0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(3):1391–1397

doi: 10.1210/en.2004-1152

Dexamethasone Induces Apoptosis in Proliferative Chondrocytes through Activation of Caspases and Suppression of the Akt-Phosphatidylinositol 3′-Kinase Signaling Pathway

Dionisios Chrysis, Farasat Zaman, Andrei S. Chagin, Masaharu Takigawa, and Lars Sävendahl

Department of Woman and Child Health (D.C., F.Z., A.S.C., L.S.), Pediatric Endocrine Unit, Astrid Lindgren Children's Hospital, Karolinska Institutet, SE-17176 Stockholm, Sweden; and Department of Biochemistry and Molecular Dentistry (T.M.), Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8525, Japan

Although glucocorticoids are known to induce apoptosis in chondrocytes, the mechanisms for this effect and the potential antiapoptotic role of IGF-I are unknown. To address this, we studied the effects of dexamethasone (Dexa) on apoptosis in the HCS-2/8 chondrocytic cell line. Dexa (25 μM) increased apoptosis (cell death ELISA) by 39% and 45% after 48 and 72 h, respectively ($P < 0.01$ and $P < 0.05$, respectively). IGF-I (100 ng/ml) decreased Dexa-induced apoptosis to levels similar to control cells. Apoptosis was associated with cleavage of poly-ADP-ribose polymerase (PARP) and α-fodrin and activation of caspases-8, -9, and -3 (Western), an effect that was counteracted when chondrocytes were cocultured with Dexa + IGF-I. Inhibitors for caspases-8, -9, and -3 (50 μM each) equally suppressed Dexa-induced apoptosis ($P < 0.01$). Time-response experiments showed that caspase-8 was activated earlier (at 12 h) than caspase-9 (at 36 h). We studied the phosphatidylinositol 3′-kinase (PI3K) pathway to further investigate the mechanisms of Dexa-induced apoptosis. Dexa decreased Akt phosphorylation by 93% ($P < 0.001$) without affecting total Akt and increased the p85α subunit 4-fold. The Akt inhibitor SH-6 (10 μM) increased apoptosis by 54% ($P < 0.001$). When combining Dexa with SH-6, apoptosis was not further increased, showing that Dexa-induced apoptosis is mediated through inhibition of the PI3K pathway. Addition of IGF-I to SH-6- or Dexa + SH-6-treated cells decreased apoptosis by 21.2% ($P < 0.001$) and 20.6% ($P < 0.001$), respectively. We conclude that Dexa-induced apoptosis is caspase dependent with an early activation of caspase-8. IGF-I can rescue chondrocytes from Dexa-induced apoptosis partially through the activation of other pathways than the PI3K signaling pathway. Based on our *in vitro* data, we speculate that *in vivo* treatment with glucocorticoids may diminish longitudinal growth by increasing apoptosis of proliferative growth plate chondrocytes. (*Endocrinology* 146: 1391–1397, 2005)

THE USE OF glucocorticoids in humans and animals is well known to be associated with impaired longitudinal growth (1, 2). The observed growth retardation is accompanied by morphological changes in the growth plate, the structure responsible for longitudinal growth. It becomes thinner as a result of decreased chondrocyte proliferation and increased apoptosis, mainly of terminal hypertrophic chondrocytes (1, 3–5). *In vivo* and *in vitro* studies have shown that glucocorticoids decrease chondrocyte proliferation, alter their synthetic capacity, and subsequently affect the cartilage matrix (3, 4, 6).

We have previously shown in rats that *in vivo* administration of dexamethasone (Dexa) increases apoptosis mainly in terminally differentiated hypertrophic chondrocytes (5). In these experiments, we also observed that Dexa increased apoptosis of resting and proliferative chondrocytes even though the magnitude was less than in hypertrophic cells. A premature loss of early proliferative growth plate chondrocytes by apoptosis could diminish the growth potential and contribute to the incomplete catch-up growth often observed after long-term treatment with glucocorticoids (7, 8).

The apoptotic pathway is complex with multiple components. Important steps in this cascade are the caspase enzymes, a family of proteins responsible for the degradation of targeted cells to undergo apoptosis. Apoptosis can occur after the activation of the initiator caspase-8, which subsequently activates caspase executors (extrinsic) or after the activation of the initiator caspase-9 (intrinsic), or through interplay between these two pathways (9). In addition to these two caspase-dependent pathways, there is also caspase-independent apoptosis (10). The phosphatidylinositol 3′-kinase (PI3K)/Akt pathway is viewed as a key player for cell survival in different cell systems (for reviews, see Refs. 11 and 12). Inhibition of the PI3K pathway and subsequently Akt phosphorylation seem to be important mechanisms of Dexa-induced apoptosis (13, 14). Moreover, growth retardation has been reported in knockout mice for *Akt1*, which demonstrates that Akt is also important for normal growth (15).

IGF-I promotes growth by increasing cell proliferation and survival. IGF-I counterbalances glucocorticoid-mediated growth retardation and increases body weight in Dexa-treated rats (16), most likely by affecting proliferation, hy-

First Published Online December 2, 2004

Abbreviations: Dexa, Dexamethasone; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; PARP, poly-ADP-ribose polymerase; PI3K, phosphatidylinositol 3′-kinase; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

pertrophy, and cell survival. The antiapoptotic actions of IGF-I are well established mainly *in vitro* but also *in vivo* (17–19). IGF-I exerts these actions in a variety of cells challenged to different apoptotic stimuli, mainly through the activation of the Akt signaling pathway (20).

This study was designed to investigate whether Dexa causes apoptosis in proliferative chondrocytes, the mechanisms for this action, and the potential antiapoptotic role of IGF-I. Because of technical limitations of *in vivo* experiments in the growth plate, we chose to study the effects of Dexa in proliferative HCS-2/8 cells. This is a well-described human chondrocytic cell line that maintains all the characteristics of chondrocytes and can be differentiated into hypertrophic chondrocytes (21).

Materials and Methods

Reagents

Dexa, IGF-1, and BSA were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). The stock solution of Dexa (25 mM) was initially prepared in ethanol and diluted 1:1000 to reach a final medium concentration of 25 μM. Human recombinant IGF-I was prepared as a stock solution (100 μg/ml; in PBS with 0.1% BSA) and diluted 1:1000 (100 ng/ml in test medium). Inhibitors for caspase-3 (Z-DEVD-FMK), caspase-8 (Z-IETD-FMK), and caspase-9 (Z-LEHD-FMK) were all purchased from R&D Systems (Minneapolis, MN), dissolved in dimethylsulfoxide and diluted (1:1000) to reach a final concentration of 50 μM in the medium. Wortmannin KY12420 (PI3K inhibitor) and SH-6 (Akt inhibitor) were purchased from Calbiochem (San Diego, CA) and both used at a final concentration of 10 μM. Polyvinylidene difluoride membrane, and the ECL^{Plus} chemiluminescent system for Western immunoblots were purchased from Amersham Biosciences UK Ltd. (Buckinghamshire, UK). Protein molecular standards from Bio-Rad Laboratories (Hercules, CA).

Chondrocyte cell culture

The chondrocytic cell line, HCS-2/8 (human chondrosarcoma derived) established by Takigawa *et al.* (21), was maintained in DMEM mixed with F12 media (DMEM/F12) supplemented with 20% fetal bovine serum (FBS) and 20 μg/ml gentamycin in humidified air with 5% CO_2. The cells were subcultivated every week and given fresh medium every 3–4 d. For Western immunoblotting, the cells were seeded in 75-cm^2 tissue culture flasks (1.5×10^6 cells/flask) in DMEM/F12 medium containing 20% FBS. After 72 h, when cells were approximately 80% confluent, they were washed with 1× PBS and the medium was changed to test medium specific for each experiment. Trypsine, PBS, EDTA, FBS, and DMEM/F12 medium were all purchased from Invitrogen Life Technologies (Paisley, Scotland, UK).

Cell proliferation assay

We used the cell proliferation reagent 4-[3-(4-iodophenyl)-2(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) kit purchased from Roche Diagnostics GmbH (Mannheim, Germany), which is a colorimetric assay for the quantification of cell proliferation and cell viability. This assay is based on a water-soluble tetrazolium salt, which is cleaved to formazan by mitochondrial enzymes. The amount of the formazan dye formed is directly correlated to the number of metabolically active cells. The cells were plated in 96-well plates (2×10^4 cells/well), and cultured for 72 h in DMEM/F12 with 20% FBS added. The cells were then washed once with 1× PBS before the medium was changed to test medium, which contained 1% FBS and for each experiment specific concentrations of effectors. After treatment, WST-1 was added, 20 μl/well (final dilution 1:10), and the reaction mixture was incubated for 1 h at 37 C before reading absorbance at 450 nm. Each experiment included three wells and was repeated four times.

Cell death detection ELISA

Apoptosis was studied with the detection and quantification of cytoplasmic histone-associated DNA fragments (mono- and oligonucleasomes) by photometric enzyme immunoassay (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics). The cells were first treated as described for the proliferation assay and then lysated (80 μl lysis buffer) and centrifuged. The supernatant was removed for analysis of cytoplasmic histone-associated DNA fragments according to the manufacturer's instructions. In preliminary experiments, we excluded necrosis to occur under our experimental conditions by the negative determination of mono- and oligonucleasomes in the supernatant of the cell cultures. Three samples were analyzed for each experiment, which was repeated four times.

RT-PCR for type II and X collagens

Extraction of total RNA from HCS-2/8 proliferative chondrocytes treated for 24, 48, and 72 h with and without Dexa (25 μM) was performed by using QIAGEN RNeasy mini kit (VWR International AB, Stockholm, Sweden). The following pair of primers were used to generate the respective cDNAs: type II collagen, 5′-ATGACAATCTGGCTCCCAACACTGC-3′ and 5′-GACCGGCCCTATGTCCACACCGAAT-3′ giving a PCR product of 364 bp; human type X collagen, 5′-AGCCAGGGTTGCCAGGACCA-3′ and 5′-TTTTCCCACTCCAGGAGGGC-3′ (387 bp). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CAATgACCCCTTCATTgACC-3′ and 5′-CCTgCTTCACCACCTTCTTg-3′ (694 bp), was used as control to normalize the data. RT-PCR was performed as it has been previously described (22). The amplification conditions for type II and type X collagen were as follows: 96 C for 5 min, 96 C for 30 sec with 35 cycles of denaturation, annealing for 1 min at 55 C (30 sec at 54 C, 30 cycles for GAPDH) extension for 1 min at 72 C (1 min at 72 C for GAPDH) and final extension for 5 min at 72 C. PCR products were separated by electrophoresis in a 2% agarose gel with ethidium bromide (15 μg/10 ml). RNA from HCS-2/8 chondrocytes, 18 d in culture under differentiation conditions was used as a positive control for type X collagen.

Western immunoblotting

After each treatment, both floating and adherent cells were lysated in RIPA buffer [150 mM NaCl, 10 mM Tris (pH 7.2), 0.1% sodium dodecyl sulfate, 1.0% Triton X-100, 1% deoxycholate, and 5 mM EDTA] and the protein concentration was measured by the Bradford protein assay (Bio-Rad Laboratories AB, Sundbyberg, Sweden). Proteins were separated on 7.5% Tris-HCl or 4–20% gradient acrylamide gels (Bio-Rad Laboratories). Proteins were transferred on Hybond-P polyvinylidene difluoride-transfer membrane. The membranes were blocked with 5% Blotto, nonfat dry milk (Santa Cruz Biotechnology Inc., Santa Cruz, CA) in Tris-buffered saline with 0.1% Tween 20, for 1 h at room temperature. The membranes were probed overnight at 4 C (gentle shaking) with primary antibodies against PARP (1:1000 dilution), caspase-3 (1:1000 dilution), Akt 1/2 (1:1500 dilution) or the p85α subunit of PI3K (1:1000 dilution), all purchased from Santa Cruz Biotechnology Inc. Additional primary antibodies including caspase-8 (1:1000 dilution), human-specific caspase-9 (1:500 dilution), α-fodrin (Asp 1185, 1:500 dilution), and phospho-Akt (Ser-473, 1:1000 dilution) were purchased from Cell Signaling Technology (Beverly, MA). The blots were probed with the corresponding secondary antibody, goat-antirabbit Ig-horseradish peroxidase (HRP) (Transduction Laboratories, Lexington, KY, 1:10000 or 1:15000 dilutions), goat-antimouse IgG-HRP (1:10000), or rabbit-antigoat IgG-HRP (1:5000 dilution), all purchased from Santa Cruz Biotechnology, Inc. The ECL^{Plus} Western blotting detection system was used according to the manufacturer's instructions. The resulting bands were confirmed by comparing the size of the protein in the cell extract with known molecular weight markers. The antigen-antibody complexes were then detected by chemiluminescence. After films had been developed, blots were stained with Coomassie Blue to ensure equal loading of total protein. Each experiment was repeated at least three times.

Statistical analysis

Results are presented as the mean ± SEM. Differences between the groups were tested by one-way ANOVA. All P values were calculated using the Newman-Keuls posttest. A P value <0.05 was considered significant.

Results

Effect of Dexa on chondrocyte proliferation and apoptosis

HCS-2/8 chondrocytes were cultured under semiconfluent conditions for 24, 48, and 72 h in the presence of 25 μM Dexa. The treatment with Dexa decreased cell proliferation rate after 24 h (60.4 ± 10.90%, $P < 0.05$ *vs.* control), 48 h (61.30 ± 0.65%, $P < 0.01$ *vs.* control), and 72 h (63.70 ± 1.85%, $P < 0.001$ *vs.* control). Apoptosis was significantly increased after treatment with Dexa (25 μM) for 48 h (139.5 ± 8.90%; $P < 0.01$ *vs.* control) and 72 h (145.1 ± 15.47%; $P < 0.05$ *vs.* control) but not after 24 h (114.1 ± 6.24%; $P > 0.05$ *vs.* control; Fig. 1). The Dexa-induced decrease in chondrocyte proliferation and increase in apoptosis showed clear dose-dependencies (Fig. 2, A and B).

To rule out whether our experimental conditions may influence the differentiation process of HCS-2/8 cells, we analyzed by PCR the expression of type II and X collagen after 24, 48, and 72 h of culture. We found that type II collagen was expressed at all time points and that type X collagen, a marker of differentiating chondrocytes, was not expressed at any time point for both control and Dexa-treated cells. These data indicate that Dexa treatment did not influence the rate of chondrocyte differentiation when exposed for up to 72 h. Control experiments showed that HCS-2/8 chondrocytes can express type X collagen when cultured under conditions promoting cell differentiation for 18 d (data not shown).

We then investigated whether IGF-I can counteract the antiproliferative and apoptotic actions of Dexa in cultured HCS-2/8 chondrocytes. Initial experiments with IGF-I only (100 ng/ml) showed that cell proliferation was stimulated (112.5 ± 3.4% of control; $P < 0.05$ *vs.* control; Fig. 3A) and spontaneous apotosis was decreased (85.0 ± 5.8% of control; $P < 0.05$ *vs.* control; Fig. 3B). When IGF-I (100 ng/ml) was combined with Dexa (25 μM), the antiproliferative effect of Dexa was partially counteracted ($P < 0.05$ *vs.* Dexa; Fig. 3A). Whereas cells cultured with Dexa only demonstrated increased apoptosis (134.8 ± 1.28%; $P < 0.001$ *vs.* control), those cocultured with IGF-I plus Dexa had less apoptosis (110.2 ± 4.42% of control cells; $P < 0.001$ *vs.* Dexa; $P > 0.05$ *vs.* control; Fig. 3B).

To confirm our apoptosis data (cell death detection ELISA), we studied the proteins PARP and α-fodrin, both well known to be cleaved during caspase-dependent apoptosis. Indeed, Dexa-induced apoptosis was accompanied by

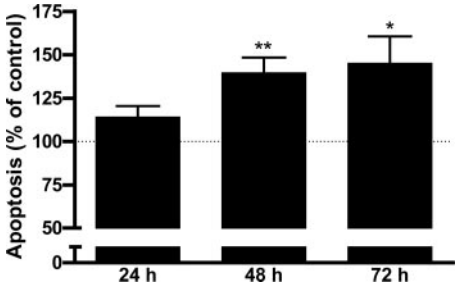

FIG. 1. HCS-2/8 cells were treated with 25 μM Dexa for 24, 48, and 72 h. Apoptosis was measured with cell death ELISA, and the levels are expressed as percent of control-treated cells. Dexa increased apoptosis after 48 h (**, $P < 0.01$) by 39.5%, and after 72 h (*, $P < 0.05$) by 45% when compared with the control group.

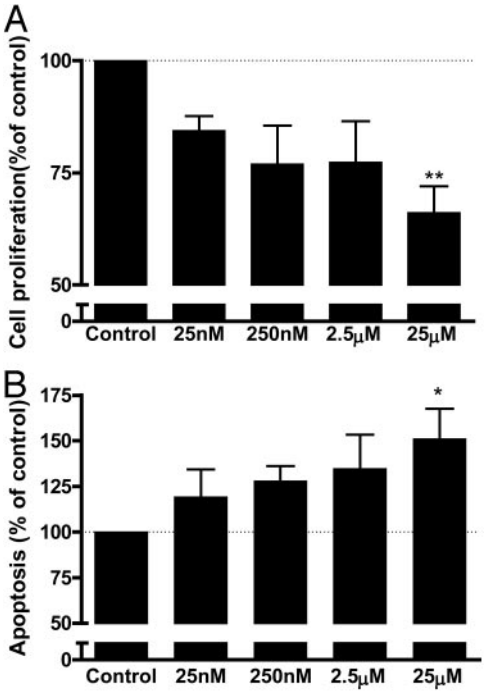

FIG. 2. Dose-response effects of Dexa (72 h) on cell proliferation (A) and apoptosis (B). Cell proliferation was measured with the WST-1 cell proliferation assay and apoptosis with a cell death ELISA; see *Materials and Methods*. Results are expressed as percent of control-treated cells. Dexa had a clear dose-dependent inhibitory effect on cell viability (A) and a dose-dependent proapoptotic effect (B). *, $P < 0.05$; **, $P < 0.01$ *vs.* control, respectively.

a 2.5-fold increase in cleaved PARP (28.0 ± 1.3 and 11.0 ± 0.5 optical units in Dexa and control groups, respectively; $P < 0.001$ *vs.* control; Fig. 4A) and a 3-fold increase in cleaved α-fodrin (40.0 ± 2.4 and 13.0 ± 0.6 optical units in Dexa and control groups, respectively; $P < 0.001$ *vs.* control; Fig. 4B). In contrast, IGF-I prevented Dexa-induced cleavages of PARP and α-fodrin, and the levels were similar as in control-treated cells (Fig. 4, A and B).

Dexa activates the caspase cascade

We then studied whether Dexa-induced apoptosis is associated with caspase activation (Western immunoblotting). We found that Dexa (25 μM; 72 h incubation) increased cleavage of caspase-3, whereas significantly less cleaved caspase-3 was detected in cells treated with Dexa + IGF-I (16.0 ± 0.9 *vs.* 4.0 ± 0.2 optical units; $P < 0.001$; Fig. 4C). Cells treated with IGF-I (100 ng/ml) alone had no detectable cleaved caspsase-3 (Fig. 4C). Our next step was to analyze the activation of the caspase initiators, caspases-8 and -9. We found that Dexa (25 μM; 72 h) activated both these caspase initiators and that cotreatment with IGF-I (100 ng/ml) decreased the levels to what was detected in control cells. The 41-kDa fragment of caspase-8 was increased 3-fold in the Dexa group when compared with control (34.0 ± 1.5 and 11.0 ± 0.8 optical units, respectively; $P < 0.001$; Fig. 4C) and the addition of IGF-I returned it to control levels. Similarly, the cleaved product of caspase-9 (37-kDa fragment) was increased 2.7-fold by Dexa and cotreatment with IGF-I significantly decreased cleaved caspase-9 (100.0 ± 4.8 and 37.0 ± 2.9 optical

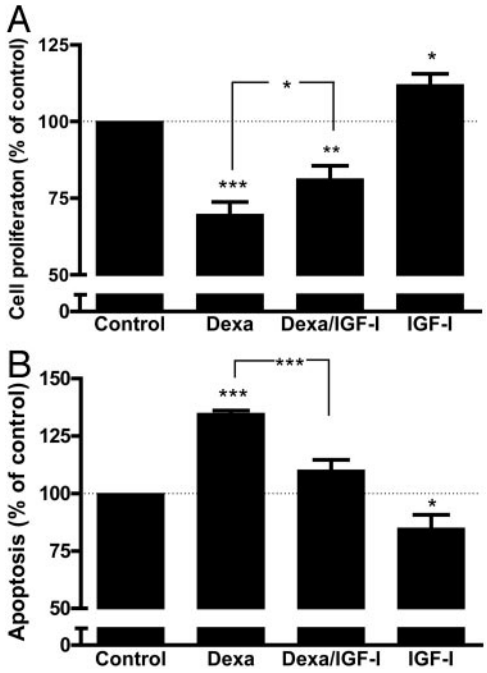

FIG. 3. Effects of IGF-I on HCS-2/8 cell proliferation (WST-1 cell proliferation assay) and apoptosis (cell death ELISA) after 72 h of cotreatment with or without Dexa. A, IGF-I (100 ng/ml) alone increased basal cell proliferation by 13% (*, $P < 0.05$ *vs.* control). The addition of IGF-I to Dexa (25 μM)-treated cells improved cell proliferation by 17% (*, $P < 0.05$ *vs.* Dexa), but still cell viability was significantly less than in control (**, $P < 0.01$). B, IGF-I alone decreased basal apoptosis by 15% (*, $P < 0.05$ *vs.* control). Cotreatment with Dexa and IGF-I decreased apoptosis by 18% (***, $P < 0.001$ *vs.* Dexa) and reached levels similar to control ($P > 0.05$), whereas Dexa increased apoptosis by 35% (***, $P < 0.001$ *vs.* control).

units, respectively; $P < 0.001$) to levels similar as in control cells (Fig. 4C).

We performed experiments with caspase-inhibitors to further confirm that Dexa-induced apoptosis is caspase dependent (Fig. 5). Cells treated with Dexa alone (25 μM, 72 h), had increased apoptosis compared with control-treated cells (151.0 ± 4.1% of control, $P < 0.001$). On the other hand, cells cotreated with Dexa and caspase-3 inhibitor had less apoptosis (72.7 ± 4.1% of control; $P < 0.001$ *vs.* Dexa, $P > 0.05$ *vs.* control). Similar results were obtained with the inhibitors for caspase-8 (100.2 ± 17.5% of control, $P < 0.01$ *vs.* Dexa), and caspase-9 (108.7 ± 13.9% of control, $P < 0.01$ *vs.* Dexa). None of the caspase inhibitors affected cell proliferation (data not shown). When the HCS-2/8 chondrocytes were treated with caspase inhibitors alone, apoptosis was slightly decreased when compared with control-treated cells (caspase-8 inhibitor: 71.6 ± 3.1%, caspase-9 inhibitor: 87.9 ± 5.4%, and caspase-3 inhibitor: 79.1 ± 10.5%, $P < 0.01$ *vs.* control).

To rule out which caspase-initiator is activated first, we determined the levels of caspase-8 and -9 in HCS-2/8 chondrocytes treated with Dexa (25 μM) for 0, 12, 24, 36, or 48 h. As seen in Fig. 6, these studies showed a clear time dependency where caspase-8 was activated earlier (12 h) than caspase-9 (36 h).

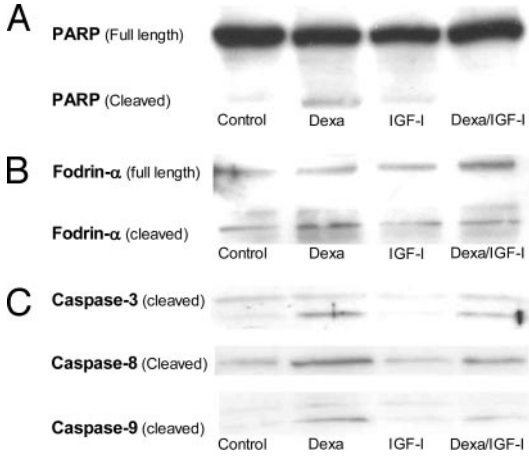

FIG. 4. Cells were treated for 72 h with Dexa (25 μM), IGF-I (100 ng/ml), or the combination of Dexa and IGF-I. A, Western immunoblots revealed that Dexa induced cleavage of PARP, whereas the addition of IGF-I restored it. B, The same results were obtained with another protein substrate for caspases, fodrin-α. C, Representative Western blots for caspases-8, -9, and -3. The cleaved products of all these caspases were increased after treatment with Dexa and decreased by the addition of IGF-I.

Inhibition of Akt phosphorylation by Dexa induces apoptosis

The PI3K/Akt pathway is considered as a key component of cell survival in different cell systems. To investigate whether the PI3K pathway is important for the apoptotic actions of Dexa in chondrocytes, Akt phosphorylation was studied. Phosphorylated Akt was significantly decreased in Dexa- (7.0 ± 2.9% of control, $P < 0.001$ *vs.* control) and Dexa/IGF-I-treated cells (26.7 ± 2.9% of control, $P < 0.001$ *vs.* control, $P < 0.01$ by *t* test *vs.* Dexa), whereas total Akt did not change (Fig. 7A). IGF-I alone increased phosphorylated Akt by 23% ($P < 0.01$ *vs.* control). Moreover, the p85α subunit of PI3K, well known to inhibit Akt phosphorylation, was increased more than 4-fold after treatment with Dexa (21.0 ± 3.4 and 5.0 ± 1.0 optical units in Dexa and control groups, respectively; $P < 0.01$).

Our next step was to study apoptosis after blocking Akt phosphorylation with the specific Akt inhibitor SH-6. Under our experimental conditions, phosphorylation of Akt was

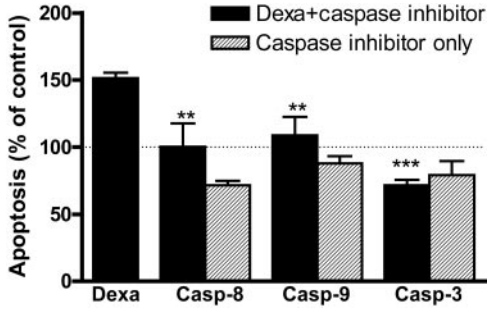

FIG. 5. Cells were treated for 72 h with Dexa (25 μM) alone, with Dexa and caspase inhibitors (50 μM; *filled bars*), or with each caspase inhibitor alone (*hatched bars*). Dexa alone increased apoptosis by 51.3%, whereas all caspase inhibitors in coculture with Dexa decreased apoptosis to control levels (**, $P < 0.01$ *vs.* Dexa; ***, $P < 0.001$ *vs.* Dexa). Caspase inhibitors alone slightly decreased spontaneous apoptosis compared with control-treated cells ($P < 0.01$).

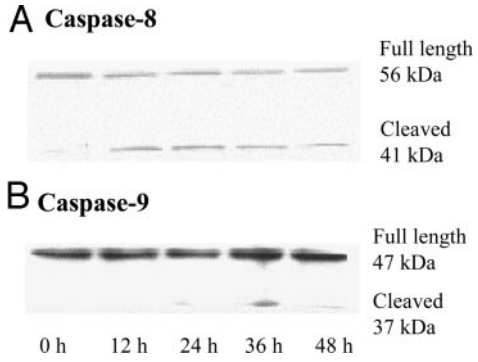

FIG. 6. To assess which caspase initiator is activated first, HCS 2/8 cells were cultured with 25 μM Dexa for 12, 24, 36, and 48 h. The control was untreated cells at time point 0. Western blots were performed at all time points for caspase-8 (A) and caspase-9 (B). Cleaved products of caspase-8 (p41 kDa) were detected as early as after 12 h, whereas those from caspase-9 (p37 kDa) were detected first after 36 h.

almost abolished when cells were treated with SH-6, whereas IGF-I weakly increased Akt phosphorylation (Western immunoblotting; data not shown). As seen in Fig. 7B, SH-6 alone (10 μM) increased apoptosis compared with control-treated cells (154.0 ± 0.7% of control, $P < 0.001$), an effect that was counteracted when SH-6 was combined with IGF-I (121.2 ± 0.7% of control, $P < 0.001$ *vs.* SH-6). On the other hand, the addition of Dexa to SH-6-treated cells had no further effect on apoptosis (156.6 ± 1.4% of control, $P > 0.05$ *vs.* SH-6; Fig. 7B). In contrast, the addition of IGF-I to SH-6/Dexa-treated cells decreased apoptosis compared with SH-6/Dexa (124.2 ± 1.4% of control; $P < 0.001$ *vs.* SH-6/Dexa; Fig. 7B), but still apoptosis remained higher than in control ($P < 0.001$). As expected, Dexa alone increased apoptosis (151.6 ± 4.6%; $P < 0.001$ *vs.* control), whereas IGF-I decreased apoptosis (82.6 ± 3.1%; $P < 0.01$ *vs.* control).

We used another PI3K inhibitor, Wortmannin, to confirm that IGF-I is able to exert antiapoptotic actions despite the Akt pathway had been blocked. After 72 h in culture with 10 μM Wortmannin, apoptosis was significantly increased compared with control-treated cells (327.0 ± 3.2% of control; $P < 0.001$ *vs.* control), an effect that was partially prevented by IGF-I (239.5 ± 5.7% of control; $P < 0.001$ *vs.* Wortmannin) but still remained higher than in control ($P < 0.001$). As expected, total Akt was not changed, but phosphorylation of Akt was almost abolished by Wortmannin and partially restored by IGF-I (data not shown).

Discussion

We here show that Dexa not only decreases chondrocyte proliferation but also increases apoptosis, whereas IGF-I as a growth-promoting factor counteracts both these effects. The observed apoptosis is caspase dependent and includes activation of both caspase initiators: caspase-8 and -9. We also demonstrate that inhibition of Akt phosphorylation is a crucial step involved in Dexa-induced apoptosis.

Or data show that Dexa induces apoptosis in the human HCS-2/8 chondrocytic cell line in a dose-dependent manner. In addition, this was not an early effect because increased apoptosis was detected first after 48 h treatment, as it has been observed in bovine articular chondrocytes (23). Moreover, human growth plate proliferative chondrocytes are known to express caspase-8 (24), and rat proliferative chondrocytes express caspases-3 and -6 (25). Altogether, this indicates that these cells have the machinery for apoptosis and have the potential to activate it after being exposed to a suitable stimulus. Loss of proliferative chondrocytes by apoptosis after treatment with glucocorticoids could have important clinical significance because it may diminish growth potential in children. This premature loss of proliferative chondrocytes could account for the incomplete catch-up

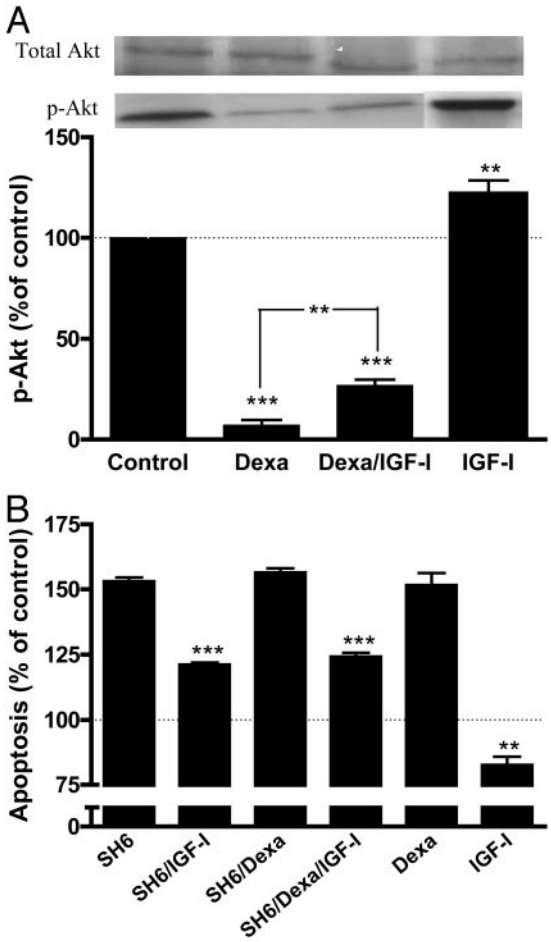

FIG. 7. Dexa caused apoptosis in HCS-2/8 cells through inhibition of Akt phosphorylation. A, Cells were treated for 72 h with 25 μM Dexa, with Dexa plus IGF-I (100 ng/ml), or IGF-I alone. Western immunoblots revealed no changes of total Akt. In contrast, Dexa inhibited phosphorylation of Akt (***, $P < 0.001$ *vs.* control) and the addition of IGF-I had only a weak effect (***, $P < 0.001$ *vs.* control, **, $P < 0.01$ by *t* test *vs.* Dexa). IGF-I alone, increased phosphorylation of Akt compared with control cells (**, $P < 0.01$ *vs.* control). B, The PI3K pathway was blocked with the specific inhibitor of Akt phosphorylation SH-6 (10 μM) and apoptosis was studied by cell death ELISA. SH-6 caused a significant increase in apoptosis ($P < 0.001$ *vs.* control) and cotreatment with IGF-I (100 ng/ml) decreased apoptosis (***, $P < 0.001$ *vs.* SH-6). On the other hand, addition of Dexa (25 μM) to SH-6-treated cells did not change apoptosis ($P > 0.05$ *vs.* SH-6), but again IGF-I added to SH-6/Dexa-treated cells decreased apoptosis (***, $P < 0.001$ *vs.* SH-6/Dexa) to a similar level as in cells treated with SH-6/IGF-I ($P > 0.05$). Dexa alone increased apoptosis to the same degree as SH-6- or SH-6/Dexa-treated cells. IGF-I alone (100 ng/ml) decreased spontaneous apoptosis compared with control cells (**, $P < 0.01$ *vs.* control).

growth and diminished final height which has been reported after glucocorticoid excess (8). In this study, we focused on a pharmacological dose of Dexa (25 μM), not only because it reflects the clinical use of glucocorticoids and therefore their side effects, but also because Dexa has been reported to have anabolic effects at lower concentrations (26). Although our present *in vitro* data are in correlation with other *in vitro* (23) and *in vivo* studies (5), one should be careful when drawing definitive conclusions from *in vitro* data and extrapolating these to human disorders.

We here show that apoptosis is caspase dependent in HCS-2/8 chondrocytes because the caspase initiators, caspase-8 and -9, and the executor caspase-3 are all activated. This is further supported by our finding that coincubation with each caspase inhibitor totally blocked Dexa-induced apoptosis. Furthermore, our experiments reveal that caspase-8 is activated earlier than caspase-9. This suggests that caspase-9 is activated through caspase-8 as it has been reported in other cell systems after Dexa treatment (27). The delay in caspase-9 activation could be because it requires the cleavage of Bid by caspase-8, which then translocates to the mitochondria and there releases cytochrome *c* (28).

Several mechanisms have been reported by which Dexa causes apoptosis in different cell lines, including inhibition of the PI3K pathway and decreased phosphorylation of Akt (13, 14, 29–31). Our experiments clearly show that Dexa inhibits Akt phosphorylation in HCS-2/8 chondrocytes. In addition, when we blocked the Akt signaling pathway, Dexa had no additive effect on apoptosis. The p85α subunit, which inhibits the catalytic activity of the PI3K, has previously been reported to be increased by Dexa (14, 32). We investigated weather the abrogation of Akt phosphorylation is a result of an increase in the p85α subunit. Indeed, we found that Dexa causes a dramatic increase in the p85α subunit, thus providing a mechanism for Dexa-induced inhibition of the PI3K signaling pathway and subsequent inhibition of Akt phosphorylation.

IGF-I has antiapoptotic effects in many cell types in response to different apoptotic stimuli mainly through the activation of the PI3K/Akt pathway (17, 20). Therefore, we hypothesized that IGF-I can rescue chondrocytes from Dexa-induced apoptosis. Our data demonstrate that IGF-I counteracts the antiproliferative and proapoptotic actions of Dexa in the human chondrocytic cell line HCS-2/8. The IGF-I prevention of Dexa-induced apoptosis was associated with restoration of PARP and α-fodrin cleavage, which are products of caspase activation, and abrogation of caspases-8, -9, and -3 activation. Several studies have shown that the PI3K signaling pathway and subsequently the phosphorylation of Akt are very important targets for the antiapoptotic actions of IGF-I (17, 20, 33, 34). The involvement of other pathways facilitating the antiapoptotic actions of IGF-I have been demonstrated also in other cell types (35–37). Our findings in HCS-2/8 chondrocytes clearly demonstrate that IGF-I protects chondrocytes from Dexa-induced apoptosis. This effect is most likely partially mediated through other pathways than the PI3K/Akt signaling pathway because, when we blocked this pathway with SH-6, IGF-I was able to significantly reduce apoptosis. On the other hand, we observed some restoration of Akt phosphorylation after IGF-I cotreatment and Wortmannin significantly reduced the antiapoptotic actions of IGF-I, suggesting that the PI3K/Akt signaling pathway may mediate some of the antiapoptotic actions of IGF-I.

In summary, our results show that Dexa decreases not only proliferation of chondrocytes but also increases apoptosis through inhibition of Akt phosphorylation and therefore inhibition of the PI3K/Akt signaling pathway. IGF-I counteracts these actions of Dexa resulting in increased chondrocyte proliferation and decreased apoptosis. Based on our data in HCS chondrocytes, we speculate that treatment with IGF-I or GH may rescue growth plate chondrocytes from apoptosis and thereby prevent loss of growth potential during long-term treatment with glucocorticoids. Further *in vivo* studies are needed to confirm whether this is the case.

Acknowledgments

Received August 30, 2004. Accepted November 24, 2004.

Address all correspondence and requests for reprints to: Dionisios Chrysis, Pediatric Endocrinology Unit, Q2: 08, Astrid Lindgren Children's Hospital, Karolinska Hospital, SE-17176 Stockholm, Sweden. E-mail: Dionisios.Chrysis@kbh.ki.se.

References

1. **Altman A, Hochberg Z, Silbermann M** 1992 Interactions between growth hormone and dexamethasone in skeletal growth and bone structure of the young mouse. Calcif Tissue Int 51:298–304
2. **Allen DB** 1996 Growth suppression by glucocorticoids therapy. Endocrinol Metab Clin North Am 25:699–717
3. **Annefeld M** 1992 Changes in the rat epiphyseal cartilage after treatment with dexamethasone and glycosaminoglycan-peptide complex. Pathol Res Pract 188:649–652
4. **Kember NF, Walker KV** 1971 Control of bone growth in rats. Nature 229: 428–429
5. **Chrysis D, Ritzen EM, Sävendahl L** 2003 Growth retardation induced by dexamethasone is associated with increased apoptosis of the growth plate chondrocytes. J Endocrinol 176:331–337
6. **Dearden LC, Mosier HD, Brundage Jr M, Thai C, Jansons R** 1986 The effects of different steroids on costal and epiphyseal cartilage of fetal and adult rats. Cell Tissue Res 246:401–412
7. **Baron J, Klein KO, Colli MJ, Yanovski JA, Novosad JA, Bacher JD, Cutler Jr GB** 1994 Catch-up growth after glucocorticoid excess: a mechanism intrinsic to the growth plate. Endocrinology 135:1367–1371
8. **Magiakou MA, Mastorakos G, Chrousos GP** 1994 Final stature in patients with endogenous Cushing's syndrome. J Clin Endocrinol Metab 79:1082–1085
9. **Barnhart BC, Alappat EC, Peter ME** 2003 The CD95 typeI/type II model. Sem Immunol 15:185–193
10. **Lockshin RA, Zakeri Z** 2004 Caspase-independent cell death? Oncogene 23: 2766–2773
11. **Datta SR, Brunet A, Greenberg ME** 1999 Cellular survival: a play in three Akts. Genes Dev 13:2905–2927
12. **Kandel ES, Hay N** 1999 The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. Exp Cell Res 253:210–229
13. **Leis H, Page A, Ramirez A, Bravo A, Segrelles C, Paramio J, Barettino D, Jorcano JL, Perez P** 2004 Glucocorticoid receptor counteracts tumorigenic activity of Akt in skin through interference with the phosphatidylinositol 3-kinase signailing pathway. Mol Endocrinol 18:303–311
14. **Singleton JR, Baker BL, Thorburn A** 2000 Dexamethasone inhibits insulin-like growth factor signaling and potentiates myoblast apoptosis. Endocrinology 141:2945–2950
15. **Chen WS, Xu PZ, Gottlob K, Chen ML, Sokol K, Shiyanova T, Roninson I, Weng W, Suzuki R, Tobe K, Kadowaki T, Hay N** 2001 Growth retardation and increased apoptosis in mice with homozygous disruption of the akt1 gene. Genes Dev 15:2203–2208
16. **Tomas FM, Knowles SE, Owens PC, Chandler CS, Francis GL, Read LC, Ballard FJ** 1992 Insulin-like growth factor-I (IGF-I) and especially IGF-I variants are anabolic in dexamethasone-treated rats. Biochem J 282:91–97
17. **Butt AJ, Firth SM, Baxter RC** 1999 The IGF axis and programmed cell death. Immunol Cell Biol 77:256–262
18. **Chrysis D, Calikoglu AS, Ye P, D'Ercole AJ** 2001 Insulin-like growth factor-I overexpression attenuates cerebellar apoptosis by altering the expression of Bcl family proteins in a developmentally specific manner. J Neurosci 21:1481–1489

19. **Wilkins HR, Ohneda K, Keku TO, D'Ercole AJ, Fuller CR, Williams KL, Lund PK** 2002 Reduction of spontaneous and irradiation-induced apoptosis in small intestine of IGF-I transgenic mice. Am J Physiol 283:G457–G464
20. **Vincent AM, Feldman EL** 2002 Control of cell survival by IGF signaling pathways. Growth Horm IGF Res 2002 12:193–197
21. **Takigawa, M, Tajima K, Pan HO, Enomoto M, Kinoshita A, Suzuki F, Takano Y, Mori Y** 1989 Establishment of a clonal human chondrosarcoma cell line with cartilage phenotypes. Cancer Res 49:3996–4002
22. **Martensson K, Chrysis D, Savendahl L** 2004 Interleukin-1β and TNF-α act in synergy to inhibit longitudinal growth in fetal rat metatarsal bones. J Bone Miner Res 19:1805–1812
23. **Van Offel JF, Schuerwegh AJ, Bridts CH, Stevens WJ, De Clerck LS** 2002 Effect of biphosphonates on viability, proliferation, and dexamethasone-induced apoptosis in articular chondrocytes. Ann Rheum Dis 61:925–928
24. **Cetin E, Girsch W, Brand G, Thurnher D, Cetin EM, Trieb K** 2004 Distinct expression of APO-1/Fas and caspase-8 in the human growth plate. Calcif Tissue Int 74:181–186
25. **Chrysis D, Nilsson O, Ritzen EM, Savendahl L** 2002 Apoptosis is developmentally regulated in rat growth plate. Endocrine 18:271–278
26. **Kubota S, Moritani NH, Kawaki H, Mimura H, Minato M Takigawa M** 2003 Transcriptional induction of connective tissue growth factor/hypertrophic chondrocyte specific 24 (CTGF/Hcs24) gene by dexamethasone in human chondrocytic cells. Bone 33:694–702
27. **Marchetti MC, Di Marco B, Cifone G, Migliorati G, Riccardi C** 2003 Dexamethasone-induced apoptosis of thymocytes: role of glucocorticoids receptor-associated Src kinase and caspase-8 activation. Blood 101:585–593
28. **Li H, Zhu H, Xu CJ, Yuan J** 1998 Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94:491–501
29. **Distelhorst CW** 2002 Recent insights into the mechanism of glucocorticoids-induced apoptosis. Cell Death Differ 9:6–19
30. **Ogawa M, Nishiura T, Oritani K, Yoshida H, Yoshimura M, Okajima Y, Ishikawa J, Hashimoto K, Matsumura I, Tomiyama Y, Matsuzawa Y** 2002 Cytokines prevent dexamethasone-induced apoptosis via the activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways in a new multiple myeloma cell line. Cancer Res 60:4262–4269
31. **Hsu JH, Shi Y, Hu L, Fisher M, Franke TF, Lichtenstein A** 2002 Role of the AKT kinase in expansion of multiple myeloma clones: effects on cytokine-dependent proliferative and survival responses. Oncogene 21:1391–1400
32. **Giorgino F, Pedrini MT, Matera L, Smith RJ** 1997 Specific increase in p85α expression in response to dexamethasone is associated with inhibition of insulin-like growth factor I stimulated phosphatidylinositol 3-kinase activity in cultured muscle cells. J Biol Chem 272:7455–7463.
33. **Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME** 1997 Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 275:661–665
34. **Oh CD, Chun JS** 2003 Signaling mechanisms leading to the regulation of differentiation and apoptosis of articular chondrocytes by insulin-like growth factor-1. J Biol Chem 278:36563–36571
35. **Parizzas M, Saltiel AR, LeRoith D** 1997 Insulin-like growth factor I inhibits apoptosis using the phosphatidylinositol 3′-kinase and mitogen-activated protein kinase pathways. J Biol Chem 272:154–161
36. **Grey A, Chen Q, Xu X, Callon K, Cornish J** 2003 Parallel phosphatidylinositol-3 kinase and p42/44 mitogen-activated protein kinase signaling pathways subserve the mitogenic and antiapoptotic actions of insulin-like growth factor I in osteoblastic cells. Endocrinology 144:4886–4893
37. **Thimmaiah KN, Easton J, Huang S, Veverka KA, Germain GS, Harwood FC, Houghton PJ** 2003 Insulin-like growth factor I-mediated protection from rapamycin-induced apoptosis is independent of Ras-Erk1-Erk2 and phosphatidylinositol 3′-kinase-Akt signaling pathways. Cancer Res 63:364–374

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**