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Chondromodulin-I expression in the growth plate of young uremic rats

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Background. Growth retardation of chronic renal failure is associated with alterations in the growth plate suggestive of a disturbed chondrocyte maturation process and abnormal vascular invasion at the chondro-osseous interphase. Chondromodulin I (ChM-I) is a potent cartilage-specific angiostatic factor. Its pattern of expression in the uremic rat growth plate is unknown. Persistence of ChM-I synthesis and/or imbalance between ChM-I and vascular endothelial growth factor (VEGF) expressions might play a role in the alterations of uremic growth plate.

Methods. Growth cartilage ChM-I expression was investigated by immunohistochemistry, in situ hybridization, and reverse transcription-polymerase chain reaction (RT-PCR) in growth-retarded young uremic rats (UREM), control rats, fed ad libitum (SAL) or pair-fed with the UREM group (SPF), and uremic rats treated with growth hormone (UREM-GH). VEGF expression was analyzed by immunohistochemistry.

Results. ChM-I and ChM-I mRNA were confined to the proliferative and early hypertrophic zones of growth cartilage. A similar number of chondrocytes per column was positive for ChM-I in the 4 groups. In accordance with the elongation of the hypertrophic stratum in uremia, the distance (X±SEM, μm) between the extracellular ChM-I signal and the metaphyseal end of growth cartilage was higher ($P < 0.003$) in UREM (236 ± 40) and UREM-GH (297 ± 17) than in SAL (92 ± 7) and SPF (113 ± 6). No differences in ChM-I expression were appreciated by RT-PCR. Similar VEGF positivity was observed in the hypertrophic chondrocytes of all groups.

Conclusion. In experimental uremia, expansion of growth cartilage does not result from increased or persistent expression of ChM-I or from reduced VEGF expression at the cartilage-metaphyseal bone interphase. GH treatment does not modify ChM-I and VEGF expressions.

Growth retardation is a major manifestation of chronic renal failure in the pediatric age [1–3]. Several lines of evidence indicate that renal failure causes peripheral resistance to growth hormone action as a result not only of the decreased renal function itself but also of the associated malnutrition [4–14]. However, the pathogenetic mechanism of growth impairment in chronic renal failure remains to be determined. To clarify it, the study of growth plate in the uremic rat model has progressively gained in interest over the last years because it is the organ where longitudinal growth takes place and it is hardly available in children [15–17].

Growth failure induced by uremia is associated with marked changes in the morphology of the growth plate. Findings from our laboratory [18, 19] and others [20] have shown that the tibial growth cartilage height of severely uremic rats is much greater than that of control rats with normal glomerular filtration rate (GFR), either fed ad libitum or pair-fed with the subtotally nephrectomized animals. This indicates that this finding cannot be attributed to the malnutrition associated to chronic renal failure. Likewise, it was not observed in rats with metabolic acidosis, a metabolic disorder frequently found in renal failure, and normal renal function [21]. Expansion of the chondrocyte columns in the growth plate of uremic rats results from marked elongation of the hypertrophic zone and, as inferred from a detailed histomorphometric analysis [19], is related to alterations in the kinetics of chondrocytes and an imbalance between cartilage formation and its replacement by bone. Increased hypertrophic zone height, reduced size of the chondrocytes adjacent to the metaphyseal bone, reduced expression of type X collagen, and irregular cartilage-bone interphase all suggest a disturbed maturation process of the growth cartilage cells of uremic rats [19, 22, 23].

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Key words: chondromodulin-I, chronic renal failure, growth plate, VEGF, growth hormone.

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Chondromodulin I (ChM-I) is a 25-kD glycoprotein initially isolated from fetal bovine epiphyseal cartilage [24]. ChM-I is specific of cartilage, stimulates the growth of chondrocytes [24, 25], and inhibits endothelial cell growth [26]. ChM-I is a potent antiangiogenic factor expressed in the avascular zone of cartilage in developing bone of mammalian and avian embryos, but absent in calcifying cartilage [27–32].

Formation of new capillary sprouts is a key and early step required for replacement of chondrocytes by osseous cells at the growth cartilage-primary spongy bone junction of the long bones [33, 34]. Because of the potential role of ChM-I as important regulator of angiogenesis and the above-described findings indicative of disturbed chondrocyte maturation at the growth plate, it is tempting to hypothesize that, in uremia, persistent synthesis of ChM-I by the hypertrophic chondrocytes might lead to inhibition of vessel arrival and the ensuing expansion of growth cartilage. This might be important to understand the changes induced by chronic renal failure in the growth plate dynamics, and to provide some insight on the underlying mechanism of growth retardation in this disease. Similarly, the beneficial effect of growth hormone (GH) administration on growth [35–37] might bring about significant changes in the pattern of expression of ChM-1 in the growth cartilage of uremic rats.

METHODS

Experimental protocol

Female Sprague-Dawley rats aged 30 ± 2 days and weighing 100 ± 5 g were obtained from the breeding area of the animal facility building of the University of Oviedo. Rats were housed in individual cages and maintained at an environmental temperature of 22°C and a 12-hour light-dark cycle. After 3 days of acclimation to the experimentation area, the animals were classified in the following groups of 10 individuals each: rats with chronic renal failure induced by 5/6 nephrectomy, untreated (UREM) or treated with GH (UREM-GH); normal renal function, sham-operated rats, either fed ad libitum (SAL) or pair-fed with the uremic group (SPF). All animals had free access to tap water and received standard rat chow with a protein content of 17.2% (A04®; Panlab, Barcelona, Spain).

Subtotal nephrectomy or sham operation was performed in two stages on days 0 and 4 of the protocol as formerly described [17]. From day 4 on, animals and food were weighed daily using an electronic balance (Ohaus GT 2100®; Florham Park, NJ, USA). Between days 11 and 17, animals were treated with 3.3 mg/kg/day (10 IU/kg/day) of recombinant human GH (Norditropin®, Novo Nordisk Pharma, Madrid, Spain) injected intraperitoneally at approximately 9 a.m. and 5 p.m. Rats were sacrificed under anesthesia on day 18 of the protocol. Two days before sacrifice each animal received 20 mg/kg of calcein (Sigma, St. Louis, MO, USA) by intraperitoneal route.

Samples

Samples were collected at sacrifice. Serum samples were stored at −20°C until measurement of urea nitrogen (SUN) and creatinine with a Kodak Ektachem DT60® analyzer (Rochester, NY). Tibias were removed and processed as follows. Immediately after sacrifice, the proximal end of the tibiae, including the growth plate, was dissected. Tibias from four animals randomly chosen per group were removed, kept in liquid nitrogen, and stored at −80°C for further RNA extraction. From the remaining six animals per group, the right tibia was fixed in 40% ethanol, and the left one in 4% neutral formalin, at 4°C. Then, both tibias were dehydrated in graded solutions of ethanol and embedded in methylmethacrylate (MMA) [38] for further histomorphometric, immunohistochemical, and in situ hybridization studies.

Growth and nutrition

Weight gained between days 11 and 18, the period of GH treatment, was calculated. Longitudinal growth rate was measured in 10-μm thick frontal sections of proximal end of tibias obtained using a rotary microtome (HM355S®; Microm, Barcelona, Spain) fitted with tungsten carbide blades. Sections were examined under an Olympus incident light fluorescence microscope (Olympus BX41®; Barcelona, Spain) coupled to a digital camera (Olympus DP11®, Olympus Optical España, Barcelona, Spain) to detect calcein label. Images were captured and the distance between the chondro-osseous junction and the calcein label was measured using an image analysis system (Scion Image®; Scion Corporation, Frederick, MD, USA). The average value of these measurements divided by 2 (days) was considered as the daily longitudinal bone growth rate in each animal. Food efficiency was calculated as the ratio between weight gained and food consumed (g/g) by each animal between days 11 and 18 of the protocol.

Morphometric analysis of growth plate

Heights of growth cartilage and its hypertrophic zone, as well as height and area of the three most distal hyperthrophic chondrocytes, were measured in 5-μm thick sections stained with alzian blue and safranine at 20 locations on each section, using the above mentioned image analysis system.

Immunohistochemical analysis for ChM-I and vascular endothelial growth factor (VEGF)

Immunohistochemical staining for ChM-I or VEGF was performed in ethanol- or formalin-fixed sections, respectively. A 15-minute incubation in fresh 100% acetone

was used to remove MMA from the tissue sections. After hydration, the sections for ChM-I analysis were treated with hyaluronidase [2 mg/mL, 1 h, 37°C in phosphate-buffered saline (PBS) at pH 5.0]. Then, all sections were treated with 3% hydrogen peroxide and 25% goat serum and incubated overnight (18 h) at 4°C with 1/500 solution of polyclonal anti-ChM-I antibody [32] or 6/100 polyclonal anti-VEGF (Neo Markers, Westinhouse, CA, USA). After 30-minute incubation with antirabbit secondary conjugated antibody (EnVision +TM®; Dako Corporation, Carpinteria, CA, USA), the final reaction product was revealed with 3–3′-diaminobenzidine (DAB) (Sigma), (20 mg of DAB in 50 mL of 0.05 mol/L Tris-hydrochloric buffer plus 50 μL of 30% hydrogen peroxide). Sections for VEGF immunohistochemistry were counterstained with alzian blue.

Quantification of immunohistochemical staining for ChM-I

Labeling index for ChM-I was calculated as the number of ChM-I–positive cells per column of chondrocytes. To estimate the proximity of ChM-I signal to the metaphyseal bone, the distance between the lower limit of the extracellular signal for ChM-I and the chondro-osseous junction was measured at 20 locations on each section.

To compare the signal of ChM-I observed in the present experiment with that of rats of different ages, immunohistochemical analysis of ChM-I expression was carried out in tibias collected from 3-week and 12-week-old healthy Sprague-Dawley rats.

In situ hybridization for ChM-I

The ChM-I antisense and sense DNA probes were synthesized with EcoR1 and BamH1 linearized plasmid pGEM 4Z, respectively, which contains a 499-base pair fragment [32]. The plasmid was used as a template for synthesis of antisense and sense digoxigenine-UTP-labeled probes with T7 and SP6 polymerases. After hydration, sections were treated with 300 μg/mL proteinase, fixed in 4% paraformaldehyde, and washed in PBS containing 0.1% diethylpirocarbonate (DEPC) and 0.3% Triton X-100. After incubation in 5 × standard sodium citrate (SCC), prehybridization was performed for 1 hour at 42°C. The prehybridization solution consisted of 50% deionized fomamide, 4 × SCC, 50 × Denhardt's solution, 5 g polyvinylpyrrolidone, 5 g bovine serum albumin (BSA), and 250 μg/mL yeast tRNA. Hybridization solution was performed overnight at 42°C. The hybridization solution consisted of all components of the prehybridization mixture in which the digoxigenine-UTP-labeled probes were diluted and covered with plastic cover slips. After washing in 2 × SSC at room temperature, and 2 × SSC and 0.1 × SSC at 42°C, nonspecific binding was blocked by 1 × blocking buffer (Blocking Reagent®, Boehringer Mannheim GmbH, Mannheim, Germany) for 1 hour, and 20% goat serum in buffer A (1 mol/L TrisHCl, 5 mol/L NaCl, pH 7.5) for 30 minutes. Slides were placed in a moist chamber (ISO 20®; Grant Instruments, Cambridgeshire, UK) and were incubated overnight with alkaline phosphatase–conjugated anti-digoxigenine (1:1000; Boehringer Mannheim GmbH) in buffer A at 4°C. Sections were rinsed in buffer A and then in buffer B (1 mol/L TrisHCl, 5 mol/L NaCl, $MgCl_2$, pH 9.5) for 10 minutes. Alkaline phosphatase was then revealed with a substrate solution containing 0.16 mg/mL 5-bromo-4-chloro-3-indolylphosphatase (Sigma) and 0.33 mg/mL nitroblue tetrazolium (Sigma) in buffer B for 15 minutes at room temperature. The enzymatic reaction was stopped with a rinse in tap water. Sections were dried at 37°C and mounted with Vectamount® (Vector Laboratories, Burlingame, CA, USA).

Two parallel sections served as negative controls: one section hybridized with a labeled-sense riboprobe, and a second section incubated without adding any probe to the hybridization mixture. No hybridization signal was found in any of these negative controls. All solutions, when appropriate, were prepared with 0.1% DEPC-treated water. Sections were counterstained with nuclear red.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA from tibial growth plates stored at −80°C was extracted using acidic phenol extraction of guanidine isothiocyanate lysate, according to the method of Chomczinsky and Sacchi [39], with minor modifications. The quality of RNA was analyzed by agarose electrophoresis. One pool of RNA was made for each group. The amount of RNA was estimated by measuring the absorbancy at 260 nm.

PCR primers were deduced from rat ChM-I sequence. RT-PCR was performed using the GeneAmp® RNA PCR Core Kit (Applied Biosystems, Foster City, CA, USA) following the instructions of the manufacturer. In brief, 1 μg of total RNA were reverse transcribed into cDNA using random hexamers. The resulting cDNA was amplified by PCR using specific primers pairs (ChM-I F: 5′-AAGCAGTGCTCCCTCTACCA-3′ and R: 5′-CTCTCTCCTTCCTGCTGGTG-3′) and 32 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds after an initial 5-minute denaturation step at 94°C. The amplified products (153 bp) were resolved on a 2% agarose gel and visualized by ethidium bromide staining and ultraviolet transillumination. As control, β-actin was used following the same protocol that yielded a PCR product of 294 bp. The PCR products were verified by sequencing. Control reactions performed by omitting reverse transcriptase or template RNA showed no reaction product. The optical densities of the PCR products were

Table 1. Growth and nutrition in the four groups of animals

	SAL ($N = 10$)	SPF ($N = 10$)	UREM ($N = 10$)	UREM-GH ($N = 10$)	P value
Weight gain Day 11 to 18 *g*	26.1 ± 1.6	21.2 ± 3.0[d]	18.7 ± 2.3[a,d]	32.9 ± 2.5	0.001
Food intake Day 11 to 18 *g*	121.8 ± 3.6	95.3 ± 6.8[a]	95.3 ± 6.8[a]	108.5 ± 5.1	0.001
Food efficiency day 11 to 18 *g/g*	0.21 ± 0.01	0.22 ± 0.03	0.20 ± 0.03	0.30 ± 0.02[a,b,c]	0.006
Longitudinal bone growth rate μ*m/day*	143 ± 12	108 ± 10	60 ± 13[a,b,d]	132 ± 16	0.001

Abbreviations are: SAL, sham-operated rats fed ad libitum; SPF, sham-operated rats pair-fed with the UREM group; UREM, uremic rats; UREM-GH, uremic rats treated with growth hormone. Data are given as mean ± SEM. Weight and food measurements are expressed from day 11 to 18 because this was the period of the protocol corresponding to growth hormone treatment. Longitudinal growth rate was measured over the two days before sacrifice by calcein labeling.
[a]Different from SAL group; [b]different from SPF group; [c]different from UREM group; [d]different from UREM-GH group.

Table 2. Morphologic features of proximal tibia growth plates in the four groups of animals

	SAL ($N = 6$)	SPF ($N = 6$)	UREM ($N = 6$)	UREM-GH ($N = 6$)	P value
GP cartilage height μ*m*	358 ± 14	351 ± 18	571 ± 37[ab]	657 ± 55[ab]	0.001
HZ height μ*m*	160 ± 7	157 ± 7	360 ± 20[ab]	365 ± 20[ab]	0.001
HZ/GP height ratio μ*m*/μ*m*	0.45 ± 0.02	0.45 ± 0.02	0.60 ± 0.02[ab]	0.57 ± 0.04[ab]	0.001
Area of most distal chondrocytes μm^2	477 ± 23	465 ± 32	360 ± 23[abd]	541 ± 21	0.003
Height of most distal chondrocytes μ*m*	21 ± 1	20 ± 1	16 ± 2[abd]	22 ± 1	0.001

Abbreviations are: SAL, sham-operated rats fed ad libitum; SPF, sham-operated rats pair-fed with the UREM group; UREM, uremic rats; UREM-GH, uremic rats treated with growth hormone; GP, growth plate; HZ, hypertrophic zone. Data are given as mean ± SEM.
[a]Different from SAL group; [b]different from SPF group; [c]different from UREM group; [d]different from UREM-GH group.

analyzed by a computer program (Scion Corporation). Results were normalized for the density of β-actin.

Statistical analysis

Values of each group are given as mean ± SEM. Comparisons among groups were performed by analysis of variance (ANOVA) followed by the Newman-Keuls multiple range test or Kruskal Wallis test as appropriate. A P value <0.05 was considered as indicative of significant difference.

RESULTS

Renal function

Serum concentrations of urea nitrogen and creatinine were higher ($P < 0.001$) in the UREM (50 ± 4 mg/dL and 0.9 ± 0.0 mg/dL) and UREM-GH (47 ± 3 mg/dL and 1.0 ± 0.0 mg/dL) groups than in SAL (12 ± 1 mg/dL and 0.5 ± 0.0 mg/dL) and SPF animals (13 ± 1 mg/dL and 0.3 ± 0.0 mg/dL). There were no differences between the subtotally nephrectomized rats or between the sham-operated groups.

Growth and nutrition

As shown in Table 1, UREM rats exhibited severe growth retardation as demonstrated by less weight gain, lower food consumption, reduced food efficiency, and decreased longitudinal bone growth rate than SAL group. To a certain extent, the growth impairment was caused by the depressed food intake because SPF animals also grew worse than the SAL controls. However, particularly in terms of longitudinal growth, poor growth of UREM animals could not be exclusively attributed to the reduced food intake because their osseous front advance was markedly lower than that of SPF rats. The beneficial effect of GH treatment on growth became manifest by increased weight gain and longitudinal growth rate in UREM-GH compared with UREM animals. This growth-promoting action was related to a better utilization of consumed food.

Morphometry of growth plate

Heights of growth plate cartilage and its hypertrophic zone were much higher in UREM than SAL rats (Table 2). In addition, chondrocytes adjacent to the metaphyseal bone were smaller in UREM animals. These alterations were not found in the SPF group. GH administration normalized chondrocyte size but not growth plate cartilage height. An outstanding characteristic of growth plate of UREM rats was the marked irregularity of the chondro-osseous junction and the disturbed arrangement of the chondrocyte columns.

Expression of ChM-I in the growth plate cartilage

A similar pattern of intracellular and extracellular immunohistochemical staining for ChM-I was observed in the four groups of animals (Fig. 1). In the chondrocytes, the protein was localized in the cytoplasma with a granular aspect. Within the growth plate, intracellular and extracellular signals were confined to the transitional zone between proliferative and hypertrophic chondrocytes. Neat positivity for ChM-I was also appreciable in articular cartilage (data not shown). Quantitative assessment of intracellular ChM-I signal revealed a small and similar number of labeled cells per column in all groups: 1.5 ± 0.1 in SAL, 1.5 ± 0.3 in SPF, 2.1 ± 0.3 in UREM, and 1.6 ± 0.1 in UREM-GH. As the growth plate cartilages were much higher in the chronic renal failure animals and

the ChM-I signal did not extend beyond the early hypertrophic zone, the distance between the lower limit of the extracellular ChM-I signal and the metaphyseal bone was much greater ($P < 0.003$) in UREM (236 ± 40 μm) and UREM-GH (297 ± 17 μm) than in SAL (92 ± 7 μm) and SPF (113 ± 6 μm) groups.

The distribution of ChM-I mRNA as detected by in situ hybridization was quite similar to that of immunohistochemistry. Expression of ChM-I mRNA was mostly confined to the transitional area between proliferative and hypertrophic zones, no differences being found among the four groups (Fig. 1). The specificity of the signal was confirmed by its absence in the sections hybridized with the sense probe, and without adding any probe.

Immunohistochemistry for ChM-I performed in growth plate sections from healthy control rats served to confirm that the small number of ChM-I–labeled chondrocytes was to a certain extent a function of age because ChM-I labeling index significantly decreased from 4.2 ± 0.7 cells/column in weaning rats of 3 weeks of age to 0.3 ± 0.0 cells/column in 12-week-old adult rats. Interestingly, the distribution pattern of ChM-I expression in the growth plate cartilage changed with age. In adult individuals, ChM-I–positive cells were almost absent and confined to the distal hypertrophic chondrocytes. By contrast, in 3-week-old rats, there was a strong extracellular signal and a greater number of labeled cells in the resting, proliferative, and early hypertrophic zones.

ChM-I mRNA expression was confirmed in all groups by RT-PCR (Fig. 2).

RT-PCR yielded a 153-bp amplification product for the ChM-I mRNA and a 294-bp product for β-actin mRNA. No significant differences in the ChM-I/β-actin mRNA ratios were observed among the four groups analyzed (SAL: 0.85 ± 0.06; SPF: 1.13 ± 0.15; UREM: 0.68 ± 0.03; UREM-GH: 0.85 ± 0.19).

Immunohistochemical analysis of VEGF expression in growth plate

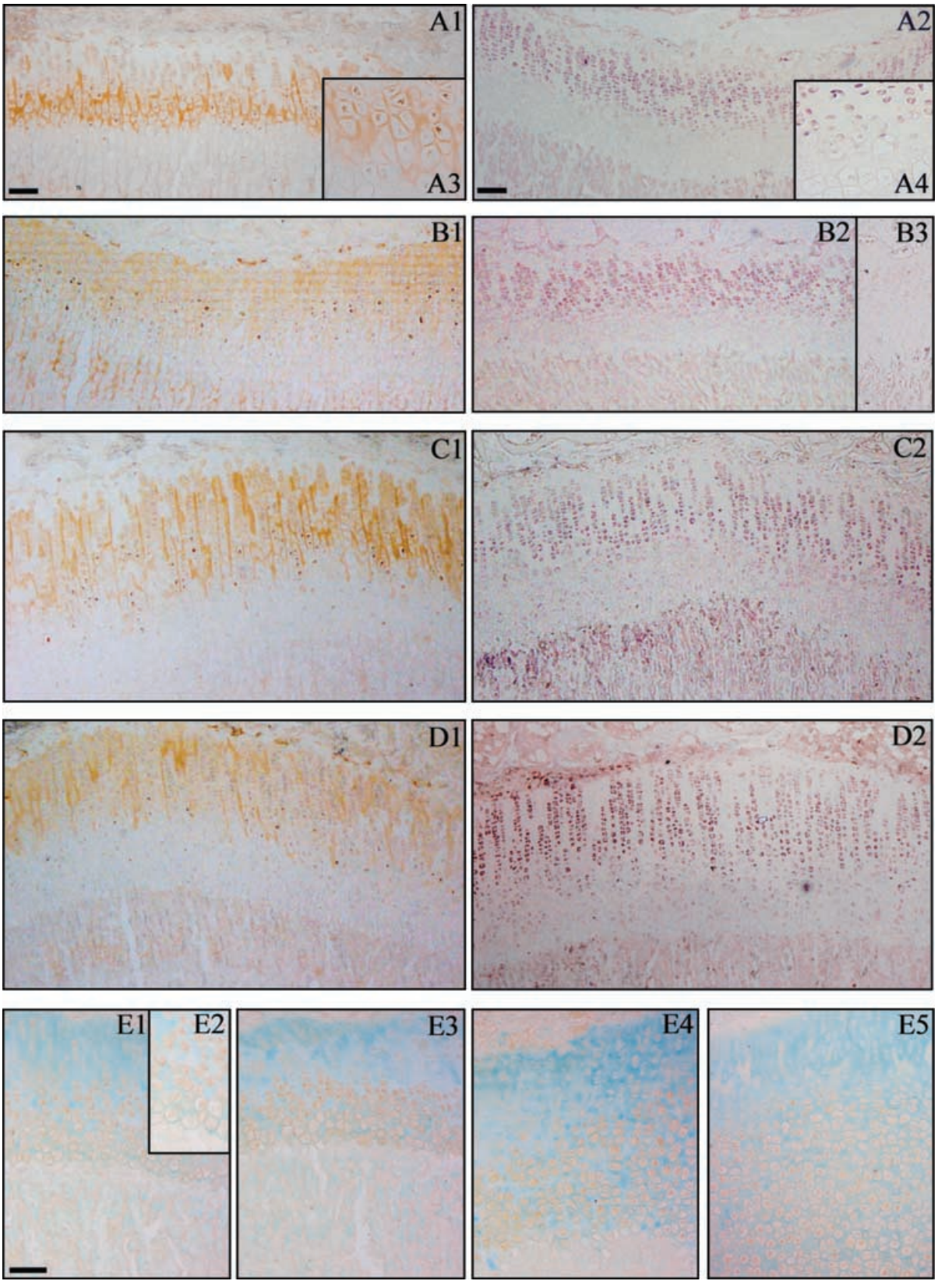
As shown in Figure 1, the VEGF immunohistochemical signal was strong and no differences in the distribution pattern or intensity were apparent among the four groups of rats. The VEGF signal was mostly intracellular and localized within the cytoplasm of hypertrophic zone chondrocytes. Staining was also found in the peripheral rim of some chondrocyte lacunae. The majority of articular chondrocytes was also positive for VEGF (data not shown).

DISCUSSION

The experimental protocol used in this study resulted in sustainedly low growth rate in UREM rats as shown by longitudinal bone growth rate, assessed by calcein labeling, significantly lower than that of SAL and SPF animals. This indicates that the degree of induced chronic renal failure was severe enough to produce a maintained adverse effect on growth velocity. The finding that growth rate of UREM rats was lower than that of SPF indicates that the impairment of growth in those animals was caused not only by the low food intake but also by a uremia-derived specific effect. As demonstrated in former studies [35, 40, 41], treatment with high doses of GH resulted in marked acceleration of growth velocity even in the absence of a stimulatory effect on appetite. This is concordant with the known anabolic effect of the hormone.

Growth retardation of UREM rats was associated with evident alterations in the histologic aspect of tibial growth plate. Epiphyseal cartilage increased in height as a result of an expansion of the hypertrophic zone, and the chondrocytes adjacent to the spongy metaphyseal bone were reduced in size. These findings are in agreement with former studies of our group [18, 19] and others [20]. Other authors have reported no modification [35, 40, 42], or even decrease [43–45], of growth cartilage height in uremic rats. The reasons for this lack of uniformity are not clear, although the severity and duration of chronic renal failure [46] and the calcium content of diet [47] have been shown to exert an influence on growth plate morphology in experimental uremia. In our study, the beneficial effect of GH treatment on growth was not accompanied by significant additional changes in the height of the growth cartilage and its hypertrophic zone but the aspect of most distal chondrocytes normalized. It has been hypothesized that expansion of chondrocyte columns in uremia results from a disequilibrium between cartilage production at the epiphyseal end of growth plate and bone apposition at the metaphyseal end [19]. In uremic animals treated with GH, the increased growth rate with persistence of expanded growth plate cartilage indicates that GH stimulates both bone formation rate and cartilage production. On the other hand, the normalization of the size of hypertrophic chondrocytes, as shown by the greater area and height of the most distal growth cartilage cells of UREM-GH rats in comparison with UREM group (Table 2) and a more organized arrangement of chondrocyte columns suggest a beneficial action of GH treatment on the process of chondrocyte maturation. Although a detailed study on the modifications induced by GH treatment on growth plate has not been published, administration of GH to uremic rats has been shown to result in increased expression of insulin-like growth factor I and its mRNA in proliferative chondrocytes [40], as well as type X collagen mRNA in hypertrophic chondrocytes [22, 23] of growth plate.

The morphologic changes observed in the uremic growth plate as well as former reports showing decreased expression of markers of chondrocyte maturation, such as type X collagen [22, 23] or parathyroid hormone/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parathyroid hormone–related peptide receptor [22, 45], in the growth plate of nephrectomized rats point to a uremia-induced disorder of chondrocyte maturation. Growth cartilage is avascular. It is tempting to speculate that, in order to facilitate vascular and bone invasion, as chondrocytes mature and approach the metaphyseal end of cartilage column, genes encoding angiostatic factors become suppressed, and genes encoding angiogenic factors become activated. The results of the study presented here show that, in the four groups of animals, ChM-I was expressed in the proliferative and early hypertrophic strata, but not by the more mature chondrocytes located closer to the metaphyseal bone front. On the contrary, VEGF-positive cells were observed in the area of the hypertrophic cartilage next to the primary spongiosa. This agrees with the above hypothesis that in order to facilitate the vascular invasion and, subsequently, the arrival of new osseous tissue, the angiostatic activity of cartilage must cease in the distal part adjacent to metaphyseal bone.

Our results do not support the assumption that defective chondrocyte maturation might lead to persistent expression of ChM-I and reduced VEGF synthesis and explain the mechanism of growth plate elongation and low bone formation rate of uremic individuals. There were no significant modifications in the levels of ChM-I and VEGF expression in the growth cartilage of uremic rats in comparison with normal renal function rats either fed ad libitum or pair-fed with the uremic animals. It may be argued that, as the growth plate of uremic animals was markedly enlarged, the growth cartilage of these animals produced substantially more ChM-I in absolute terms than that of control animals. The increased amount of ChM-I might exert a strong inhibition on the arrival of the vascular invasion front. However, cartilage is avascular and, consequently, ChM-I, a matrix peptide, should exert its action by paracrine or autocrine mechanisms. In our study, the distance between the lower limit of the extracellular ChM-I signal and the metaphyseal bone was greater in the uremic animals in consonance with the expansion of the hypertrophic growth cartilage zone. Thus, a persistent antiangiogenic effect of ChM-I is not likely to be responsible for the delayed vascular and bone invasion. Likewise, the growth-promoting effect of GH treatment was not associated with noticeable changes in the expression of ChM-I or VEGF. This also questions the role of these peptides in the pathogenesis of growth retardation in chronic renal failure.

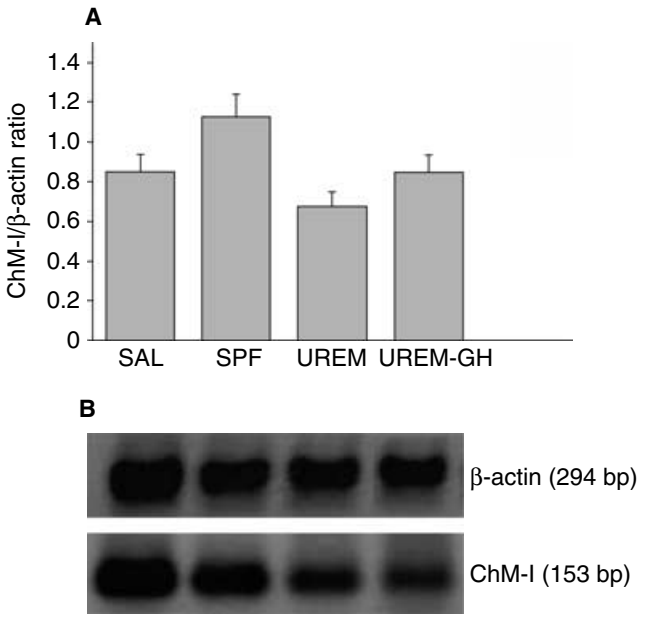

Fig. 2. Analysis of ChM-I mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR) in the four groups of rats. The values of the ChM-I/β-actin ratio (mean ± SEM of three separate experiments) were not significantly different among the four experimental groups (*A*). Representative example of the RT-PCR amplified products (*B*).

The variable intensity and distribution of ChM-I expression found in our groups of rats of different ages indicates that the role of this protein may change as the individual becomes older, and it is also in agreement with the progressive reduction of growth plate size with age. Kitahara et al [48] found gradual decrease of ChM-I expression with age in the articular cartilage of rats. However, in their study, the intensity of ChM-I extracellular signal in the epiphyseal cartilage remained unchanged. Little is known on the function of ChM-I as regulator of postnatal endochondral bone formation because most investigations on ChM-I have been performed in cell cultures or embryos [24, 26–31, 49]. Recently, it has been shown that ChM-I knockout mice [50] exhibit normal growth and fertility, but abnormal osseous remodeling in the long run [51]. Therefore, it would be of high interest to examine the pattern of expression of ChM-I in the bone

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Fig. 1. Representative images showing immunohistochemical and in situ hybridization analysis performed in proximal tibial growth plates of SAL (*A1*, *A2*, *A3*, *A4*, *E1*, *E2*), SPF (*B1*, *B2*, *B3*, *E3*), UREM (*C1*, *C2*, *E4*), and UREM-GH (*D1*, *D2*, *E5*) groups. Strong and similar in intensity and distribution, extracellular immunohistochemical staining for ChM-I was observed in the intercellular matrix of proliferative and upper hyperthrophic zones in all groups (A1, B1, C1, D1). Intracellular cytoplasmic signal was observed in few chondrocytes of these regions (A3). ChM-I mRNA was strongly expressed, following a pattern of distribution similar to that of the protein (A2, A4, B2, C2, D2). Negative hybridization control with sense probe is shown in B3. Most hyperthrophic chondrocytes showed an intense cytoplasmic immunohistochemical staining for VEGF without differences among the groups (E1, E3, E4, E5). Calibration bar is 100 μm. Greater magnification of ChM-I, ChM-I mRNA, and VEGF signals are shown in A3, A4, and E2. Abbreviations are: SAL, sham-operated rats fed ad libitum; SPF, sham-operated rats pair-fed with the UREM group; UREM, uremic rats; UREM-GH, uremic rats treated with growth hormone.

of uremic animals with distinct forms of renal osteodystrophy and different concentrations of serum parathyroid hormone.

CONCLUSION

The study presented here characterizes the pattern of ChM-I expression in the growth cartilage of uremic rats. Our results do not support a key role of ChM-I or VEGF in the pathogenesis of growth plate alterations induced by uremia, and in the mechanism responsible for growth retardation in young individuals with chronic renal failure. Further studies are needed to clarify this issue, as well as the function of ChM-I in the postnatal life.

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