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Collagen Metabolism Is Markedly Altered in the Hypertrophic Cartilage of Growth Plates from Rats with Growth Impairment Secondary to Chronic Renal Failure

JESÚS ÁLVAREZ,[1,2] MILAGROS BALBÍN,[2,3] MARTA FERNÁNDEZ,[1] and JOSÉ M. LÓPEZ[1,2]

ABSTRACT

Skeletal growth depends on growth plate cartilage activity, in which matrix synthesis by chondrocytes is one of the major processes contributing to the final length of a bone. On this basis, the present work was undertaken to ascertain if growth impairment secondary to chronic renal insufficiency is associated with disturbances of the extracellular matrix (ECM) of the growth plate. By combining stereological and in situ hybridization techniques, we examined the expression patterns of types II and X collagens and collagenase-3 in tibial growth plates of rats made uremic by subtotal nephrectomy (NX) in comparison with those of sham-operated rats fed ad libitum (SAL) and sham-operated rats pair-fed with NX (SPF). NX rats were severely uremic, as shown by markedly elevated serum concentrations of urea nitrogen, and growth retarded, as shown by significantly decreased longitudinal bone growth rates. NX rats showed disturbances in the normal pattern of chondrocyte differentiation and in the rates and degree of substitution of hypertrophic cartilage with bone, which resulted in accumulation of cartilage at the hypertrophic zone. These changes were associated with an overall decrease in the expression of types II and X collagens, which was especially marked in the abnormally extended zone of the hypertrophic cartilage. Unlike collagen, the expression of collagenase-3 was not disturbed severely. Electron microscopic analysis proved that changes in gene expression were coupled to alterations in the mineralization as well as in the collagen fibril architecture at the hypertrophic cartilage. Because the composition and structure of the ECM have a critical role in regulating the behavior of the growth plate chondrocytes, results obtained are consistent with the hypothesis that alteration of collagen metabolism in these cells could be a key process underlying growth retardation in uremia. (J Bone Miner Res 2001;16:511–524)

Key words: growth plate, uremia, collagen, collagenase, bone growth

INTRODUCTION

CHRONIC ILLNESS in childhood very often interferes with normal growth and leads to diminished stature and delayed bone ages. This is the case of children with chronic renal disease, in which stunted growth is seen in approximately one-third of the children at the time of diagnosis, and normal growth rate usually is not restored by chronic hemodialysis or even after successful renal transplantation.[1–3] The pathogenesis of this growth failure is complex and multifactorial including nutritional, electrolyte, and hormonal disturbances. Although there have been numerous reports on the pathogenesis of this growth retardation, most of them have been focused on the endocrinological disturbances and

[1]Departamento de Morfología y Biología Celular, Facultad de Medicina, Universidad de Oviedo, Asturias, Spain.
[2]Instituto Universitario de Oncología del Principado de Asturias (IUOPA), Asturias, Spain.
[3]Departamento of Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Oviedo, Asturias, Spain.

only very few attempts have been made to characterize the effective response of the growth plate cartilage to uremia. Based on such low knowledge, we performed a first histomorphometric study to characterize modifications of the growth plate in rats with growth impairment secondary to chronic renal failure.[4] Results from this former study indicated that uremia depressed both the activity of the growth plate cartilage and the replacement of cartilage by bone at the metaphyseal end. The present study is a continuation of this previous work; because chondrocytes are specialized connective-tissue cells in which their main function is the production of a specific collagenous extracellular matrix (ECM), we examine if growth failure induced by uremia is associated with modifications of ECM of the growth plate.

The ECM of growth plate cartilage is an inextensible network of collagen fibrils embedded in a highly hydrated complex of aggregating proteoglycan and hyaluronic acid, which has a main role in affecting the behavior of cells that contact it.[5–8] Type II and type X collagen are the most abundant proteins found in the growth plate cartilage. Type II is the predominant structural collagen, accounting for up to 25% of the dry weight. It is present in all zones of the growth plate and is the major protein responsible for providing the tissue with a fibrillar framework to arrange chondrocytes as well as other ECM components. Type X collagen is a specific ECM component of the hypertrophic-preossifying cartilage. The half-life of collagen fibers in the growth plate is much shorter than in other tissues, being the control of collagen production and turnover of major importance for chondrocyte differentiation and bone growth. Degradation of collagens primarily depends on collagenases, which share the unique ability to cleave the native helix of fibrillar collagens. Sequence comparisons have proven that rat and mouse collagenases are homologous to the human collagenase-3 referred to as matrix metalloproteinase 13 (MMP-13).[9] This enzyme degrades very efficiently the native helix of fibrillar collagens, with preferential activity on type II collagen. Likewise, it also is able to degrade the initial cleavage products of collagenolysis to smaller fragments as well as cartilage aggrecans.[10–13] Based on such a wide range of action and considering that its expression in physiological conditions is restricted to the growth plate of bones, collagenase-3 has been considered to play a major role during endochondral ossification.[14–17] Recently, we have provided additional support to this suggestion by showing that the pattern of collagenase-3 expression in the growth plate of young rats varies in the function of the animal's growth rate.[18]

The present study tests the hypothesis that chronic renal failure could induce alteration of the synthetic and degradative activities of the growth plate cartilage collagens and such alteration could be a key process underlying growth alteration. To this end, we have examined the expression patterns of the two major collagens of the growth plate cartilage (types II and X collagens) and of the essential enzyme for the beginning of the collagenolysis (collagenase-3) in tibial growth plates of severely uremic rats. In situ hybridization techniques were coupled with electron microscopy to analyze possible changes in the ultrastructure of the collagen fibril architecture.

MATERIALS AND METHODS

Animals and experimental protocol

Male Sprague–Dawley rats, 21 days old, were housed in individual cages and fed a standard 23.9% protein rat chow containing 0.85% calcium and 0.75% phosphorus (AO3; Panlab SL, Barcelona, Spain). After a 4-day adjusted period, the animals were divided into three groups: 5/6 nephrectomized (NX), sham-operated fed "ad libitum" (SAL), and sham-operated pair-fed (SPF) NX animals. At the beginning of the experiment, rat's weights ranged from 70 to 90 g, the mean weight being no different among the three groups of rats. Subtotal NX or sham operation was performed in two stages, on day 0 and day 4, as reported previously.[4] SPF animals were subjected to pair feeding with NX animals. Rats' body growth was assessed by weight and length gained between day 4 and 14. Rats and food consumption were weighed daily. Nose to tip tail length was measured under anesthesia on day 4 and day 14. After 14 days, the experiment was ended and rats were killed by exsanguination under anesthesia. At that moment, blood was obtained for measuring serum concentrations of urea nitrogen, with an autoanalyzer Kodak EktachemR (Eastman Kodak, Rochester, NY, USA).

Tissue collection and processing

Twenty-four rats (8 per group) were used for histological studies. All animals were injected intraperitoneally with calceine (15 mg/kg body weight) (Sigma, St. Louis, MO, USA) 4 days before death. Tibias were isolated immediately after death and cut through the sagittal plane of the epiphysis into two equal-sized parts, obtaining four tibial halves from each animal. Tissues were processed according to four different protocols. One tibial half per animal was processed for determination of both the rate of longitudinal bone growth and the histomorphometric parameters, a second tibial half was processed for messenger RNA (mRNA) in situ hybridization, a third tibial half per animal was processed for histochemical studies, and the last tibial half was processed for electron microscopy. All tissue collections were performed between 9:30 and 11:30 a.m.

Determination of the rate of longitudinal bone growth

Tibial halves for determination of the rate of longitudinal bone growth were cut additionally in five prismatic blocks, fixed in 40% ethanol for 3 days at 4°C, and subsequently dehydrated in ethanol, embedded in Durkupan-ACM (Sigma), and cut on a Ultracut E ultramicrotome (Reicher, Vienna, Austria) parallel to the tibial vertical axis. Sections 1 μm thick were obtained in three blocks chosen in an unbiased manner per animal and were viewed in an incident-light fluorescence microscope (Leitz, Wetzlar, Germany) equipped with a micrometric eyepiece. Measurements of the distance between the zone of vascular invasion in the growth plate and the proximal endpoint of the calcein front were obtained at four randomly determined locations on each of the three sections per animal, and the mean of

these measurements divided by four (days between calceine injection and killing) was considered the longitudinal bone growth per day in each animal.

Histomorphometry

One section 1 μm thick was obtained in each of the three randomly chosen samples per animal and was used for histomorphometric analysis. Sections were stained with toluidine blue and analyzed with an Olympus light microscope (Olympus, Shinjuku-ku, Japan) interfaced via a Sony camera (Sony, Tokyo, Japan) to an image analysis system (Qwin Pro; Leica, Heidelberg, Germany). Detailed histomorphometric analysis has been reported previously.[18] Growth plates were divided arithmetically proximodistally in five horizontal strata of equal height (S1–S5) to obtain cell populations with differing degrees of maturation.

The height of the growth plate was estimated by measuring its height at four randomly chosen locations on each section. Axial cell columns, over a short time period can be considered as steady-state systems in which cell production and elimination rates are constant.[19,20] On this basis, the chondrocyte location turnover and the mean cell height increase per hour in each stratum were estimated. Chondrocyte location turnover is defined as the time that a chondrocyte remains in a certain position of the column. This time is equal to the time a hypertrophic cell remains in the most distal position of the column and was estimated by the inverse of the number of chondrocytes leaving the growth plate per column per day.[21] This last (the number of chondrocytes leaving the growth plate per column per day) could be estimated by dividing the daily linear growth rate by the chondrocyte height in the distal stratum (S5). The mean chondrocyte height, defined as the length of the intercept bisecting the cell profile measured parallel to the long axis of the bone, was estimated in each stratum by direct measurements of the vertical axis of 50 chondrocytes cut centrally (through the nucleus) per section.[20] Thus, a total of 150 cells were measured to give a mean value per animal and stratum. The mean cell height increase per hour of a stratum was estimated by dividing the difference between the mean chondrocyte height in this stratum and that of the preceding one by the time required for a chondrocyte to place across it. The time each chondrocyte spends in a stratum is equal to the time required for replacement of the total number of cells in such stratum and can be estimated by dividing the mean number of cells per column in this stratum by the number of chondrocytes leaving the stratum per column per hour.[21] The number of chondrocytes per column in each stratum was obtained by dividing the height of the stratum by the mean chondrocyte height. Because the growth plate is a steady-state system, the mean number of chondrocytes leaving each stratum per column per hour is approximately the same for all the strata and can be estimated by the number of chondrocytes leaving the growth plate per column per day.[19]

In situ hybridization

Tibias for in situ hybridization were fixed by immersion in 4% paraformaldehyde at 4°C for 12 h, rinsed in phosphate-buffered saline (PBS), decalcified in 10% EDTA at pH 7.0 for 48 h, dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin. Sections were cut at a thickness of 5 μm and mounted on Superfrost Plus slides (Menzel-Glaser, Braunschweig, Germany). Blocks were cut parallel to the tibial vertical axis and at random orientation relative to the horizontal plane. Digoxigenin-11-uridine triphosphate (DIG-11-UTP)–labeled single-stranded RNA probes were prepared with DIG RNA labeling mix and the appropriate RNA polymerases to transcribe either sense or antisense probes according to the manufacturer instructions (Boehringer, Mannheim, Germany). Rat collagenase-3 probe was a 314-base pair (bp) fragment corresponding to nucleotides 350–653 of the rat collagenase-3 gene.[22] The type II collagen probe was a 550-bp *Pst*I fragment from the aminoterminal portion of rat pro-α1(II) chain cloned in PGEM 3Zf-vector.[23] The type X collagen probe was a 650-bp *Hin*dIII fragment containing 360 bp of noncollagenous (NC1) domain and 290 bp of 3′-untranslated sequence of the mouse type X collagen gene, subcloned into the *Hin*dIII site of pBluescript.[24] Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a 1.2-kilobase (kb) *Eco*RI fragment obtained from American Type Culture Collection (ATCC; Manassas VA, USA). In situ hybridization was performed using methods described in detail elsewhere.[18] Likewise, quantification of in situ hybridization signals was done as previously reported.[18] Briefly, a first approach was used to estimate expression time lengths for the three proteins studied. Expression time length was defined as the time a growth plate chondrocyte expresses a protein in its lifetime during the differentiation cycle. A second approach consisting in semiquantitative comparisons of cell mRNA content was used to estimate expression levels in chondrocytes with differing degree of maturation.

Histochemistry

Tissues for histochemistry were fixed by immersion in 4% paraformaldehyde at 4°C for 12 h, rinsed in PBS, decalcified in 10% EDTA at pH 7.0 for 48 h, embedded in Tissue-Tek O.C.T. compound, and snap-frozen. Cryostat sections were cut at a thickness of 5 μm and mounted on Superfrost Plus slides. Alkaline phosphatase (ALP) activity was detected in frozen sections pretreated with a 10-mM $MgCl_2$ solution to reactivate enzyme activity and subsequently incubated with a substrate solution containing 0.16 mg/ml 5-bromo-4-chloro-3-indolylphosphate and 0.33 mg/ml nitroblue tetrazolium in 100 mM Tris, 100 mM NaCl, and 50 mM $MgCl_2$, pH 9.5, for 30 minutes at room temperature. Quantification of cytochemical stain intensity was performed by the two approaches described previously on two serial sections per animal.

Table 1. Serum Urea Nitrogen, Cumulative Food Intake, Weight and Length Gain, Growth Rate, Growth Plate Height, Mean Number of Chondrocytes per Column, and Time for Chondrocyte Location Turnover of the Three Groups of Rats[a]

	SAL	*SPF*	*NX*
SUN (mg/dl)	11.3 ± 1.9	8.1 ± 1.7	74.0 ± 10.7*,†
Food intake (g)	195.0 ± 23.8	121.8 ± 16.6*	121.9 ± 20.3*
Weight gain (g)	66.4 ± 11.6	29.7 ± 5.7*	26.9 ± 10.2*
Length gain (cm)	3.5 ± 1.1	3.1 ± 0.2	2.4 ± 1.4
Longitudinal growth rate (μm/day)	319.5 ± 21.4	280.8 ± 21.4*	169.5 ± 27.4*,†
Growth plate height (μm)	592.7 ± 35.1	454.4 ± 64.0*	862.7 ± 102.6*,†
Chondrocytes per column	31.5 ± 3.0	24.7 ± 3.3*	49.5 ± 5.9*,†
Time for chondrocyte turnover (h)	3.0 ± 0.4	3.3 ± 0.3	4.9 ± 0.8*,†

[a] Each point is the mean value of 8 animals ± SD.
SUN, serum urea nitrogen.
* $p < 0.05$ when compared with SAL; † $p < 0.05$ when compared with SPF.

Electron microscopy

Tibial halves for electron microscopy were cut additionally in thin vertical sections. Alternate cartilage blocks were prepared using two different fixation procedures. Three samples per animal were fixed in a solution of 2% glutaraldehyde and 0.7% ruthenium hexamine trichloride (RHT; Strem Chemicals, Newburyport, MA, USA) in 0.05 M cacodylate buffer, pH 7.4, for 3 h at 4°C. They were then washed in buffer and postfixed in a solution of 1% osmium tetroxide and 0.7% RHT in cacodylate buffer for 2 h at room temperature. The other three samples per animal were fixed in a solution of 4% glutaraldehyde and 1 mM calcium chloride in 0.05 M cacodylate buffer, pH 7.4, for 4 h at 4°C. They were then washed in buffer and postfixed in a solution of 1% osmium tetroxide and 2% potassium ferrocyanide in cacodylate buffer for 2 h at room temperature. Both types of fixed blocks were dehydrated with a graded series of acetone and embedded in Durkupan-ACM (Sigma). Ultrathin sections were cut on a Reicher Ultracut E ultramicrotome, stained with lead citrate and viewed with a JEM-2000 EX II electron microscope (Jeol, Tokyo, Japan).

RNA extraction and reverse-transcription polymerase chain reaction

Nine rats (3 per group) were used for preparation of total cellular RNA. Total RNA was isolated by the guanidinium isothiocyanate procedure and separated by electrophoresis in 1.2% agarose-formaldehyde gels to verify the RNA integrity. Complementary DNA (cDNA) synthesis and polymerase chain reaction (PCR) of total RNA were performed with an RNA-PCR kit (Perkin-Elmer Cetus, Foster City, CA, USA). Reverse transcription (RT) was carried out for 45 minutes at 58°C with 2 μg of total RNA and random hexamers as primers in a total volume of 20 μl. For the amplification, a 2-μl aliquot of each RT reaction was amplified in a volume of 50 μl with the next oligonucleotides as primers:

Rat type II collagen: 5′TTGGTGTGGACATAGGGCCT3′ and 5′GTCTGCCCAGTTCAGGTCTC3′.

Rat type X collagen: 5′GCATTAGCACCCAAGATCTG3′ and 5′CGAGTGGACGTACTCAGAGG3′.

Rat GAPDH: 5′TGCATCCACTGGTGCTGCCA3′ and 5′GAGGGCCTCTCTCTTGCTCT3′.

Rat collagenase-3: 5′ATTGTGAACTACACCCCTG3′ and 5′TGGCCAAGCTCATGGGCA3′.

PCR was carried out in a GeneAmp 2400 PCR system (Perkin-Elmer Cetus) with cycles of 94°C (15 s), 60°C (15 s), and 72°C (45 s) for both type II collagen and GAPDH and with 94°C (15 s), 58°C (15 s), and 72°C (45 s) for type X collagen and collagenase-3. The reaction product was removed after 30 cycles for collagenase-3, 25 cycles for GAPDH and type II collagen, and 42 cycles for type X collagen and analyzed by agarose gel electrophoresis. Previously, a 10-μl aliquot of each reaction product was removed after 24, 30, 35, and 42 cycles and analyzed by agarose gel electrophoresis to assess that the reaction was in the exponential phase of amplification. Bands were scanned by using a densitometer (Scion Image based on National Institutes of Health Imager for Apple Computer, Frederick, MD, USA) and the signals were obtained for collagens, and collagenase-3 in the three samples of each group were corrected to the signals obtained for GAPDH in the corresponding samples.

Statistical analysis

A mean value for each of the tested parameters was computed on a per animal basis in the group. These values were used to calculate a mean and SD in each group. Data were compared among the three groups using a one-way analysis of variance (ANOVA), and, when the *f* test was significant ($p < 0.05$), differences between specific means were tested by Student-Newman *t*-test.

RESULTS

Renal failure of NX rats was confirmed by concentrations of serum urea nitrogen about seven to nine times higher than

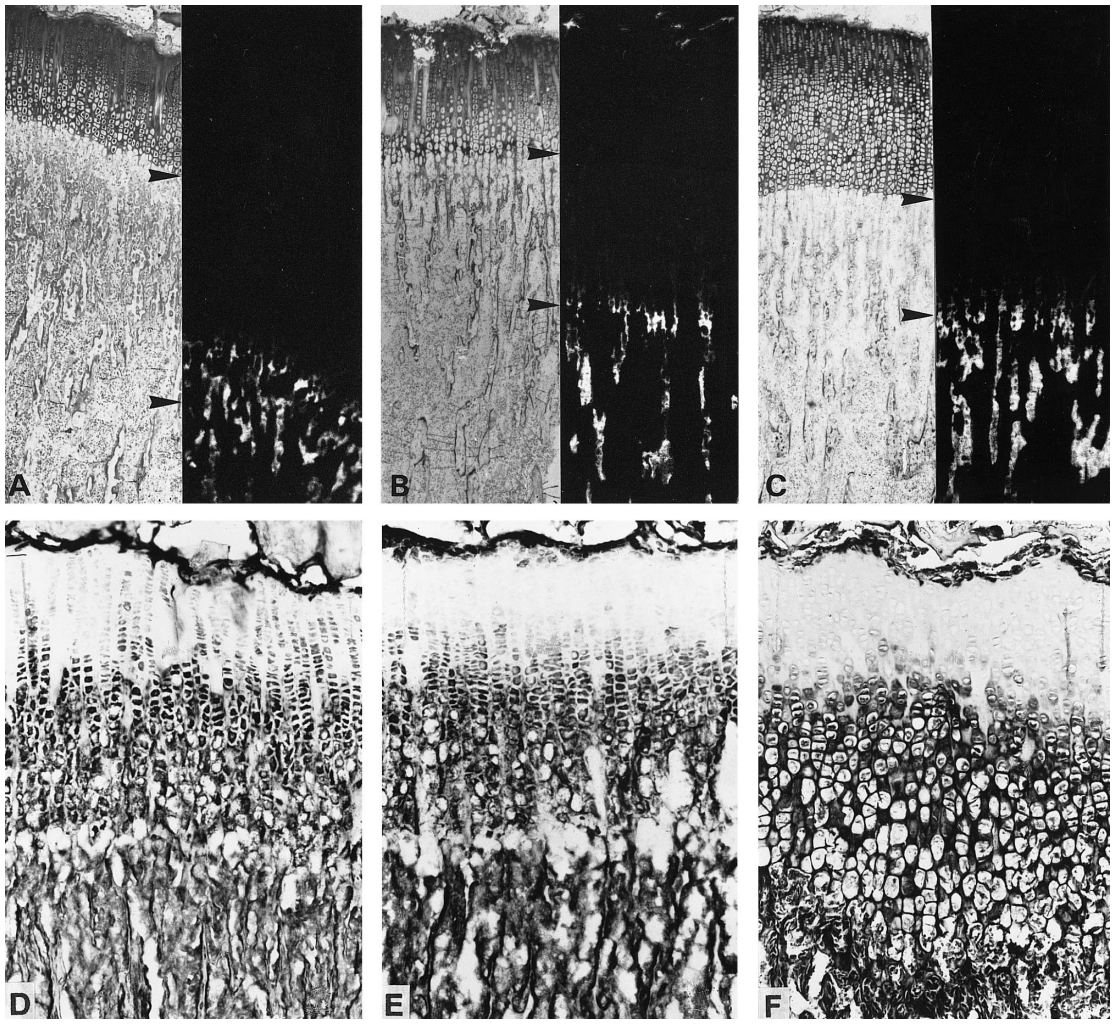

FIG. 1. Growth plate histology and longitudinal growth. (A–C) Parallel sections of the proximal tibial growth plate viewed by bright-field microscopy after toluidine blue staining (left) and by incident-light fluorescence microscopy (right) of (A) SAL rats, (B) SPF rats with 5/6 nephrectomized rats, and (C) 5/6 nephrectomized rats NX rat at the same magnification (×26). The distance between the lower border of the growth plate and the fluorochrome-labeled front (space between arrowheads) appears clearly different between NX rats and the two control groups. Please note that in lateral zones of the tibia both the growth plate and the calcein front appear curved (A) and that growth plate height is markedly increased in NX rats. (D–F) Histochemical detection of ALP activity in growth plates from (D) SAL, (E) SPF, and (F) NX rats. It is observed that histological differences between NX rats and control groups are restricted to the hypertrophic zone, which is characterized by positive ALP activity (magnification ×65).

in SAL and SPF rats (Table 1). Food intake of NX and SPF animals was reduced to 63% of that of SAL (Table 1). NX rats gained less length than SPF rats, but differences were not statistically significant (Table 1). However, differences became significant when the rate of longitudinal bone growth was considered. By using the calcein labeling method, growth rate in NX rats was found to be significantly lower than in SPF rats and even more so than in SAL rats, whereas SPF rats grew significantly lower than SAL rats (Table 1; Fig. 1). Growth plates from NX rats became altered, being greater and more irregular than those of both groups of control rats. It is remarkable that growth plates from NX and SPF rats showed a divergent alteration in the sense that, when compared with SAL, their heights were increased in NX rats and decreased in SPF rats (Table 1). In the NX rats, the height of the proliferative zone, characterized by no ALP activity, was not noticeably different from the control groups (Fig. 1). It showed the normal features with proliferating chondrocytes arranged regularly in axial columns. By contrast, the zone of hypertrophy, characterized by ALP activity was significantly wider (Fig. 1). Hypertrophic chondrocytes in NX rats showed a less regular distribution in vertical columns and the chondro-osseous junction appeared more irregular than in either SAL or SPF rats. Both the height of the growth plate and the number of chondrocytes per column were significantly increased in NX rats (Table 1), whereas cell height increase per hour at the distal strata and terminal chondrocyte height were sig-

TABLE 2. CELL HEIGHT AND CELL HEIGHT INCREASE PER HOUR AT THE DIFFERENT STRATA OF GROWTH PLATES IN THE THREE GROUPS OF RATS[a]

	SAL	*SPF*	*NX*
Cell height (μm)			
S1	8.2 ± 0.7	8.1 ± 0.6	8.5 ± 0.5
S2	9.2 ± 0.7	9.9 ± 0.9	9.6 ± 0.6
S3	20.3 ± 2.1	22.0 ± 3.3	18.9 ± 1.4†
S4	35.5 ± 2.5	37.1 ± 2.4	33.4 ± 3.0†
S5	39.5 ± 2.8	38.4 ± 2.6	34.2 ± 3.2*,†
Cell height increase rate (μm/h)			
S1	—	—	—
S2	0.03 ± 0.02	0.08 ± 0.03*	0.02 ± 0.01†
S3	0.81 ± 0.24	1.13 ± 0.49	0.26 ± 0.09*,†
S4	1.92 ± 0.38	2.28 ± 0.49	0.70 ± 0.27*,†
S5	0.57 ± 0.23	0.21 ± 0.11	0.04 ± 0.02*,†

[a] Each point is the mean value of 8 animals ± SD.
* $p < 0.05$ when compared with SAL; † $p < 0.05$ when compared with SPF.

nificantly decreased (Table 2). Morphometric data indicated that uremia affected differentiation from prehypertrophic chondrocytes to chondrocytes that were properly hypertrophic.

The expression of mRNAs of type II and X collagens and collagenase-3 was analyzed by in situ hybridization (Fig. 2) and quantitated by horizontal stratum (Fig. 3). In normal SAL rats, type II collagen expression was observed in all growth plate zones although changes in the level of expression were found for chondrocytes located at different horizontal strata of the same vertical column (Fig. 2A). Type II collagen expression was low in the most proximal stratum (S1), increased in S2, peaked in S3, and then showed a progressive decrease to reach relatively low values in the most distal stratum (S5; Fig. 3). In SPF rats, the pattern of type II collagen expression along the vertical columns of chondrocytes was basically the same as in SAL rats (Fig. 2D), but values for expression levels, as estimated by cytochemical stain intensities, were significantly lower (Fig. 3). Type II collagen expression in NX rats was not noticeably different from the control groups at the proliferating and early hypertrophic chondrocytes (Fig. 2G). However, it was significantly decreased in the two distal strata (S4 and S5) and in the last stratum (S5) it was extremely low (Figs. 2G and 3). Thus, chondrocytes located at the abnormally extended zone of hypertrophic cartilage of the NX rats' growth plates showed little or even no expression of type II collagen. Hypertrophic chondrocytes showing no expression of type II collagen were metabolically active, as proven by expression of GAPDH, a constitutively active housekeeping gene (Fig. 4). Because the spatial arrangement of chondrocytes within columns reflects their development in a temporal fashion, the occurrence of negative chondrocytes at the distal layers of a growth plate column means in terms of temporal sequence that type II collagen expression was turned off at a specific point of the abnormally extended process of hypertrophy in NX rats. Type II collagen expression was estimated to cease about 24.7 h before ossification in NX rats.

Comparable results were obtained when type X collagen expression was studied. In SAL rats, type X collagen expression suddenly appeared at the early hypertrophic zone (S3), peaked in S4, and declined to moderate values in the most distal chondrocytes (S5) (Fig. 2B). Differences between SAL and SPF rats were found in expression levels (greater in SAL rats; Fig. 3) but not in the duration of the period of expression (Table 3). Taken together, these results indicate that chondrocytes from SAL rats expressed higher levels of type X collagen during a similar period of time than those from SPF rats. In spite of such differences, type X collagen expression extended from early hypertrophic chondrocytes to chondrocytes located at the site of vascular invasion in both groups and then it could be considered that the expression pattern along vertical columns of chondrocytes was not changed substantially (Figs. 2B and 2E). By contrast, the pattern of type X collagen expression in growth plates from NX rats did vary. Expression of type X collagen was high only in a narrow stripe of cartilage beginning at the early hypertrophic zone and extending to a few layers of chondrocytes. Beyond this point, type X collagen expression suddenly dropped and was hard to recognize at the most distal layers of hypertrophic chondrocytes (Fig. 2H). The occurrence of negative chondrocytes located at both the proximal and the distal edges of the positive cells in a growth plate column means in terms of a temporal sequence that type X collagen expression was turned on at the beginning of the hypertrophy and subsequently was turned off at a later step of the abnormally extended hypertrophic process in NX rats. It was estimated that in NX rats type X collagen expression ceased about 19.8 h before ossification. Nevertheless, the estimated duration during which each chondrocyte expressed type X collagen in NX rats was found to be significantly higher than in both SAL and SPF rats (Table 3).

Unlike collagens, the expression pattern of collagenase-3 was not remarkably changed by uremia. In SAL rats, collagenase-3 expression was very low at the proliferating chondrocytes, increased at the early hypertrophic chondro-

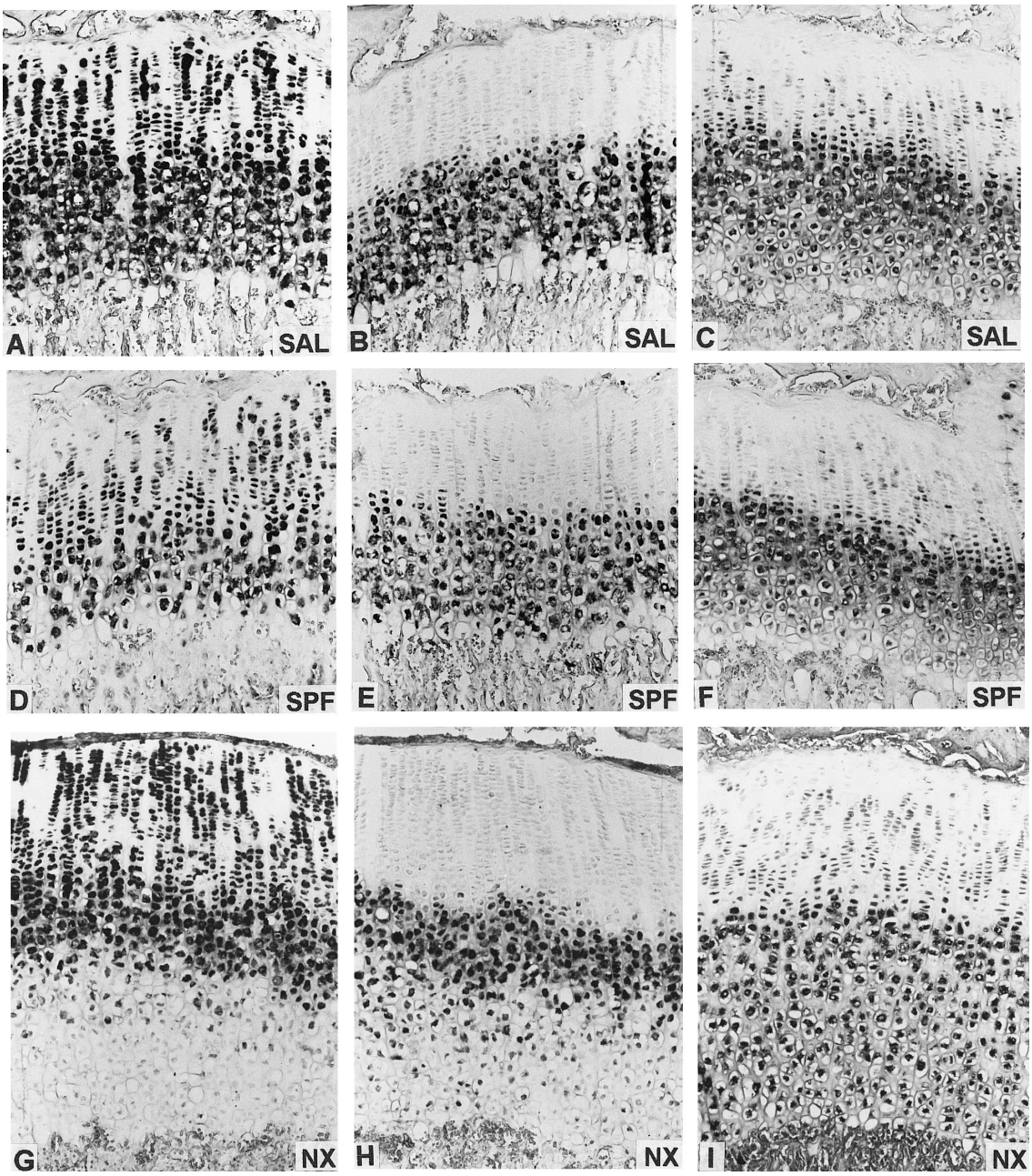

FIG. 2. In situ hybridization of type II and X collagens and collagenase-3 in growth plates from (A–C) SAL, (D–F) SPF, and (G–I) NX rats. Note that no substantial differences between SAL and SPF rats are found in the expression patterns along vertical columns of chondrocytes for the three probes studied. Type II collagen expression in (A) SAL and (D) SPF rats is observed on the whole extent of the growth plate. Type X collagen expression suddenly changes from zero to relatively high levels at the early hypertrophic zone in both (B) SAL and (E) SPF rats. Collagenase-3 expression in both (C) SAL and (F) SPF rats was located mainly at the hypertrophic cartilage. There are substantial changes in the expression pattern of both collagen types in NX rats. Type II collagen expression in NX rats (G) is not noticeably different from the control group at the proliferating and early hypertrophic chondrocytes but is significantly decreased at the distal zone of the growth plate. Chondrocytes found in the abnormally extended zone of hypertrophic cartilage show little or even no expression. Type X collagen expression in growth plates from (H) NX rats is significantly decreased at the lower layers of hypertrophic chondrocytes. By contrast, collagenase-3 expression in (I) NX rats is observed in chondrocytes located at the abnormally extended hypertrophic cartilage of these animals (magnification is the same for all micrographs, ×105).

cytes, and reached maximal values in chondrocytes located at S4, which corresponded to the stratum in which cell height increase per hour was maximal (Table 2). Subsequently, the expression of the enzyme reached moderate values in the most distal chondrocytes (Figs. 2C and 3). Although there were differences between SAL and SPF rats,

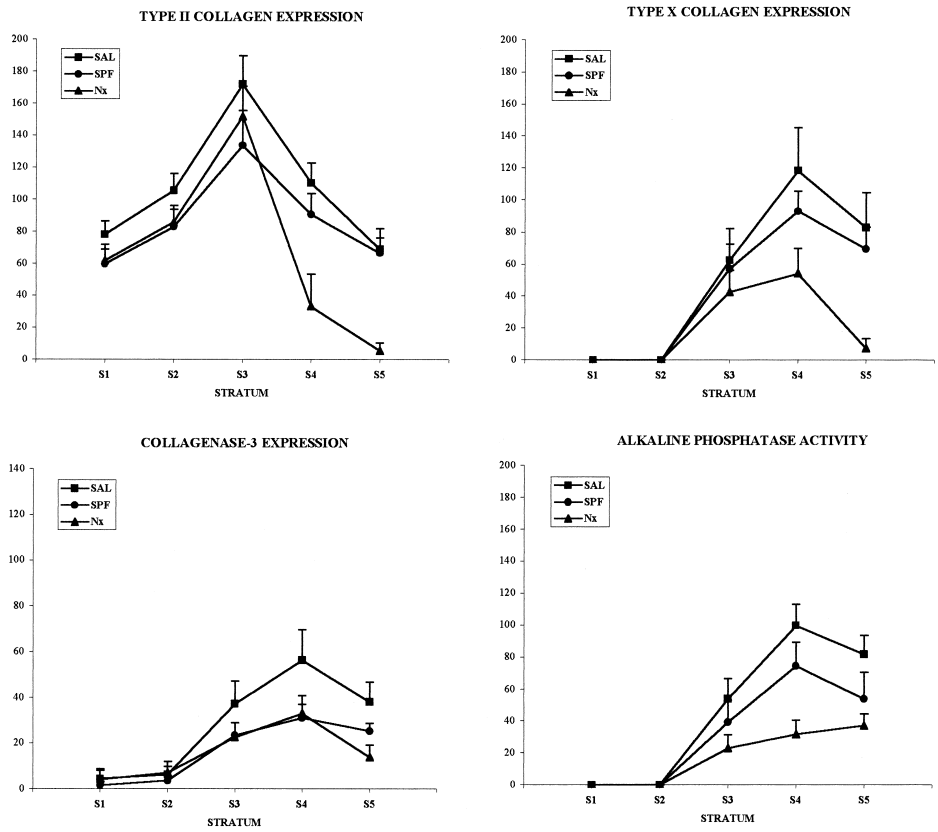

FIG. 3. Changes in chondrocytic expression at different levels of the tibial growth plate. Expression levels for type II collagen, type-X collagen, and collagenase-3 widely varied in chondrocytes located at different horizontal strata of the same vertical column. Although values for expression levels significantly vary, expression profiles are basically the same in SAL and SPF rats. NX rats had significant decreases in collagen expression at the distal region of the growth plate with less impairment of both collagenase-3 expression and ALP activity.

the expression pattern was similar in these two groups of control rats (Figs. 2C and 2F). In NX rats, the expression pattern of collagenase-3 was basically the same as in control groups; that is, it was highly expressed at the early hypertrophic zone and then the signal gradually decreased along the distal end (Fig. 2I). Nevertheless, collagenase-3 expression was detected on the whole extent of the hypertrophic cartilage. Thus, a weak but specific signal for collagenase-3 was detected even at chondrocytes closed to the osseochondral junction, those located at the abnormally extended zone of the growth plate of these animals (Fig. 2I). As a result, the estimated time for the duration of the period during which each chondrocyte expressed collagenase-3 was 98.7 h (Table 3), a value much higher than those estimated for either SAL or SPF rats (39.3 and 33.7, respectively). As for collagenase-3, ALP activity was detected on the whole extent of the hypertrophic cartilage of NX rats (Fig. 1). Nevertheless, it was significantly lowered when compared with control rats (Fig. 3).

Results on mRNA levels obtained by PCR amplification agreed with those obtained by in situ hybridization on tissue sections. Levels of type II and X collagens in NX rats were lower than in SPF rats and even more so than in SAL rats (Fig. 5). By contrast, collagenase-3 levels were higher in NX rats than in SPF rats.

The result that collagen expression was depressed in growth plate chondrocytes from NX rats, together with the demonstration that collagenase-3 expression was affected

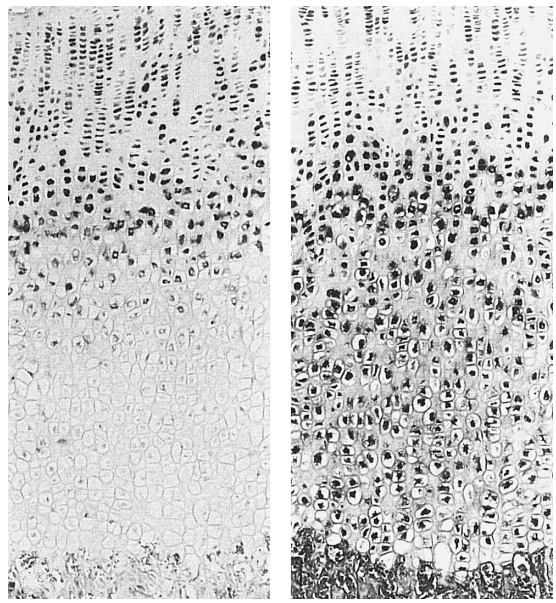

FIG. 4. Parallel serial sections of growth plates from NX rats hybridized to the type II collagen probe (left) and to the GAPDH probe (right). Hypertrophic chondrocytes not expressing for type II collagen are metabolically active because they express the constitutive enzyme GAPDH (magnification is the same for the two micrographs, ×105).

TABLE 3. ABSOLUTE NUMBERS AND PERCENTAGES OF POSITIVE CELLS TO TYPE II COLLAGEN, TYPE X COLLAGEN, ALP ACTIVITY, AND COLLAGENASE-3 IN A VERTICAL CELL COLUMN AND ESTIMATED EXPRESSION TIME LENGTHS IN THE THREE GROUPS OF RATS[a]

	SAL	*SPF*	*NX*
Type II collagen			
Cells/column	28.5 ± 2.3	21.6 ± 3.8*	35.0 ± 5.5*,†
Percentage	90.7 ± 3.1	87.1 ± 8.3	70.5 ± 3.7*,†
Type X collagen			
Cells/column	9.7 ± 1.0	8.9 ± 1.4	11.0 ± 2.1†
Percentage	30.8 ± 1.5	35.8 ± 2.8*	22.2 ± 2.7*,†
Expression time length (h)	29.1 ± 5.0	29.3 ± 6.4	54.7 ± 14.3*,†
Collagenase-3			
Cells/column	13.2 ± 0.9	10.3 ± 1.4*	19.8 ± 2.7*,†
Percentage	41.9 ± 2.6	42.3 ± 8.2	39.9 ± 1.8
Expression time length (h)	39.3 ± 5.3	33.7 ± 4.9	97.8 ± 20.4*,†
ALP activity			
Cells/column	8.3 ± 0.9	7.5 ± 1.2	12.6 ± 2.0*,†
Percentage	26.5 ± 1.9	30.3 ± 2.4*	25.4 ± 2.1†
Expression time length (h)	25.0 ± 4.5	24.8 ± 5.2	62.3 ± 14.2*,†

[a] Each point is the mean value of 8 animals ± SD.
* $p < 0.05$ when compared with SAL; † $p < 0.05$ when compared with SPF.

scantily strongly suggested that uremia might yield a imbalance between collagen formation and degradation and this could lead to changes in the content and/or structure of the ECM. To test this last possibility, the ultrastructure of growth plate matrix was studied by transmission electron microscopy (Fig. 6). The ECM of the growth plate from SAL and SPF rats shared most of their ultrastructural features. Both groups exhibited a well-defined structural organization in which three different zones could be defined according to collagen fibril architecture: a pericellular matrix forming a narrow mantle around each chondrocyte in which collagen fibrils were virtually absent; a territorial matrix, located between adjacent chondrocytes in the same axial column, composed by a network of intermingled collagen fibrils; and a interterritorial matrix, located between cell columns, formed by large bundles of longitudinally oriented collagen fibrils (Figs. 6A–6C). Fibril diameter was fairly uniform in the different zones (about 20 nm) except at the resting zone, which contained some thicker fibrils (about 50–100 nm). Clusters of electron-dense calcified granules were first observed within the interterritorial matrix of the early hypertrophic zone. In the lower region, the interterritorial matrix appeared almost completely calcified forming mineralized longitudinal septa (Fig. 6A). Such septa protruded into the primary spongiosa and served as a scaffold to osteoblasts, which formed a single cell layer around them and produced the first osteoid. In growth plates from NX rats, the ultrastructure of the ECM basically was unchanged at the proliferating and early hypertrophic chondrocytes. However, several alterations were observed at the distal zone of the hypertrophic cartilage, where the vertical diameter of chondrocytes was significantly reduced (Fig. 6D). At this zone, the parallel orientation of collagen fibrils in the interterritorial matrix changed to a more network-like arrangement (Figs. 6E and 6F). Therefore, distinction between territorial and interterritorial matrix was greatly attenuated. Moreover, the interterritorial matrix appeared poorly calcified, being mineral deposits were scarce in number and small in size (Figs. 6D and 6F). As a result, longitudinal septa appeared rather ill defined at the hypertrophic cartilage and appeared not to protrude into the primary spongiosa, in which osteoblasts formed disorganized clusters.

DISCUSSION

Present results indicate that uremia alters the normal patterns of chondrocyte maturation and the rates and degree of substitution of hypertrophic cartilage with bone as well as the pattern of expression of type II and X collagen genes. In NX rats, there was a generalized decrease of collagen expression but this was especially marked in the abnormally extended zone of the hypertrophic cartilage, where chondrocytes showed very low or even no collagen expression. On the other hand, collagenase-3 expression was affected to a lower degree, suggesting an imbalance between synthetic and degradative activities for cartilage collagens. Changes in gene expression were coupled to both modifications in the collagen fibril architecture and alteration of the mineralization pattern. Because the growth plates of uremic and SPF rats were substantially different, modifications observed in these animals cannot be attributed to the nutritional deficit associated with renal failure. These results are consistent with the hypothesis that collagen metabolism is disturbed specifically in the growth plates of uremic rats.

The ECM of cartilage plays a critical role in regulating the diffusion of nutrients, metabolites, and hormones between the chondrocytes and the relatively distant blood vessels of connective tissue. As a result, the ECM influences

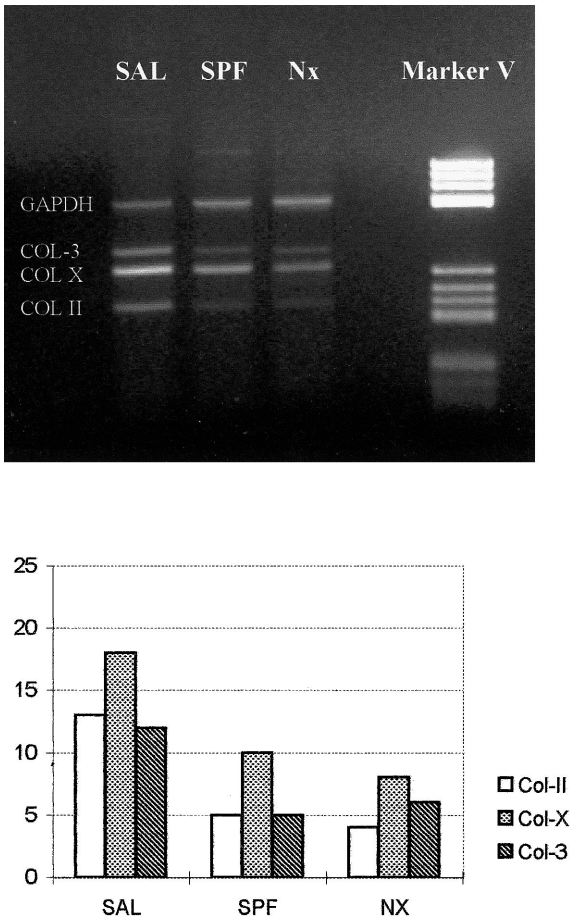

FIG. 5. RT-PCR amplification of GAPDH, collagenase-3, and type II and type X collagen mRNAs from SAL, SPF, and NX rats. PCR reactions were stopped at the exponential phase of amplification. Products were separated on a 2% agarose gel electrophoresis in Tris-borate buffer. DNA molecular weight marker was marker V (Boehringer Mannheim). Bands were scanned by using a densitometer, and the signals obtained for collagens and collagenase-3 in the three samples of each group were corrected to the signals obtained for GAPDH in the corresponding samples.

the behavior of the chondrocytes that contact it, regulating their proliferation, migration, shape changes, gene expression, and even cell survival.[25–26] Therefore, changes in the synthetic capabilities of chondrocytes directly influence the properties of the ECM, which in turn exerts a feedback effect on the activities of the cells. On this basis, it could be suggested that chondrocytes might partially regulate their activity indirectly via changes in the composition and structure of the ECM. In support of this idea is the finding that many endocrine factors exert their effect affecting the genetic expression of matrix components.[27–34] Because chondrocyte differentiation in the growth plate is associated closely with profound changes in the nature and structure of the ECM and considering that present data proved that uremia induced changes in the expression pattern of the major structural matrix proteins, it is reasonable to suggest that alteration of collagen metabolism in growth plate chondrocytes could be a key process underlying growth retardation in uremia. Moreover, because collagens are the components responsible for providing the ECM with a fibrillar network to arrange chondrocytes as well as other matrix components, changes in collagen expression observed in uremic rats may likely be a primary determinant of the abnormal chondrocyte differentiation process rather than a secondary consequence. Accordingly with this suggestion, it has been reported that both collagen type II and X play a critical role in the terminal differentiation of the hypertrophic cartilage, because they interact with the matrix vesicles activating Ca^{2+} loading and initiating mineral deposition.[35,36] Data reported in the this article are in good accordance with these former findings because hypertrophic cartilage of uremic rats showed both decreased collagen expression and defective mineralization and this resulted in the generation of an abnormal terminal differentiation stage consisting of a distinct cell population having size and shape alterations, which is accumulated at the metaphyseal surface of the growth plate. It is of note that ALP, an enzyme implicated in cell-mediated mineralization, was found in hypertrophic chondrocytes of uremic rats at relatively high levels. Therefore, defective mineralization cannot be attributed to lack of activity of this enzyme.

The fact that uremia caused decreased collagen expression and enlargement of the growth plate cartilage may appear contradictory, especially if it is considered that malnutrition alone (SPF animals) induced a decrease in both longitudinal bone growth and growth plate height (when compared with SAL animals). However, it may be explained in light of the fact that an increased height of the growth plate does not necessarily correspond to a higher metabolic activity of this cartilage.[20] Growth plate height is the result of two antagonistic processes: chondrogenesis and ossification. In isolation, chondrogenesis would lead to progressive widening of the growth plate but, simultaneously, the metaphyseal border of the growth plate is invaded by blood vessels and bone cell precursors, which remodel the cartilage into bone. During normal growth, chondrogenesis and ossification are tightly coupled so that the height of the growth plate remains basically constant. We have previously reported that this dynamic equilibrium is disturbed in severely uremic rats.[4] In the same way, it recently has been reported that the size of the growth plate in NX rats varied depending of the ingest of calcium.[37] These authors have proven that the growth plate was enlarged in NX rats ingesting a high-calcium diet whereas it appeared unmodified in NX rats given low-calcium levels. Furthermore, growth plate enlargement was associated with diminished tibial growth, low tartrate-resistant acid phosphatase activity (a marker of osteoclasts), and decreased osteocalcin mRNA expression. By contrast, these authors did not find significant changes in the expression levels of types II and X collagens. Data of the present study do not agree with that last result, because a significant decrease in the expression of types II and X collagens has been found in chondrocytes located at the abnormally extended zone of hypertrophic cartilage of the NX rats. This result provides

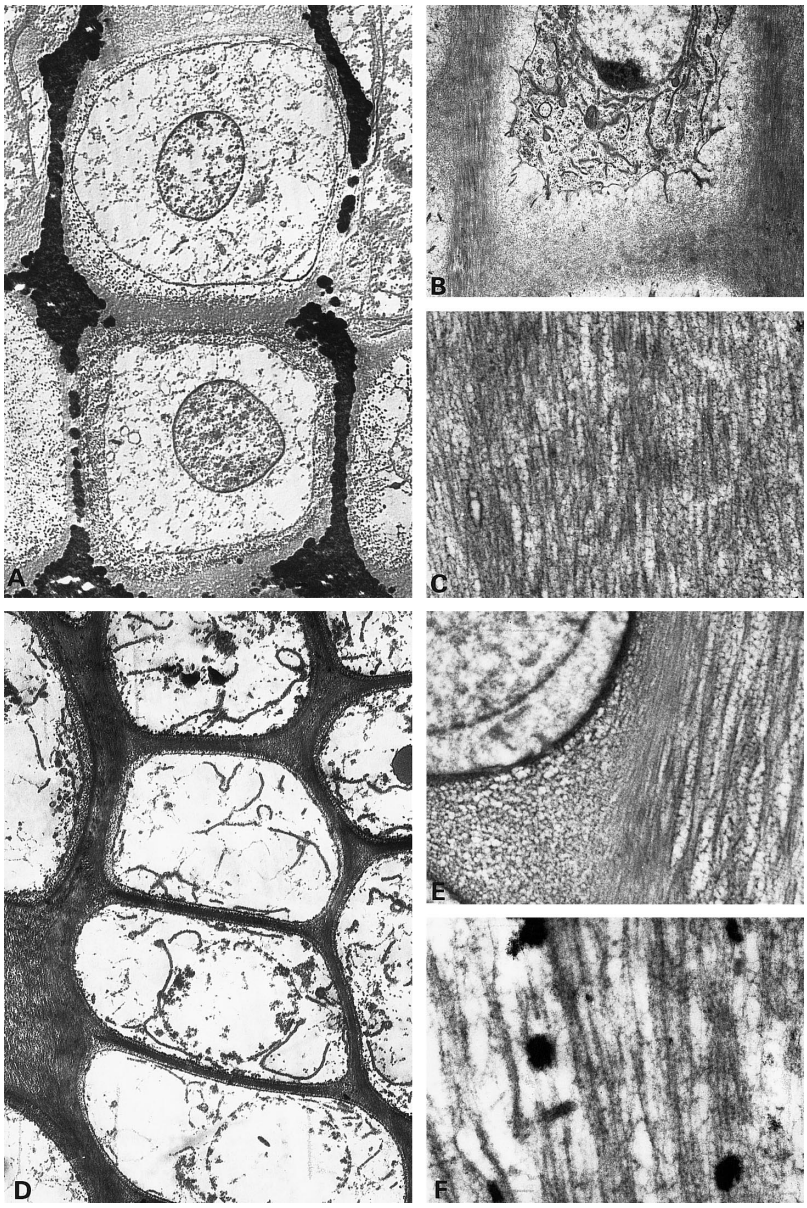

FIG. 6. Electron microscopy of growth plates from (A–C) SAL and (D–F) NX rats. (A) Chondrocytes of the lower hypertrophic zone of a SAL rat. The interterritorial matrix appears almost completely calcified-forming mineralized longitudinal septa. (B) Pericellular, territorial, and interterritorial compartments of the matrix in the early hypertrophic zone. (C) Higher magnification of the interterritorial matrix showing the large bundles of longitudinally oriented type II collagen fibrils. (D) Chondrocytes of the lower hypertrophic zone of an NX rat. Chondrocytic height is significantly reduced and the interterritorial matrix appears poorly calcified. (E) Territorial matrix at the left and the interterritorial matrix at the right in the lower hypertrophic zone of NX rats. Note that the parallel orientation of collagen fibrils in the interterritorial matrix changes to a more network-like arrangement. (F) Higher magnification of the interterritorial matrix of an NX rats showing low-compacted collagen fibrils and small mineral deposits. (A and D) Fixed in 2% glutaraldehyde and 0.7% RHT; (B, C, E, and F) fixed in 4% glutaraldehyde and 1 mM calcium chloride [magnifications: (A and D) ×900; (C) ×12,200; (E) ×4500; (F) ×19,800].

support for the assumption that changes in the morphology and dynamics of the growth plate of uremic rats are correlated to disturbances in expression pattern of matrix collagens.

Uremia also had an effect on the expression of collagenase-3 but it was different to that observed on collagen expression. Collagenase-3 levels in NX rats were lower than in SAL but higher than in SPF rats. Furthermore, in situ studies proved expression of the enzyme on the whole extent of the hypertrophic cartilage. Because the hypertrophic zone was enlarged, the number of positive cells for collagenase-3 in uremic rats was even significantly higher than in controls. These results are basically coincident with those observed in rachitic rats in which decreased bone growth rate was coupled to increases of both hypertrophic cartilage volume and collagenase activity.[38] In the same way, we also have reported in normal growing rats that collagenase-3 expression is low in the most distal zone of the hypertrophic cartilage, that adjacent to the vascular invasion.[18] The relatively minor affectation of collagenase-3 expression in growth plates, in which the replacement of hypertrophic cartilage by bone is depressed severely, and its physiologically weak expression at the ossification front suggest that this enzyme could be associated more directly with chondrocyte hypertrophy than with the replacement of cartilage by bone at the metaphyseal end. However, this does not rule out a role of collagenase-3 in the ossification process. Because both vascularization and ossification are highly dependent on previous chondrocyte hypertrophy, collagenase-3 could be required to generate primary

changes in the cartilage matrix and/or to activate signaling factors that would be essential for the subsequent action of other proteinases. In this way, collagenase-3 has been reported to play a major role in bone formation.[39] Accordingly, matrix remodeling during endochondral ossification is thought to result from a combined action of several proteinases, including metalloproteinases like gelatinase A (MMP-2), gelatinase B (MMP-9), stromelysin 1 (MMP-3), stromelysin 2 (MMP-10), membrane-associated metalloproteinase (MMP-14),[40–45] and other proteinases like cathepsin B and cathepsin K.[46–48]

Growth plate activity is regulated by a complex interplay of endocrine, paracrine, and autocrine signaling substances. Growth hormone/insulin-like growth factor I (GH/IGF-I) axis and calciotropic hormone metabolism, two hormonal systems likely involved in the control of growth plate activity, are markedly impaired in chronic renal failure.[3] It is difficult to speculate on the role played by these hormonal disorders in the growth plate alterations observed in our uremic rats. Like chronic renal failure, malnutrition and acidosis produce resistance to GH and IGF-I. However, the modifications induced by malnutrition (SPF rats) and metabolic acidosis (unpublished data of our laboratory) in the growth plate are significantly different from those found in uremic rats. Thus, although the role of IGF-I has been to increase chondrocyte type II collagen mRNA levels and the local synthesis of IGF-I has been reported to be depressed in growth plate chondrocytes of uremic rats,[49–52] our findings cannot be solely explained by a defective local production of IGF-I.

On the other hand, growth plate chondrocytes have receptors for calcitriol, parathyroid hormone (PTH), PTH-related protein (PTHrP), and calcium, but the physiological effects of most of these signaling substances on chondrocyte metabolism are not understood completely. It has been proven that PTHrP acts on the growth plate to regulate negatively hypertrophic differentiation.[53,54] Accordingly, overexpression of this factor has been reported to suppress the development of hypertrophy, calcification, and endochondral bone formation.[53] These last effects are basically coincident with our results on uremic rats and then this model would provide a possible explanation for inhibition of the hypertrophy in our animals. However, this explanation is not consistent with previous works reporting that PTH/PTHrP receptor mRNA is down-regulated in the growth plate of uremic rats.[55] In this way, recent work has reported that growth plate height as well as mRNA levels for the PTH/PTHrP receptor are highly dependent on the severity of the secondary hyperparathyroidism of the uremic rats.[56] Nevertheless, little is known about the influences of the hormonal disturbances caused by chronic renal failure on the altered metabolism of growth plate cartilage and this subject requires further investigation.

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Address reprint requests to:
Dr. José Manuel López
Departamento de Morfología y Biología Celular
Facultad de Medicina
C/Julián Clavería 6
33006 Oviedo, Asturias, Spain

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