Expression of CCAAT/enhancer-binding protein-beta (C/EBP β) and CHOP in the murine growth plate

TWO POSSIBLE KEY MODULATORS OF CHONDROCYTE DIFFERENTIATION

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Our aim was to evaluate the expression of transcription factors CCAAT/enhancer-binding protein-beta (C/EBP β) and C/EBP-homologous protein (CHOP) in the growth plate. Proximal tibial epiphyseal growth plates from ten 15-day-old Wistar rats were used. Additionally, anti-proliferating cell nuclear antigen (PCNA), anti-5-bromo-2'-deoxyuridine (BrdU) immunostaining, terminal transferase dUTP nick end-labelling (TUNEL) and nucleolar organiser region-associated proteins (AgNOR) techniques were performed. The histological morphology of the growth plate from C/EBP β knock-out mice was also analysed.

The normal growth plate showed that C/EBP β and CHOP factors are expressed both in the germinative/upper proliferative and in the lower proliferative zones. Furthermore, BrdU+ and PCNA+ cells were present exclusively in the germinative and proliferative zones, while TUNEL+ and AgNOR+ cells were seen in all three zones of the growth plate. Acellular areas, hypocellularity, the increase in cell death and anomalies in the architecture of the cell columns were observed in the growth plates of C/EBP β (-/-) knockout mice.

We suggest that C/EBP β and CHOP transcription factors may be key modulators participating in the chondrocyte differentiation process in the growth plate.

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The growth plate is an integral structure of hyaline cartilage and is responsible for the longitudinal growth of long bones. Two distinct processes may be distinguished in chondrogenesis in the growth plate: a patterning process, which determines the architecture of the growth plate, and a differentiation process, which determines the phenotype and cellular activity of the chondrocytes. Both are genetically characterised by a complex cascade of specific gene activations and inactivations which are still poorly understood.¹ The tissue architecture of the growth plate is based on a columnar (isogenic) and row arrangement of cells. Moreover, depending on the nature of the different subpopulations of chondrocytes, three zones are distinguished in the growth plate from the epiphysis to the metaphysis, namely, the germinative, proliferative and hypertrophic zones. This zonal distinction is based on phenotypic changes that chondrocytes undergo within the same column as a result of a still poorly understood, process of proliferation and differentiation which gives rise to different zones according to the different stages of maturation.²⁻⁷ It has been suggested that this differentiation process involves a switch to a previous phenotype of the chondrocyte,^{8,9} which requires the expression of a new genetic programme. "At present, there is more evidence available about the growth factors and signalling molecules regulating the differentiation of each chondrocyte subpopulation than about the transcriptional regulation of these processes".¹⁰

The processes of cell differentiation are related to the expression of transcription factors. The C/EBP (CCAAT/enhancer-binding protein) family of transcription factors acts as a regulator of adipocyte cellular differentiation.^{11,12} The common mesenchymal origin of the adipocyte and chondrocyte make it conceivable that the chondrocyte differentiation programme may also involve members of the C/EBP family.¹³ C/EBPs are members of the basic region/leucine zipper (bZIP) class of leucine zipper transcription factors, which are some of the most important factors involved in the regulation of cell proliferation and differentiation. Their role has been previously described in other tissues.¹⁴ CHOP is a member of the C/EBP family, acting as a dominant-negative inhibitor of DNA binding.¹⁵ However, the expression of C/EBP β and CHOP transcription factors within the growth plate is not fully understood.

Our aim was to investigate whether the transcription factors C/EBP β and CHOP are expressed in growth-plate

chondrocytes. Growth plates of C/EBP β knockout mice also underwent histological analysis. In addition, the cellular activity of every growth-plate zone, including assays for cell proliferation (anti-PCNA and anti-BrdU immunostaining), the TUNEL technique, and the activity of nucleolar organiser regions (NORs) identified with ribosomal activity was analysed.

Materials and Methods

We selected at random ten 15-day-old male Wistar rats which were killed using CO₂, following EU guidelines for the care of animals used for experimentation and other scientific purposes (Guideline 86/609/CCC and RD 223/88 BOE). All had received a single intraperitoneal injection of 30 mg/kg of BrdU (5-bromo-2'-deoxyuridine, Sigma Chemical Co, St Louis, Missouri) in sterile isotonic saline two hours before being killed. Both proximal tibial epiphyseal growth plates of each animal were obtained using a stereoscopic lens. Growth plates from the right tibiae were fixed in 4% neutral buffered formaldehyde for a day, and then processed for routine histology. Thereafter, serial 5 μ m longitudinal sections were cut and placed on poly-L-lysine treated slides; three sections were used for each technique.

In all assays, the cell nuclei were counted throughout three microscopic fields (40 x) of the central region of each specimen of growth plate. Only dramatically dark brown (not light brown) nuclei were considered as positive in these counts. The labelling index (LI) or percentage of positive nuclei in each zone was calculated by dividing the number of positive chondrocytes in each zone by the total number of cells counted in that particular zone (germinative, proliferative and hypertrophic zones), and multiplying by 100. For evaluation of AgNOR a x100 oil-immersion lens was used. The mean value of the three sections of each specimen was determined.

Immunohistochemistry for transcription factor assessment. The method of Maytin and Haberner¹⁵ was followed for the immunohistochemical assays on transcription factors. The growth plates from the left tibiae were used for this assay. Briefly, tissue specimens were frozen in Tissue-Tek@OCT™ compound (Miles Inc, Elkhart, Indiana) cut into 5 μ m sections and rinsed in methanol followed by 0.1% Triton X-100 to achieve nuclear permeabilisation. In this study, primary rabbit antisera directed against C/EBP β or CHOP/gadd153 (mAb, 1:50, Santa Cruz Biotechnology, Santa Cruz, California) were used.

Immunohistochemistry for assessment of cycling cells. Immunostaining of S-phase and cycling cells (BrdU+ cells, and PCNA+ cells, respectively) was performed with a ZYMMED LAB-SA system. We used anti-BrdU (mAb+nuclease, working solution; Amersham Biosciences UK Ltd, Little Chalfont, UK) or anti-PCNA (mAb, 1:200, Biomed V 1006, Biomed Co, Foster City, California) as the first antibody, and a mixture of anti-mouse and anti-rabbit biotinylated immunoglobulins as the second antibody

(ZYMMED), followed by peroxidase-conjugated streptavidin. Antigens were tracked after reaction with diaminobenzidine and H₂O₂. Nuclear counterstaining was carried out with Carazzy's haematoxylin.

TUNEL assessment. For *in situ* visualisation of TUNEL+ cells, a TUNEL assay (terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphatase (dUTP)-biotin nick-end labelling) was performed, following the method described by other authors.¹⁶

Assessment of NOR activity. To analyse the activity of the nucleolar organiser region (NOR), the AgNOR technique was performed as described by Crocker and Nar.¹⁷ Two variables were assessed: 1) the percentage of labelled cells (AgNOR+), and 2) a mean of the number of black dots per cell.

C/EBP β knockout mice. Bone samples from C/EBP β (-/-) mice, which do not express a C/EBP β transcription factor, were kindly provided by R. W. Hanson (Case Western Reserve University School of Medicine, Cleveland, Ohio).¹⁸ The animals studied in this work belong to the phenotype A of C/EBP β (-/-) mice, with no overt disruption of glucose homeostasis. The anomaly of glucose homeostasis defines the B phenotype in C/EBP β (-/-) mice, which die of hypoglycaemia within one to two hours after birth.^{18,19}

The collection of samples, which contained both proximal tibial epiphyses with their growth plates, belonging to 12-day-old mice (eight growth plates from C/EBP β (+/+) mice which express C/EBP β transcription factor, control group, six growth plates from C/EBP β (-/-) mice, assay group), were fixed in 4% formaldehyde for a day, and then processed and embedded in paraffin. Thereafter, serial 5 μ m longitudinal sections were cut. The cellularity and the presence of complete and incomplete cell columns were assessed in both groups. Columns were considered to be 'complete' when cells from the three zones were present or 'incomplete' when any of these three zones were absent.

Statistical analysis. The mean and SEM of each variable were calculated. Comparison between groups was performed using the Mann-Whitney U test, which tests differences in distribution of ranks. For the C/EBP knockout mice data are given as mean percentages. Fisher's exact test was used to evaluate statistical differences.

Results

Normal Wistar growth plate. Our results show that C/EBP β +, CHOP+, BrdU+ and PCNA+ cells were exclusively present in the germinative and proliferative zones (Fig. 1) whereas TUNEL+ and AgNOR+ cells were present in all three zones of the growth plate (Tables I and II).

LI analysis for transcription factors. Both germinative and proliferative zones showed a nuclear immunostaining positivity for C/EBP β and CHOP factors (Fig. 1I to Fig. 1L). The germinative and the upper proliferative layers showed a predominancy of C/EBP β compared with CHOP (NS and $p < 0.05$, respectively), while the lower proliferative layer

Fig. 1

Photomicrographs of a tibial epiphyseal growth plate from a 15-day-old Wistar rat. Figure 1A – PCNA+ cells are seen in the germinative and proliferative zones, but not in the hypertrophic zone (x 100); Figure 1B – Enlargement of the rectangular area shown in Figure 1A (x 400); Figure 1C – BrdU+ cells are observed in the germinative and proliferative zones. The absence of BrdU immunostaining in the hypertrophic zone is evident. The number of BrdU+ cells is lower than that of PCNA-positive cells (x 100); Figure 1D – Enlargement of the rectangular area in Figure 1C (x 400); Figure 1E – TUNEL+ cells in the germinative and upper proliferative zones of the growth plate (x 400); Figure 1F – TUNEL+ cells in the lower proliferative and hypertrophic zones of the growth plate (x 400); Figure 1G – AgNOR staining in the hypertrophic zone. Some nuclei display several AgNOR black dots (arrow) (x 400); Figure 1H – Enlargement of some hypertrophic chondrocytes showing several black dots (x 1000); Figure 1I and 1J – C/EBP β + and CHOP+ cells, respectively, are seen in the germinative and proliferative zones but not in the hypertrophic zone (x 100); Figures 1K and 1L – Enlargement of the rectangular areas of Figures 1H and 1I respectively (x 400), showing germinative and upper proliferative zones. There is a higher immunoreactivity of C/EBP β compared with that of CHOP in these zones (x 400).

Table I. Mean (SEM) LI for transcription factors according to the growth plate zone

Growth plate zone	C/EBP β LI	CHOP LI
Germinative (n = 10)	2.6 (0.4)	1.5 (0.4)
Upper proliferative (n = 10)	10.1 (0.9)	7.5 (0.8)*
Lower proliferative (n = 10)	6.7 (0.6)†	7.7 (0.4)*

*statistically significant differences ($p < 0.05$) with respect to C/EBP β LI†statistically significant differences ($p < 0.05$) with respect to the upper proliferative zone**Table II.** Mean (SEM) LI for BrdU+, PCNA+, TUNEL+ and AgNOR+ according to the growth plate zone

Zone	BrdU LI	PCNA LI	TUNEL LI	AgNOR LI
Germinative (n = 10)	6.4 (0.6)	34.4 (1.7)	12.3 (1.7)*	35.6 (0.6)
Proliferative (n = 10)	8.0 (0.3)	46.5 (0.7)†	27.9 (1.0) †	32.6 (3.1)
Hypertrophic (n = 10)	0	0	21.0 (1.0)	17.4 (2.7)

*statistically significant differences ($p < 0.05$) with respect to PCNA†statistically significant differences ($p < 0.05$) with respect to the germinative zone

showed the opposite ($p < 0.05$). Furthermore, C/EBP β immunostaining was higher in the upper proliferative layer than in the lower proliferative layer ($p < 0.05$) (Table I).

LI analysis throughout the different zones of the growth plate. The germinative zone showed an AgNOR LI and PCNA LI greater than 34% (Table II). The proliferative zone showed a PCNA LI greater than 45% (Figs 1A and 1B). BrdU+ cells (8%) appeared mainly in the upper proliferative zones (Figs 1C and 1D). In the proliferative zone, the TUNEL LI was lower than the PCNA LI ($p < 0.05$; Figs 1E and 1F). The hypertrophic zone showed no BrdU immunostaining and only a residual PCNA cytoplasmatic immunostaining which was not considered to be significant in this study (Figs 1A and 1C). The TUNEL LI was particularly predominant in the upper hypertrophic zone, appearing as a black ring inside the nucleus. On the other hand, the

AgNOR LI in the hypertrophic zone was lower than that in the germinative and proliferative zones ($p < 0.05$). However, the number of AgNOR black dots per chondrocyte was higher in the hypertrophic zone (n = 10, mean = 5.7, SEM = 0.2) than in the germinative (n = 10, mean = 2.8, SEM = 0.2) ($p < 0.05$) and the proliferative zones (n = 10, mean = 5.2, SEM = 0.3; NS) (Figs 1G and 1H).

Analysis of C/EBP β knockout mice. The bone marrow of C/EBP β knockout mice showed a decrease in the number of adipocytes, as well as a predominance of myeloblasts and megakaryocytes. The growth plate of C/EBP β (-/-) mice showed a decrease in the number of chondrocytes, disarray in chondrocyte columns, global hypocellularity and interspersed acellular areas (Figs 2A and 2B).

Incomplete columns in the C/EBP β (+/+) group were generally defined by their lack of one of the characteristic zones within the growth plate. In the C/EBP β (-/-) group they were defined by their lack of two zones within the growth plate. The percentage of incomplete columns in the growth plate of the C/EBP β (-/-) group (68.5%) was higher than that of the C/EBP β (+/+) group (13.6%) ($p < 0.0001$) (Figs 2A and 2B). In the C/EBP β (-/-) group, the degree of disruption of columns was higher in the proliferative/hypertrophic zones than in the germinative/proliferative zones. In addition, the TUNEL+ cell density in the C/EBP β (-/-) group (37.1%) was higher than that of the C/EBP β (+/+) group (17%) ($p < 0.005$). TUNEL+ cells were also observed in articular cartilage of both groups, being much more apparent among the chondrocytes of the articular cartilage of the C/EBP β group (-/-) (Figs 3A and 3B).

Discussion

Our results show that C/EBP β and CHOP transcription factors are expressed in two layers of the growth plate. First in the germinative/upper proliferative layers the expression of

Fig. 2

Photomicrographs of growth plates of proximal tibial epiphyses from A) C/EBP β (+/+) and B) C/EBP β (-/-) knockout mice. There is hypocellularity and a higher number of incomplete columns in the C/EBP β (-/-) group (haematoxylin and eosin staining x 400).

Fig. 3

Photomicrographs of TUNEL staining of sections of proximal tibial epiphyseal articular cartilage from A) C/EBP β (+/+) and B) C/EBP β (-/-) knockout mice. The number of TUNEL+ cells is higher in the C/EBP β (-/-) group (x 400).

C/EBP β predominates over that of CHOP. It is known that C/EBP β is a transcription factor which is involved in cell differentiation and cellular activity.^{11,12} In the germinative/upper proliferative layers we have observed the presence of PCNA and BrdU activity, both resulting from the presence of cycling cells in that area. BrdU, in addition, reflects the presence of mitotic chondrocytes as previously described in the literature.^{3,4} Secondly, the lower proliferative layer shows a predominant expression of CHOP over C/EBP β . CHOP is a transcription factor associated with cell death.²⁰ From the lower proliferative layer to the end of the growth plate in the metaphysis, no BrdU activity was observed and hypertrophy of chondrocytes had begun.

Given that those areas in which both transcription factors are expressed are also areas characterised by well-known phenotypic changes,²⁻⁴ we hypothesise that the growth plate presents two areas where the expression of transcription factors run parallel to cell differentiation. These two areas are the germinative/upper proliferative and the lower proliferative layers.

Our findings in the growth plate of C/EBP β (-/-) knockout mice show that in the absence of C/EBP β transcription factor, the growth plate is still present. Nevertheless, the differentiation and cellular activity of the chondrocytes are impaired, as seen by the global hypocellularity and the presence of acellular areas. Our results suggest that the C/EBP β defect observed in our knockout mice affects the differentiation process of the chondrocytes in the growth plate.

The fact that the growth plate is present indicates that the patterning process is normal. Genes liable to disturb this patterning process have been described in the initial process of chondrogenesis. It has been suggested that without transcription factor Sox-9, cells committed to chondrocyte lineage are unable to form pre-cartilaginous condensations and to activate a number of cartilage markers, including Col2a1 and aggrecan.¹

To date, the specific mechanism of action of transcription factor C/EBP β in chondrocytes is still poorly understood.

Within the members of the C/EBP family, however, it is known that C/EBP α protein is present in the germinal layer of the growth plate co-localised with GH-receptor, and that proliferative chondrocytes do not express C/EBP α .¹³

However, our results for AgNOR activity show that the percentage of labelled cells decreases from the germinative to the hypertrophic zone. Additionally, in the hypertrophic zone there are fewer stained cells (about 17% only), but they display a greater activity of NOR per cell than the rest of chondrocytes of the growth plate. Therefore, this ribosomal activity indicator suggests that there is residual cellular activity in this zone, even if its function has still to be elucidated. This may be supported by recent observations which show that when transcription factor Sox-4 is highly expressed in the zone of mineralising cartilage, these chondrocytes undergo hypertrophy.²¹

Our data show that positivity of the TUNEL assay appears in the germinative, proliferative and hypertrophic zones. It is well known that TUNEL is characterised by the cleavage of DNA into discrete fragments, which seems to be the earliest sign of apoptosis.¹⁶ Cleavage of DNA may yield double-stranded, low-molecular-weight DNA fragments as well as single-strand breaks (nicks) of DNA. These breaks of DNA can be detected by enzymatic labelling of the free 3'-OH termini with modified nucleotides. In the TUNEL technique, the TdT enzyme introduced labelled nucleotides in each of the 3'-ends of the DNA strand. These 3'-ends appear whenever the DNA strand is cleaved or restructured. In our present study, in order to confirm that TUNEL+ cells are actually owing to apoptosis, electrophoresis on agarose gels and pulsed field were performed with the DNA from growth-plate chondrocytes. Interestingly, the characteristic DNA ladder (200 base pair fragments or multiples) of apoptotic cells was not present in our samples. By contrast, large fragments of more than 1 kb were observed (data not shown). This could be interpreted in one of three ways. Either the apoptotic phenomenon began with larger fragments, or there were very few cells in the final stages of

apoptosis and thus the 200 base pair-ladder or its multiples had not been observed, or there was some other phenomenon taking place, different from apoptosis, but still responsible for the TUNEL+ signal or at least a part of it.

TUNEL+ chondrocytes associated with apoptosis have been described at random sites throughout the epiphyseal plate.²² However, under which conditions and for what particular purpose do cells fragment or restructure their DNA? Could it be that throughout the transdifferentiation process, the DNA strand becomes restructured? It has been suggested that the TUNEL labelling may refer to sites of active gene transcription.^{8,23} This would explain our finding of a high percentage of TUNEL+ cells observed in the rat growth plate zones.

Our results also reveal an increase in apoptosis in the C/EBP β (-/-) group of mice compared with C/EBP β (+/+) mice. How can this observation be explained? C/EBP β (-/-) (phenotype A) mice typically live until they are four to six months of age, dying from problems associated with their severely compromised immune system and developing lesions very similar to those appearing in animals which overexpress interleukin (IL)-6.^{18,19} CHOP is involved in programmed cell death and the induction of CHOP was evident in C/EBP β (-/-) mice.²⁰ CHOP contributes to the IL-1 growth-inhibitory signals through increasing production of IL-6,²⁴ and IL-1 inhibits replication of cartilage cells and stimulates production of matrix metalloproteinases, among other actions.²⁵

We conclude that although the initial function of the growth plate is only beginning to be elucidated, it is appropriate to consider that the phenotypical and functional changes undergone by the growth plate chondrocytes require the involvement of a new set of genes, with subsequent expression of transcriptional factors. Furthermore, C/EBP β and CHOP transcription factors may be key modulators participating in the chondrocyte differentiation in the growth plate.

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