

Expression of HIF Prolyl Hydroxylase Isozymes in Growth Plate Chondrocytes: Relationship Between Maturation and Apoptotic Sensitivity

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The overall goal of the current study was to examine the functional activity of the prolyl hydroxylases (PHDs) in maturing chondrocytes. Herein, we show for the first time that the PHDs are expressed in the maturing zone of the growth plate, and by a chondrocytic cell line. We determined if this protein and its substrate, hypoxia inducible factor (HIF)-1 α , modulated the induction of apoptosis. Using a chondrocyte cell line that matured in culture, we inhibited HIF-1 α expression using siRNA technology and pharmacologically blocked PHD activity. We noted that PHD suppression sensitized the cells to an apoptotic challenge with H₂O₂. We next examined the interplay between the PHDs and HIF-1 α by suppressing HIF-1 α and blocking PHD activity. We noted reduced killing when the mature HIF-silenced cells were challenged with H₂O₂. In contrast, there was limited change in the viability of immature cells. Based on these differences in chondrocyte susceptibility, it is concluded that HIF-1 α sensitizes maturing cells to H₂O₂-mediated killing. We next determined if this change in the viability of the PHD-inhibited cells was linked to changes in activation of caspase-3. It was noted that there was a minimal change in enzyme activity of the PHD-inhibited HIF-1 α suppressed cells. Finally, we found that as the chondrocytes mature, the activities of catalase and SOD were significantly reduced and that there was a decrease in the levels of Bcl-2 and Bcl_{XL}. This loss of protective activity together with the changes mediated by HIF would be expected to generate conditions that would favor the induction of chondrocyte apoptosis. *J. Cell. Physiol.* 210: 257–265, 2007. © 2006 Wiley-Liss, Inc.

In skeletal tissues, cartilage serves a number of critical functions including locomotion, support, and growth. This latter activity is mediated by a specialized tissue, the endochondral growth plate. Following a number of rounds of proliferation, chondrocytes in the plate initiate a maturation program that is characterized by changes in the phenotypic characteristics of the cells and the attainment of a hypertrophic state (Cancedda et al., 1995). Additionally, the maturing chondrocytes display a unique change in metabolic activity in which there is a loss of mitochondrial function. Although hypertrophic chondrocytes are very active biosynthetically, they generate almost all of their metabolic energy through glycolysis. The glycolytic state reflects the limited vascularity of the cartilage and a low tissue oxygen tension (pO₂) (Shapiro et al., 2005).

A number of metabolic strategies have been developed to protect cells from hypoxic stress. For example, a decrease in O₂ concentration promotes the stabilization of hypoxia inducible factor (HIF) (Wenger, 2002). HIF activates the transcription of genes encoding proteins that will either increase O₂ delivery (through the synthesis of VEGF or erythropoietin) or promote metabolic adaptation (by the enhanced expression of glucose transporters and glycolytic enzymes) (Wenger and Gassmann, 1997). Under normoxic conditions, HIF-1 α is constitutively synthesized and subsequently degraded in the proteasome (Cockman et al., 2000; Ivan et al., 2001). Cells sense the pO₂ by a family of at least three prolyl hydroxylases (PHD) (Semenza, 2001; Huang et al., 2002; McNeill et al., 2002; Appelhoff et al., 2004). We have shown that these enzymes respond to the pO₂ by hydroxylating proline residues on HIF-1 α , thereby enhancing its subsequent degradation (Srinivas et al., 1999).

While it is known that the pO₂ in the epiphyseal cartilage is low (Shapiro et al., 1997; Schipani et al.,

2001), to date, there is very little information available concerning the mechanism by which this molecule regulates chondrocyte maturation. However, our group has shown that HIF is expressed in hypertrophic cells of the growth plate (Rajpurohit et al., 1996), while Schipani et al. have reported that HIF deletion causes massive tissue disorganization and increased chondrocyte apoptosis (Schipani et al., 2001). Other studies have indicated that HIF-1 α activity and sensitivity to apoptosis is regulated by generation of reactive O₂ species (ROS) (Brune et al., 2003).

The goal of the current investigation is first to evaluate PHD expression in chondrocytes and relate expression levels to attainment of the hypertrophic state. Second, we explore the relationship between PHD expression and HIF activity, and chondrocyte survival. Lastly, we test the hypothesis that the induction of the death response is linked to the generation and dismutation of ROS and activation of caspase-3. Based on this information, we hypothesize that during chondrocyte maturation, microenvironmental changes in pO₂ serve to modify functional activities of the terminally differentiated cells.

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MATERIALS AND METHODS

Experimental design

We used an *in situ* hybridization technique and immunohistochemistry to define the PHD expression profile of maturing chondrocytes embedded within the tibial growth plate. Isozyme expression was related to histological stages of chondrocyte maturation. In a parallel study, we examined PHD expression in N1511 chondrocytes undergoing maturation *in vitro*. Maturation was activated by treating the cells with BMP-2 and the differentiated state was determined using a number of common markers of hypertrophy. To explore the functional role of the PHDs, we pharmacologically suppressed the protein and measured cell death, the ultimate fate of the hypertrophic cell. In these experiments, apoptotic sensitivity was examined in the presence of H₂O₂. Since PHD activity is directed at stabilizing HIF-1 α , we next examined the importance of both HIF and PHD proteins in relationship to apoptotic sensitivity. We suppressed HIF-1 α using siRNA technology and blocked PHD activity pharmacologically. Both the HIF-1 α silenced cells and the PHD-inhibited chondrocytes were treated with H₂O₂ and sensitivity to the apoptogen was determined. We next evaluated the activities of redox-sensitive proteins that serve to protect chondrocytes from H₂O₂ and other apoptotic agents. For these studies, to activate the death response, maturing chondrocytes were treated with H₂O₂. We then measured catalase and superoxide dismutase (SOD) activities and the expression of the redox-sensitive antiapoptotic proteins Bcl-2 and Bcl-X_L proteins. Finally, we related apoptosis to caspase-3 activity in HIF-1 α -suppressed cells. This latter experiment permitted us to assess how changes in oxidative metabolism were related to the activity of a critical late stage protein that serves as the executioner enzyme in the chondrocyte apoptotic pathway.

In situ hybridization studies

PHD expression in the growth plate was assessed by an *in situ* hybridization procedure. An embryonic human proximal femoral growth plate was provided by National Disease Research Interchange under ethical guidelines that protect human subjects and donor confidentiality. The tissue was paraffin-embedded, serially sectioned (5 μ M), permeabilized with proteinase K (10 μ g/ml), and fixed in 4.0% paraformaldehyde. Plasmids used to generate the antisense probes have been previously described (Shimazu et al., 1996). Briefly, digoxigenin (DIG)-labeled antisense riboprobes were synthesized from linearized PHD templates (Epstein et al., 2001). The inserts were labeled using RNA polymerase (T7 for sense strand and SP6 for anti-sense strand). The labeled probe was re-suspended in 0.1 M DTT in DEPC water containing RNase inhibitor, and integrity was assessed by gel electrophoresis. Sections were treated with 0.2 M HCl–0.1 M triethanolamine (TEA) in 0.25% acetic anhydride for 10 min at room temperature and dehydrated with ethanol. Samples were incubated overnight at 50°C with the hybridization probe (1:50) in a buffer containing 50% formamide, 10 mM Tris-HCl [pH 7.6], 1 \times Denhardt's solution [2% BSA, 2% polyvinylpyrrolidone, 2% Ficoll 400, DEPC water], 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, and 1 mM EDTA. This medium was replaced with 2 \times sodium chloride, sodium citrate (SSC), 50% formamide, and TNE (1 M Tris-HCl, 5 M NaCl, and 1 mM EDTA) at 37°C for 10 min. Unhybridized probe was removed by treating sections with RNase A (10 μ g/ml) in TNE at 37°C for 10 min followed by a wash with TNE at 37°C for 10 min. To increase stringency, sections were subjected to sequential washes with SSC at 2 \times –0.2 \times at 50°C for 20 min. Detection was achieved by treatment with DIG buffer 1 (1 M Tris [pH 7.5] and 5 M NaCl) for 5 min, followed by treatment with DIG buffer 2 (1.5% blocking reagent [DakoCytomation, Glostrup, Denmark] in DIG buffer 1 in 1% BSA) for 60 min. Sections were treated with the anti-DIG antibody (1:1,000) overnight at 4°C. Subsequently, samples were washed twice with DIG buffer 1 for 15 min and then washed with DIG buffer 3 (1 M Tris [pH 9.5], 5 M NaCl, and 1 M MgCl₂). To detect the hybridized probe, sections were incubated with nitro blue tetrazolium (450 μ g/ml) and 5-bromo-4-chloro-3-indoyl phosphate (175 μ g/ml) in DIG buffer 3 at 37°C

for 60 min. After 3 min, the reaction was stopped with DIG buffer 4 (1 M Tris [pH 7.6] and 500 mM EDTA pH 8.0), counterstained with 0.2% methyl green in water, and mounted in Crystal Mount (Abcam, Cambridge, MA). Bound probe was visualized with an Olympus IK-ILL 30 light microscope connected to a Spot camera.

In vivo immunolocalization studies

Expression of PHD-1, -2, and -3 was assessed by immunohistochemistry in longitudinal sections of the embryonic (E18.5) mouse tibial growth plate (34). All animal studies were performed in accord with Thomas Jefferson University IACUC guidelines. The tissue was paraffin-embedded, serially sectioned (5 μ M), permeabilized with proteinase K (10 μ g/ml), and fixed in 4.0% paraformaldehyde. Sections were then deparaffinized and rehydrated. Next, serial sections were treated with PHD-1, -2, and -3 antibodies, respectively (PHDs: Novus, Littleton, CA). Following treatment with the primary antibody, sections were treated with Alexafluor 594-labeled secondary antibodies (Molecular Probes, Carlsbad, CA), counterstained with DAPI, and visualized with a fluorescent microscope. For the immunolocalization, immunohistochemistry, and *in situ* hybridization studies, collagen type X expression was used as a marker of the hypertrophic zone (not shown).

Cell culture

N1511 mouse chondrocytes were obtained from Dr. Motomi Enomoto-Iwamoto. The cells were maintained in culture using the method described previously (Kamiya et al., 2002). Briefly, the cells were plated at a concentration of 50,000 cells/ml in α -MEM containing 10% FBS, 0.2% L-glutamine, and penicillin/streptomycin. To induce maturation, after 24 h, the adherent cells were treated one time with 200 ng/ml recombinant BMP-2 (Alpha Diagnostic Intl., San Antonio, TX). On day 5, β -glycerophosphate (5 mM) was added to the culture media. This treatment promotes maturation of the chondrocytes. Untreated cells maintained an immature phenotype and served as a control (Kamiya et al., 2002). Assessment of the hypertrophic state was performed at day 8, by measuring alkaline phosphatase activity, expression of a number of key transcripts, and the presence of biological mineral. To inhibit PHD activity, 1 mM dimethylxalyglycine (DMOG) was added to the media on day 7.

Alkaline phosphatase activity

Following culture, total protein was collected and total protein concentration was determined using the Bradford Assay. Alkaline phosphatase activity was measured using the Sigma Fast™ p-Nitrophenyl phosphate kit (Sigma, St. Louis, MO). Readings were taken at 0 and 30 min, and known concentrations of p-nitrophenol were used to quantitate results. The results are expressed as percent control (μ mole p-nitrophenol/min/ μ g protein). This method has been described in detail in an earlier publication (Golub et al., 1992).

Expression of mature chondrocyte transcripts by RT-PCR

Cells were washed and total RNA was isolated using the Qiagen RNeasy[®] Mini kit. RNA yield was determined spectrophotometrically and integrity confirmed by gel electrophoresis. RNA was reverse-transcribed and then amplified using the Superscript™ One-Step RT-PCR with Platinum Taq[®] (Invitrogen, Carlsbad, CA) kit. PCR products were analyzed by 1.0% agarose gel electrophoresis. The forward (F) and reverse (R) primers used:

Aggrecan:	(F) gatgttagtgacagccatt	(R) agactctccactgcccatt
Collagen II:	(F) gtgagccatgacccgc	(R) gaccaggatttccagg
Collagen X:	(F) cgtgtctgttttactgta	(R) actctgctattttctgtgag
Osteocalcin:	(F) ctctctctgctactctgct	(R) gctctcaagcactactgctc
β -Actin:	(F) gtgctatgttgcctggatt	(R) tgctaggctgtgatctct

Assessment of mineralization

The medium was supplemented with 5 mM β -glycerophosphate for the last 3 days of the culture period. To ascertain whether the chondrocytes had generated biological apatite, cultures were harvested and mineralization was assessed by FT-IR in the reflectance mode (Yagami et al., 1999).

Expression of chondrocyte PHD transcripts by RT-PCR

Total RNA was isolated from immature and maturing chondrocytes using the Qiagen RNeasy[®] Mini kit as described above. RNA was reverse-transcribed and then amplified using the Superscript[™] One-Step RT-PCR with Platinum Taq[®] (Invitrogen) kit. PCR products were analyzed by 1.0% agarose gel electrophoresis. The forward (F) and reverse (R) primers used:

PHD1: (F) gaccagattgctgggtaga (R) aacacctttctgtcccagatg
 PHD2: (F) agccatgggtgctgttacc (R) ccttcacacctttctcact
 PHD3: (F) caggttatgttcgcatgtg (R) gattcagttttctagttaaattcctg

Cellular viability assay (MTT)

To assess sensitivity to an apoptogen, immature and maturing chondrocytes were maintained for 8 days and then treated with 10–100 μ M H₂O₂ (Fisher, Pittsburgh, PA). The cells were challenged with H₂O₂ for 8 h. Cells were washed, treated with MTT (120 μ g/ml), and incubated for 3 h at 37°C. The reagent was removed, and 400 μ l of 0.04 M HCl in isopropanol was added to each well. The optical density of the solution was read at 595 nm.

Measurement of catalase and SOD activity

Following culture, total protein was collected from maturing and immature chondrocytes and total protein concentration was determined using the Bradford Assay. Catalase activity was measured using the Fluoro Catalase[™] kit (Cell Technologies, Mountain View, CA). Samples were treated with 20 μ M H₂O₂ for 1 h and following a 10-min incubation with the detection reagent color development was activated by the addition of horseradish peroxidase. Fluorescence was determined at 540 nm and 590 nm. Known concentrations of active catalase were used as a positive control. SOD activity was measured using a SOD Assay kit (Cell Technologies). Samples were treated with WST-1 and xanthine oxidase solution following the manufacturers protocol for 20 min and absorbance detected at 450 nm. Known concentrations of SOD were used as a positive control.

Immunoblot analysis

Cells were isolated with MPER[®], 5 M NaCl, complete protease inhibitor cocktail, DTT, 100 μ M NaF, and 100 μ M Na₃VO₄ and quantitated using a Bradford assay. Protein (100 μ g) was then loaded onto 4–20% Tris gradient gels. Samples were transferred to PVDF membranes and subsequently blocked with Blotto (5.0% TBS-T +5.0 g dried milk). Blots were treated overnight with one of the following antibodies: Bcl-2 (1:500, Santa Cruz, Santa Cruz, CA), Bcl-xL (1:500, Santa Cruz), HIF-1 α (1:500, R&D Systems, Minneapolis, MN), Lamin-A/C (1:500, BD Biosciences, San Jose, CA), or Tubulin (1:1,000, Santa Cruz). Membranes were then washed and treated with HRP-labeled secondary antibody (1:500, Pierce, Rockford, IL). Blots were visualized with Lumigen[™] TMA-6 (Amersham Biosciences, Piscataway, NJ).

Immunofluorescence analysis of HIF-1 α

Expression of HIF-1 α in cells was assessed by immunofluorescence analysis. The silenced cells were cultured at 2% O₂ in a Hypoxia Work Station (in VIVO₂, Ruskin Technology, West Yorkshire, UK). Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton-X100. The samples were then blocked with 10% FBS and treated

overnight with HIF-1 α (BD Biosciences). Following treatment with the primary antibody, sections were treated with Alexafluor 594 labeled secondary antibodies (Molecular Probes, OR). The cells were examined by confocal microscopy (Olympus, Center Valley, PA). Vector transfected cells were used as a control.

siRNA plasmid construction

An siRNA construction kit (Gene Silencer Kit, BioCarta, San Diego, CA) was utilized to downregulate HIF-1 α . The following phosphorylated oligonucleotides were used (Forward [F], Reverse [R]):

F: 5'tcgaggagatgcagcaagatctccgagtactgcgagatcttgcgcatctcttttt
 R: 5'ctagaaaaagagatgcagcaagatctccgagtactccgagatctgctgcatctctcc

Permanent cell lines were generated using 80% confluent monolayers transfected with the HIF-1 α siRNA vector followed by clonal selection using 800 μ g/mL of hygromycin B (Invitrogen). A cell line with backbone vector containing scrambled sequences served as a control (pSHH). Experiments were done before passage 30, and suppression was verified by RT-PCR (data not shown) and immunoblotting.

Measurement of caspase-3 activity

Caspase-3 activity was measured using the fluorescent caspase substrate, PhiPhiLuxG1D2 (OncoImmune, Inc., Gaithersburg, MD). Cells were matured for 8 days in culture and then incubated with 10 μ M PhiPhiLux-G1D2 for 1 h at 37°C. The substrate was removed and the cellular fluorescence captured using confocal microscopy.

RESULTS

Expression of PHD isozymes and HIF-1 α in the growth plate

Figure 1A shows that all three PHD isozymes are expressed in chondrocytes of the human growth plate. Low levels of PHD expression are evident in the resting (reserve) and proliferating zones. Moreover, as chondrocytes hypertrophy and become terminally differentiated, there is a progressive increase in PHD expression. The distribution of PHD protein was determined immunohistochemically on mouse tibial growth plate sections. Figure 1B shows that while the three PHDs are expressed by all chondrocytes in the growth plate, there is an elevated level of expression in the hypertrophic region. These results indicate that there is a close correlation between the pattern of PHD expression in cells of the tibial growth cartilage and maturation status.

Maturation of N1511 chondrocytes in culture

When treated with BMP-2 and β -glycerophosphate for 7 days, chondrocytes displayed a maturing phenotype. Thus, these cells exhibit a significant increase in alkaline phosphatase activity (Fig. 2A). To confirm that the maturing cells were terminally differentiated, we evaluated the expression of collagen type II and X, osteocalcin and aggrecan by RT-PCR. Figure 2B shows that there is an increase in expression of collagen type II by day 7, and elevation in the expression of collagen type X and osteocalcin transcripts. Also, FT-IR analysis indicates the presence of a carbonated apatite (data not shown). In contrast, in the same time period, the untreated cells maintain an immature phenotype. Thus, these cells exhibit low alkaline phosphatase activity and the expression levels of collagen type II and type X are low. Moreover, these cells fail to mineralize their

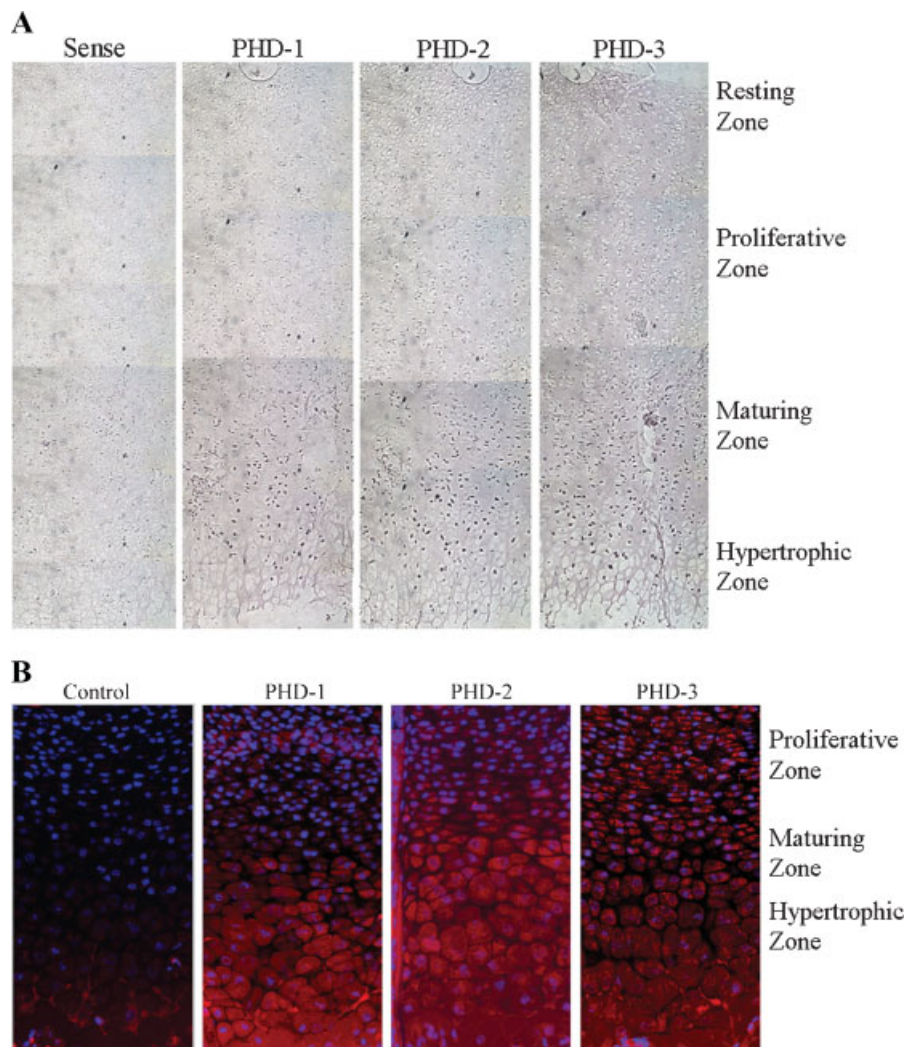


Fig. 1. Analysis of PHD expression in the growth plate. **A:** Paraffin-embedded sections of human proximal tibial growth plate were hybridized to digoxigenin-labeled antisense riboprobes that were synthesized from linearized PHD 1–3 templates. Hybridization was visualized by light microscopy. Digoxigenin-labeled PHD-1 sense strand riboprobe was used as a negative control. For all three PHD isozymes, cells in the hypertrophic zone displayed the highest level of

staining (mag. 200 \times). **B:** Paraffin-embedded sections of murine proximal tibial growth plate were treated with antibodies against PHD 1–3. Subsequently, the sections were treated with Alexafluor 594-labeled secondary antibodies and counterstained with DAPI. Rabbit IgG was used as a negative control. All three PHD isozymes were expressed primarily in the hypertrophic zone (mag. 200 \times).

extracellular matrix. Based on these criteria, after 7 days in culture, these cells are termed *immature*; in contrast, in the same time period, the BMP-2- and β -glycerophosphate-treated cells are termed *mature*.

PHD expression by maturing chondrocytes

We next evaluated the expression of the PHDs in the maturing chondrocytes by RT-PCR analysis. Figure 2C shows that although the levels of expression are different, all three PHDs are expressed by this cell line. Thus, PHD-1 and -2 are expressed at higher levels than PHD-3. However, upon maturation, there is an elevated level of expression of all three PHDs; PHD-1 and -2 are the most highly expressed isozymes. While PHD-3 is expressed by the maturing cells, the level of expression is much lower than the other two isozymes.

Maturation-dependent modulation of apoptotic sensitivity

We examined the apoptotic sensitivity of the maturing chondrocytes. Figure 3A shows that when challenged with H_2O_2 for 8 h, the maturing cells are exquisitely sensitive to the apoptogen. Thus, at 10 μ M, there is a

40% decrease in viability of the mature chondrocytes. At higher concentrations (50 and 100 μ M), almost all of the mature cells are dead. In contrast, the immature cells exhibit a decrease in sensitivity to the apoptogen. Thus, at 50 and 100 μ M respectively, 40 and 20% of the cells remain viable. Since maturation resulted in the sensitization of chondrocytes to the H_2O_2 challenge, we measured the activities of enzymes involved in dismutating ROS. Figure 3B shows that with maturation, there is a profound decrease in both SOD and catalase activities. In both cases, the activity of each enzyme is decreased by 30–40%. Since Bcl-2 and Bcl-XL also provide protection against redox-active apoptogens, we measured the expression of both proteins in maturing and immature chondrocytes. Western blot analysis (Fig. 3C) shows that with maturation, there is a decrease in both Bcl-2 and Bcl-XL protein levels.

Modulation of chondrocyte apoptotic sensitivity by PHD and HIF-1 α

To explore the relationship between HIF expression and sensitivity to apoptosis, we inhibited PHD activity using dimethylxalylglycine (DMOG). Figure 4A shows

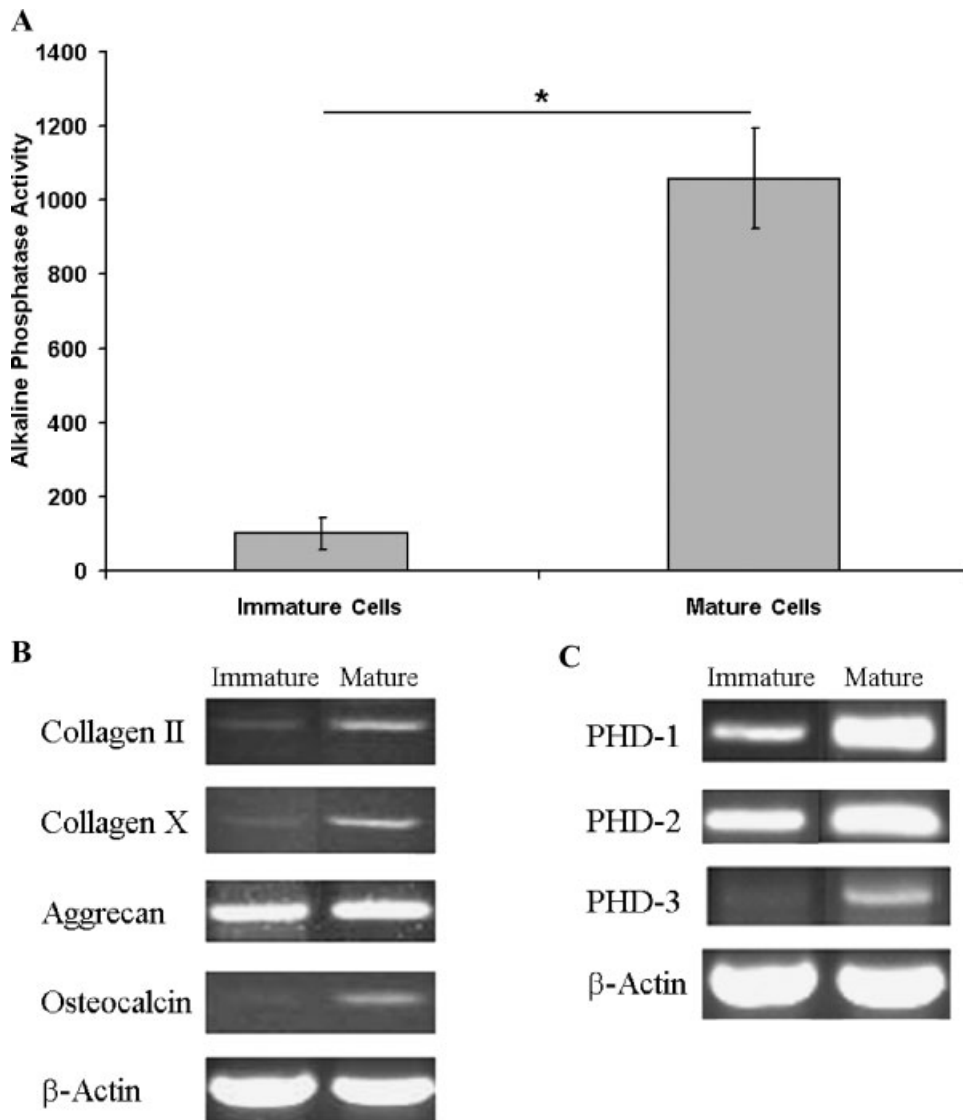


Fig. 2. Expression of maturation markers and PHDs in N1511 chondrocytes. **A**: N1511 cells were treated with 200 ng/ml BMP-2 and cultured for 7 days. Total protein was extracted and alkaline phosphatase activity was assessed. Note there was a marked increase in enzyme activity in matured chondrocytes when compared with the immature cells (set to 100). Values shown are mean \pm SEM; *significantly different from control; $P < 0.05$. **B**: N1511 chondrocytes were maintained in culture for 7 days and RNA was isolated. Collagen

type II and X, aggrecan, and osteocalcin expression was assessed by RT-PCR. β -actin served as a loading control. There was a temporal induction of collagen type II and X, and osteocalcin in mature cells. **C**: The expression of the PHDs was assessed by RT-PCR analysis. β -actin served as a loading control. Note, there was a marked induction of PHD-1 and -3 in maturing chondrocytes. PHD-2 was moderately increased in the maturing cells.

that as DMOG concentration is raised from 0.1 to 1.0 mM, there is a dose-dependent increase in HIF-1 α protein levels. Accordingly, when the inhibitor is present, about 50–60% of both mature and immature chondrocytes are killed when treated with 10 μ M H₂O₂ (Fig. 4B). There is no statistical difference in the percent of immature and maturing cells killed by H₂O₂. Note that immature cells are killed by 10 μ M H₂O₂ only when the inhibitor is present (compare killing of immature cells in Fig. 3A,B).

A similar experiment was performed using HIF-1 α -silenced cells. Immunofluorescence analysis of the silenced cells indicates that there is a profound decrease in HIF-1 α levels. In contrast, vector transfected control cells (pSHH) evidenced a raised level of HIF fluorescence (Fig. 4C). To examine the sensitivity of the HIF suppressed cells to the apoptogen, they were challenged with H₂O₂. Figure 4D shows that that the maturing

silenced cells are almost resistant to the H₂O₂ challenge. In contrast, about 50% of mature pSHH-transfected cells are killed when treated with H₂O₂. This finding suggests that HIF-1 α sensitizes the mature cells to H₂O₂.

Next, we explored the interaction between PHD and HIF-1 α in terms of sensitization to H₂O₂. Using the HIF-silenced cells, we blocked hydroxylase activity using DMOG. Figure 4E shows that suppression of PHDs sensitizes immature chondrocytes to H₂O₂. Unlike the control cells (pSHH), there is only a small change (about 20%) in viability of the immature silenced cells. Thus, when HIF-1 α is suppressed, there is a minimal decrease in viability and the immature cells appear to be resistant to apoptogen treatment (Fig. 4E). A similar study was performed with the mature chondrocytes. That suppression of PHDs sensitizes mature cells to the H₂O₂ is shown in Figure 4F. While the HIF-1 α suppressed

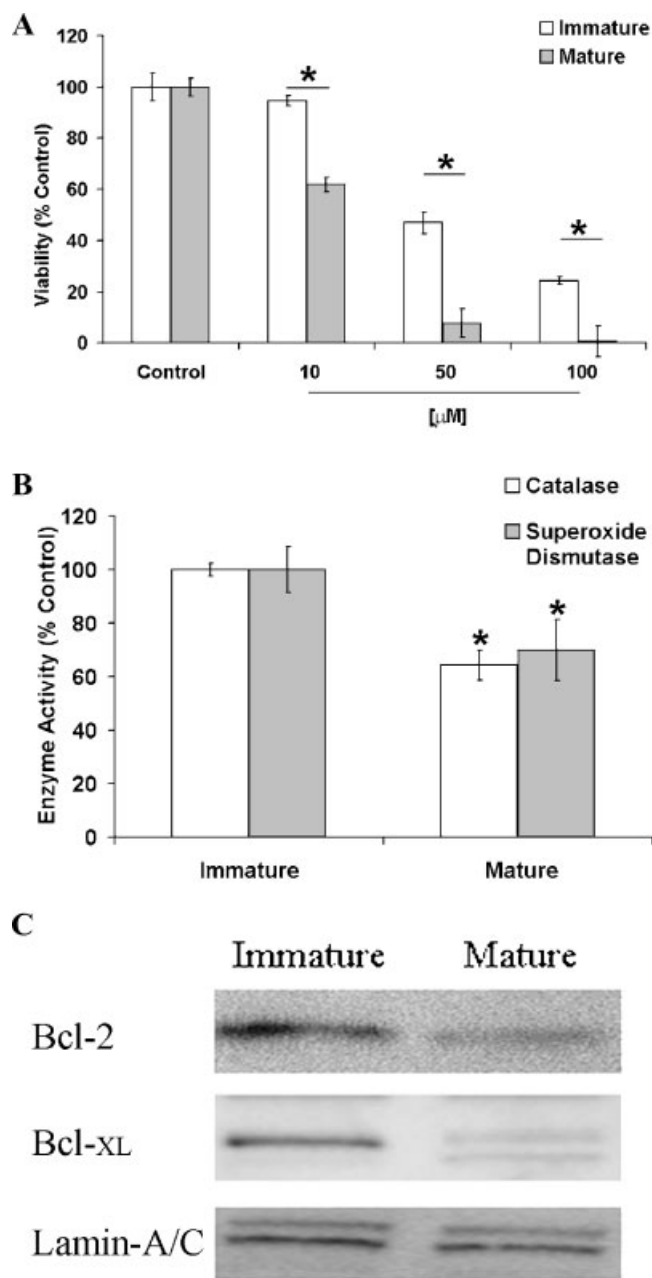


Fig. 3. Maturation-dependent changes in susceptibility to H₂O₂ and expression of catalase, superoxide dismutase, and anti-apoptotic proteins. **A:** Sensitivity of immature and maturing chondrocytes to increasing concentrations of H₂O₂. Cells were treated with the apoptogen for 8 h and viability was measured by the MTT assay. Note the increased susceptibility of maturing chondrocytes to H₂O₂. At 50 and 100 µM, the mature cells were all killed by the apoptogen. Viability was expressed as a % of untreated immature to maturing cell values. Data shown is the mean ± SEM of three experiments. * Significantly different from mature MTT values; *P* < 0.05. **B:** Activities of catalase and SOD in immature and mature chondrocytes were determined chemically. Enzyme activities are expressed as a function of total protein concentration and normalized to 100% of immature values. In mature chondrocytes, a significant decrease in both catalase and SOD activities were observed. Values are mean ± SEM of three experiments; * significantly different from immature cells; *P* < 0.05. **C:** Expression of Bcl-2 and Bcl-XL in immature and mature chondrocyte by Western blot analysis. Both Bcl-2 and Bcl-XL protein levels were decreased in maturing chondrocytes. Lamin-A/C served as the loading control.

chondrocytes remain viable in the face of the apoptogen challenge, when PHDs are depleted by DMOG, about 40% of the mature control (pSHH) cells are killed. Again, as was seen with the immature chondrocytes, when

HIF-1 α is suppressed, they remain refractory to H₂O₂-mediated cell death.

Caspase-3 activity in relationship to PHD and HIF-1 α expression

Since most forms of death are linked to upregulation of caspase-3 activity, we evaluated the activity of this protein in relationship to PHD and HIF-1 α expression in immature chondrocytes. Inhibition of PHD expression by DMOG results in stimulation of caspase-3 activity (Fig. 5). When HIF-1 α is suppressed, the level of fluorescence of the immature HIF-1 α -suppressed cells is similar to the immature vector-treated chondrocytes.

DISCUSSION

The overall goal of the current study was to examine the functional activity of the PHDs in maturing chondrocytes. Herein, we show for the first time that the PHDs are expressed in the maturing zone of the growth plate, and by a chondrocytic cell line. Since PHDs are maximally expressed at a late developmental stage, we determined if this protein and its substrate, HIF-1 α , modulated a late stage activity of chondrocytes, the induction of apoptosis. For these studies, we pharmacologically blocked PHD activity and inhibited HIF-1 α expression using siRNA technology. When challenged with H₂O₂, we noted that PHD suppression sensitized the cells to the apoptotic challenge. In contrast, HIF suppression decreased cell death. While these findings indicate that the PHDs are not directly involved in the induction of apoptosis, it is concluded that HIF-1 α serves two functions: it metabolically enhances survival, while at the same time sensitizing the chondrocytes to pro-apoptotic signals.

Initially, we used *in situ* hybridization and immunohistochemical procedures to explore the PHD expression profile in the growth plate. We detected the presence of the three PHD isozymes in both mouse and human tissues. While the immunohistochemical studies indicated that each of the three PHDs were present in all regions of the plate, we noted that expression of the isozymes was most marked in the hypertrophic zone. We also determined the expression of the PHD transcripts in N1511 chondrocytes, a cell line that undergoes rapid maturational changes in culture. RT-PCR analysis indicated that the three PHDs were expressed in the immature cells: PHD-1 and -2 were expressed at higher levels than PHD-3. When the cells were matured in culture, there was a dramatic increase in both PHD-1 and -3, while PHD-2 levels evidenced a modest change in expression. These findings suggest that maturational events may play a critical role in regulating PHD expression. One clue to this role is the observation that PHD-3 enhances the sensitivity of neuronal cells to growth factor depletion (Straub et al., 2003). This observation is relevant to the physiology of chondrocytes in the growth plate, since the tissue is avascular and growth factor depletion is possible. In this case, the PHD isozymes may play dual roles in growth: O₂ sensors and as mediators of late stage events in the chondrocyte life cycle.

To evaluate the interaction between HIF and PHD, we inhibited PHD expression using DMOG and measured the susceptibility of the cells to an apoptogen. In the presence of DMOG, when treated with a low concentration of H₂O₂, both maturing and immature chondrocytes were killed. In contrast, when control cells (no DMOG),

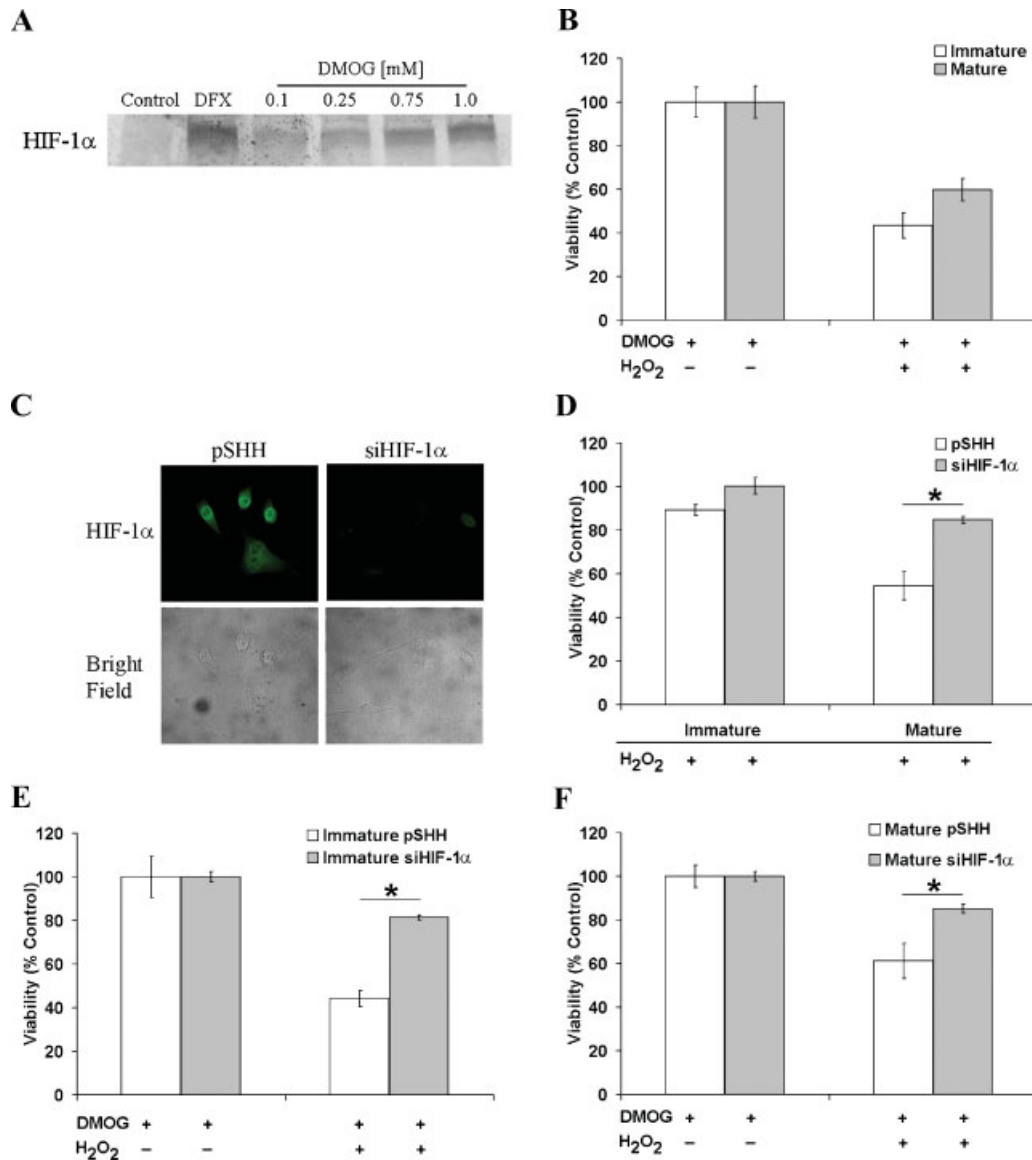


Fig. 4. Relationship between HIF expression and H₂O₂-mediated cell death **A:** Chondrocytes were treated with DMOG (0.1–1.0 mM) for 6 h and HIF-1 α levels were measured by Western blotting. Note there was a dose-dependent increase in HIF-1 α levels in the immature DMOG-treated cells. As an internal control, cells were treated with desferrioxamine (130 μ M) for 6 h to elevate HIF-1 α levels. **B:** Immature and maturing cells were treated with the PHD inhibitor DMOG and cell viability in response to H₂O₂ was assessed. When treated with DMOG, H₂O₂ causes a significant decrease in the viability of both immature and maturing chondrocytes in the presence of DMOG. **C:** Immunofluorescence analysis of HIF-1 α expression in silenced chondrocytes. There is a marked decrease of fluorescence in the siHIF-1 α silenced cells when compared to the vector transfected controls (pSHH). **D:** The effect of H₂O₂ on the viability of immature

and mature siHIF-1 α silenced chondrocytes. Note, the maturing silenced cells appear to be almost refractory to the apoptogen challenge. In contrast, there is a decrease in viability of the vector treated (pSHH) cells. **E:** The effect of H₂O₂ on the viability of PHD inhibited immature HIF-1 α silenced chondrocytes. Immature HIF-1 α suppressed cells were treated with DMOG to block PHD activity and sensitivity to H₂O₂ determined using the MTT assay. Note there was only a small change in viability of the silenced cells. However, suppression of PHDs increased the sensitivity of the control (pSHH) cells. **F:** The effect of H₂O₂ on the viability of PHD-inhibited mature chondrocytes. In concert, with the immature cells (see E above) there was only a small change in viability of the silenced cells. All values shown are the mean \pm SEM of three experiments; * significantly different; $P < 0.05$.

were challenged with the H₂O₂, there was a maturation-dependent change in apoptotic sensitivity. Thus, mature cells died when treated with low doses of apoptogens. Furthermore, when mature cells were treated with DMOG and challenged with H₂O₂, the extent of killing remained the same when compared with untreated mature cells. Accordingly, suppression of PHD activity does not further enhance the susceptibility of the maturing cells to the apoptogen. Thus, from a functional viewpoint, during the maturation process, PHDs play a role in protecting chondrocytes from local apoptogens.

We examined the interplay between the PHDs and HIF-1 α by suppressing HIF-1 α and measuring chondrocyte apoptotic sensitivity. We noted that when the mature HIF-1 α silenced cells were challenged with H₂O₂, there was a 30% reduction in killing. In contrast, there was limited change in viability when the immature cells were treated with the apoptogen. Based on these differences in chondrocyte susceptibility, it is concluded that HIF-1 α sensitizes maturing cells to H₂O₂-mediated killing.

As was noted earlier, by increasing HIF-1 α stability, the microenvironmental modulation of PHD activity

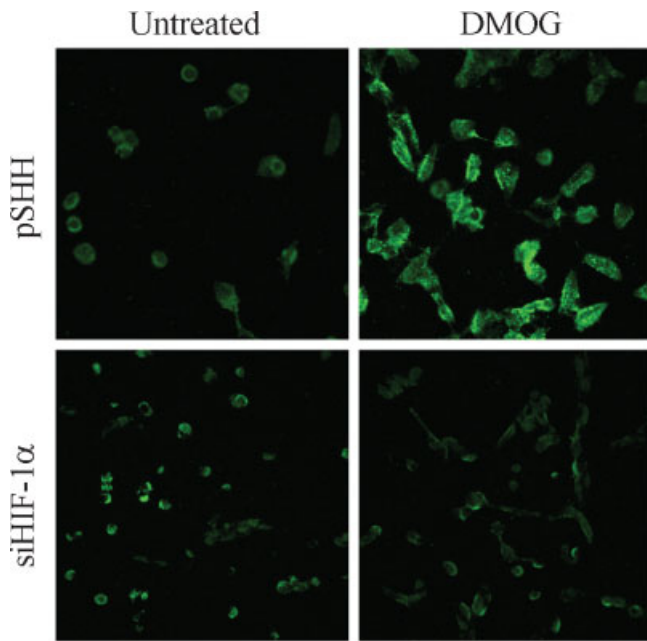


Fig. 5. Caspase-3 activity in siHIF-1 chondrocytes. Immature pSHH and HIF-1 silenced chondrocytes were treated with DMOG and caspase-3 activity was assessed by immunofluorescence. However, DMOG treatment did not alter the basal level of caspase-3 activity in the HIF-1 silenced cells. In contrast, control (pSHH) cells evidenced a marked increase in caspase-3 activity when treated with DMOG.

influences chondrocyte sensitivity to H_2O_2 . While it is not yet known how HIF promotes apoptotic sensitivity, it may change survival activity by promoting Akt phosphorylation (Gort et al., 2006), decreasing the mitochondrial membrane potential and depleting thiol reserves (Zamzami et al., 1998), and causing a loss of respiratory function (Matsumoto et al., 1988; Rajpurohit et al., 1999). To assess if this decrease in viability was linked to activation of the executioner protein, caspase-3, we measured the activity of the protein in DMOG-treated cells. We found that while the DMOG-treated control (pSHH) cells exhibited a marked increase in caspase-3 activity, the DMOG-treated HIF-1 α -suppressed cells exhibited no change in activity. These findings suggest that caspase-3-mediated cell death is linked to HIF stabilization. This conclusion is supported by the results of the HIF-1 α silencing study discussed above and adds credence to the view that this protein serves a number of differing functional roles. From a metabolic viewpoint, HIF-1 α mediates a survival role, promoting chondrocyte metabolic activity in a hypoxic environment. In the context of the investigations described here, and in line with a number of parallel studies, HIF-1 α is pro-apoptotic (Carmeliet et al., 1998; Bruick, 2000).

Finally, it is important to note that while a considerable number of agents have been linked to chondrocyte apoptosis, we chose to challenge the cells with low concentrations of H_2O_2 . In a previous study, we have shown that maturing chondrocytes generate ROS (Matsumoto et al., 1991); moreover, in many tissues including cartilage, H_2O_2 is a potent physiological apoptogen (Lo and Kim, 2004). One mechanism by which cells protect themselves from ROS is through expression of catalase and SOD, two powerful dismutating enzymes (Valko et al., 2006). We found that during maturation, there was decreased activity of both enzymes. Thus, in N1511 cells, as the chondrocytes

mature the activities of catalase and SOD were reduced by more than 50%. It would not be unreasonable to assume that the increased sensitivity of the cells to apoptogens during the maturation process is linked in part to the fall in activity of both of these proteins. In parallel with this study, we also examined the levels of two proteins that confer protection against killing, Bcl-2 and Bcl_{XL}. In line with earlier studies of the growth plate (Rajpurohit et al., 1999; Cheung et al., 2003), we found that both of these anti-apoptotic proteins were both expressed by N1511 cells in culture, and with maturation, there was a profound decrease in expression. It is likely that loss of the protective activity, provided by these two members of the Bcl-2 family of proteins, together with the loss of dismutating activities, provided by catalase and SOD, would sensitize the maturing chondrocytes to local apoptogens. Experiments are now underway to determine the mechanism by which maturing chondrocytes are sensitized to an apoptotic challenge through the activities of both PHD and HIF.

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