Inhibition of Mitogen-activated Protein Kinase Kinase Induces Apoptosis of Human Chondrocytes*

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We previously have reported that the mitogen-activated protein kinase (MAPK) pathway is stimulated by adhesion of human chondrocytes to anti-β1-integrin antibodies or collagen type II in vitro. These mechanisms most likely prevent chondrocyte dedifferentiation to fibroblast-like cells and chondrocyte death. To investigate whether this pathway plays an essential role for the differentiation, phenotype, and survival of chondrocytes, we blocked mitogen-activated protein kinase/extracellular signal-regulated kinase (Erk) (MEK), a kinase upstream of the kinase Erk by using U0126. Exposure of chondrocytes to U0126 caused activation of caspase-3 in a dose-dependent manner. Western blot analysis with an antibody specific for dually phosphorylated Erk shows that collagen type II induced phosphorylation of Erk1/2 was specifically blocked by U0126 in a dose-dependent manner. Immunohistochemical analysis showed that treated chondrocytes were caspase-3 positive. In treated chondrocytes, the cleavage of 116-kDa poly(ADP-ribose)polymerase resulted in the 85-kDa apoptosis-related cleavage fragment and was associated with caspase-3 activity. Analysis by electron microscopy showed typical morphological signs of apoptosis, such as crescent-shaped clumps of heterochromatin, and a degraded pericellular matrix. Thus, these results indicate that the MEK/Erk signal transduction pathway is involved in the maintenance of chondrocyte differentiation and survival. These data stimulate further investigations on the role of mitogen-activated protein kinase pathways in human chondrocytes.

Apoptosis, or programmed cell death, plays a key role in embryogenesis, immunological competence and tissue homeostasis for cell removal and can distinguished biochemically and morphologically from cell necrosis, which is a passive, energy-stasis for cell removal and can distinguished biochemically and morphologically from cell necrosis, which is a passive, energy-dependent independent of cell death. Chondrocyte degradation and death occurs in enchondral ossification as well as in age-associated athropathies such as osteoarthritis (1, 2). Chondrocyte apoptosis, can be induced in vitro by a variety of agents, such as nitric oxide, oxygen radical scavengers (3), tumor necrosis factor (4), and interleukin-1β (5).

It is known that many cell types including chondrocytes require integrin mediated interactions with the extracellular matrix to survive, differentiate, and proliferate (6, 7). Cells undergo a specific cell death or apoptosis in the absence of specific matrix components (8). Interaction between chondrocytes and cartilage matrix components or anti-β1-integrin antibodies leads to a rearrangement of cytoskeletal and signaling proteins localized at focal adhesions and focal adhesion kinase (6–9). These stimulate docking proteins such as Src-homology collagen. Src-homology collagen then associates with growth factor receptor-bound protein 2, and extracellular signal-regulated kinase (Erk) (7). These mechanisms most likely prevent chondrocyte dedifferentiation to fibroblast-like cells and chondrocytes death (7–8, 10).

It is well known that one of the early reactions occurring in the cell after the damage of its DNA is the activation of poly(ADP-ribose)polymerase (PARP), a nuclear enzyme present in eukaryotes. Several lines of evidence show that PARP is involved in different cellular functions including DNA repair (11, 12), DNA replication (13), and programmed cell death (14, 15). During apoptosis, clumps of heterochromatin and nuclear fragmentation appear and PARP is one of the earliest proteins to be specifically cleaved from 116 kDa to two fragments of ~85 and 25 kDa (16).

A group of cysteine proteases called “caspases” play a central role in apoptosis. They are synthesized as proenzymes, containing an N-terminal prodomain. Activation occurs by cleaving this prodomain at a specific aspartic acid cleavage site that is localized between the prodomain and each subunit of the caspase. One caspase can process and activate the other in a cascade reaction beginning by initiator caspases, which interact with specific adaptor proteins. Initiator caspases activate effector caspases (e.g. caspase-3, -6, and -7) either directly or indirectly that cleave a number of structural and regulatory proteins including DFF45 (DNA fragmentation factor/inhibitor of caspase-activated DNases, PARP, lamin, and cytokertaines (17).

Mitogen-activated protein kinase (MAPK) p44 (Erk1) and p42 MAPK (Erk2) are important mediators of cellular responses to intracellular signaling proteins. MEK1 and MEK2 regulate the activity of Erk1/2 by phosphorylating threonine and tyrosine residues, and further upstream MAPK kinase

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1. The abbreviations used are: Erk, extracellular signal-regulated kinase; PARP, poly(ADP-ribose)polymerase; MAPK, mitogen-activated protein kinase; MEK, MAPK/Erk; IGF, insulin-like growth factor.
kinases (Raf) regulate the activity of MEK by phosphorylation of two serine residues. The activated Erk1/2 then translocates into the nucleus and regulates the activities of several nuclear transcription factors (18–22).

Furthermore, it is known that the MEK/Erk signaling pathway is involved in the cell growth and differentiation in many cell types. However, the role of MEK/Erk signaling pathway in chondrocyte function and differentiation is at present not fully understood. Therefore, in this study, we examined the essential role of Erk pathway on chondrocyte differentiation and survival by treating chondrocytes with the inhibitor U0126, which has been shown to specifically block mitogen-activated protein kinase/Erk kinase (MEK), the kinase upstream of Erk (23). Upon treatment with U0126, chondrocytes undergo apoptosis, suggesting that a functional death-mediating signaling cascade is associated with the extracellular matrix protein. Inhibition of MEK with inhibitors is a relatively new approach to clarify the role of these kinases in cellular functions.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Polyclonal anti-phospho-p42/p44 Erk1/2 antibodies and U0126 were purchased from Promega (Mannheim, Germany). Polyclonal anti-active caspase-3 antibody and monoclonal anti-PARP antibody were purchased from Becton Dickinson (Heidelberg, Germany). Anti-pan Erk1/2 antibodies were purchased from Transduction Laboratories (Heidelberg, Germany). Secondary antibodies conjugated with alkaline phosphatase were purchased from Roche Molecular Biochemicals (Mannheim, Germany).

Collagen type II, trypsin, pronase, and collagenase were purchased from Sigma (Munich, Germany). IGF-I was purchased from Biomol (Hamburg, Germany). Polyclonal anti-phospho-p42/p44 Erk1/2 antibodies and horseradish peroxidase-conjugated secondary antibodies (anti-active caspase-3 and normal rabbit IgG 1:30) in a moist chamber overnight at 4 °C; 7) rinsing; 8) incubation with dual system-APAAP-complex (1:50) for 30 min at room temperature; 9) rinsing; 10) new fuscin staining for 30 min at room temperature. Subsequently, the specimens were washed and dried, covered with Kaisers glycerin-gelatine and examined using a Zeiss Axiophot light microscope.

**Western Blot Analysis**—A detailed description of the culture technique used for the following experiments has been published previously (25). Briefly, the cells were fixed for 10 min. After counting, the cells were diluted to 1.5 × 10^6/ml in serum-free medium, plated on dishes coated with collagen type II stimulated with IGF-I and then treated with U0126 (0.1, 1, 10, 100 μM) for 1 h or left untreated. After rinsing, the cells were immunolabeled as follows: 1) incubation with serum (1:20 in Tris buffer) at room temperature for 10 min; 2) incubation with primary antibodies (anti-active caspase-3 and normal rabbit IgG 1:30) in a moist chamber overnight at 4 °C; 3) rinsing; 4) incubation with mouse-anti-rabbit IgG antibodies (1:50) at room temperature for 30 min; 5) rinsing; 6) incubation with dual system bridge antibodies (1:50) for 30 min at room temperature; 7) rinsing; 8) incubation with dual system-APAAP-complex (1:50) for 30 min at room temperature; 9) rinsing; 10) new fuscin staining for 30 min at room temperature. Subsequently, the specimens were washed and dried, covered with Kaisers glycerin-gelatin and examined under a Zeiss Axiophot 100 light microscope.
Effects of U0126 on Chondrocyte Adhesion to Collagen Type II—Human chondrocytes were cultured on collagen type II-coated Petri dishes, serum starved, stimulated with IGF-I and then either treated with U0126 or left untreated for the indicated times. The chondrocytes grown on collagen type II without U0126 exhibited a round to oval shape and numerous small ridge-like or cuspidal surface processes from the beginning of cultivation onwards (Fig. 3A). The chondrocytes grown on collagen type II with U0126 showed cell detachment, loss of cell processes, and membrane shrinkage. The number and density of the chondrocytes cultivated on collagen type II and in the absence of U0126 was significantly higher than those cultivated on collagen type II and treated with U0126. After a culture period of 1, 2, 4, 6, 8, 10, 12, and 14 h, the total number of treated chondrocytes grown on collagen type II was reduced by about 13% (p = 0.06), 35% (p = 0.009), 42% (p = 0.01), 55% (p = 0.008), 64% (p = 0.006), 66% (p = 0.002), and 71% (p = 0.005) compared with those cultivated on collagen type II and in the presence of IGF-I (Fig. 1).

Electron Microscopy of U0126-induced Changes in Chondrocytes—Fig. 2A shows an untreated human chondrocyte cultured on collagen type II and stimulated with IGF-I. Typical cartilage cells were mainly round to oval, contained a well-developed rough endoplasmic reticulum, a large Golgi apparatus, and other organelles or structures, such as mitochondria and small vacuoles. The nucleus was large and round, with minimal heterochromatin. The cells formed a pericellular freshly matrix, which was closely attached to the cell membrane. Cytoplasmatic processes were present. In contrast, a representative chondrocyte from an U0126-treated culture (Fig. 2B) had a cellular morphology typical of apoptosis, with nuclear changes including chromatin condensation into dense areas along the nuclear membrane and nuclear fragmentation. The cellular membrane was irregular (bleb formation), and cytoplasmic vacuoles (dilatation of mitochondria and endoplasmic reticulum) can be seen.

Induction of Chondrocyte Apoptosis by U0126—Human chondrocytes cultured on collagen type II with exposure to IGF-I undergo apoptosis after treatment with U0126. Immunohistochemical analysis with antibodies against cellular factors involved in apoptosis showed that treated chondrocytes were active caspase-3 positive (Fig. 3). In addition, to verify that the U0126-induced increase of chondrocyte apoptosis is dose-dependent, serum-starved human chondrocytes cultured on collagen type II were treated with various concentrations of U0126 (0.1, 1, 10 μM) and immunolabeled with anti-active caspase-3 antibodies. The results showed a marked dose-dependent increase in induction of activated caspase-3 in chondrocytes cultured on collagen type II after 1 h (Fig. 3B–D). In contrast, control cultures of chondrocytes treated with IgG maintained their nuclear morphology throughout the culture period (Fig. 3A). The total number of caspase-3 positive chondrocytes grown on collagen type II, treated with IGF-I was increased in the presence of 0.1 μM U0126 by about 27% (p = 0.0076), of 1 μM U0126 by 55% (p = 0.094), and of 10 μM U0126 by about 74% (p = 0.095) compared with those cultivated on collagen type II, treated with IGF-I and in the absence of U0126.

U0126 Changes in the Activity of Extracellular Signal-regulated Kinase 1 and 2—It has been shown that a reduction in the Erk signaling pathway stimulates the apoptotic pathway in different cell types (26). For this reason, we examined whether the inhibition of Erk1/2 activity plays a role in transmitting the apoptotic signals in human chondrocytes. Anti-pan Erk1/2 antibody recognizes both inactive and active forms of Erk1/2 and indicates the expression level of total Erk1/2. Anti-phospho-Erk1/2 recognizes only phosphorylated Erk1/2 and indicates the activation of Erk1/2. Western blot analysis with anti-activated Erk1/2 from chondrocytes cultured on collagen type II stimulated with IGF-I in the presence of 1 μM U0126 demonstrated a significant decrease in Erk phosphorylation (44-kDa band completely abolished), compared with chondrocytes cultured on collagen type II stimulated with IGF-I and in the absence of U0126 (Fig. 4B). In contrast, Western blot analysis with a pan-Erk antibody, which recognizes both the phosphorylated and nonphosphorylated forms of Erk1 and 2, did not change in response to U0126 treatment (Fig. 4A). Densitometric analysis of the results from immunoblotting performed in triplicate from adhesion of IGF-I-treated chondrocytes to colla-
gen type II with antibodies against phospho-Erk1/2 showed that in the presence of 0.1 μM U0126 the relative Erk1/2 proteinform expression had fallen by 43%/51% (p<0.002/p<0.01), in the presence of 1 μM U0126 the relative Erk1/2 proteins expression had fallen by 89%/99.9% (p=0.0063/p=0.0048), and in presence of 10 μM U0126 the relative Erk1/2 proteins expression had fallen by 97%/99.9% (p=0.0019/p=0.0031) (compared with adhesion of untreated chondrocytes to collagen type II).

**Discussion**

Signaling by integrins or transmembrane G proteins leads to activation of the Ras-MAP kinase signaling pathway. Erk1/Erk2, a downstream kinase of MAPK pathway, regulates the expression of various transcription factors. Activation of MAPK pathway may be a mechanism by which integrins regulate gene expression (6). This study addresses the important role of the Ras-MAP kinase signaling pathway in the stimulation of human chondrocyte differentiation. In a previous study (7), we could show that activation of MAPK pathway regulates the activity of a number of intracellular signaling proteins through phosphorylation. We demonstrated that collagen binding integrins and activated IGF-I receptor coimmunoprecipitate with intracellular signaling adaptor proteins such as Src-homology collagen, and this common target forms the Src-homology collagen/Erk2/Erk-complex leading to Ras-mitogen-activated protein kinase signaling pathway activation (7). We suggested that this mechanism most likely prevents chondrocyte dedifferentiation to fibroblast-like cells and chondrocyte death.

To investigate the consequences of inhibition of MAP kinase pathway, we used a specific inhibitor of the MAPK signaling pathway, U0126, which can inhibit the phosphorylation and

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**Figure 3. Immunodetection of active caspase-3 by APAAP method.** Serum-starved human chondrocytes were plated on dishes coated with collagen type II stimulated with IGF-I and then treated with various concentrations of U0126: 0, 0.1, 1, and 10 μM for 1 h. The cells were immunolabeled with anti-active caspase-3 antibodies. The results showed a marked dose-dependent increase in induction of activated caspase-3 (arrows) in chondrocytes (B-D). In contrast, control cultures of chondrocytes treated with IgG maintained their nuclear morphology throughout the culture period (A).

**Figure 4. Activity profiles of Erk1/2 after treatment with U0126.** Serum-starved human chondrocytes were plated on dishes coated with collagen type II stimulated with IGF-I and then treated with various concentrations of U0126: 0, 0.1, 1, and 10 μM for 1 h. The cells were immunolabeled with anti-Erk1/2 (A) and anti-phospho-Erk1/2 (B). The results showed a significant dose-dependent decrease of phosphorylated Erk1/2.

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**Figure 5. Detection of PARP and its apoptotic fragment in chondrocytes exposed to U0126.** Serum-starved human chondrocytes were plated on dishes coated with collagen type II stimulated with IGF-I and then treated with various concentrations of U0126: 0, 0.1, 1, 10, 100, and 1000 μM for 1 h. Intact PARP and the 85-kDa fragment were analyzed by Western blotting using the monoclonal anti-PARP antibody. After 1 h of treatment with different doses of U0126, the 85-kDa fragment was visible, and this process was dose-dependent.

**Blot:** anti-PARP

increased with increasing concentration of U0126 in culture. Taken together, these results indicate that the proteolysis of PARP by U0126 is dose-dependent.

**Discussion**

Inhibition of MEK
activation of the Erk1/Erk2 in a dose-dependent manner. We demonstrated that specific inhibition of Erk1/Erk2 resulted in activation of caspase-3 and cleavage of PARP in human chondrocytes in vitro. Therefore the specific inhibition of Ras-mitogen activated kinase leads to apoptosis because activation of caspase-3 and cleaving of PARP are common features of apoptosis (27, 28).

After 1 h of treatment with U0126, we found caspase-3 labeling in the nucleus of chondrocytes. In fact, it is suggested according to recent experiments that pro-caspase-3 is localized to the nucleus of chondrocytes. In fact, it is suggested that caspase-3 and cleaving of PARP are common features of apoptosis (31). It is known that activated caspase-3 cleaves PARP into an NH₂-terminal 24-kDa fragment containing the DNA-binding domain and an about 85-kDa COOH-terminal fragment containing the automodification and catalytic domains, and PARP cleavage seems to be characteristic of later “irreversible” stages of apoptosis (32). This was used to distinguish apoptotic and necrotic cell death (15). According to other investigators (32), the PARP cleavage product was accompanied by the reduction of intact PARP. Caspase-3, in its activated form is the main cause of apoptotic modification, such as PARP degradation. Intact PARP inhibits endonucleases that cleave DNA during apoptosis (33).

At the moment, one can only speculate about the particular mechanisms leading to caspase-3 activation. As mentioned above Erk1/Erk2 translocates into the nucleus and regulates the activities of several nuclear transcription factors. Inhibition of Erk1/Erk2 prevents translocation of it into the nucleus, because of that the expression of genes that code for proapoptotic proteins may increase or genes that code for antiapoptotic proteins may be repressed. Interruption of the MAPK pathway by inhibition of Erk1/Erk2 may also prevent the activation of proapoptotic factors as previously described (34). Indeed, they have further reported that apoptosis correlated with a reduced activity of Erk signaling for ovarian granulosa cells. A group of proteins regulating apoptosis are members of the Bcl2 superfamily, which comprises a large number of pro- and antiapoptotic molecules mainly located in the mitochondrial outer membrane and therefore partly inducing mitochondrial changes, e.g. cytochrome c release (35–36). The ratio of pro- (Bax, Bak, Bid, Bad . . . ) versus antiapoptotic (Bcl2, Bcl-xl , Bcl-w ), Bcl2 superfamily members determines how cells respond to death signals (35). In osteoarthritic cartilage the expression of Bcl2 is lower than in healthy cartilage (37). Bcl2 is also down-regulated in serum-free cultured human articular chondrocytes (38) and in articular chondrocytes in transgenic mice lacking the expression of type II collagen (39).

Several observations suggest that pathological conditions that cause degradation of cartilage may induce apoptosis (40, 41). It seems that chondrocyte apoptosis is involved in several cartilage diseases, e.g. osteoarthritis (37, 40), rheumatoid arthritis (42), chondrodysplasias, and chondrosarcomas (38). Recently it was reported that the pathogenesis of osteoarthritis may also result from the inappropriate response of chondrocytes to catabolic and anabolic stimuli from mitogens, i.e. growth factors and hormones (43). The effects of particular growth factors (e.g. IGF-I) are mediated by MAPK pathway (7). It was found that the mRNA of a special protein Egr-1 (early growth response-1) was significantly down-regulated in samples from osteoarthritic cartilage. The Egr-1 protein binds to a specific GC-rich sequence in the promoter region of many target genes hence regulating their expression (43). Several studies have indicated that interruption of integrin/extracellular matrix interactions especially between integrins and collagen type II results in apoptosis of chondrocytes (10, 39).

It is important to know the apoptosis pathways of chondrocytes because inhibition of chondrocyte apoptosis may be of therapeutic value after cartilage injury and in arthritis. Apoptosis is also important in physiological conditions such as chondrogenesis and during enchondral ossification. It was recently reported (44) that growth-arrest-specific gene 2 (gas 2) might play an important role in regulating chondrocyte proliferation and differentiation. They observed further that exactly at the same time when mesenchymal cells of mouse limb bud interdigital tissues die by apoptosis, the gene product of gas 2 was cleaved by caspases and suggested that caspase-3 might be involved in gas 2 cleavage. Expression of gas 2 is coupled to increased susceptibility to apoptosis. Furthermore, it has been suggested that apoptosis of chondrocytes during development may control chondrocyte number in cartilage tissue (45).

In the present study, we demonstrate that chondrocytes underwent apoptosis after treatment with U0126, a novel inhibitor of MEK1/2, suggesting that MAP kinase pathway has a regulatory function in chondrocytes differentiation and survival.

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Inhibition of MEK