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ARTICLES

Regulation by Glucocorticoids of Cell Differentiation and Insulin-Like Growth Factor Binding Protein Production in Cultured Fetal Rat Nasal Chondrocytes

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Abstract Glucocorticoids (GCs) modulate insulin-like growth factor action in cartilage through mechanisms that are complex and insufficiently defined, especially in the context of cranio-facial growth. Because the family of IGF-binding proteins (IGFBP-1 to -6) is important in the regulation of IGF availability and bioactivity, we examined the effect of GCs on chondrocyte differentiation in correlation with IGFBP production in cultured fetal rat chondrocytes isolated from nasal septum cartilage of fetal rat. Dexamethasone (DEX) effects were tested before and at the onset of extracellular matrix maturation. DEX induced a dose-dependent increase in the size of cartilage nodule formed, ^{45}Ca incorporation into extracellular matrix, alkaline phosphatase activity, and sulfatation of glycosaminoglycans, maximal effects being obtained with a 10-mM DEX concentration. The IGFBPs produced by cultured chondrocytes were characterized in culture medium which had been conditioned for 24 h under serum-free conditions by these cells. Western ligand blotting with a mixture of [^{125}I]IGF-I and -II revealed bands of 20, 24, 29, a 31–32 kDa doublet and a 39–41 kDa triplet which were differently regulated by DEX. Immunoblotting showed that following DEX exposure, IGFBP-3 and -6 were up-regulated whereas IGFBP-2, -5, and the 24 kDa band were down-regulated. The effect of DEX on both differentiation and IGFBP production showed a same dependence, and developed when extracellular matrix maturation had been just induced. The results obtained in this chondrocyte culture system show that production of IGFBPs is modulated by DEX at physiological concentrations thus regulating IGF availability and action, a control which could promote the primordial role of the rat nasal septum in craniofacial growth. J. Cell. Biochem. 88: 911–922, 2003.

Key words: chondrocyte; dexamethasone; IGFBP; mineralization

Glucocorticoid (GC) hormones play an essential role in tissue differentiation during development, by regulating the expression of genes that control development [Rousseau, 1984]. The influence of the glucocorticoids on expression of cartilage phenotype has been shown to be diverse in several model systems. For example, it has been demonstrated that glucocorticoids, at physiological concentrations, are essential for cartilage phenotype expression and maintain the integrity of cartilage matrix in organ culture, isolated primary cell populations, and rat chondrosarcoma cells [Jennings and Ham, 1983; Kato and Gospodarowicz, 1985; Takano et al., 1985; Horton et al., 1988; Bellows et al., 1989; Grigoriadis et al., 1989]. Conversely, some studies have documented glucocorticoid-induced suppression of cartilage phenotype markers, both in vitro and in vivo [Silbermann et al., 1987; Grigoriadis et al., 1996; Jux et al., 1998]. In that respect, the fetal rat system is particularly interesting: (i) there is a peak of fetal secretion of glucocorticoids between 17 and 20 days of gestation [Cohen, 1973; Henning,

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1978], and (ii) during this particular period, the first centers of ossification arise [Johnson, 1933]. GCs have been shown to be implicated in the maturation of several fetal tissues and could mediate these effects by modulating the IGF/IGFBP system. We have previously shown that the IGF system participates both to cell proliferation and glycogen storage, in cultured fetal rat hepatocytes [Menuelle et al., 1999]. Furthermore, both in vivo and in vitro studies demonstrate participation for the IGF system in liver maturation (see Discussion in Menuelle et al. [1999]). Indeed during fetal life, serum levels of IGF-II are elevated [Moses et al., 1980], together with the strong expression of IGF-II mRNA in the liver [Daughaday and Rotwein, 1989], the main site of IGF production. The liver IGF-II production declines rapidly after birth, as well as the IGF-II serum level which is progressively replaced by IGF-I, a factor mediating GH action [Daughaday and Rotwein, 1989; Trippel, 1992].

Despite extensive research, no specific endocrine mechanism has as yet been identified in fetal growth which would play a role similar to GH established in postnatal growth. Consequently, several autocrine and paracrine actions of growth factors, especially the IGFs, were proposed to control postnatal fetal growth (reviewed in Chard [1994]). IGFs (IGF-I and -II) play essential role in cell metabolism, proliferation, and differentiation, hence have major effects on fetal and postnatal development and organogenesis in mammals [Underwood and D'Ercole, 1984; Humbel, 1990]. Historically, the developmental impact of insulin-like growth factors (IGFs) has been initially discovered in cartilage by their ability to promote sulfate incorporation into proteoglycans [Salmon and Daughaday, 1957]. High levels of IGF-II gene expression are reported in growth plate chondrocytes in embryonic rodents [Wang et al., 1995] and chondrocytes in culture [Froger-Gaillard et al., 1989]. IGF-II stimulates clonal growth of human fetal chondrocytes [Vetter et al., 1986], as well as DNA and proteoglycan synthesis in cultured rabbit costal chondrocytes [Kato and Gospodarowicz, 1985; Trippel, 1992; Takigawa et al., 1997]. On the other hand, the actions of both IGF-I and -II are modulated by non-covalent association with high affinity insulin-like growth factor binding proteins (IGFBP-1 to -6) [Clemmons, 1998]. IGFBP-2 to -5 can be submitted to limited proteolysis which modulates IGF availability [Binoux et al., 2000]. The regulation of the expression of IGFBPs has been characterized in chondrocytes of many species and several culture conditions: articular chondrocytes in human [Olney et al., 1995; DiBattista et al., 1996], rabbit [Froger-Gaillard et al., 1989], bovine [Olney et al., 1993; Morales, 1997], ovine [Sunic et al., 1998], and rat species [Matsumoto et al., 1996b], as well as human growth plate cartilage [Matsumoto et al., 2000], rabbit [Koedam et al., 2000], bovine [Olney et al., 1993; Olney and Mougey, 1999], ovine [Sunic et al., 1995; Borromeo et al., 1996; De los Rios and Hill, 2000], and mouse species [Bhaumick, 1993]. Despite these characterizations, no information concerning IGFBPs produced by rat growth plate chondrocytes is available. The developmental pathway of skeleton and growth diverge depending on its anatomical site. The cranio-facial skeleton derives for cephalic neural crest while the axial and appendicular ones is of mesodermal origin. All previously reported studies were performed on mesoderm-derived chondrocytes. Our work focuses on rat nasal septum cartilage, this cartilage acts as a pacemaker for the growth of the skull and the face [Scott, 1953; Wang et al., 1999]. Nasal septum cartilage is derived from a primordial cartilagenous mass called the chondrocranium, which originates from cephalic neural crests. The present study was performed in order to investigate whether glucocorticoids together with IGFs could participate to this skeletal maturation.

We used cultured chondrocytes isolated from nasal septum cartilage of fetal rat which recapitulate the in vivo progressive acquisition of the chondrocyte phenotype until extracellular matrix mineralization is achieved [Sautier et al., 1993; Kergosien et al., 1998]. The secretion and regulation of IGFBP production by fetal chondrocytes from nasal septum cartilage of 17.5-day-old fetal rats were characterized in correlation with the influence of Dexamethasone (DEX) on the biomineralization of the extracellular matrix of these cells.

MATERIALS AND METHODS

Materials

Culture Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco (Grand Island, NY) and fetal calf serum from Biosys (Compiegne, France). Antibiotics were obtained

from Boehringer (Mannheim, Germany) and $^{45}CaCl_2$ (10–40 mCi/mg calcium) and [^{35}S] sulfate (20–40 Ci/mg of sulfur), from Amersham (Buckinghamshire, England). Collagenase, hyaluronidase, and DEX were purchased from the Sigma Chemical Co (St. Louis, MO). Rabbit antisera raised against rat anti-IGFBP-2, -3, and -6 were kindly provided by S. Shimasaki (La Jolla, CA). hIGFBP-4 ad -5 goat polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (CA). Antirabbit IgG polyclonal antibody coupled to horseradish peroxydase was purchased from the Sigma Chemical Co (St. Louis, MO). Antigoat IgG polyclonal antibody coupled to horseradish peroxidase was purchased from Santa Cruz Biotechnology, Inc.

Culture Procedure

Chondrocytes were isolated as previously described [Sautier et al., 1993]. Briefly, nasal septa cartilages from 17.5-day-old fetal rats (Sprague–Dawley) were dissected and dissociated enzymatically in a mixture of 0.25% collagenase (type I) and 0.1% hyaluronidase in phosphate-buffered saline (PBS) for 2 h at 37°C. Cells were then dissociated from partially digested cartilage fragments by repeated pipetting, total cell dissociation being rapidly obtained. After three washes in PBS, cells were counted and seeded at a final density of 2×10^4 cells/cm^2 in DMEM culture medium supplemented with 10% fetal calf serum, 50 UI/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml ascorbic acid. The non-adhering cells were removed 4 h after plating. Cells were cultured in a humidified atmosphere containing 95% ambiant air and 5% CO_2 at 37°C. The medium was replaced every 48 h and experiments were initiated at two different times of culture. After 6 and 10 days of culture (time zero of the experiments), i.e., before or at the beginning of the initiation of matrix nodule mineralization, the medium was replaced by a medium deprived of serum and containing or not DEX. Twenty-four hours later, a source of Pi as β-glycerophosphate at 10 mM was added, and cells were cultured for a further 16 h period. At time 40 h for each set of experiments, i.e., at 8 and 12 days of culture, biochemical tests were performed.

Histological Analysis

Observations of cell cultures were performed with an inverted phase contrast microscope (Leitz Diavert). For Von Kossa staining, cultures were fixed in situ with 5% formaldhehyde for 5 min. Then cells were successively incubated in 2.5% silver nitrate for 20 min in dark, 0.5% aqueous hydroquinone solution for 2 min, and 5% sodium hyposulfite for 1 min. Concerning Alcian Blue staining, cultures were fixed in situ in a solution of acetone:methanol (1:1) for 15 min. Then cells were rinsed twice in 3% acetic acid and incubated in a mixture of Alcian Blue (1%), acetic acid (3%), pH 2.5 for 30 min at room temperature.

Calcium Incorporation Into Cell Layer

Experiments were performed in the presence of $^{45}CaCl_2$ (5 μCi/ml), the total calcium concentration of the medium being 2 mM. Cells were incubated for 6 h with $^{45}CaCl_2$ in serum-free medium supplemented with DEX. At the end of the incubation, cells were rapidly washed four times with serum-free medium and incubated at 37°C for five successive periods of 4 min. This chase step allowed the release of rapidly exchangeable ^{45}Ca [Anagnostou et al., 1996]. The high rate of the early ^{45}Ca release progressively decreased and might correspond to the slow turnover rate of ^{45}Ca deposits (result not shown). Cells were then washed once with PBS at 4°C, and the radioactive material of the cell layers was extracted in 10% formic acid for 24 h at room temperature [Bellows et al., 1991]. Aliquots were used to determine ^{45}Ca counts by liquid scintillation spectrometry. The results are expressed as micromole of calcium per milligram of cell protein.

Determination of the Rate of Proteoglycan Synthesis

Proteoglycan synthesis was determined by measuring the incorporation of [^{35}S]sulfate in glycosaminoglycans, as previously described [Kato et al., 1980]. Briefly, the cells were labeled with 5 μCi/ml [^{35}S]sulfate for 6 h. After labeling, the cells were digested by pronase E, and the radioactivity in the material precipitated with cetylpyridinium chloride was measured in a scintillation counter.

Alkaline Phosphatase Activity

Alkaline phosphatase activity was determined in cell extracts and culture medium by measuring the *p*NPPase activity as previously

described [Anagnostou et al., 1996]. Briefly, cells were harvested using a rubber policeman and solubilized in the assay buffer containing 0.2% Nonidet P-40. Then they were sonicated with 0.6 s/35W pulses at 4°C, and the pNPPase activity was determined on aliquots.

Western Ligand Blotting and Immunoblotting

The conditioned media obtained from cultures conducted in parallel with morphological and biochemical studies, were analyzed by Western ligand blotting and immunoblotting according to previously described method [Hossenlopp et al., 1986]. Lyophilized samples (0.750 μl equivalent of conditioned medium which contained the same amount of proteins, close to 40 μg, within the range of glucocorticoids used) were submitted to 12.5% SDS–PAGE under non-reducing conditions, followed by electrotransfer to nitrocellulose. The different IGFBP species were first detected by incubation with a mixture of [^{125}I]IGF-I and [^{125}I]IGF-II and revealed by autoradiography. Then the blots were probed as previously described [Hossenlopp et al., 1986] using rabbit anti-rat IGFBP-2, -3, and -6 at 1/400 dilution (generous gift from Shunichi Shimazaki [Liu et al., 1993] and goat anti-human IGFBP-5 and -4 at 1/100 dilution. The anti-rabbit IgG antibody-horseradish peroxydase conjugate at 1/5000 dilution and the anti-goat IgG antibody-horseradish peroxydase conjugate at 1/1000 dilution were added to bind the immunocomplexes, which were then visualized by chemiluminescence (ECL) and Hyperfilm ECL film (Amersham, Les Ulis, France). Molecular weights were calculated using broad range markers (Bio-Rad SA, Marnes-la-Coquette, France). Quantification of the bands on the blots was performed by phospho-imaging.

Definitions

For the responses to DEX, a "stimulation index" was used, defined as the ratio of the values obtained for treated cultures to the values obtained for controls. For each protocol considering biochemical tests at least three independent experiments were performed on different cell preparations. Data are expressed as mean ± SD of triplicate culture measurements. Student's test for paired samples was used for statistical analyses of treated cultures and corresponding controls. For Western blotting, at least two samples from two different experiments were measured and a representative experiment is shown.

RESULTS

Biomineralization During Cartilage Cell Culture

^{45}Ca incorporation into cell layers, previously used as an index of mineralization [Bellows et al., 1991; Anagnostou et al., 1996], was investigated at different times of culture using a 6 h pulse in the presence of ^{45}Ca. ^{45}Ca incorporation was measured during the last 6 h of the 16 h period of β-glycerophosphate presence. ^{45}Ca incorporation was not detectable at day 5 of culture, and became measurable at day 8 and increased 2.5 times between day 8 and 12 to decrease after day 16 (Fig. 1A). Alkaline phosphatase activity in cell extracts and culture medium followed the same pattern as ^{45}Ca incorporation (Fig. 1B,C). The culture periods of day 6–8 and day 10–12 were thus chosen to study the DEX effect on extracellular matrix mineralization.

Effect of DEX on Morphological Aspects

Day 6 and 10 culture times corresponded to stages preceeding and just at the onset of chondrocyte extracellular matrix maturation, respectively (Fig. 2a,e). After 40 h of culture in the absence of DEX, an increase of matrix volume appeared as refringent material in phase contrast (Fig. 2b,f) and stained by Alcian Blue (Fig. 3a,c), concomitantly to appearance of von Kossa staining revealing the onset of extracellular matrix mineralization (Fig. 3e,g). DEX induced an enlargement of three dimensional nodules formed and increased their refringent aspect at 1 and 10-mM DEX, while the beneficial effect of DEX was attenuated for upper concentrations, as seen by phase contrast examination (data not shown). These observations were completed by Alcian Blue staining, in presence of the more efficient concentration of 10-mM DEX. Obtained results supported the increase of extracellular matrix volume and proteoglycan accumulation (Fig. 3b,d). Finally, von Kossa staining confirmed a stage- and DEX-dependent nodule size increase regarding the extent of extracellular matrix mineralization, mainly at day 12 (Fig. 3f,h). Thus, DEX-induced increase of matrix maturation was associated with biomineralization in chondrocyte cultures.

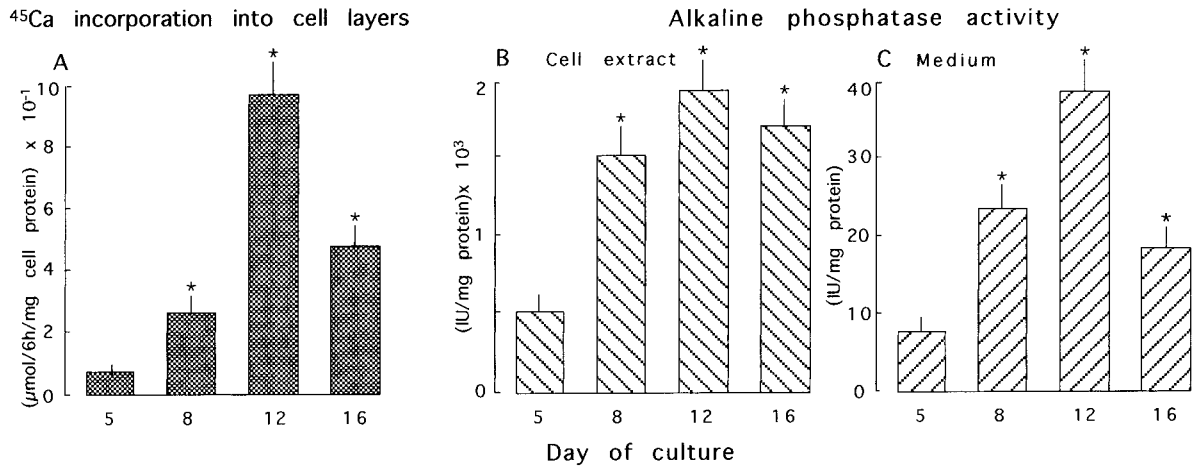

Fig. 1. ^{45}Ca incorporation into cell layers and ALP activity during the culture period. At different times of the culture performed in the absence of DEX (5–16 days), the medium was replaced by a fresh medium deprived of serum and containing 10 mM β-glycerophosphate. 12 h later, the medium was renewed by an identical one but supplemented with ^{45}Ca (5 µCi/ml). ^{45}Ca incorporation into cell layers was measured 6 h later (**A**). In parallel cultures grown in the absence of ^{45}Ca, ALP activity in cell extracts (**B**) and culture medium (**C**) was measured. Statistical significance is represented by * for $P < 0.001$, as referred to corresponding cultures for 5 days.

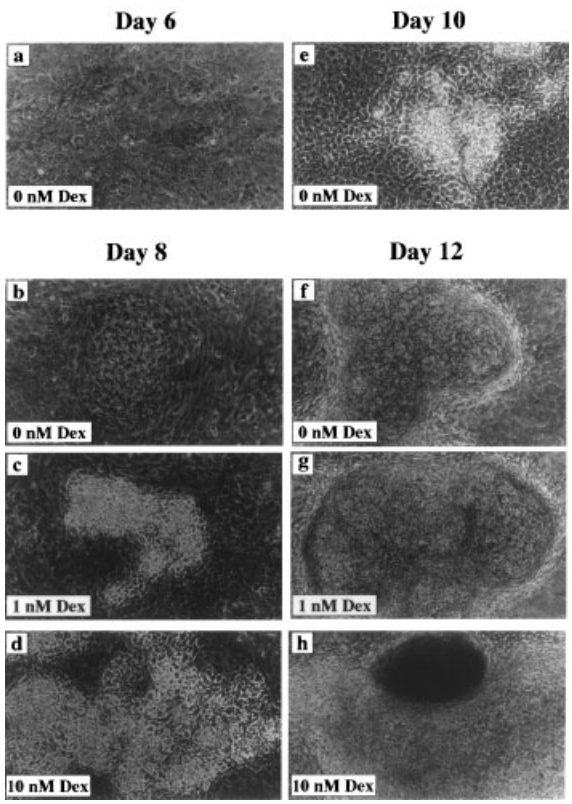

Fig. 2. Phase contrast study of mineralizing rat chondrocyte cultures in the absence and presence of DEX. At day 6 or 10 of culture, the medium was replaced by a fresh medium deprived of serum and containing or not 1 or 10-mM DEX, 10 mM β-glycerophosphate being added 24 h after DEX addition. Phase contrast study was performed at day 6 and 10 of culture (**a** and **e**), and after 40 h of presence of DEX solvent (**b** and **f**) or DEX at 1 nM (**c** and **g**) and 10 nM (**d** and **h**), i.e., at day 8 and 12 of culture (×100).

Effect of DEX on Biomineralization

Increasing concentrations of DEX were added in culture medium at day 6 and 10 for a period of 40 h. When ^{45}Ca incorporation was measured for the last 6 h at day 12, a clear stimulation by DEX was already observed at 1 nM and maximal at 10 nM (stimulation index: 1.71 ± 0.20) (Fig. 4A). In parallel experiments, the incorporation of [^{35}S]sulfate into newly synthesized proteoglycans, which is known to be a biochemical marker of differentiated phenotype of chondrocytes, and cellular alkaline phosphatase activity were measured (Fig. 4B,C). Like ^{45}Ca incorporation they showed a maximal stimulation at 10-mM DEX (stimulation index: 1.55 ± 0.19 and 1.24 ± 0.13, respectively). At day 8, all the biological effects studied appeared not to be significantly stimulated. It must be noted that in all these experiments the protein content was not modified by physiological concentrations of DEX, suggesting an absence of DEX effect on cell proliferation (result not shown). Thus the three parameters of chondrocyte biomineralization were equally regulated considering either the stage of culture or the dose dependence of the DEX stimulation.

Analysis of IGFBPs Secreted Into the Culture Media

The IGFBPs secreted into the culture media in the presence or absence of DEX were analyzed by Western ligand blotting at the two

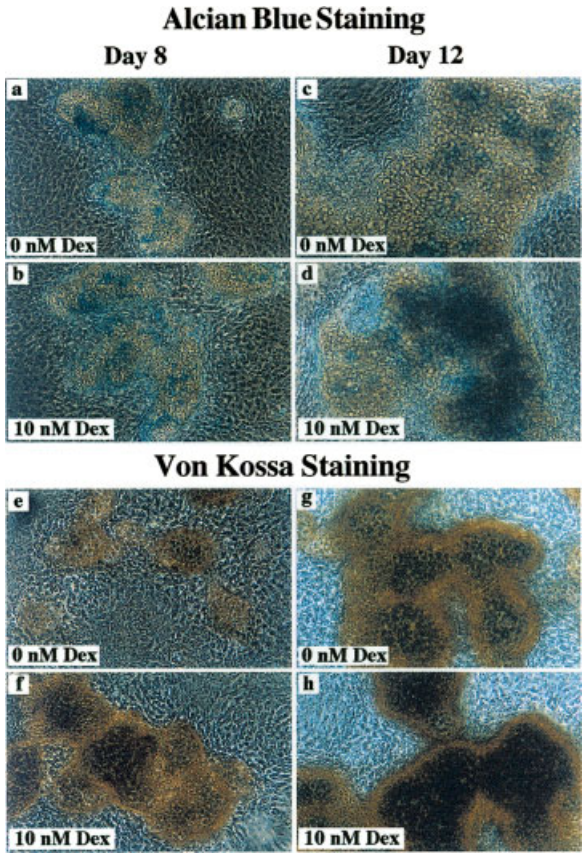

Fig. 3. Influence of Dex on Alcian Blue and von Kossa staining of chondrocyte cultures. At day 6 or 10 of culture, the medium was replaced by a fresh medium deprived of serum and containing 10 mM DEX or ethanol vehicle, 10 mM β-glycerophosphate being added 24 h after DEX addition. After 40 h of presence of DEX solvent (**a**, **c**, **e**, **g**) or 10-nM DEX (**b**, **d**, **f**, **h**), Alcian Blue (a–d), and von Kossa staining (e–h) was performed (×100).

critical stages of culture differentiation. In media conditioned in the absence of DEX, IGFBPs appeared as two major bands corresponding to a 39–41 triplet and a 31–32 kDa doublet and two minor-ones at 24 and 20 kDa, respectively (Fig. 5A). At day 12, a weak 29 kDa band was present. In the presence of DEX, the 39–41 kDa triplet showed a biphasic effect at day 12 of culture with a maximal stimulation at 10 nM. Such an effect was not evidenced at day 8 (Fig. 5A,B). The 31–32 kDa doublet and the 24 kDa band revealed a progressive dose-dependent decrease in the presence of DEX which was more pronounced at day 12 than at day 8. Reversely, the 20 kDa band showed a progressive increase as a function of DEX concentration, an effect even more accentuated in more differentiated cells. Thus, a differential DEX regulation for IGFBP expression was presently identified, which progressively emerged during chondrocyte maturation. According to the literature, the 39–41 kDa triplet corresponded probably to IGFBP-3, the 31–32 kDa doublet either to IGFBP-2 or -5, and the 24 kDa band to IGFBP-4 [Hossenlopp and Binoux, 1994].

In order to further characterize IGFBPs, the same conditioned media were also subjected to Western immunoblot analysis. As reported in other cell types and culture systems, native and degraded entities were identified based on their molecular weight profiles: a 32 kDa and a 18 kDa for IGFBP-2, a 39-41 triplet and a 27–29 doublet for IGFBP-3, a 29 kDa and a 20 kDa for IGFBP-5, a 27 kDa band as IGFBP-6. The 24 kDa band whose apparent molecular weight is analog to that of the nonglycosylated form of IGFBP-4 [Grigoriadis et al., 1989; Sunic et al., 1998] was not recognized by the IGFBP-4 antiserum tested and presented no cross-reactivity with any of the other antisera used. In basal conditions, the IGFBP-2 to -6 were expressed by chondrocytes. It should be noted that it is the first time that IGFBP-6 was identified in fetal rat cartilage, although it has been revealed in organotypic cultures of adult bovine articular cartilage [Morales, 1997]. Among the degraded entities, the 15 kDa IGFBP-2 fragment may be specific to mineralized tissues as it was not evidenced in non-mineralizing articular cartilage [Borromeo et al., 1996] and reported in fetal rat calvaria cells [McCarthy et al., 1994]. The 27–29 kDa doublet IGFBP-3 degradation product is found in human osteoblast-like cells [Lalou et al., 1994; Binoux et al., 2000]. The 20 kDa IGFBP-5 fragment is found in cultured fetal rat osteoblast cells [Chen et al., 1995] and in adult articular rat chondrocytes [Matsumoto et al., 1996a]. In regard to the maintained capacity to bind [^{125}I]IGFs (as shown in Fig. 5) and its molecular mass, it can be postulated the 20 kDa IGFBP-5 degradation fragment (Fig. 6) corresponds to the amino-terminal IGFBP-5$^{1-169}$ fragment present in chronic renal failure serum and implicated in adult rat growth plate chondrocyte proliferation [Kiepe et al., 2001].

The two identified entities of IGFBP-2 appeared to show a dose-dependent decrease by DEX (1–100 nM) while those of IGFBP-3 presented, at day 12, a maximal increase at 10 nM DEX. Reversely, intact IGFBP-5 was decreased by DEX while the 20 kDa degradation product accumulated correlatively. DEX regulated

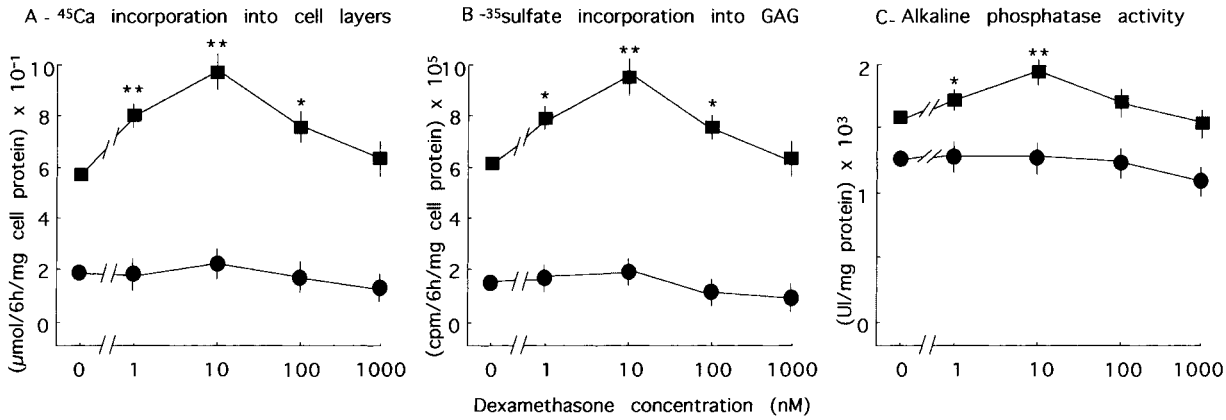

Fig. 4. Dose dependence of DEX effect on ^{45}Ca incorporation into cell layers, proteoglycan synthesis and ALP activity. At day 6 or 10 of culture, the medium was replaced by a fresh medium deprived of serum and containing increasing concentrations of DEX from 1 nM to 1 μM or ethanol vehicle, β-glycerophosphate at 10 mM being added 24 h after DEX addition. Thirty-four hours after DEX addition, the medium was renewed by an identical one but supplemented either with ^{45}Ca (5 μCi/ml) or [^{35}S]sulfate (5 μCi/ml). ^{45}Ca incorporation (**A**), [^{35}S]sulfate incorporation (**B**), and ALP activity (**C**) was measured 6 h later. The symbols correspond to day 6 (●) or day 10 (■) culture times followed by 40 h treatment with DEX. Statistical significance is represented by * for $P < 0.01$, and ** for $P < 0.001$, as referred to corresponding cultures grown in the absence of DEX.

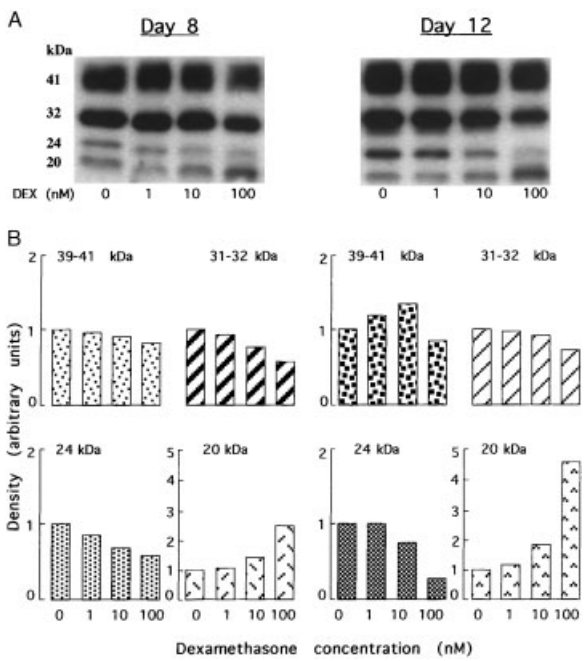

Fig. 5. Analysis of IGFBPs in media conditioned by fetal rat chondrocytes cultured in the presence or absence of DEX. From day 6 or 10 of culture, cells were cultured in medium deprived of serum with and without increasing concentrations of DEX (1 to 100 nM) for 40 h, serum-free medium being renewed after 16 h. Samples of media conditioned during the last 24 h were submitted (0.750 ml eq/slot) to SDS–PAGE (12.5%) under nonreducing conditions and transferred to nitrocellulose. Autoradiogram of the Western ligand blot are shown in (**A**), the migrations of molecular size markers being indicated on the left. Lower panels show the quantification of separate bands on the blot by phospho-imaging (**B**). Two independent experiments performed on different cell preparation gave similar results.

diversely IGFBP-6 and -4, with an increased and decreased expression, respectively. This complementary characterization of IGFBPs by Western blotting in our culture system validated the dose-dependent effects of DEX shown by Western ligand. Thus, a differential DEX

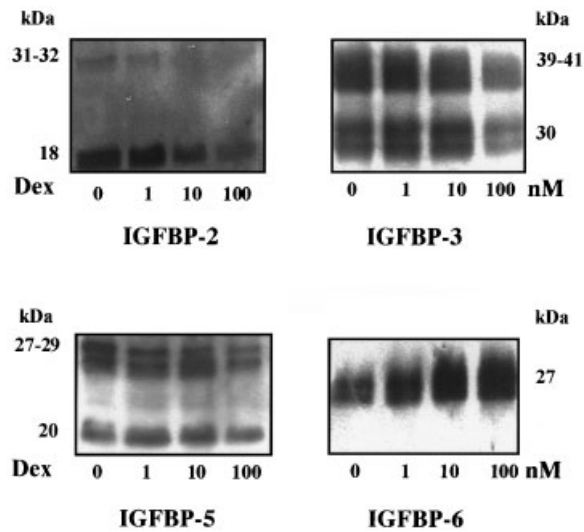

Fig. 6. Immunoblotting of IGFBPs. From day 10 of culture, cells were cultured in medium deprived of serum with and without increasing concentrations of DEX (1–100 nM) for 40 h, serum-free medium being renewed after 16 h. Samples of media conditioned during the last 24 h were submitted (0.750 ml eq/slot) to SDS–PAGE (12.5%) under nonreducing conditions and transferred to nitrocellulose. The blots were probed with IGFBP-2, -3, -5, and -6 antibodies as described in Materials and Methods. Two independent experiments performed on different cell preparation gave similar results.

regulation for IGFBP expression was presently identified, which progressively emerged during chondrocyte maturation.

DISCUSSION

Primary cultures of chondrocytes derived from fetal rat nasal septum cartilage, when cultured for less than 2 weeks in the presence of ascorbic acid and organic phosphate, reveal cell growth, and differentiation resulting in the formation of macroscopic three-dimensional nodules of calcified cartilage [Sautier et al., 1993]. Using this system, we showed that the synthetic glucocorticoid DEX increased proteoglycan sulfatation, alkaline phosphatase activity, and calcium deposition, three parameters of chondrocyte extracellular matrix maturation and mineralization, DEX effects being greater in more differentiated cells. In these chondrocyte primary cultures, it is obvious that DEX could not exert its differentiating effects before the extracellular matrix was engaged into its maturation program. Such a result is in agreement with previous reports in which glucocorticoids have been shown to increase proteoglycan synthesis in rabbit costal chondrocytes [Kato and Gospodarowicz, 1985; Takano et al., 1985], a model which conversely to ours, does not mineralize. Furthermore, the present maximally effective concentration (10 nM) and EC_{50} (1 nM) place this response unambiguously within the physiological range. Indeed, DEX appears to have a more efficient glucocorticoid activity when compared to corticosterone, the major glucocorticoid circulating in rat [Thompson et al., 1980]. The concentration found to cause a maximal response in 17.5-day-old-fetal rat chondrocytes was therefore similar to the plasma level of free glucocorticoids they would have been exposed to in vivo [Cohen, 1973]. Thus, this in vitro study suggests that glucocorticoids are implicated in endochondral mineralization process during late gestation period. One pathway of glucocorticoid action is the regulation of the IGF/IGFBP system. As IGFBP modulation plays a primordial role in IGF biological effects, the characterization of the IGFBP production was performed in our culture model.

To the best of our knowledge, the analysis of IGFBP released by chondrocytes isolated from fetal rat nasal septum has never been performed before; in rat, only IGFBPs secreted by cultured adult articular chondrocytes has been analyzed [Kiepe et al., 2001]. In the present study, under serum-free conditions, in conditioned media obtained in basal conditions, IGFBP-2 to -6 were detected. In situ reports in murine rib growth plate cartilage suggest that only mRNAs of IGFBP-5 and -6 are expressed, primarily in the early stages of chondrogenesis [Wang et al., 1995]. But more recent studies, concerning ovine fetal epiphyseal growth plate, revealed the presence of mRNA for IGFBP-2 to -6 [De los Rios and Hill, 1999]. These results do not preclude the specific site of IGFBP production at the level of rat nasal septum. Further study will be necessary to clarify the origin of the differences observed in various species, types of cartilage, maturation stage or experimental procedure. In the present work important amounts of IGFBPs (-2 to -6) were secreted by cultured rat nasal septum chondrocytes, even in basal conditions, conversely to that found in other chondrocyte models, as for example in newborn rabbit growth plate [Koedam et al., 2000]. Thus differences may be related either to species-specific variations, rabbit [Koedam et al., 2000], versus rat (this study) or to site-specificity of cartilage associated with their differential origins, neural crest (this study) versus mesoderm (all other studies).

GCs as well as IGFs have important effects on mineralized tissues, often opposite on IGFBP regulation, and thus the IGF/IGFBP system is believed to be implicated in the pathway of glucocorticoid action [Canalis, 1998; Jux et al., 1998]. The physiological roles of IGFBPs in many cell types have been reported but the regulation by physiological glucocorticoid concentrations of released IGFBPs has not been investigated before in rat chondrocytes in culture. We found that Dex addition, at the initiation period of extracellular matrix maturation, induced in a dose-dependent manner a biphasic effect on IGFBP-3 (stimulatory until 10 nM at physiological concentrations), a down-regulation of IGFBP-2, -5 and the 24 kDa band (possibly IGFBP-4), and an up-regulation of IGFBP-6. In newborn rabbit costal chondrocyte cultures when stimulated by DEX, a similar regulation pattern of IGFBP-5 and the peculiar biphasic one of IGFBP-3 are found [Koedam et al., 2000]. In fetal ovine chondrocyte cultures, cortisol decreased the abundance of IGFBP-2 and -5 mRNAs and peptide release and increased IGFBP-3 mRNAs, but observed only at supra-physiological concentrations of glucocorticoids

[De los Rios and Hill, 2000] when compared to the circulating concentrations in ovine fetus [Li et al., 1993]. Thus, a local modulation of IGFBP balance by glucocorticoids within the septum nasal cartilage could regulate IGF bioavailability and action, if we referred to already described IGF/IGFBP mode of action [reviewed in Collett-Solberg and Cohen, 2000]. IGFBP proteolysis generated IGFBP degradation products. The residual ability of these IGFBP fragments to bind IGF-I and -II may or may not be physiologically significant depending on their concentration. Indeed, despite their weak affinities for IGFs, they might allow a competition with IGF-receptor and IGFBP native forms or develop their intrinsic activities [Binoux et al., 2000].

In our cultured fetal chondrocytes undergoing terminal differentiation, the down-regulation of IGFBP-2 by DEX, which was mainly on its 18 kDa degraded form, may have a physiological significance during chondrogenic differentiation. Little data are available about IGFBP-2 regulation in growth plate in vivo as well as in vitro. The precise regulation by DEX of IGFBP-3 and its 30 kDa carboxy-truncated fragment, in the present study, may be relevant and related to an important role of this IGFBP during extracellular matrix mineralization. IGFBP-3 can promote or inhibit growth, effects being either IGF-mediated or IGF-independent [Collett-Solberg and Cohen, 2000]. In a mesenchymal chondrogenic cell line, IGF-independent and IGF-dependent effects of IGFBP-3 on chondrocyte proliferation associated with chondrocyte differentiation are evidenced [Spagnoli et al., 2000]. The present maturation stage-specific results suggest that only the IGF-dependent effect of IGFBP-3 was DEX-regulated. Indeed, IGFBP-3 DEX regulation was absent when matrix maturation was not engaged and IGFBP-3 DEX regulation was present when matrix maturation was engaged. Besides, the increase of chondrocyte biomineralization correlated with IGFBP-3 regulation and occurred apparently without affecting proliferation. Also, conversely to IGFBP-3, IGFBP-5 was decreased by DEX and its degradation fragment accumulated. Although the role of IGFBP-5 in articular chondrocytes is uncertain, it is possible that the IGFBP-5 protease acts on IGFBP-5 to release bioactive IGF peptides. In cultured epiphyseal rat chondrocytes, the 20 kDa degradation fragment inhibits cell proliferation whereas intact IGFBP-5 stimulates this process [Kiepe et al., 2001]. Divergent results were obtained in cultured human osteoblast-like cells, where the 23 kDa carboxy-truncated fragment of IGFBP-5 stimulated mitogenesis [Andress and Birnbaum, 1992]. Thus, in our culture model, where the amino-terminal form of IGFBP-5 accumulated under DEX stimulation, the ratio between intact and degraded IGFBP-5 forms may be important in preserving the cartilage-to-bone developmental sequence necessary for endochondral mineralization. The opposite regulation of IGFBP-3 and -5 by DEX is in agreement with the results obtained in immortalized human chondrocytes where the balance IGFBP-3/-5 influences chondrocyte proliferation and thereby cartilage metabolism [Matsumoto et al., 1996b]. Concerning IGFBP-6, its increase by DEX is in agreement with the one found in rat osteoblasts [Gabbitas and Canalis, 1996]. *O*-glycosylation of IGFBP-6 impedes its degradation [Bach, 1999]. Prenatally, IGFBP-6 mRNA identification in rat and mouse development shows that IGFBP-6 mRNAs are expressed in sites of cell differentiation and proliferation [Cerro et al., 1993]. However, it is noteworthy that IGFBP-6 is associated with quiescence or non-proliferation in many in vitro systems, showing its possible role as an autocrine inhibitor of cell proliferation [reviewed in Bach, 1999]. Our results suggest that DEX could act as a differentiating agent by increasing IGFBP-6 levels which may tightly regulate the effects of IGF-II.

In conclusion, in primary cultures of chondrocytes derived from fetal rat nasal septum cartilage, the precise regulation of IGFBPs and their degradation fragments considering their physiological functions, seemed to be in favor of Dex differentiating effects. The differential regulation of IGFBPs expressed by cartilage may be relevant to the pleiotrophic effects of glucocorticoids. The nasal septum being essential for the growth of the face, the developmental DEX regulation of IGFBP balance could allow an equilibrated balance between proliferation and differentiation and thus promote a normal craniofacial growth.

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