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Effect of relaxin on the phenotype of collagens synthesized by cultured rabbit chondrocytes

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The effect of porcine relaxin on rabbit articular and growth plate chondrocytes in primary culture was investigated by measurement of total collagen production and analysis of the phenotypes of newly synthesized collagen chains. A 24-h treatment of monolayer articular and multilayer growth plate chondrocytes with 2 μg per ml relaxin had no effect on total DNA and did not significantly modify the amount of [^{3}H]proline-labelled collagen chains secreted by the cells. However, polyacrylamide gel electrophoresis demonstrated relevant modifications in relaxin treated chondrocytes. A significant increase was observed in the proportion of type III collagen and in the intensity of the band corresponding to α_2I chains. Two-dimensional peptide mapping of CNBr-cleaved molecules indicated that the band that was identified as α_1II on monodimensional gels contained a significant proportion of α_1I collagen chains, as demonstrated by the presence of α_1I cyanogen bromide-digested peptides. The intensity of this band was increased by relaxin treatment. Furthermore, total RNA analysis by slot blot and Northern blot techniques showed a dose-dependent stimulation of α_1I and α_1III mRNA levels after incubation with increased relaxin concentrations, but no change in the amount of α_1II mRNA. These results suggested that when added to cartilage cells in vitro, relaxin modulated the expression of type I, type II and type III collagen genes by amplifying the dedifferentiation process.

Introduction

In mammals, the polypeptide hormone relaxin is thought to promote connective tissue remodelling during pregnancy and at parturition. In addition to its effects on the uterus, cervix and mammary gland [1,2], relaxin acts on the pubic symphisis before and during parturition by softening and lenghthening the pubic cartilage and the interpubic ligament [3–6] but the precise mechanisms involved in these changes are not known.

Alterations in collagen have been reported in vivo in the presence of relaxin, but the exact relationship between the hormonal activity and the changes in collagen is poorly understood, due to contradictory results (reviewed in Ref. 7). In rodents, an elevation in collagen degradation after collagenase activation has been reported by some authors [8,9]. In studies with pregnant guinea pigs,

Abbreviations: GBSS, Grey's balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; FCS, fetal calf serum; PMSF, phenylmethylsulfonyl fluoride; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate.

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however, an increase in total collagen was observed suggesting that ultrastructural changes could be the results of a variation in collagen aggregation rather than a loss of this protein [10,11]. Studies performed with cells in culture have also provided equivocal results. Uchima and Greenwood [12] reported an increase in collagen synthesis by guinea pig fibroblasts but Sheffield and Anderson [13], using a similar system, failed to find this effect. None of these studies have included descriptions of collagen phenotypes.

Type II collagen, the major component of hyaline cartilage, has been recognized as a reliable marker of the differentiated phenotype of chondrocytes. The instability of this phenotype is largely substantiated and several compounds, such as 5 BudR [14], retinoic acid [15,16] and phorbol myristate acid (PMA) [17] have been shown to exert a dramatic effect on differentiation by inducing a transition in the expression of collagen genes and proteoglycans.

The present work was initiated to investigate the direct effect of the relaxin purified from pig ovaries [18] on cartilage metabolism in vitro, in the absence of any other additional hormones or factors. For this purpose primary cultures of chondrocytes from articular and growth plate cartilage were prepared. Total collagen synthesis and secretion were measured, and the different collagen types were analyzed at both the protein and RNA levels. Our results show that relaxin amplified the natural tendancy of cultured chondrocytes to dedifferentiate to cells producing higher amounts of type I and type III collagens.

Material and Methods

Animals and tissues. Young growing female Fauve de Bourgogne rabbits (2–3 weeks old) were killed. Cartilage slices were isolated from the articular surface of long bones as described by Green [19] and from the resting zone of the growth plate area of the scapulae. The scapulae were first cleaned by removing muscles and bone ligamentous insertions and by peeling off the periosteum on both sides. The growth plate area was cut off from the bone area. The resting zone was then separated under microscope, taking care to avoid the columnar proliferative zone on one side and the fibrous superficial area on the other side.

Cell culture. Primary cultures of chondrocytes from both types of cartilage and skin fibroblasts were prepared separately after sequential enzymatic digestion with 0.05% testicular hyaluronidase, 0.2% Trypsin and 0.2% clostridial collagenase (Boehringer Mannheim, F.R.G.) in Gey's balanced salt solution (GBSS). Cells were plated at $3.5 \cdot 10^5$ cells per 75 cm^2 flask containing 14 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Boehringer Mannheim, F.R.G.), 100 IU/ml penicillin and 100 μg/ml streptomycin. The flasks were maintained at 37 °C in a 5% CO_2 atmosphere until cells reached steady state. Articular chondrocytes reached confluency on day 8. Growth plate chondrocytes were confluent after 9 days and continued to proliferate actively during an additional period of 8 days, forming multilayered colonies of aggregated cells as previously described [20]. They were maintained in culture for 8 additional days, at the end of which cell division had ceased, the cells were then hypertrophic and surrounded by an abundant extracellular matrix.

Hormone treatment and cell culture labelling. Unless otherwise specified, the hormones were added to articular chondrocytes on day 8 or 9 and to growth plate cells on day 18–20. Relaxin (PRLX 277, Howard Florey Institute) was purified from ovaries of pregnant pigs and was determined as pure by amino acid analysis. Relaxin and bovine insulin (25.5 IU/mg, Sigma) were dissolved in 1% bovine serum albumin (BSA) (A4161 Sigma) and DMEM and added to the cultures at a final concentration of 2 μg/ml and 5 μg/ml, respectively. Before hormone treatment, the cells were washed twice with GBSS and pre-incubated in DMEM without fetal calf serum FCS for 20 h. This medium was discarded and replaced with fresh serum-free DMEM supplemented with 50 μg/ml ascorbic acid, 50 μg/ml β-aminopropionitrile fumarate. Labelling was achieved by adding 20 μCi/ml [3,4-^{3}H]proline (CEA, Saclay, France, SA 30 Ci/mmol) with either relaxin or insulin. Control flasks were prepared by adding the same volume of 1% BSA-DMEM alone. After a 24 h incubation, culture media and cell layers from two identically treated flasks were harvested

separately and used for collagen extraction. DNA content was measured according to Burton [21] in similarly treated flasks.

Isolation of radiolabelled collagenous proteins. Culture medium was collected in the presence of proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM *N*-ethylmaleimide (NEM) and 25 mM EDTA). The cell layer was scraped with a rubber policeman and extracted with 100 mM Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100 and proteinase inhibitors (1 mM PMSF, 10 mM NEM, 25 mM EDTA). Both medium and cell mixtures were then treated similarly but separately. After centrifugation for 10 min at $10000 \times g$ to eliminate cell debris, an aliquot from each pool was heated for 1 h at 60°C, dialyzed against several changes of 0.5% acetic acid, hydrolyzed with 6 M HCl, passed through an ion-exchange resin and used for [^{3}H]hydroxyproline determination. The ratio of total collagenous proteins in each compartment was expressed according to Wiestner et al. [22] as follows:

$$\frac{2\times[\text{cpm(Hypro)}]}{5\times[(\text{cpm(pro)}-\text{cpm(Hypro)}]+2\times[\text{cpm(Hypro)}]}\times 100.$$

This was based on the assumption that collagen contains about 5-times as many as amino acids as non-collagenous proteins.

The remaining culture medium and cell layer were divided into two equal fractions. One fraction was dialyzed against ammonium carbonate buffer (1 mM) supplemented with proteinase inhibitors (1 mM PMSF, 2 mM NEM, 1 mM benzamidine) for procollagen analysis and the other was dialyzed against 0.5% acetic acid before pepsin digestion. Crystalline pepsin (Sigma) was dissolved in acetic acid 0.5 M (pH 2), added to the lyophilized sample at a final concentration of 100 μg/ml and incubated for 18 h at 4°C. Enzyme action was stopped by addition of 2M NaOH to pH 7, and the mixture was dialyzed against distilled water. Before electrophoresis, the dried sample was resuspended in 200 μl sample buffer (125 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 0.01% Bromophenol blue).

Gel electrophoresis and peptide mapping. Collagen chains were electrophoresed on 5% slab gels according to the procedure of Neville and Glossman [23] in the presence or in the absence of dithiothreitol (DTT). Labelled proteins were visualized by processing the gels with EN^{3}HANCE (NEN) and exposure to X-ray films. Two-dimensional peptide mapping was performed by cyanogen bromide digestion. Gel strips were cut after migration in the first dimension on 5% gels, then equilibrated with 70% formic acid and incubated for one night at room temperature in a solution of formic acid containing 50 mg/ml cyanogen bromide (CNBr). Elimination of the reagent was achieved by equilibration of the strips in 100 mM Tris-HCl buffer (pH 6.8) supplemented with 30% glycerol and 0.01% Bromophenol blue. Strips were then placed on top of a 12.5% polyacrylamide gel and included within a 5% stacking gel. CNBr-derived peptides were revealed by fluorography using the same procedure as for the first dimension lanes.

RNA extraction and analysis. Total cellular RNA was isolated from cultured articular and growth plate chondrocytes were isolated by guanidium chloride extraction and differential precipitation according to the procedure of Kahn et al. [24]. The rRNAs (28S and 18S) were visualized by staining with Ethidium bromide.

For slot blot analysis total RNAs were denatured by treatment with a formaldehyde/20 × SSC mixture at 55°C for 15 min then deposited on a nitrocellulose filter under vacuum.

Northern blot analysis was performed by denaturing RNAs with a mixture containing formamide, formaldehyde and morpholinepropanesulfonic acid (Mops) according to the procedure described by Maniatis [25]. Two different concentrations of RNA were used. The samples were run on a 1% denaturing agarose gel and transferred to Hybond N (Amersham) in sodium citrate buffer. Blots were prehybridized for 4–6 h at 42°C in 50% formamide, 5 × sodium phosphate buffer supplemented with 0.5% SDS, 5% dextran sulfate, 5 × Denhardt' solution and 100 μg/ml heat denatured salmon sperm DNA. Hybridizations were performed in the same solution at 42°C for 24 h.

The following probes were generously provided by Dr. F. Ramirez (U.S.A.): BC7 was a 1.011 kb long cDNA encoding the C propeptide region of the bovine Proα_1II collagen [26]; Hf404 (1.8 kb

cDNA) and Hf32 (2.2 kb cDNA) encoded part of the triple helical domain of human α_1I and α_2I collagen chains [27,28]; Hf934 was a 1.8 kb cDNA encoding the COOH terminal propeptide of the human α_1III collagen [29]. The β actin probe was a 1.15 kb rat cDNA.

The probes were nicked translated to a specific activity of $(2–4) \cdot 10^8$ cpm/μg using [^{32}P]dCTP 800 ci/mmol (Amersham).

After hybridization the blots were washed at 65°C several times (three or four) with 2× SSC/0.1% SDS for 15 min then exposed to Cronex films for various times. Several exposures at −20°C or −70°C were tried in order to perform scanning densitometry.

Results

Effect of relaxin on total collagen synthesis

Tritium-labelled hydroxyproline is found almost exclusively in collagen and was used to quantitate total collagen production in non-dividing articular and growth plate chondrocytes cultures after incubation for 24 h in the presence or absence of relaxin. The results in Table I indicate that relaxin had no effect on hydroxyproline incorporation into collagenous proteins. Newly synthesized collagenous proteins represented about 10% of total protein in both monolayer and multilayer chondrocytes, and relaxin did not affect this proportion. In quiescent multilayer growth plate cells, the amount of DNA per flask was almost 5-fold more elevated than in articular monolayer cells and 3-times higher than in dividing monolayer growth plate cells. In all cases, the DNA content per flask was not significantly modified by relaxin, indicating that the hormone did not induce chondrocyte proliferation during the period of study. The collagen distribution between the two culture compartments (culture medium and cell layer) was then evaluated. An equal percentage (8–10%) of collagen was found in the two compartments of quiescent articular and growth plate chondrocytes and relaxin (either one or three injections during 3 days) had no effect on this distribution (Table I). In monolayer dividing growth plate chondrocytes, only 1–2% of collagen was found in the cell layer, whereas 10–12% was released in the culture medium. Again, relaxin did not exert significant changes on these proportions. By comparison, addition of insulin to articular or growth plate chondrocytes for 3 days induced a 4-fold increase in [^{3}H]proline and [^{3}H]hydroxyproline incorporation. This was associated to a higher DNA amount but the relative percentages of collagen were unaffected.

TABLE I

EFFECT OF RELAXIN ON [^{3}H]PROLINE INCORPORATION INTO COLLAGENOUS PROTEINS OF ARTICULAR AND GROWTH PLATE CHONDROCYTES

Relaxin was added either as a single dose (1) of 2 μg/ml or as three injections (3) of 2 μg/ml for 3 consecutive days. Cell layer and culture medium were harvested 24 h later. [^{3}H]Hydroxyproline was separated from [^{3}H]proline and the present of collagen was calculated according to Wiestner et al. [22]. Values are the mean ± S.D. of three or six primary cultures (n) derived from different animals. The variations between relaxin-treated and control cells were not significantly different.

	Treatment	Total [^{3}H]hydroxypoline cpm/μg DNA ($\times 10^{-3}$)	Collagenous proteins (% of total proteins)	
			culture medium	cell layer
Growth plate chondrocytes				
dividing	none	6.3 ± 1.1	10.5 ± 1.4 (n = 3)	1.1 ± 0.3
monolayer	relaxin (1)	7.0 ± 0.8	12 ± 1.8 (n = 3)	1.7 ± 0.4
non dividing	none	8.0 ± 1.0	8.6 ± 0.8 (n = 3)	10.8 ± 2.8
multilayer	relaxin (1)	6.1 ± 0.8	8.1 ± 0.7 (n = 3)	7.3 ± 2.6
Articular chondrocytes				
non dividing	none	6.7 ± 1.8	7.5 ± 0.8 (n = 6)	9.1 ± 3.6
monolayer	relaxin (1)	5.6 ± 1.5	7.5 ± 0.6 (n = 6)	10.0 ± 2.9
	relaxin (3)	5.8 ± 1.6	8.3 ± 1.6 (n = 3)	11.5 ± 2.1

Effect of relaxin on collagen phenotype

The radiolabelled collagen chains extracted from quiescent articular and growth plate chondrocytes were analyzed by polyacrylamide gel electrophoresis. In the absence of pepsin treatment, collagen precursors were identified. Proα_1II was predominantly detected in the culture medium where it represented 60% of the labelled proteins but was not detected in the cell layer of both types of chondrocyte which contained exclusively $P_n\alpha_1$II and the totally converted α_1II chains in the respective proportion of 30% and 60%. When the culture medium and cell layer were digested separately with pepsin and run in 5% gels, α_1II collagen represented the major component (Fig. 1). Another band with a low mobility was also visible on the gel under non-reduced conditions. After dithiothreitol treatment, this band disappeared indicating that it comigrated with α_1II (not shown). By comparison with collagen extracted from fibroblast cultures, the band was identified as the α_1III trimer. Two other faint bands were found in extracts from the chondrocyte cell layer, and corresponded to the minor cartilage collagens 1α, 2α previously reported by Burgeson [30] and now designated as type XI collagen. The 3α chains was not distinguished from α_1II because of their high structural homology. In addition, comparison with fibroblastic collagens allowed the identification of α_2I chains – sign of the presence of type I collagen.

No additional bands were seen, indicating that relaxin treatment did not induce qualitative changes in collagen phenotype. However, quantitative changes in the proportions of each collagen type were detected by scanning densitometry of the gels (Table II). Type II and type XI collagens, which are regarded as reliable markers of cartilage, represented 75% of total collagen found in culture medium and 90% in the cell layer. Slight quantitative variations were noticed between quiescent articular and growth plate cells. In the cell layer of articular chondrocytes, type XI was twice as abundant as in growth plate cells (Table

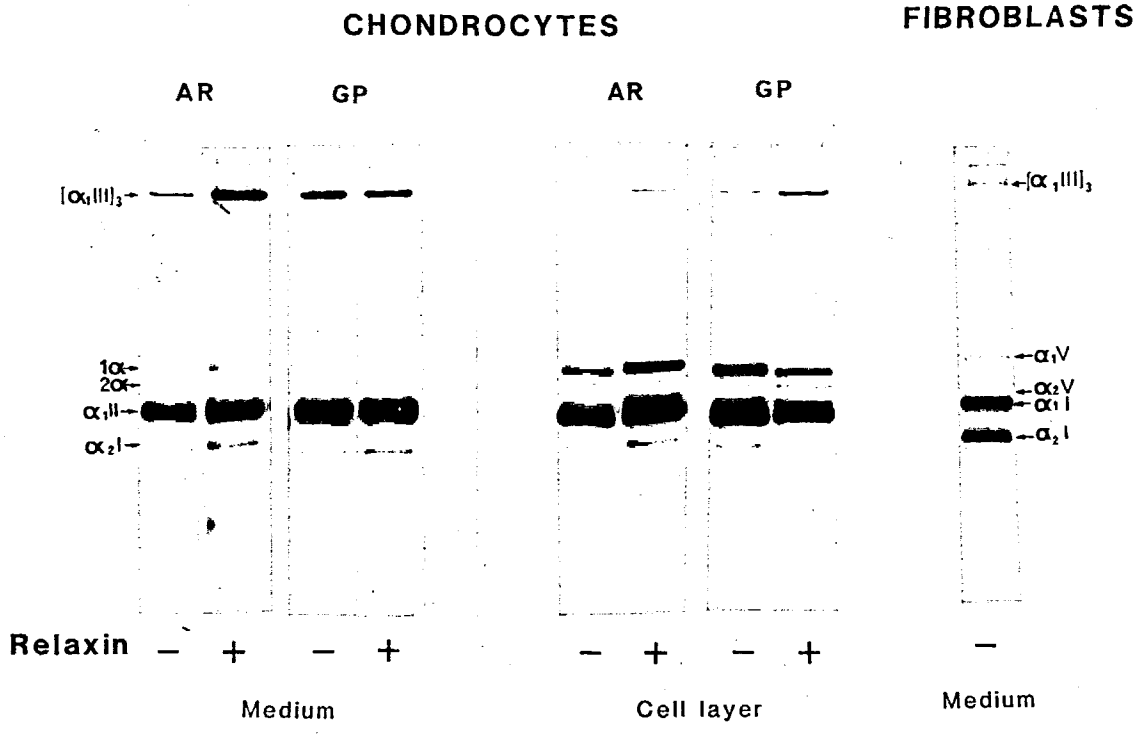

Fig. 1. Analysis on a 5% SDS-polyacrylamide gel of ^{3}H-labelled collagen chains synthesized by articular (AR) or growth plate (GP) chondrocytes cultured with (+) or without (–) relaxin. Collagen extracted from fibroblasts was used as control.

TABLE II

EFFECT OF RELAXIN ON THE RELATIVE PERCENT OF COLLAGEN CHAINS PRODUCED BY NON-DIVIDING CHONDROCYTES

Band intensities were determined by scanning densitometry. Values represent the percent of each collagen type in three separate experiments (mean ± S.D.)

Collagen	Articular chondrocytes		Growth plate chondrocytes	
	−relaxin (2μg/ml)	+relaxin (2μg/ml)	−relaxin (2μg/ml)	+relaxin (2μg/ml)
Culture medium				
1α, 2α	2.1 ±0.5	5.6 ±1.3	1.5 ±0.5	0.7±0.4
α_1II (+ α_1I)	79.0 ±3.1	65.0 ±4.0	78.4 ±5.6	71.7±4.9
α_1III	15.3 ±1.1	27.3 ±3.6 [a]	16.72±2.95	26.2±2.8 [a]
α_2I	2.8 ±0.7	6.5 ±1.1 [c]	2.1 ±1.2	3.4±1.0
Cell layer				
1α, 2α	18.6 ±1.6	16.9 ±0.6	8.7 ±2.6	11.2±3.1
α_1II (+ α_1I)	77.3 ±1.1	78.4 ±2.3	85.2 ±5.8	78.1±3.8
α_1III	0.83±0.15	1.14±0.32	4.4 ±1.5	8.6±1.9 [b]
α_2I	0.9 ±0.2	2.7 ±0.6 [c]	1.0 ±0.6	1.2±0.3

Significant differences were determined vs. non-treated flasks using a paired Student's *t*-test: [a] $P < 0.1$; [b] $P < 0.02$; [c] $P < 0.05$.

II), but no difference was seen in the culture medium where it only represented 2% of total collagens.

Relaxin essentially exerted its effect on the proportion of 'fibroblastic type' collagens (α_2I and α_1III). Type III collagen increased from 15 to 27% in culture media of both articular and growth plate chondrocytes, this stimulation remained the same when relaxin was added for three consecutive days (not shown). In the cell layers, the modification was less obvious and restricted to growth plate cells, since in articular chondrocyte type III was almost totally released in the culture medium.

Variations in the relative proportion of α_2I chains were also observed. In control culture medium it represented 3% of total collagen and only 1% in the cell layer. Relaxin treatment induced a 2–3-fold increase ($P < 0.05$) in both compartments of articular cells, but the difference was not significant in growth plate chondrocytes. It must be emphasized that the detection of α_2I chains is directly related to the presence of type I collagen and, thus, indicates that α_1I chains have been synthesized in the theoretical ratio of at least two α_1I for one α_2I on the basis that normal heterotrimers have been produced. Due to their identical electrophoretic mobility on one-dimension gels, α_1I chains cannot be distinguished from α_1II. In an attempt to demonstrate the presence of α_1I chains, two-dimensional analysis of cyanogen bromide-digested chains (CB peptides) was performed (Fig. 2). The α_1I CB peptides were detected, in culture media in association with α_1II CB peptides. The intensity of the former increased with relaxin treatment and the intensity of the α_1II CB peptides was decreased, indicating a reduction in type II collagen production. Precise quantification of the α_1I compared to the α_1II and α_2I collagen CB peptides was not possible, due to the partial superimposition of some of the peptides. Nevertheless the intensity of the α_1I CB peptides appeared to be more than 2-fold that of the α_2I CB peptides, which should have been observed if only synthesis of $(\alpha_1\text{I})_2\alpha_2$I heterotrimers had occurred, thus suggesting that some α_1I trimers were produced.

Effect of relaxin on the level of collagen RNAs

Two concentrations (2 and 4 μg) of total cellular RNA isolated from chondrocytes and fibroblasts were used to quantitate the relative amount of α_1II, α_1III, α_1I and α_2I mRNA. Since a cross hybridization between the different collagen probes was possible due to the strong conservation

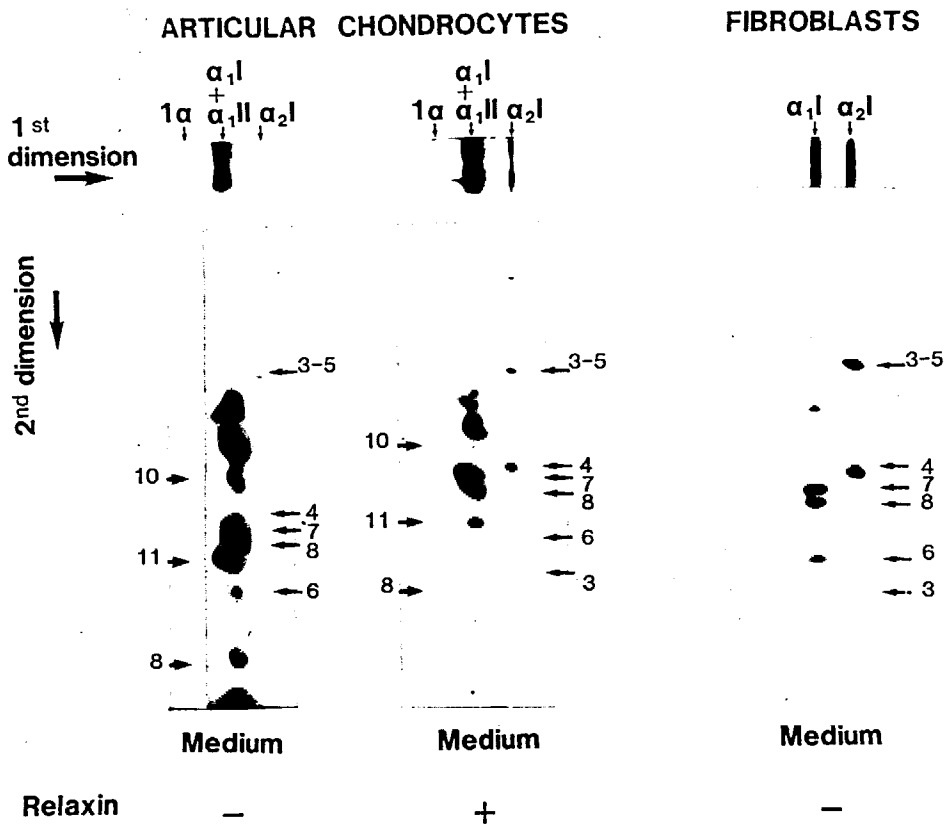

Fig. 2. Two-dimensional CNBr peptide maps of collagen chains extracted from culture medium of articular chondrocytes treated with or without 2 μg/ml relaxin and separated in the first dimension on 5% gels. The CB peptides corresponding to α_1II are indicated by small arrows on the left side of the panels, those corresponding to α_1I and α_2I are indicated by long arrows on the right side. CB peptides from fibroblasts culture medium were used for type I collagen chains identification.

of collagen genes, filters were washed using high stringency conditions.

The cDNA probe BC7 coding for α_1II was first tested on RNA extracted from quiescent articular chondrocytes and fibroblasts. As expected, RNA from chondrocytes gave single transcripts of 5 kb specific of the proα_1II gene, and the two specific proα_1I transcripts of 5.8 and 4.8 kb normally found in fibroblasts were only detected after a longer exposure to X-ray film (Fig. 3a). This slight cross-hybridization has been previously reported by Sangiorgi et al. [26]. In contrast, two strong signals corresponding to the latter transcripts were identified in fibroblasts hybridized with the α_1I probe Hf404. These two transcripts were also detected in RNA extracted from chondrocytes (Fig. 3b). A slight degree of cross-hybridization may have occurred, but would not be sufficient to explain the intensity of these signals. It seemed more likely that chondrocytes contained a significant amount of α_1I mRNAs.

Hybridization with two other cDNA probes, Hf32 and Hf934 coding from proα_2I and pro α_1III collagen chains, demonstrated the presence of significant levels of their respective transcripts (Figs. 4a and 5b). However α_1III transcripts were almost undetectable in cartilage tissue. The effect of increasing concentrations of relaxin (0.5–2 μg/ml) was evaluated on the RNA levels of quiescent articular chondrocytes. Three different collagens genes were tested and the intensities of their transcripts were measured by scanning densitometry. Table III demonstrated that the stimulation of α_1I and α_1III mRNA amounts was

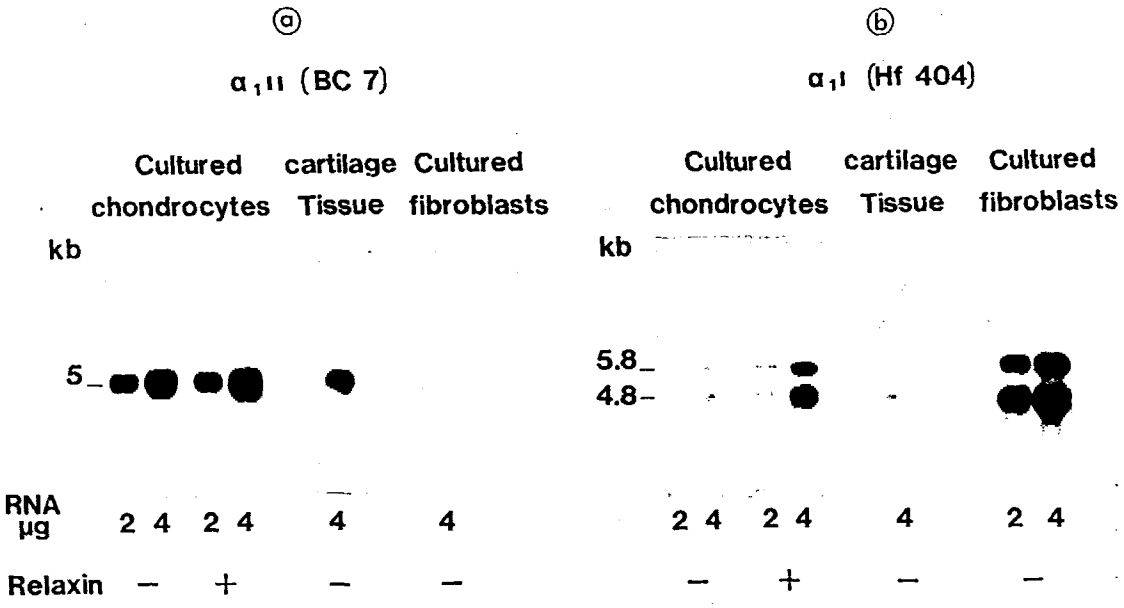

Fig. 3. Northern blot analysis of the mRNA levels of cultured articular chondrocytes treated with or without 2 µg/ml relaxin and hybridized with either α_1II (a) or α_1I (b) cDNA probes. RNA extracted from articular cartilage tissue and cultured human fibroblasts were used as controls. Filters were exposed for an identical length of time (24 h) except for fibroblast RNA hybridized with α_1II (3 days).

dose dependent but, surprisingly, no significant modifications of the proportion of type II collagen were noticed. This result was subsequently confirmed with non-dividing growth plate chondrocytes (Fig. 5a).

In contrast to α_1I mRNA, the stimulation of

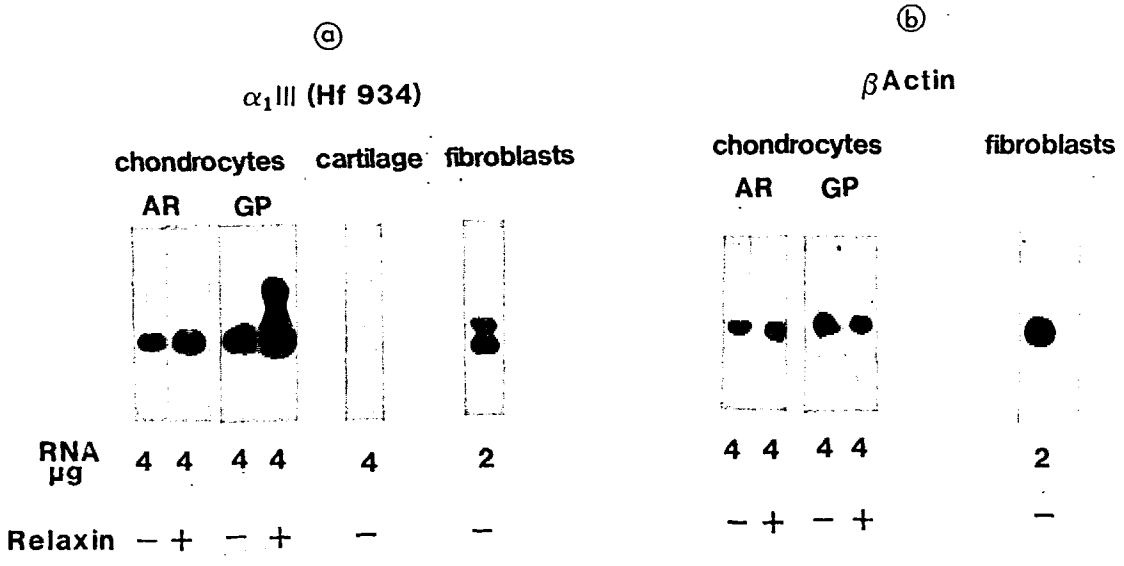

Fig. 4. Northern blot analysis of the mRNA levels of articular and growth plate chondrocytes treated with or without 2 µg/ml relaxin and hybridized with either α_1III (a) or β actin (b) cDNA probes. Filters were exposed for an identical length of time (2 days) except for articular cartilage (4 days).

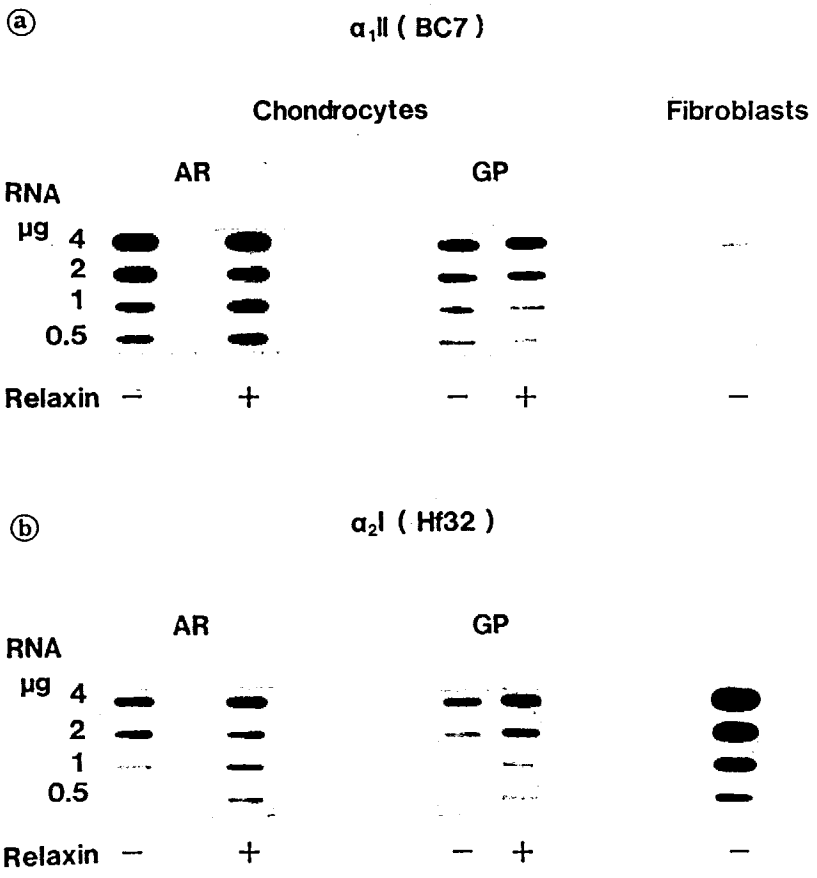

Fig. 5. Slot blot analysis of total RNA extracted from articular (AR) and growth plate (GP) chondrocytes cultured in the presence or absence of relaxin. Hybridization was performed with α_1II (a) or α_1I (b) cDNA probes. Total RNA from cultured human fibroblasts were used as control.

α_2I mRNA was limited to 1.8 when relaxin was present at 2 μg/ml (Table III). β-Actin was used as an internal standard, since the intensity of its transcript (Fig. 4b) remained unaffected by relaxin (Table III).

Discussion

The present data indicate that relaxin had a direct effect on the phenotype of cartilage cells in vitro. This effect was observed using cultures of chondrocytes from two different anatomical origins, long bones and scapulae. Because relaxin is known to act on the pubic symphysis in vivo, we first undertook experiments in vitro using rabbit cartilage cells from this tissue, but were unable to obtain sufficient chondrocytes in primary cultures. Rather than using serial chondrocyte subcultures, which are known to lose progressively their differentiated state [31,32], we chose to use epiphyseal chondrocytes.

Cultured growth plate chondrocytes issued from cartilage possessed the capacity to proliferate for a longer period than articular cells. They constituted a monolayer during the first 10 days of culture, then continued to proliferate for at least 2 additional weeks, forming multilayered colonies, and became quiescent after 23–25 days [20]. At that time they were surrounded by an abundant extracellular matrix containing essentially type II collagen and proteoglycans in a situation that could be close to the in vivo conditions of intact cartilage. On the other hand, articular chondrocytes grew as a monolayer and stopped their divi-

TABLE III

EFFECT OF INCREASING CONCENTRATIONS OF RELAXIN ON THE LEVELS OF COLLAGEN mRNAs IN ARTICULAR CHONDROCYTES

Band intensities for RNA were determined by scanning of Northern blots or slot blots and the areas were expressed as arbitrary units. Values represent the mean ± S.D. of two or three experiments.

Gene	cDNA probe	Relaxin concentration (μg/ml)	Stimulation index: RNA in relaxin treated cells / RNA in control cells
α_1II (COL2A1)	BC7	2	1.1 ± 0.2
	BC7	1	1.0 ± 0.2
	BC7	0.5	1.1 ± 0.2
α_1I (COL1A1)	Hf404	2	3.9 ± 0.1 [a]
	HF404	1	2.9 ± 0.2 [b]
	HF404	0.5	2.4 ± 0.3
α_1III (COL3A1)	Hf934	2	3.3 ± 0.3 [b]
	Hf934	1	3.0 ± 0.2 [b]
	Hf934	0.5	1.8 ± 0.4
α_2I (COl1A2)	Hf32	2	1.8 ± 0.3
β-actin	β-actin	2	0.9 ± 0.2

Significant differences were determined vs. a β-actin cDNA probe using the Student's t-test: [a] $P < 0.001$; [b] $P < 0.01$.

sion when they reached confluency, after 9 days in culture. Such a system allowed us to study the possible influence of the matrix on relaxin activity. We raised the hypothesis that in multilayer cells, this matrix could modify the accessibility of the hormone to the cell membrane or that it could protect the chondrocytes against a relaxin effect through a stabilization of its phenotype.

Total collagen biosynthesis was not modified by relaxin treatment of either type of chondrocyte and the distribution of total collagen between culture media and cell layers was not affected by relaxin in either dividing or non-dividing cells. The mitogenic activity of relaxin could not be measured accurately, because the media contained serum. However, incubation for 24 h in a serum-free medium was sufficient to allow protein synthesis in the absence of a complete cell cycle. Indeed, recent results from Narayan and Page [33] demonstrated that collagen synthesis was not coupled to the mitogenic activity of serum.

Consistent with differences in their known biological actions, but in spite of their structural similarity, insulin and relaxin appeared to act differently on chondrocyte metabolism. Insulin stimulated total protein synthesis, whereas relaxin was without effect.

In spite of its lack of effect on total collagen synthesis, relaxin induced changes in the relative proportion of collagen types. These changes were first noticed by analysis of collagen phenotype on one-dimensional gels. However the results were somehow complicated, because of the comigration of cartilage-specific chains (α_1II and 1α) with their fibroblastic counterpart (α_1I and α_1V).

A significant increase was found in the proportion of type I and type III collagens which were essentially secreted in the culture medium. Type III was also present at a higher level in the pericellular environment of growth plate cells. Although the exact meaning of this difference remains unclear, it could be related to the presence of the matrix, which could entrap α_1III molecules after their secretion out of the cell. They would therefore be less rapidly released in the culture medium than in articular cells. Since both types of chondrocyte responded similarly to relaxin by secreting higher amounts of type III, we must conclude that the matrix did not prevent the hormone reaching the cell.

Until now, the presence of type III collagen in cultured chondrocytes has been controversial. Several authors have failed to detect α_1III trimers in chicken chondrocytes even after incubation with BrdU [14], but a recent study from Yasui [15] has demonstrated conclusively that retinoic acid-treated chicken chondrocytes produced type III. Since it has been shown to be secreted in culture medium before type I [15], its presence is now interpreted as the first indicator of chondrocyte dedifferentiation in monolayer cultures. RNA data confirmed the presence of type III in our system, the dose-dependently increased level of α_1III mRNA transcripts in relaxin-treated cells supported the hypothesis that relaxin stimulated the expression of the α_1III gene.

The detection of α_2I chains on polyacrylamide gels is an indicator of the occurrence of type I collagen. Its presence at high levels has been demonstrated in subcultured chondrocytes [32], in virally transformed chondrocytes [34] and in long-term cultures of articular chondrocytes [35] where the amount of α_1I chains was 2-fold that of α_2I. However, when retinoic acid was added to chondrocyte cultures, no synthesis of α_2I chains was detected, but a high amount of α_1I trimers was found [16]. In our system, relaxin had a moderate effect on α_2I synthesis, and the two-dimensional mapping allowed identification of the characteristic α_1I CB peptides (CB7, CB8 and CB6) in a proportion that appeared to be more than twice that of α_2I CB4 and α_2I CB3-5. This observation, in agreement with previous findings [32], suggested that some α_1I trimers have been produced. This assessment was supported by the demonstration of a dose-dependent stimulation of α_1I mRNA transcripts by relaxin, when the increase in α_2I mRNA was more limited (1.8-fold). The presence of α_1I mRNA in untreated cultured chondrocytes was not surprising, as they have been detected as early as 1 day after initiation of cultures [36], although transcripts for α_2I were demonstrated only 7 days later in this system.

Indeed, α_1I mRNAs have been recognized in bovine articular cartilage [26], and in intact rabbit articular cartilage itself (Fig. 3), but were not translated into type I collagen chains (results not shown). Consistent with the findings of Adams et al. [34], we suggest that some α_1I transcripts could already be present in cartilage cells in a non-translatable form. Initiation of primary cultures would induce a restoration of the ability of these RNAs to be translated.

Northern hybridization data indicated that the steady state of α_1II mRNA transcripts was not significantly altered by relaxin treatment. On the other hand, the peptide mapping showed a decrease in the amount of Type II collagen. One possible explanation for this discrepancy would be that a certain proportion of the α_1II mRNA remained in the cytoplasm without being translated into proteins. This is equivalent to the situation in vivo during the differentiation of prechondrogenic cells into mature chondroblasts, since these cells contain α_2I mRNAs, but do not synthesize detectable type I collagen [37,38]. Although run-off transcription and in vitro translation assays remain to be performed to obtain further insight into the mechanism of regulation by relaxin, we suggest that both transcriptional and post transcriptional controls are involved. Several hypotheses based on recent findings can be proposed: (i) a transcriptional activation of the α_1III and α_1I collagen genes by relaxin could be modulated at the promoter level in a similar way to that of β-TGF, which has been shown to exert its effect through the mediation of a nuclear factor [39]. (ii) mRNA stabilities could be affected by relaxin, since growth factors were demonstrated to stabilize type I mRNA [40], whereas glucocorticoids accelerated mRNA degradation [41]. (iii) The presence of some cytoplasmic factor(s) acting at the translation elongation level to modulate the mRNAs translatabilities could also play an important role [37,42]. Further investigations will be required to substantiate these speculations and the precise mechanism could, in fact, be the result of an interplay of several parameters.

The physiological significance of the in vitro results obtained with relaxin will have to be confirmed in an in vivo system; nevertheless, it was of interest to observe that the presence of an abundant extracellular matrix around the cells did not significantly modify the response of growth plate chondrocytes to relaxin when compared to flattened articular cells. This suggests that in vivo relaxin could proceed similarly and that the matrix itself has no protective effect against the de-

differentiating capacities of relaxin, supporting its possible role in the remodelling of the connective tissue framework in the pubic symphisis.

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