-IA, BMP-IB
CMAX	cell-matrix attachment complex
FAK	focal adhesion kinase
F-actin	filamentous actin
FN	fibronectin
Dia	p140 Diaphanous
ECM	extracellular matrix
erk	extracellular signal regulated protein
GAP	GTPase activating protein
GEF	Guanine exchange factors
Grb2	growth-factor-receptor-bound protein 2
ICE	interleukin-1 converting enzyme
IGF	insulin growth factor
Ihh	Indian hedgehog
JNK	Jun N-terminal kinase
MAPkinase	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase also known as MEK
MAPKKK	mitogen-activated protein kinase kinase kinase also known as MEKK
PTH	parathyroid hormone
PTHrP	PTH-related peptide
PI3	phospatidylinositol 3 kinase
PKC	protein kinase C
ROCK	Rho associated coiled-coil containing protein kinase
SAPK	stress-activated protein kinase
SH2	Src homology domain 2
SH3	Src homology domain 3
TGFB	transforming growth factor beta
TUNEL	*T*dt-mediated d*U*TP-X *n*ick *e*nd *l*abeling)

with integrin cytoplasmic domains are α-actinin, focal adhesion kinase (FAK), talin, tensin, zyxin, and other signal transduction proteins (Fig. 3).

Integrins and cytoskeletal proteins were concentrated in cell matrix attachment complexes (CMAX) in proliferative and hypertrophic chondrocytes from hyaline cartilage. The identification of CMAX proteins in freshly isolated sterna, however, only allowed structural and not functional analysis. To address functional questions an organ culture model for whole sterna was developed that recapitulated normal development in a completely defined media (Hirsch and Svoboda, 1998).

The CMAX proteins maintained a normal distribution after the embryonic sterna were cultured for 8 days. In single confocal optical sections near the cell surface, β_1 integrin subunits (punctate spots) were present near the ends of F-actin bundles (Fig. 2A). Similarly, vinculin appeared to associate near the ends of actin bundles and was observed as "streaks" (Fig. 2D) instead of punctate distribution detected for β_1 integrin. F-actin had a normal distribution at the surface of the hypertrophic chondrocytes, similar to freshly isolated sternar chondrocytes (Hirsch et al., 1996). In confocal optical sections scanned through cell nuclei, actin, β_1 integrin subunits (Fig. 1E,F) and vinculin appeared to associate with plasma membranes and did not stain nuclear regions.

FAK, zyxin, paxillin, and p-tyr (Fig. 2) were also present in hypertrophic chondrocytes. The punctate distribution of FAK (Fig. 2B) was similar to freshly isolated sternum chondrocytes (Hirsch et al., 1996), and appeared to colocalize with β_1 integrin subunits. Paxillin (Fig. 2C) and zyxin (Fig. 2E), two focal adhesion proteins implicated in integrin mediated signal transduction (Parsons, 1996) and actin polymerization (Crawford and Beckerle, 1991; Crawford et al., 1992; Golsteyn et al., 1997), had punctate distributions throughout the cytoplasm of hypertrophic chondrocytes. The distribution of paxillin was more diffuse than zyxin, consistent with that observed in chondrocytes from freshly isolated sternum (Hirsch et al., 1996).

Lastly, an antibody that recognizes all proteins with phosphorylated tyrosine residues had a staining pattern similar to FAK and paxillin. As tyrosine kinases have been implicated in integrin signaling (Parsons, 1996), the punctate distribution of these proteins near cell peripheries (Fig. 2F) indicates that CMAX proteins may have a functional role in the signal transduction pathway.

INTEGRIN-MEDIATED SIGNAL TRANSDUCTION PATHWAYS

The evidence for specific integrin-mediated signal transduction pathways in chondrocytes is circumstantial. As shown previously, some of the proteins have been immunolocalized to the cell-matrix interaction region. Attachment and blocking experiments have shown that the integrins mediate chondrocyte attachment and spreading in vitro. We have shown in our whole sternum model that blocking integrin matrix interactions decreases growth and differentiation (Hirsch et al., 1997). In addition, the transgenic mice lacking type II collagen had dwarfism and increased chondrocyte apoptosis (Yang et al., 1997) indicating that specific matrix molecules or their interactions may be necessary for chondrocyte survival. Extensive integrin-mediated signal transduction analysis of chondrocytes is not available; therefore, other cell types that have been studied in more detail will be discussed and compared to recent chondrocyte specific experiments.

Cellular signaling initiated by integrin activation is a complex ballet of molecules interacting and stimulating surrounding proteins, lipids and ions resulting in cytoskeletal reorganization, modulation of differentiation, and induction of gene expression. The choreography of events and players of this signaling pathway ballet has been a hot topic in the past few years. Each new review paper has a new schematic diagram of this pathway. I urge the readers to view the intermolecular reactions as a fluid dynamic process in which multiple events may be occurring at the same time. The present state of the analysis art of these events continues to view snapshots of individual events such as tyrosine phosphorylation without the benefit of viewing the whole stage simultaneously. It has been established that following receptor-ligand binding, molecular events include tyrosine phosphorylation, alkalization, and changes in intracellular calcium concentrations (Hannigan et al., 1996; Keely et al., 1998; Parsons, 1996; Schwartz, 1992). Although specific signaling caused by the binding of collagen integrin receptors to ligands has not been demonstrated in chondrocytes, the downstream effects of integrin binding has been shown in other cell types (Hannigan et al.,1996; Keely et al., 1998; Parsons, 1996; Schwartz, 1992).

The exact sequence of events is unknown. However, both ligand occupancy and receptor clustering are critical for the activation of intracellular integrin-mediated responses (Dedhar and Hannigan, 1996). Integrin receptor engagement and clustering leads to the formation of focal adhesions in cultured cells, where integrins link to intracellular cytoskeletal complexes and bundles of actin filaments. Though much is known about the extracellular interactions between integrins and their ligands, significantly less is known about the intracellular biochemical pathways that integrins regulate and the cellular functions controlled. The signaling pathways activated by integrins have been identified through the analysis of biochemical events that are triggered by integrin engagement. Protein phosphorylation (tyrosine, specifically) is one event detected in response to integrin stimulation. Integrin signaling pathways synergize with other receptor pathways to enhance or dampen signals elicited by each receptor.

Collagen activation of the integrin receptor, $\alpha_2\beta_1$, in platelets has been shown to stimulate second messenger pathways and protein tyrosine phosphorylation (Parsons, 1996). The $\alpha_3\beta_1$ integrin receptor, which binds fibronectin, laminin, and collagen, has also been shown to stimulate tyrosine phosphorylation of proteins when triggered in carcinoma cells and NIH 3T3 fibroblasts. In addition, it has been determined that integrin receptor binding to collagen is highly specific and dependent on collagen molecule conformation (Kuhn and Eble, 1994). Future studies of chondrocytes may demonstrate that collagen integrin receptor binding may or may not have signaling effects similar to that observed in other cell types as chondrocytes are surrounded by a complex extracellular matrix. A new subclass of fibrillar collagen receptors with homology to

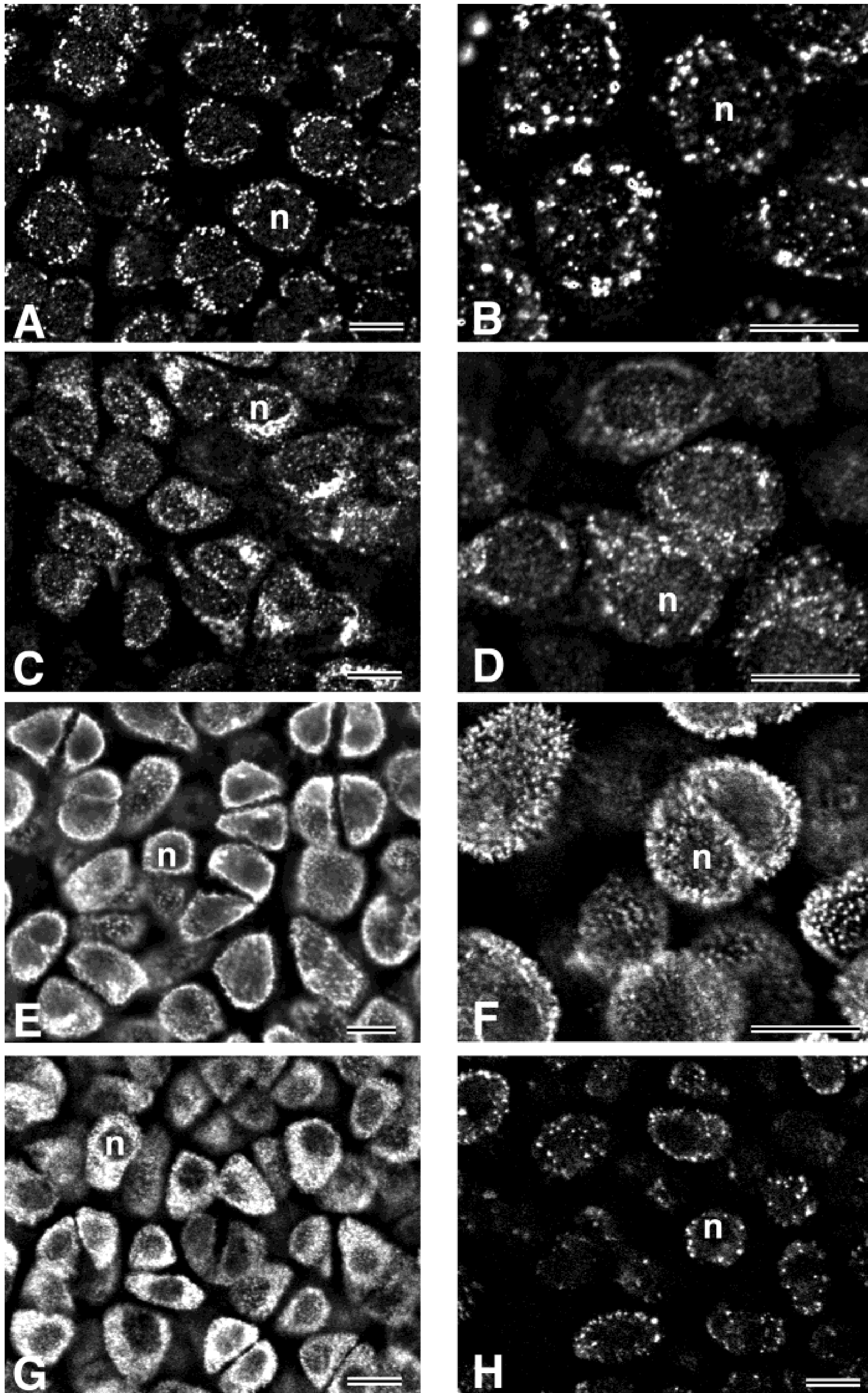


Fig. 1.

the Dictyostelium discoidem protein discoidin-1 (DDR1 and DDR2) has recently been described on tumor cells that have tyrosine kinase activities (Shrivastava et al., 1997; Vogel et al., 1997). These transmembrane receptors are 135 kDa and become phosphorylated by 5 minutes with maximum phosphorylation at 90 minutes that was sustained for 18 hours following stimulation by types I, II, III, and V collagen (Vogel et al., 1997; Kosher, 1998).

The integrin-cytoskeletal assemblies (CMAX) form the foundation for the construction of signaling complexes that include kinases (Fig. 3) (Dedhar and Hannigan, 1996). These complexes have FAK as the core activator. When ECM ligands bind to integrin, FAK associates with the cytoplasmic tail of the β integrin subunit, autophosphorylates, then phosphorylates surrounding proteins including the actin-associated protein, paxillin. The Src homology (SH) domains of FAK allow the formation of supramolecular complexes of signaling proteins. These proteins include the tyrosine kinase Src and/or csk, the adapter protein Grb2, and the guanine nucleotide exchange factor SOS. The actual signaling pathways invoked by integrin binding are still hypothetical. In our diagram (Fig. 3) several hypothetical pathways are illustrated (multiple steps may be indicated by a single arrow). The Src (Ras) activation pathway and the tyrosine kinase (Rho) pathway are thought to cross talk at several steps, such as both may have a role in the activation of MAP kinase cascades.

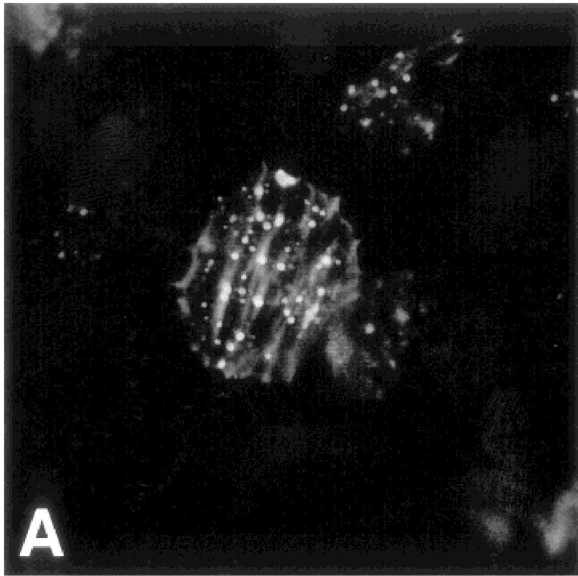
In fibroblast adhesion studies with fibronectin, integrin cross-linking causes the adapter proteins, Grb2 and mSOS1, to associate with FAK. The SH2 domain of Grb2 binds to a phosphotyrosine in the carboxyl terminus of FAK. For example, in T lymphoblastoid cells, Ras activation occurs after collagen binds the $\alpha_2\beta_1$ integrin receptor (Kapron-Bras et al., 1993). This finding suggests a connection between integrins, FAK, and the classic Ras-MAP kinase pathway (Hildebrand et al., 1996). Another way that integrins can possibly regulate the MAP kinase pathway is through Crk, another adapter protein whose SH2 and SH3 domains interact with FAK, mSOS1, and paxillin (Clark and Brugge, 1995; Schaller and Parsons, 1995). In many cell types (fibroblasts and vascular endothelial cells), integrin receptor clustering also activates the phosphorylation of phosphatidylinositol phosphate (PIP) to PIP_2, making it available for the production of IP_3 and subsequent increases in calcium concentrations (Sastry and Horwitz, 1993; Schwartz et al., 1995).

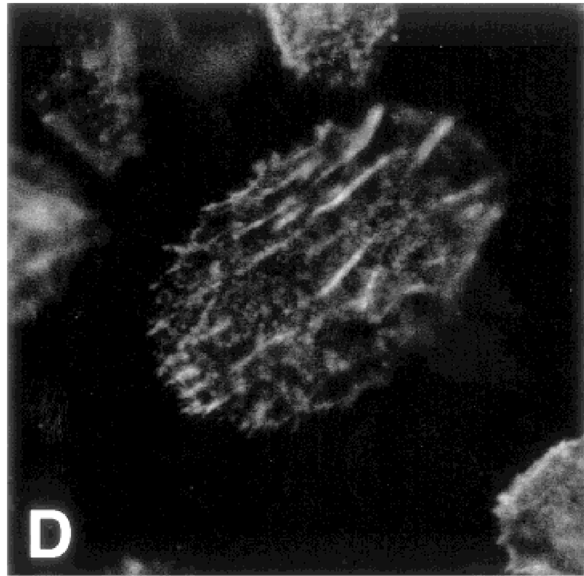
These two apparently independent events may be linked through the small GTP-binding proteins that include Rho, Rac, and Cdc42. These proteins are important players in focal adhesion assembly and actin cytoskeletal formation. Rac is involved primarily with the formation of lamellopodia and membrane ruffling while Cdc42 is found in filopodia (Hall, 1998). Rho is involved in cell adhesion and actin stress fiber formation in cultured Swiss 3T3 fibroblast cells. In addition to its GTP binding activity, Rho can be phosphorylated on tyrosine, serine, or threonine residues simultaneously or individually (Hall, 1998; Ridley and Hall, 1992, 1994).

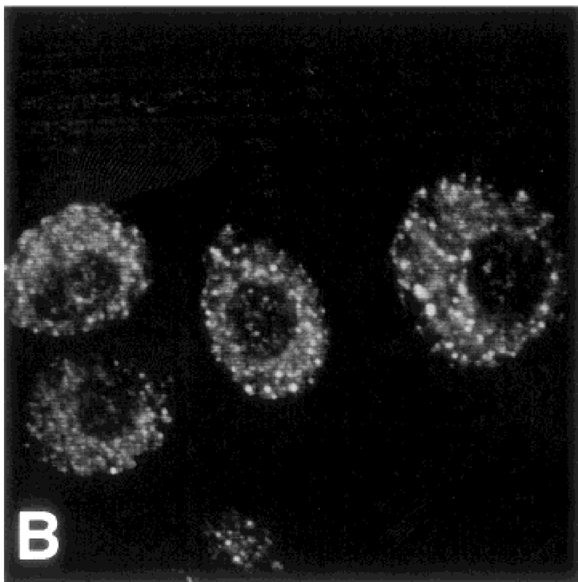
Rho shuffles between its GTP-bound active form and its GDP-bound inactive form. GTP-activating proteins (GAPs) bind Rho and hydrolyze GTP to GDP inactivating Rho. Guanine exchange factors (GEFs) release GDP from Rho-GDP, enabling it to bind GTP. Several GEF and GAP proteins have been identified in many cell systems (Hall, 1998). We have shown that p190RhoGAP becomes tyrosine phosphorylated very quickly in embryonic epithelia in response to ECM stimulation (Svoboda et al., 1998). Settleman's group demonstrated that Src phosphorylates p190RhoGAP (Cheng et al., 1995; Hu and Settleman, 1997). Phosphorylated p190RhoGAP binds to p120RasGAP (Foster et al., 1994) to form a complex with an additional unidentified 14kDa target protein (Hu and Settleman, 1997) in GST fusion protein assays. When this complex is formed, antibodies specific for phosphorylated tyrosines do not immunoprecipitate p190RhoGAP as the epitope is occupied by p120RasGAP. We hypothesize that this complex may decrease or slow down the GAP function (changing RhoGTP to RhoGDP) of these proteins, allowing RhoGTP to stay activated longer. The activated RhoGTP will have longer to interact with downstream kinases to increase actin polymerization and contraction. Further evidence that p190 influences Rho dependent actin bundle formation came from microinjection experiments in which p190 preferentially inhibited Rho-mediated stress fiber formation in fibroblasts (Ridley et al., 1993).

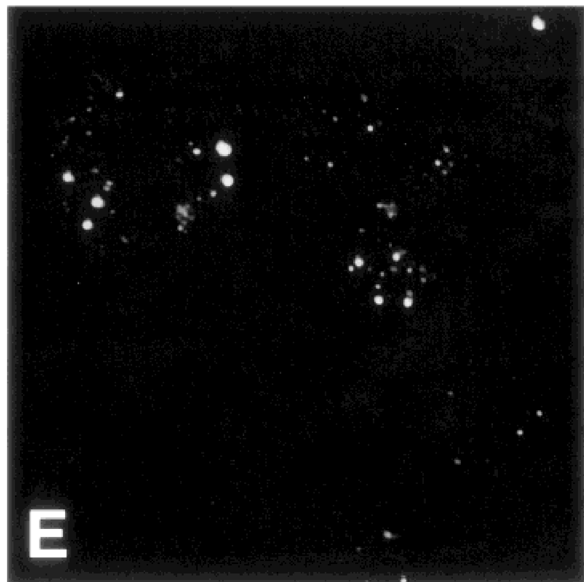
The yeast two-hybrid selection and affinity purification methods have shown that at least 20 different target proteins bind to RhoGTP (Hall, 1998). Several of these RhoGTP target proteins are serine/threonine kinases that have effects on actin polymerization, actin myosin contraction, and pH changes. We will concentrate our discussion to two specific target kinases: Rho associated coiled-coil containing protein kinase

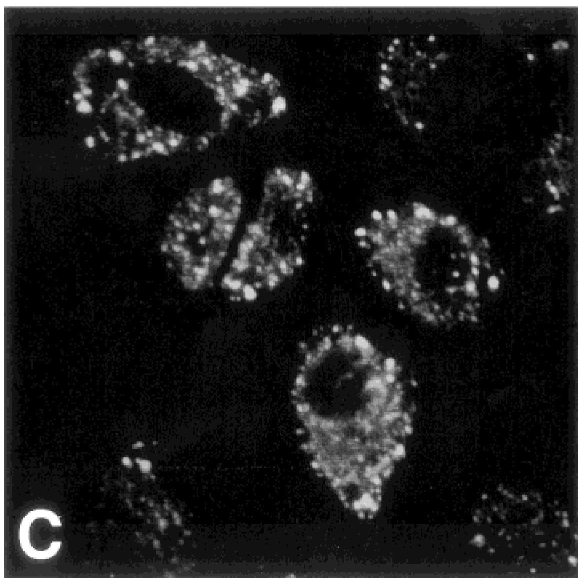
Fig. 1. The distribution of integrin receptor subunits in chondrocytes. Lateral sternal plates from day 14 and 17 sterna were incubated in specific monoclonal primary antibodies to the integrin subunits, α_3, α_2, and β_1, and detected with a secondary antibody conjugated to FITC. Whole lateral plates were mounted onto slides, then optically sectioned with a Leica confocal laser scanning microscope. No difference in protein distribution was observed between day 14 chondrocytes (A–H) and day 17 chondrocytes or hypertrophic chondrocytes. Nuclei (n) that did not stain were used to orient the tissue. At lower (**A**) and higher (**B**) magnifications, chondrocytes fixed and permeabilized in cold methanol demonstrated a punctate distribution of the α_3 integrin subunit (clone P1B5). This distribution appeared to be associated with chondrocyte plasma membranes. Distribution of the α_2 integrin subunit (clone P1E6) in chondrocytes fixed and permeabilized in cold methanol (**C**) was also associated with chondrocytes plasma membranes, although this protein was also observed in Golgi associated areas (C). The α_2 integrin subunit distribution was more obvious at a higher magnification and did not appear to differ when the chondrocytes were fixed in paraformaldehyde without subsequent permeabilization (**D**). Chondrocytes, fixed in paraformaldehyde and incubated in an anti-chicken β_1 antibody, demonstrated a plasma membrane associated distribution of the integrin subunit (**E,F**). A more punctate distribution of the β_1 integrin subunit (clone W1B10) was observed associated with plasma membranes and between dividing cells at a higher magnification (F). The distribution of the β_1 integrin subunit was also determined with the anti-human β_1 antibody (clone P4C10) (G,H). In permeabilized chondrocytes, the β_1 integrin subunit appeared cytoplasmic and associated with cell plasma membranes (**G**). In contrast, non-permeabilized chondrocytes demonstrated a punctate distribution of the β_1 integrin subunit associated with plasma membranes, and was not detected in the cytoplasm (**H**). Scale bars = 10 μm. (Reproduced from Hirsch et al., 1996, with permission of the publisher).











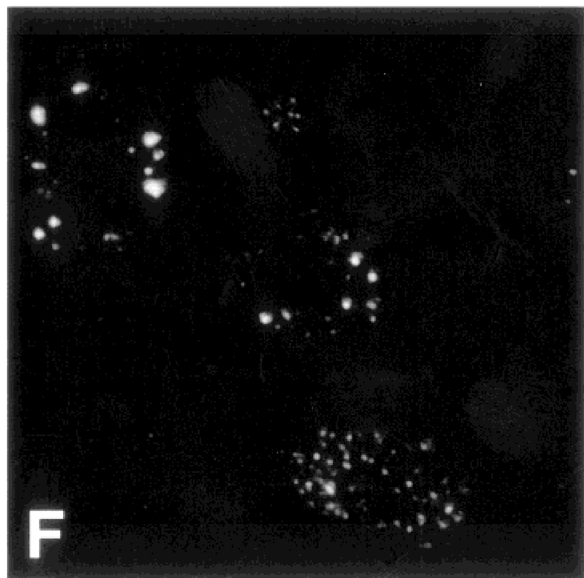


Fig. 2.

(p160ROCK) and p140Diaphanous (p140Dia). ROCK has been shown to activate myosin light chain kinase to increase actin-myosin contraction (Amano et al., 1997; Kimura et al., 1996). p140Dia has been shown to increase actin polymerization by binding to the actin binding protein, profilin, that works through the PIP_2 pathway (Watanabe et al., 1997).

One possible sequence of events may be that first contact with ECM causes integrin-mediated FAK phosphorylation, that in turn phosphorylates the surrounding proteins such as paxillin and Src. It is known that Src phosphorylates p190RhoGAP and inactivates or slows its GAP function, allowing RhoGTP to stay active longer. Activated RhoGTP binds to downstream kinases such as p160ROCK and p140Dia to increase actin polymerization and contraction. The actin reorganization aids integrin clustering, allowing more ECM binding to increase FAK and other signal transduction events (Fig. 3) that decrease apoptosis. As stated previously, as chondrocytes reside within an enriched matrix environment, this scenario would imply that they are constantly stimulated via the integrin mediated pathways; therefore, chondrocytes may have alternative negative feedback loops to keep from being over stimulated.

The general MAP kinase pathway is complicated, but briefly described for this review (Denhardt, 1996; Robinson and Cobb, 1997). Upstream signaling (either by integrins or transmembrane G-proteins) leads to an activation of kinases that activate small GTPases (Ras, Rac, Cdc42, Rho) via adapter proteins. The G-proteins activate Raf also known as mitogen-activated protein kinase-kinase-kinase (MAPKKK) or MEKK. Activated Raf stimulates (via serine/threonine phosphorylation) mitogen-activated kinase-kinase (MAPKK) or MEK. MEKs then in turn stimulate (via tyrosine and threonine phosphorylation) mitogen-activated protein kinase (MAPK) or extracellular signal regulated protein (erk). In the MEKK family, there are several Raf isoforms including Raf-1, B-Raf, and A-Raf. There are 5 MEKs with MEK 1/2 acting specifically on erk1/2. There are now several MAPKs including erk1/2, JNK/SAPK, p38, erk-3, and several p38-related homologues (see Denhardt, 1996; Robinson and Cobb, 1997, for recent reviews). The MAP kinase family can respond to a variety of extracellular signals including osmotic stress, heat shock, cytokines, and mitogens (Kortenjann and Shaw, 1995). The MAP kinases (erk-1 and erk-2) translocate to the nucleus after activation to regulate the expression of various transcription factors (c-myc, c-fos, and c-jun, tcf, srf, elk-1, apl, atfn) (Kortenjann and Shaw, 1995). Activation of the MAP kinase pathways may be a mechanism that integrins use to regulate gene expression that lead to the changes during cell spreading or migration that involve the erk 1/2 activation (Robinson and Cobb, 1997) or alternatively cell death by activating the JNK/SAPK or p38 kinases (Frisch and Ruoslahti, 1997).

Besides potentially interacting with the MAP kinase pathway, FAK can also interact with the phospholipid signaling pathway via PI_3 kinase. PI_3 kinase phosphorylates PIP (PI(4)phosphate) or PIP_2 (PI(4,5)bisphosphate) at the D3 position to generate respectively, PI(3,4)P2 or PI(3,4,5)P3 (Clark and Brugge, 1995). These phospholipid byproducts have been implicated in downstream signaling of cytoskeletal reorganization through interactions with profilin, gelsolin, and Rac (Divecha and Irvine, 1995). In addition to its phosphorylation properties, PI3 kinase can also act as a ser/thr protein kinase

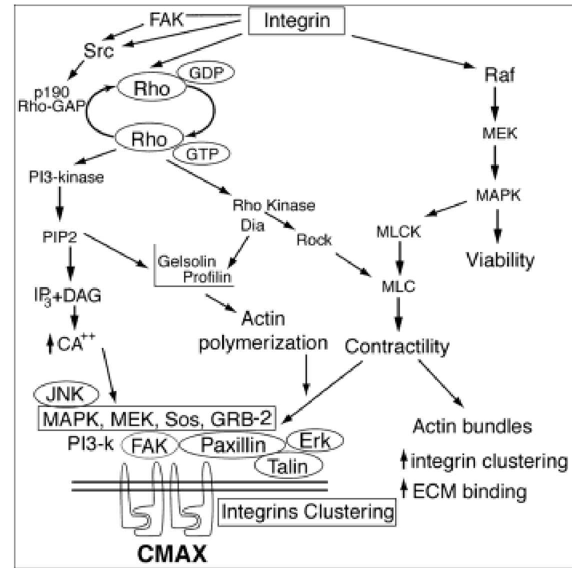


Fig. 3. Signaling pathways related to integrins and small GTPases. Pathways affecting cell motility and invasion or leading to assembly of cell-matrix attachment complexes (CMAX) are shown. RhoGTPases clearly affect cytoskeletal organization at several loci, as indicated in the diagram adapted from Keely et al. (1998). One current hypothesis is that ECM molecules bind to integrins (top); FAK autophosphorylates, then activates Src, paxillin, and Raf. Src phosphorylates p190RhoGAP that allows RhoGTP to activate p160 ROCK, p140Dia, and PI_3 kinase. p160ROCK increases actin/myosin contraction via myosin light chain kinase, while p140Dia binds profilin to increase actin polymerization. In the same time frame, PIP_2 gets converted to IP_3 and DAG, allowing release of intracellular Ca^{++}. The actin polymerization and contraction may lead to further integrin clustering, ECM binding, and amplification of the signal pathways. Abbreviations: Dia, p140 Diaphanous; ECM, extracellular matrix; FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen activated kinase-kinase; MLC, myosin light chain; MLCK, myosin light chain kinase; PI_3-K, phosphoinositide 3-kinase; PIP_2, phosphatidylinositol (4, 5)-bisphosphate; ROCK, Rho associated coiled-coil containing protein kinase.

Fig. 2. Actin associated protein distribution was normal in organ cultured chondrocytes. Single optical sections through lateral plates from whole sterna cultured for 8 days. The tissues were fixed, permeabilized, and immunolabeled using primary antibodies specific for β_1 integrin (clone W1B10, Sigma, St. Louis, MO; **A**), FAK (F15020, Transduction Laboratories; **B**), paxillin (clone 349, Transduction Laboratories; **C**), vinculin (clone VIN-11–5, Sigma; **D**), zyxin (polyclonal antibody from Dr. Mary Beckerle; **E**), or p-tyr (clone PY-20, Transduction Laboratories; **F**). The primary antibodies were detected with appropriate FITC conjugated secondary antibodies (Hirsch et al., 1996, 1997). F-actin (bundles) was stained with TRITC-phalloidin (A, D). β_1 integrin (A, punctate dots) was associated near the ends of actin filaments (A, linear bundles). Hypertrophic chondrocytes single labeled for FAK (B) demonstrated a punctate distribution throughout the cytoplasm. The distribution of paxillin (C) was diffuse throughout the cytoplasm yet demonstrated intense punctate areas of staining. Vinculin (D) was detected in “streaks” associated near the ends of F-actin (D, bundles) in a hypertrophic chondrocyte. In contrast, zyxin (E) and p-tyr (F) were detected in discrete areas of hypertrophic chondrocytes, that appeared to be close to plasma membranes. Micrographs have different magnifications. Scale bars = 5 μm.

(Divecha and Irvine, 1995). In NIH 3T3 mouse fibroblasts, FAK was found to be stably associated with PI3 kinase and tyrosine phosphorylated the p85 subunit of PI_3 kinase in vitro. The binding affinity between FAK and PI_3 kinase increased when FAK was autophosphorylated. During cell adhesion, there was increased tyrosine phosphorylation of the p85 subunit of PI_3 kinase (Chen and Guan, 1994). From these studies, it has been suggested that the SH2 domain on the p85 subunit of PI_3 kinase may play a role in its association with FAK (Clark and Brugge, 1995). In addition, activated Ras can interact directly to stimulate PI_3 kinase activity leading to increased 3-phosphorylated lipids (Divecha and Irvine, 1995).

Many of these signal transduction proteins and pathways have not been identified in chondrocytes. Chondrocytes are not motile cells, however, they do have small filapodia on the cell surface that project into the immediately surrounding extracellular matrix (Hirsch et al., 1997) termed the chondron by Poole. Articular chondrocyte chondrons contain types II, VI, IX, XI collagen, aggrecan, decorin, and fibronectin. In a recent confocal analysis of chondron development in agarose gel cultures, the proteins appeared as punctate regions near the cell membranes first, then spread into the surrounding matrix (Chang and Poole, 1997). Surface density mapping showed peaks and troughs for decorin, fibronectin, and type VI collagen, while aggrecan and type XI collagen had a uniform distribution (Chang and Poole, 1997). This provides the first evidence that matrix molecules may be "clustered" on chondrocyte cell surfaces. It has also been established that cultured chondrocytes adopt a flattened morphology and have vinculin and α-actinin at the ends and along actin bundles, respectively (Marchisio et al., 1984). Monolayer cultured chondrocytes have large actin stress fibers, produce fibronectin, and express $\alpha_5\beta_1$ and do not express α_3 or αv (Enomoto-Iwamoto et al., 1997). In our whole mount cartilage model, chondrocytes maintained a normal round morphology with actin filament bundles and CMAX protein distributions as expected for cells interacting with matrix molecules (Fig. 2) (Hirsch et al., 1996). The presence and association of these proteins in these three divergent models provide some evidence that equivalent signal transduction mechanisms may exist in chondrocytes in situ.

Recent experiments using a modified Boyden chamber system have shown that FN, types I and II collagen promoted haptotactic and chemotactic migration of chondrocytes that was tyrosine kinase dependent. In addition, the peptide GRGDSP inhibited chondrocyte migration to fibronectin but not to collagens, whereas chondrocytes migrated toward the tetra-RGD containing peptide, but not the peptide GRGDSP, in a dose dependent fashion, suggesting that cross-linking or clustering of integrins is essential to induce transmembrane signaling related to tyrosine phosphorylation (Shimizu et al., 1997).

PARATHYROID HORMONE SIGNALING IN CHONDROCYTES

Many hormones and growth factors regulate hyaline cartilage development (Tuan, 1998; Reddi, 1998; Underhill, 1998). One hormone with well-characterized signal transduction pathways that has a role in chondrocyte differentiation and survival is parathyroid hormone (PTH). PTH is an 84-amino acid protein secreted from the parathyroid gland. It acts to regulate calcium and phosphate homeostasis primarily through actions on specific receptors in kidney and bone (Juppner et al., 1991). The main function is to maintain the extracellular concentration of calcium and phosphorus. Native 1–84 PTH and the 1–34 PTH fragments equally stimulate adenylate cyclase through a G protein coupled PTH receptor (Fig. 4) (Friedlander and Amiel, 1994). PTH-related peptide (PTHrP) is a heterogeneous polypeptide having sequence homology to PTH in its 13 amino-terminal residues. While limited in homology, the peptide can bind to and activate the PTH receptor (Abou-Samra et al., 1989; Orloff et al., 1989; Friedlander and Amiel, 1994). PTH receptors have been localized on embryonic chick and rabbit cartilage cells (Kioke et al., 1990). In chondrocytes, PTH activates several second messenger systems leading to its physiological functions (Fig. 4) (Abou-Samra et al., 1994). PTH has a stimulatory effect on proliferation of chondroprogenitor cells (Lewinson and Silbermann, 1986) and inhibits collagen and matrix synthesis in avian epiphyseal cartilage via cAMP-dependent pathways (Pines and Hurwitz, 1990).

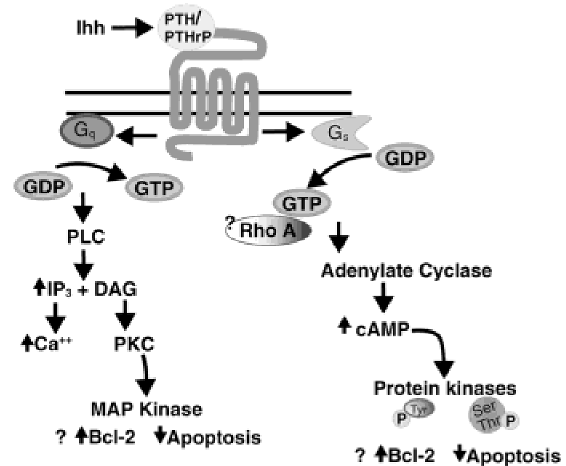


Fig. 4. The data on PTH/PTHrP influence on chondrocyte differentiation from the recent literature (Amling et al. 1997; Lanske et al., 1996; Vortkamp et al., 1996;) were assembled and placed into a hypothetical signal pathway schematic. Briefly, Vortkamp et al. (1996) demonstrated that Indian hedgehog (Ihh) is expressed in prehypertrophic chondrocytes and has a role in terminal hypertrophic differentiation. Ihh induces PTH/PTHrP to block terminal differentiation by increasing the expression of Bcl-2 (Amling et al., 1997). It is also known that the PTH/PTHrP receptor is a G-protein linked protein that increases cAMP via the Gs pathway and triggers the phospholipase C (PLC) via Gq or a related G protein (Turner et al., 1996).

PTHrP has been shown to decrease the maturation rate of embryonic articular cartilage through the homeobox protein Indian hedgehog (Ihh) (Vortkamp et al., 1996). It was recently shown that PTHrP increases the expression of the cell death inhibitor, Bcl-2 (Amling et al., 1997). Receptor gene knockout studies have shown that PTH signaling is required for normal chondrocyte differentiation and bone development (Lanske et al., 1996). Lanske et al. (1996) showed abnormal avian limb bud development when expression of the PTH/PTHrP

receptor was repressed in prehypertrophic chondrocytes. Together, the Vortkamp and Lanske studies suggest that there is stage dependent activity of the PTH/PTHrP receptor in developing cartilage. Their research suggests that PTHrP functions to suppress terminal differentiation, holding chondrocytes in a prehypertrophic stage.

The role of the perichondrium was recently studied in a whole organ culture of chick tibiotarsi (Long and Linsenmayer, 1998). Removing the perichondrium on one side of the tissue led to expansion of the type X collagen domain and increased proliferation near the site of perichondrium removal, but not near the areas that continued to have perichondrium, indicating that perichondrium has local effects on proliferation and differentiation. They also confirmed that adding PTH to the perichondrium free cultures decreased differentiation as measured by type X deposition into the matrix (Long and Linsenmayer, 1998).

Our experiments support this hypothesis as terminal differentiation was delayed in our whole sterna model (perichondrium was completely removed) when the tissue was treated with 10^{-7}M PTH. In addition, a single 24-hour treatment with 10^{-7}M PTH caused a significant growth increase early in the culture period but had no apparent effect on type X collagen expression (Lunsford et al., 1997). We also found that PTH decreased apoptosis in a dose-dependent manner predominately in the hypertrophic zone but to a lesser extent throughout the embryonic sternum (Lunsford et al., 1997).

Another study that examined the role of cell maturation on chondrocyte response to parathyroid hormone (PTH) (Schwartz et al., 1997) showed that PTH(1–34) had an effect on alkaline-phosphatase-specific activity (ALPase). The effects of PTH(1–34) on rat costochondral resting zone and growth zone chondrocytes were maturation dependent. Both cell types exhibited a rapid response to PTH, but only growth zone cells had a long-term response. Furthermore, the effects of PTH in growth zone chondrocytes were associated with changes in cyclic AMP and possibly one other pathway, whereas, in resting zone chondrocytes, the PTH effects were associated with changes in protein kinase C (PKC). (Schwartz et al., 1997).

In another related study, the expression level of the transcription factor, c-Jun, was lower in the maturing or hypertropic chondrocytes than in proliferating chondrocytes from the sterna. When hypertrophic cells were treated with parathyroid hormone (PTH), maturation was prevented and the expression levels of c-Jun and JunD were constitutively elevated. In addition, maturation-associated markers in hypertrophic cells were specifically lowered by exogenous expression of c-Jun or JunD to similar levels to those of proliferating cells or mature cells treated with PTH, indicating that Jun family proteins negatively regulate the maturation process of chondrocytes (Kameda et al., 1997).

Recently connections were made between the PTH and TGFβ superfamily. In TGFβ signaling systems, different type I receptor isoforms are dedicated to specific functions during embryogenesis. The bone morphogenetic proteins (BMPs), TGFβ superfamily members, play diverse roles in embryogenesis. Recently it was shown that two type I BMPRs, BMPR-IA and BMPR-IB, regulated distinct processes during chick limb development (Zou et al., 1997). BMPR-IB expression in the embryonic limb labeled future cartilage primordium, and its activity was necessary for the initial steps of chondrogenesis. During later chondrogenesis, BMPR-IA was specifically expressed in prehypertrophic chondrocytes. BMPR-IA regulated chondrocyte differentiation, serving as a downstream mediator of Indian hedgehog (Ihh) function in both a local signaling loop and a longer-range relay system to PTHrP. BMPR-IB also regulated apoptosis: Expression of activated BMPR-IB resulted in increased cell death, and dominant-negative BMPR-IB inhibited apoptosis. (Zou et al., 1997).

PROTEINS INVOLVED IN THE DEATH PATHWAY

During apoptosis, several transduction pathways and specific degradative enzymes become activated. The degradative enzymes are in the caspase-3 family of cysteinyl proteases also known as ICE-like as they resemble the first member described, interleukin-1 converting enzyme (ICE). These enzymes cleave essential structural components of the cell including actin cytoskeletal elements, nuclear lamins, and small nucleoproteins (Whyte, 1996). These changes eventually lead to nuclear material condensing as the nuclear DNA breaks down and cellular material pinches off, forming apoptotic bodies. The endonuclease-catalyzed, internucleosomal breakdown of nuclear DNA into 180- to 200-bp multiples is thought to signify the irrevocable commitment of cells to die via apoptosis (Wyllie, 1992). Although not all forms of physiological cell death are dependent on nuclear DNA breakdown, it has been widely used as a marker for apoptosis in many cell systems (Hughes and Gorospe, 1991; Tilly, 1994; Wyllie, 1992).

Although the degradative events that characterize apoptosis may be the same in all cell types, the specific signals that induce or suppress apoptosis may be different. Several specific proteins have been identified in cells progressing through apoptosis. Although several have been discussed previously in this review, a further discussion may clarify the role of these proteins. Some signal transduction events appear to protect cells while others lead to destruction. Bcl2 (B cell lymphoma protein) is one of the proteins that protect cells from apoptosis. This protein family was originally identified because overexpression led to increased viability and overpopulation of lympocytes causing lymphoid tumors. Bcl2 can inhibit the ICE-like proteases and interacts with a protein that promotes cell death, Bax. ICE-like proteases are expressed in a wide variety of cell types. ICE expression increases in cells that have been stressed with growth factor or extracellular matrix withdrawal (Boudreau et al., 1995).

Two members of the MAP kinase family are activated in the apoptosis pathway. The JNK (c-*j*un-*N*-terminal *k*inase) also known as the SAPK (*s*tress *a*ctivated *p*rotein *k*inase) that has been shown to be activated in apoptotic epithelial cells (Frisch et al., 1996; Frisch and Ruoslahti, 1997). This pathway appears to be modulated by Bcl-2, as the overexpression of Bcl-2 suppressed the ICE-like enzymes or caspases that were required for the activation of JNK. Recently it was

shown that the primary substrate for caspase is gelsolin, an actin severing protein (Kothakota et al., 1997). Cleavage of gelsolin created an N-terminal fragment that depolymerized actin in a Ca^{++} independent manner and was a precursor to cell death (Kothakota et al., 1997). A recent review of caspase-dependent and independent cell death explores several models with various signal transduction pathways (Green and Kroemer, 1998). As discussed previously, the pathways and mechanisms that control apoptosis have not been established for chondrocytes specifically, even though it is well known that a percentage of hypertrophic chondrocytes do expire by this mechanism (Gibson et al., 1995; Hirsch et al., 1997; Roach, 1997).

CONCLUSIONS AND FUTURE DIRECTIONS

In this brief review, several themes have become apparent. The first is that the control of chondrocyte differentiation and apoptosis pathways is complex with many different types of stimulatory and inhibitory mechanisms. Signal transduction proteins, lipids, and other factors are present in chondrocytes, however, the interactions between these elements have not been established. The chondrocyte is a unique developmental model as it has a defined sequence of maturation events that can be exploited to determine important factors. This unique tissue is surrounded by an enriched matrix and may have mechanisms in place to prevent over stimulation by secreted molecules. The research community has the tools and techniques to determine changes in gene expression as direct or indirect result of integrin or other ECM receptor stimulation. The research community has also benefited from the recent development of transgenic animals that have altered or depleted specific matrix molecules. These animals will provide a rich resource to further examine the signal transduction pathways leading to either differentiation or survival of chondroctyes as described for the type II collagen defective mouse (Yang et al., 1997). Unfortunately, most transgenic animals with disrupted signaling pathways or integrin subunits expire before the cartilage tissues develop.

In conclusion, although the research community has made strides to understand the mechanisms that control cellular response to the extracellular matrix and established that many cell types depend upon these interactions for survival, we still do not fully understand the signal transduction pathways that determine chondrocyte differentiation and survival.

NOTE ADDED IN PROOF

Two recent issues of Science contain articles relating to this review. In the June 26, 1998 issue two papers by the University of Texas Southwestern Medical Center group demonstrate that the heterotrimeric guanine nucleotide-binding proteins (G-proteins) G_{12} and G_{13} promote cell cycle progression and reorganization of the actin cytoskeleton. They further demonstrate that G_{13} directly activates a guanine nucleotide exchange factor (GEF) for the small GTPase, Rho (Kozasa et al., and Hart et al., Science 280:2109–2114, 1998). The August 28, 1998 issue of Science (vol 281) contains a special section on apoptosis including news and reviews on Death Receptors, Mitochondria, Caspases, and the Bcl-2 protein family from pages 1301 to 1326. In addition, the same issue has and Editorial, Perspective and Report on apoptosis.

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