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Apoptosis Is Developmentally Regulated in Rat Growth Plate

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Apoptosis occurs in the growth plate during normal and abnormal longitudinal growth. To investigate the role of apoptosis during growth plate maturation, apoptosis and apoptosis-related proteins were studied in rat tibial growth plates at 2, 4, 8, and 11 wk of age. Apoptosis was studied by the terminal deoxynucleotidyl transferase (TdT)–mediated deoxy-UTP nick end-labeling (TUNEL) method, and immunohistochemistry was used to detect p53, caspase-3 and -6, the antiapoptotic proteins Bcl-2 and Bcl-x, and the proapoptotic proteins Bax and Bad. In all age groups studied, most apoptotic chondrocytes were terminal hypertrophic chondrocytes (THPCs) with a significant increase during development. At 2 wk, 0.108 ± 0.026 THPCs were found to be apoptotic per millimeter of growth plate width; at 4 wk, 0.355 ± 0.048; at 8 wk, 0.394 ± 0.043; and at 11 wk, 1.084 ± 0.069 ($p < 0.001$; 11 wk vs 2, 4, and 8 wk). THPCs were negative for p53 immunoreactivity at 2 and 4 wk, whereas some THPCs were positive at 8 and 11 wk. Caspase-3 and -6 were found in proliferative and early hypertrophic cells at 2 wk, whereas mature hypertrophic cells and THPCs were negative. At later stages of development, mature hypertrophic cells and THPCs were stained for both caspase-3 and -6. Bcl-2 and Bcl-x were present in proliferative and early hypertrophic cells at 2 wk, whereas at older ages a decrease in staining was observed. At 2 wk of age, Bax and Bad immunoreactivities were localized in proliferative and early hypertrophic cells, whereas at 8 and 11 wk many mature hypertrophic cells and THPCs were immunoreactive for Bax and Bad. Our results show that apoptosis is developmentally regulated in the rat growth plate. In older animals, with decreased growth rate and growth plate height, apoptosis is significantly increased, especially in THPCs.

Key Words: Apoptosis; growth plate; development; chondrocytes.

Introduction

Normal longitudinal growth depends on a well-defined process of maturational stages that occurs within the epiphyseal growth plate. The process involves a population of stem cells in the resting zone, which proceeds through proliferation, maturation, and hypertrophy, finally giving way to bone. The ultimate fate of the terminal hypertrophic chondrocytes (THPCs) is not clear and several mechanisms have been proposed. First, THPCs are converted into bone cells *(1)*. Second, THPCs give rise to two daughter cells where one cell dies and the other cell generates osteogenic cells *(2)*. Third, THPCs die by apoptosis *(3,4)*. Finally, THPCs go through an aberrant cell death *(5–7)*.

Many studies have established the presence of apoptosis in the growth plate in normal *(8–10)* and abnormal conditions *(10–13)*, but the location and the extent of apoptosis remain controversial. Apoptotic chondrocytes have been detected in all three zones of the growth plate but the majority of investigators believe that apoptosis occurs almost exclusively in THPCs. Differences in species, age, and methods for detection of apoptosis could account for the controversial data in the literature.

In vivo studies have shown the importance of apoptosis in growth plate homeostasis. As an example, mice with targeted disruptions of both alleles for the antiapoptotic protein Bcl-2 have short limbs *(12)*, whereas similar knockouts for caspase-3 are smaller than their normal littermates *(14)*. Furthermore, two chondrodysplastic conditions, PTHrP knockout mice *(15)* and mutations of the fibroblast growth factor receptor *(16)*, are associated with increased apoptosis of epiphyseal chondrocytes. These studies strongly suggest that altered apoptosis affects growth plate homeostasis and longitudinal growth. The precise role of apoptosis in the normal growth plate is not known although apoptosis has been proposed to be the fate of THPCs *(3,4)*. A number of studies indicate that hypertrophic chondrocytes are more susceptible to apoptosis than other cells in the growth plate. For example, the antiapoptotic protein Bcl-2 is expressed mainly in proliferative chondrocytes *(17)*. In addition, initiation of hypertrophic differentiation in chick sterna chondrocytes resulted in the initiation of apoptosis, while chondrocytes not committed to hypertrophy were resistant to apoptosis *(18)*.

The growth plate undergoes morphologic changes during normal development. Early in life, when rapid longitudinal growth occurs, it is thicker, but later when growth rate

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diminishes, it is thinner and finally fuses. Whereas previous studies conducted at specific developmental stages suggest that apoptosis could be the terminal fate of the THPC *(4,10)*, developmental data are mostly lacking. A study in rabbits *(19)* indicates that growth plates from older animals had more apoptotic chondrocytes in all three zones, but the number was unusually high compared to many other reports. The magnitude and role of apoptosis in growth plate homeostasis is still controversial, and all existing data in the literature are based almost exclusively on the TUNEL method. Moreover, data on apoptosis-related proteins and caspases, which could give a better insight into the regulation of apoptosis in the growth plate, are lacking. The aim of the current study was to examine the role of apoptosis during the developmental maturation of the rat growth plate by investigating apoptosis not only by the TUNEL method, but also by immunohistochemistry, for caspases and apoptosis-related proteins.

Results

Apoptosis

At all developmental ages, most apoptotic chondrocytes were THPCs (Fig. 1). Apoptosis was also occasionally seen in proliferative and very rarely observed in resting chondrocytes. During development, the number of apoptotic chondrocytes was increased in all layers of the growth plate (Table 1). Compared with 2 wk-old animals, the number of apoptotic THPCs/mm of growth plate was increased 3.3-fold in animals at 4, 3.6-fold at 8, and 10-fold at 11 wk of age ($p < 0.001$; 11 wk vs 2, 4, and 8 wk) (Table 1).

Caspases

At 2 wk of age, caspase-3 immunoreactivity was detected in proliferative and, to a lesser extent, in hypertrophic chondrocytes (Fig. 1). In older animals, an extension of the staining was noticed towards THPCs resulting in a more even distribution of the immunostaining throughout the growth plate and significantly more immunopositive hypertrophic chondrocytes (Fig. 1, Tables 2 and 3).

At 2 wk, caspase-6 immunoreactivity was present mostly in proliferative chondrocytes (Fig. 1). As was noticed with caspase-3, several late and terminal hypertrophic cells were immunopositive for caspase-6 at 8 and 11 wk, but not at 2 wk (Fig. 1, Tables 2 and 3). Results were confirmed with a second antibody against caspase-6 (Chemicon, Temecula, CA) (data not shown).

p53

At 2 and 4 wk of age, no p53 immunoreactivity was detected in hypertrophic chondrocytes. On the other hand, at 8 and more frequently at 11 wk some THPCs were immunoreactive for p53. Their number was limited, resembling the number of the apoptotic chondrocytes (Fig. 1, Table 3). No p53 staining was detected in resting and proliferative chondrocytes.

Antiapoptotic Proteins Bcl-2 and Bcl-x

At 2 wk, Bcl-2 immunoreactivity was present mainly in resting, proliferative, and early hypertrophic chondrocytes whereas THPCs contained very little Bcl-2 immunoreactivity (Fig. 2). During development, a decrease of the Bcl-2 staining was observed which was significant at 8 wk of age and at 11 wk only a few proliferative chondrocytes contained immunoreactivity (Fig. 2, Tables 2 and 3).

In 2-wk-old rats, Bcl-x immunoreactivity was distributed throughout the growth plate although it was more prominent in late proliferative and mature hypertrophic chondrocytes (Fig. 2). In all sections studied, a significant decrease in Bcl-x immunoreactivity was observed at 8 and 11 wk (Fig. 2, Tables 2 and 3).

Proapoptotic Proteins Bax and Bad

Bax immunoreactivity was detected in proliferative and early hypertrophic chondrocytes in 2-wk-old rats (Fig. 2). During development, the intensity and distribution of the Bax staining was altered. At 8 and 11 wk, Bax immunoreactivity was significantly increased in the hypertrophic zone and many THPCs were stained with the Bax antibody (Fig. 2, Table 3). A second antibody against Bax gave similar results (Santa Cruz Biotechnology, Santa Cruz, CA) (data not shown).

At 2 wk, Bad immunoreactivity was detected in proliferative and early hypertrophic chondrocytes whereas THPCs were negative (Fig. 2). On the other hand, growth plates from older animals (8 and 11 wk) had a different pattern of Bad immunostaining. Their hypertrophic chondrocytes including THPCs clearly contained significantly more Bad immunoreactivity (Fig. 2, Table 3). These results were confirmed with another Bad antibody (Santa Cruz Biotechnology) (data not shown).

Discussion

We report that apoptosis is detected in higher frequency in growth plate chondrocytes from older compared with younger animals. The vast majority of the apoptotic cells were THPCs. In older animals, increased apoptosis was associated with increased expression of caspases-3 and -6, decreased expression of the antiapoptotic proteins Bcl-2 and Bcl-x, and increased expression of the proapoptotic proteins Bax and Bad.

Chondrocytes proliferate, differentiate, and finally THPCs are replaced by bone. It has been proposed that the fate of the THPC is death by apoptosis *(3,4,20–22)*. If all THPCs were committed to suicide, one would expect the same number of apoptotic THPC in sections from different developmental stages. On the other hand, the number of apoptotic cells per time unit would be increased in young compared with old animals because the proliferation rate is increased and therefore more cells become hypertrophic. By contrast, we found that growth plates from 11-wk-old rats had

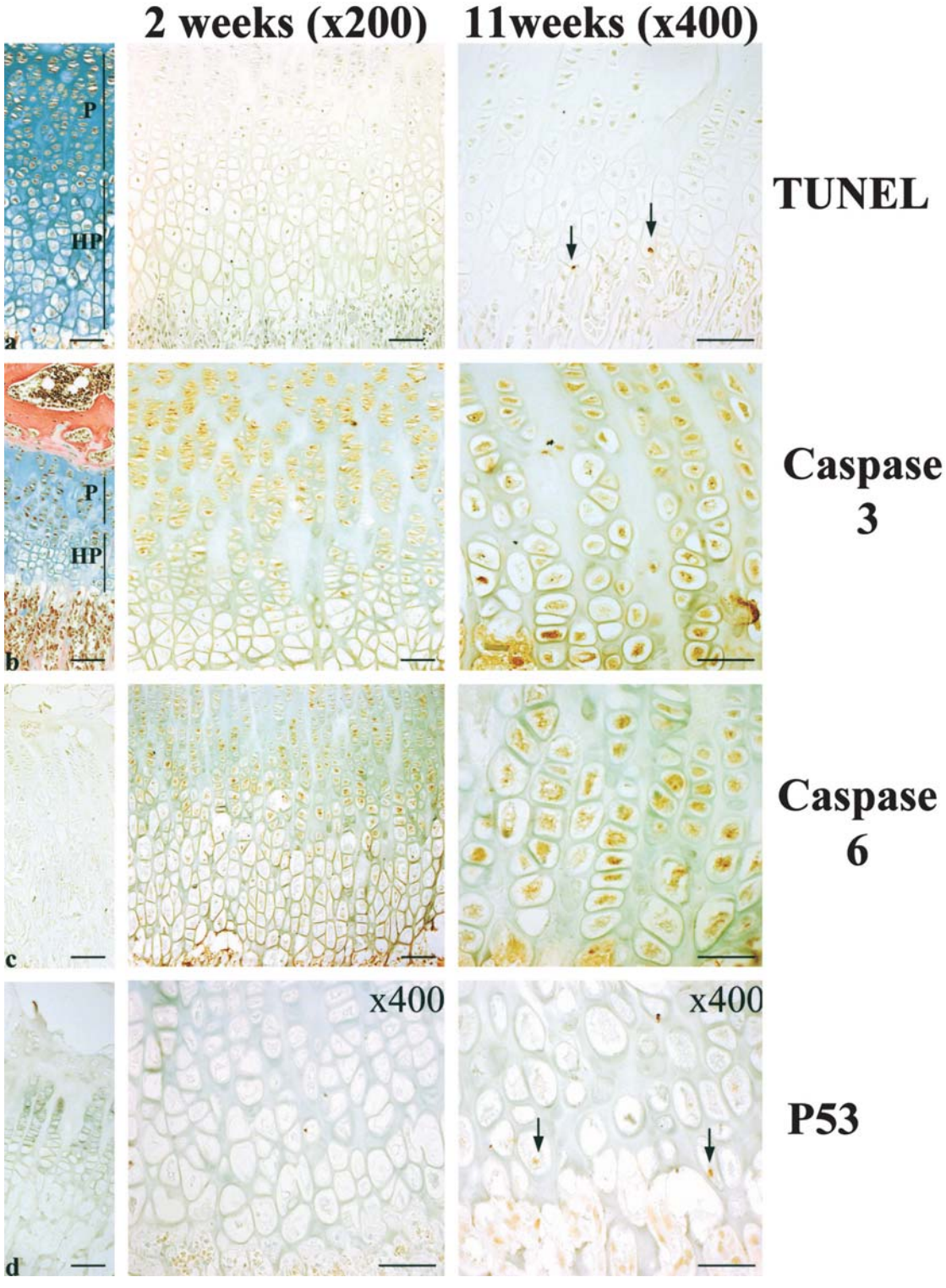

Fig. 1. Representative microphotographs of growth plates stained with Alcian blue/van Gieson show the differences in proliferative (P) and hypertrophic (HP) zones from 2- and 11-wk-old rats (**A,B**) at ×200 magnification. Assessment of the specificity of primary antibodies raised in rabbit or goat (**C,D**) by omitting the primary antibodies. TUNEL assay and immunohistochemistry for caspase-3, caspase-6, and p53 in tibial growth plates from 2- (×200 magnification) and 11-wk-old male rats (×400 magnification). Microphotographs from 11 wk are presented at ×400 magnification because the height of the growth plate is significantly smaller than at 2 wk. The TUNEL assay revealed increased apoptosis in terminal hypertrophic chondrocytes at 11 wk whereas at 2 wk apoptotic cells were rare. Arrows show TUNEL-positive cells at 11 wk in closed (**left**) and open (**right**) lacunae. The increased apoptosis in terminal hypertrophic chondrocytes at 11 wk of age was associated with positive immunoreactivity in the same population of cells for caspase-3 and caspase-6, and p53. p53 presented as ×400 magnification at both ages, 2 and 11 wk. Bars = 100 μm.

Table 1
Apoptotic Growth Plate Chondrocytes[a]

	2 wk	4 wk	8 wk	11 wk
Resting	0	0	1	4
Proliferative	1	4	8	11
THPC	12	79	92	216
THPCs/mm	0.108 ± 0.026	0.355 ± 0.048[b]	0.394 ± 0.043[b]	1.084 ± 0.069[c]

[a]Total number of apoptotic chondrocytes in the resting, proliferative, and terminal hypertrophic (THPC) zones. THPCs per millimeters of growth plate (mean ± SD) was calculated from 41 sections at each age.
[b]$p < 0.001$ vs 2 wk.
[c]$p < 0.001$ vs 2, 4, and 8 wk.

Table 2
Percentage (mean ± SD) of Immunoreactive Chondrocytes in Proliferative Zone

	2 wk	4 wk	8 wk	11 wk
Caspase-3	81 ± 8.1	78 ± 11.7	84 ± 6.4	90 ± 7.1
Caspase-6	73 ± 5.4	70 ± 6.8	77 ± 7.2	68 ± 2.0
Bcl-2	90 ± 8.6	93 ± 7.7	41 ± 11.0[a]	25 ± 2.8[a,b]
Bcl-x	80 ± 9.6	73 ± 8.2	42 ± 9.4[a]	30 ± 1.5[a,c]
Bax	86 ± 6.4	92 ± 9.4	90 ± 12.0	92 ± 4.5
Bad	87 ± 4.3	90 ± 5.8	85 ± 8.9	88 ± 3.3

[a]$p < 0.001$ vs 2 and 4 wk.
[b]$p < 0.01$ vs 8 wk.
[c]$p < 0.05$ vs 8 wk.

Table 3
Percentage (mean ± SD) of Immunoreactive Chondrocytes in Hypertrophic Zone

	2 wk	4 wk	8 wk	11 wk
Caspase-3	15 ± 6.2	13 ± 3.1	47 ± 11.4[b]	77 ± 13.6[a,b]
Caspase-6	20 ± 2.8	18 ± 1.2	52 ± 8.3[b]	81 ± 12.2[a]
p53	0	0	1.3 ± 0.1	3.4 ± 0.2
Bcl-2	25 ± 3.5	23 ± 2.0	9.0 ± 1.1[b]	6.8 ± 0.9[b]
Bcl-x	62 ± 7.2[a]	52 ± 12.8	38 ± 3.7	24 ± 2.9[b]
Bax	35 ± 8.0	40 ± 3.9	60 ± 9.4[c]	89 ± 3.2[a,b]
Bad	39 ± 5.5	43 ± 8.0	59 ± 12.3[c]	81 ± 9.7[b]

[a]$p < 0.001$ vs 8 wk.
[b]$p < 0.001$ vs 2 and 4 wk.
[c]$p < 0.01$ vs 2 wk.

more apoptotic THPCs compared with those from 2-wk-old rats. We believe that the increased number of apoptotic cells detected in growth plates from older animals reflects an increased rate of apoptosis. First, this is based on the integrated data from the Bcl family of proteins. The developmental pattern of the antiapoptotic Bcl-2 and Bcl-x proteins suggests that growth plates from older animals could be more prone to apoptosis because of the significant decrease in their immunoreactivity. Furthermore, the immunoreactivity for the proapoptotic Bax and Bad was more abundant in hypertrophic zones from the older animals (i.e., the area where apoptosis is more common). Second, immunoreactivity for caspases was almost absent in THPCs at 2 wk whereas it was present in most of the THPCs at 11 wk. These findings strongly suggest that in older animals, growth plate chondrocytes, especially the hypertrophic cells, are more susceptible to apoptosis because of decreased expression of Bcl-2 and Bcl-x, and increased expression of Bax, Bad, and caspases. Third, even though we studied an extensive number of sections, apoptotic cells were very rare at 2 wk. Finally, to the best of our knowledge, there is no direct evidence in the literature that the fate of all THPCs is death by apoptosis. This assumption is based on apoptosis detection in the growth plate at a certain point in time. The fact that some of these

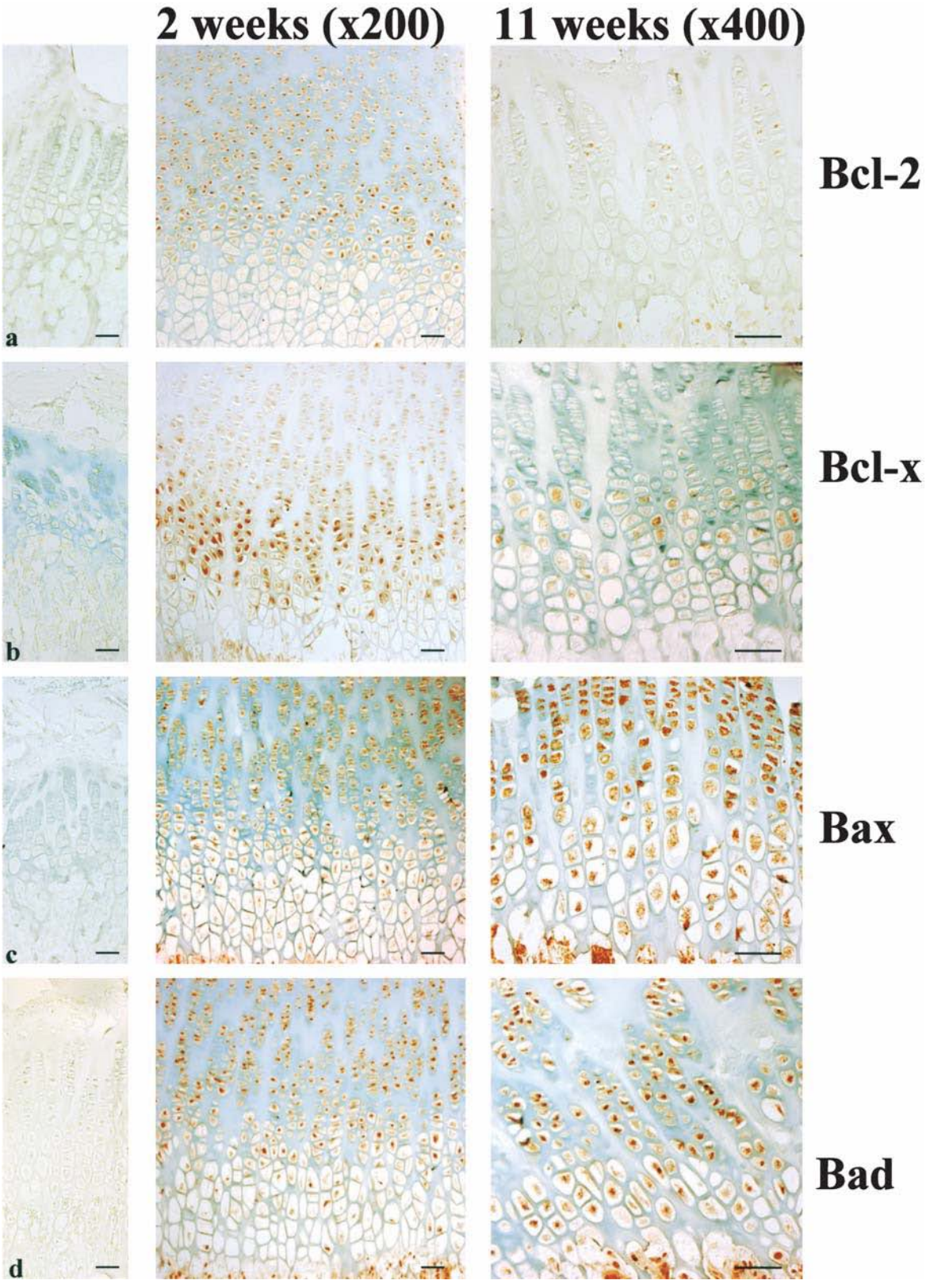

Fig. 2. Assessment of specificity of primary antibodies by replacement of primaries with normal rabbit (**A**) or goat serum (**B**), and by preincubating caspase-6 (**C**) or Bad (**D**) antibodies with corresponding peptides. A representative microphotograph from immunohistochemistry for Bcl-2, Bcl-x, Bax, and Bad in tibial growth plates from 2- (×200 magnification) and 11-wk-old male rats (×400 magnification) is shown. Immunoreactivity for the antiapoptotic Bcl-2 and Bcl-x was decreased at 11 wk in the whole growth plate, whereas immunoreactivity for the antiapoptotic Bax and Bad was increased in late and terminal hypertrophic chondrocytes, the area of increased apoptosis. Bar = 100 μm.

cells are detected as apoptotic does not provide direct evidence that this is the ultimate fate for all THPCs. In addition, the vast majority of the studies detected a limited number of apoptotic chondro-cytes in a process in which apoptosis is the proposed fate of those cells. As has been suggested, these cells could be trans-formed to osteoblastic cells *(1,2)* or die by an aberrant death *(5,7)* or by a programmed cell death, which does not have the characteristics of apoptosis.

The importance of apoptosis in the growth plate could be questioned since the number of apoptotic chondrocytes, detected by us and others *(22,23)*, is low compared with the number of THPCs that are replaced by bone during endochondral ossification. Nevertheless, for several reasons we believe that apoptosis plays a role in growth plate homeostasis, although we did not directly address this issue in the present study. First, using a special fixation method that preserves morphology, it has been found that one quarter of THPCs have morphologic characteristics of apoptosis *(3)*, suggesting that the TUNEL method can underestimate the magnitude of apoptosis because of technical limitations. Second, animal models with altered apoptosis in the growth plate (caspase-3 and PTHrP knockout mice, and mutations of the FGF-R) *(14–16)* indicate a role of apoptosis in growth plate homeostasis. Third, Bcl-2 knockout mice have skeletal deformities, with short legs and decreased growth plate thickness, but, most important, they exhibit premature maturation of growth plate chondrocytes and accelerated bone formation *(12)*. Taking into account that we mainly observed apoptosis at older ages with increased maturation and bone formation, we hypothesize that apoptosis is involved in epiphyseal senescence and closure.

We examined tibial growth plates at 2, 4, 8, and 11 wk of age because they represent different stages of longitudinal growth: fast growth before sexual maturity (2 and 4 wk); during maturation (8 wk); and during reduced growth rate (11 wk), at which time the rat tibial growth plate is reduced to a few layers of cells *(24)*. At the late stages (8 and 11 wk), we observed decreased Bcl-2 immunoreactivity and increased apoptosis. This resembles the Bcl-2 knockout mice model regarding growth plate maturation (increased apoptosis accompanied by decreased Bcl-2). Moreover, proliferation of chondrocytes is decreased in older, more mature animals *(25)* compared with younger animals, and, thus, loss of hypertrophic cells by apoptosis even in the low rate observed in our study could be important for the regulation of growth plate maturation and bone formation.

The activation of p53 leads to two cellular responses: growth arrest and apoptosis *(26)*. Studies indicate that p53 is a regulator of growth plate apoptosis. Knockout embryos for p53 exhibit alterations in bone length and width with reduced apoptosis in the hypertrophic layer of the growth plate resembling a later stage of maturation compared with normal embryos *(27)*. Furthermore, postnatally mice lacking p53 exhibit no apoptosis in the growth plate in contrast to their normal littermates *(28)*. These studies support our finding that only a few THPCs from older animals are immunostained for p53, a pattern similar to the TUNEL staining.

In our study, Bcl-2 and Bax immunoreactivities were distributed in a way similar to that previously described in 21 to 25-d-old rats *(17)*. We also report, for the first time, the expression patterns of Bcl-x, Bad, and caspase-3 and -6 immunoreactivities. Bcl-x immunoreactivity was more prominent in the transitional zone between proliferative and hypertrophic cells and extended more into the hypertrophic zone than Bcl-2. Immunoreactivity for both caspase-3 and -6 was expressed in proliferative and early hypertrophic cells at all developmental stages despite the observation that apoptosis is rare in this area of the growth plate. This suggests that these cells have the potential to die by apoptosis if they receive the appropriate stimulus, which would possibly affect longitudinal growth. Nevertheless, the integrated data we present here for the Bcl family of proteins and caspases suggest that during development a shift occurs in the growth plate—from an antiapoptotic state to a state favoring apoptosis later during development.

In summary, our study indicates that apoptosis and apoptosis-regulating proteins are developmentally regulated during growth plate maturation. When the growth plate matures and the growth rate decreases, an increased number of apoptotic terminal hypertrophic cells are detected in proliferative and mainly in mature hypertrophic chondrocytes. The developmental changes in apoptosis occurred concurrently with changes in the immunoreactivity pattern for caspases and the Bcl family of proteins, supporting the apoptosis data. Finally, we propose that apoptosis may play an active regulatory role in growth plate maturation during normal development.

Materials and Methods

Animals and Sample Preparation

All animal experiments were carried out after approval by the local ethical committee at Karolinska Institute.

Male Sprague Dawley rats (B&K Universal, Sollentuna, Sweden) were housed in 12-h light, 12-h dark cycles and fed pellets ad libitum. After an adaptation period, animals were killed with an ip injection of phenobarbital at 2, 4, 8, or 11 wk of age ($n = 5$ animals/group). Tibiae were removed and the upper part was fixed in 4% formaldehyde in 0.1 *M* phosphate buffer, pH 7.3, for 18 h at 4ºC. After fixation, specimens were decalcified in 5% EDTA and then paraffin embedded.

Five-micrometer-thick sections were cut longitudinally and mounted on histologic glass slides (Superfrost +/+; Menzel-Gloeser, Braunschweig, Germany). On each slide, four sections were mounted, one section from each group, so that all samples were treated under the same conditions. Slides were dried at 37ºC overnight.

Detection of Apoptosis

Apoptotic cells in the growth plates were identified by TUNEL, according to the manufacturer's instructions (TdT-FragEL kit, Oncogene Research Products, Boston, MA, USA) with slight modifications. Deparaffinized and rehydrated sections were treated with proteinase K (20 µg/mL) at 37ºC for 10 min instead of 20 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 min. Slides were then placed in equilibrating buffer and incubated in a reaction buffer containing TdT and deoxynucleotides at 37ºC for 90 min. Sections were rinsed several times with Tris-buffered saline (TBS); incubated with blocking buffer for 30 min to reduce nonspecific binding, and then with peroxidase streptavidin conjugate (30 min) to detect biotinylated nucleotides; and finally rinsed with TBS. The sections were then exposed to 0.5 mg/mL of diaminobenzidine (DAB) and 0.05% H_2O_2 to generate a brown reaction product. Sections were counterstained with methyl green (Vector, Burlingame, CA), dehydrated, and mounted with Mountex (Histolab, Göteborg, Sweden). At least eight sections from each animal, 25 µm apart (to avoid counting the same cells twice), were used to determine the number of apoptotic cells. The number of apoptotic chondrocytes was counted in all three zones of the growth plate (resting, proliferative, and hypertrophic). Only cells in closed lacunae were counted and expressed as their number per millimeter length of the chondroosseous junction. The length of the chondroosseous junction from each section was measured by the Image-Pro® image analysis system (Media Cybernetics, Silver Spring, MD). Treatment of sections with DNase labeled all cells, whereas sections treated with distilled water instead of TdT were negative.

Immunohistochemistry

Sections were dewaxed in xylene and rehydrated in graded alcohol baths. Antigen retrieval was performed in 0.01 *M* citrate buffer (pH 6.0) at 95–98ºC for 20 min. Slides were then washed in TBS, and endogenous peroxidase activity was quenched by incubating in 3% H_2O_2 in methanol for 5 min. Nonspecific binding was blocked by incubating with 1.0% bovine serum albumin (BSA) (Jackson, West Grove, PA) diluted in TBS for 1 h. Primary antibodies were diluted in 0.05 *M* Tris, 2.7% NaCl, 0.3% Triton-X100, and 0.1% BSA. Sections were incubated overnight at +4ºC, washed several times in TBS containing 0.1% Tween-20 (TBS-T), and then incubated with biotinylated goat anti–rabbit IgG (1:1000) (Jackson) or rabbit anti–goat IgG (1:200) (Vector) for 45 min at room temperature. Finally, sections were washed several times in TBS-T, incubated with avidin-conjugated peroxidase (Vector) for 30 min, and peroxidase activity was detected using a DAB kit that generates a brown color (Vector). Digital microphotographs were captured by a Nikon Eclipse 800 microscope (Nikon, Bergström, Stockholm, Sweden) equipped with a Hamamatsu Orca IIIf charge-coupled device camera (Hamamatsu, Stockholm, Sweden).

Bax, Bad, caspase-3, caspase-6, and p53 polyclonal antibodies were purchased from Santa Cruz Biotechnology. Bcl-2 polyclonal antibody was from Upstate Biotechnology (Lake Placid, NY), and polyclonal Bcl-x antibody was from Transduction (Becton Dickinson AB, Stockholm, Sweden). Bax antibody was used at a 1:100 dilution, Bad at 1:150, Bcl-x at 1:500, Bcl-2 at 1:600, caspase-3 at 1:100, caspase-6 at 1:100, and p53 at 1:150. Control sections were incubated with preadsorbed antibodies (blocking peptides for Bax, Bad, and caspase 6; 1:5 dilution), nonspecific rabbit IgG, or nonspecific goat IgG for 2 h at room temperature. Rat brain/cerebellum and lung were used as controls for caspase-3 and -6 *(29)*, Bcl-2 *(30)*, Bcl-x *(31)*, Bax *(31)*, and Bad *(32)*.

Immunopositive chondrocytes were detected by automatic thresholding using Image-Pro®. For each antibody, at least 300 cells were analyzed in two separate sections of each specimen. Immunopositive cells are expressed as a percentage of the total number of cells analyzed in the proliferative and hypertrophic zones, respectively.

Statistical Analyses

Results are expressed as the mean ± SD. Differences among groups were tested by one-way analysis of variance. All p values were calculated using the Newman-Keuls posttest. A value of $p < 0.05$ was considered significant.

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