Effect of retinoic acid on protein synthesis by foetal bovine chondrocytes in high-density culture: down-regulation of the glucose-regulated protein, GRP-78, and type II collagen

Anne-Marie FREYRIA,* Marie-Claire RONZIÈRE, Marguerite-Marie BOUTILLON and Daniel HERBAGE

Institut de Biologie et Chimie des Protéines, CNRS UPR 412, 7 Passage du Vercors, 69367 Lyon Cedex 07, France

The effect of 0.1–10 μ M retinoic acid (RA) on foetal bovine chondrocytes was investigated in high-density cultures (0.6×10^6 cells/cm²). After 5 days of culture in ascorbate-free medium, control chondrocytes presented a typical rounded shape and synthesized type II, IX, XI and III collagens. After RA treatment on days 2–5 of culture, the cells exhibited a fibroblast-like shape and decreased synthesis of total protein (48 %) and pepsin-resistant proteins (60 %) as determined by [³⁵S]methionine labelling. Addition of RA was not followed by the expression of type I collagen, but induced quantitative changes in the synthesis of cartilage-specific collagens (II, IX and XI) as measured by direct autoradiography of the corresponding bands after SDS/PAGE. The main change was in type II collagen synthesis, with a 80 % decrease in the cell-layer fraction and a 89 % decrease in culture-

medium fraction; inhibition of type IX and XI collagen synthesis was limited to 25 and 31 % respectively. Modifications to intracellular proteins induced by RA were determined by using two-dimensional electrophoresis associated with a computerized imaging system. Synthesis of one of the more abundant proteins (pI 4.8; 78 kDa) was decreased by 75 % after RA treatment. This protein was characterized by micro-sequencing as the glucose-regulated protein 78 (GRP 78). It was reported previously to bind denatured collagen and mutated type I procollagen molecule and to function as a molecular chaperone for collagen molecules. It remains to demonstrate whether the parallel down-regulation of GRP 78 and type II collagen observed here corresponds to a co-ordinate regulation of these two proteins.

INTRODUCTION

Chondrocytes of hyaline articular cartilage present a differentiated phenotype characterized by synthesis of cartilage-specific proteoglycan (aggrecan) and collagen type (II, IX and XI) and by a rounded or polygonal cell shape in culture (Petit et al., 1992; Mayne and Brewton, 1993). Modulation of the chondrocyte phenotype is associated with a change in collagen and proteoglycan expression and in cell shape. Further differentiation during long-bone organogenesis is accompanied by maturation of proliferating chondrocytes into hypertrophic cells and by the onset of type X collagen synthesis (Grant et al., 1987; Descalzi Cancedda et al., 1992; Iwamoto et al., 1993). De-differentiated chondrocytes present a fibroblast-like morphology and shift from type II to type I and III collagen synthesis (Hein et al., 1984; Benya and Padilla, 1986; Yasui et al., 1986; Horton et al., 1987; Dietz et al., 1993). Modulation of the chondrocyte phenotype, observed *in vivo* during cartilage embryogenesis, development or pathological degeneration such as osteoarthritis (Ronzière et al. 1990; von der Mark et al., 1992; Aigner et al., 1993; Mayne and Brewton, 1993), can be reproduced *in vitro* under various culture conditions (von der Mark, 1986; Bruckner et al., 1989; Descalzi Cancedda et al., 1992; Adolphe and Benya, 1992; Ruggiero et al., 1993) and in the presence of exogenous agents such as retinoic acid (RA), 5-bromo-2-deoxyuridine (Yasui et al., 1986; Askew et al., 1991), cytokines and growth factors. RA has been used extensively to modify the chondrocyte phenotype, either by inducing the de-differentiation of the cells in monolayer culture (Hein et al., 1984; Yasui et al., 1986; Benya and Padilla, 1986; Horton et al., 1987; Dietz et al., 1993), by altering type X collagen expression (Sanchez et al., 1991; Pacifici et al., 1991; Iwamoto et al., 1993) or by stimulating proliferation

of terminally differentiated chondrocytes and their maturation to an osteoblast-like stage (Descalzi Cancedda et al., 1992).

The mechanisms involved in the differentiation process and specifically in the action of retinoids are still unclear. In order to elucidate them, qualitative and quantitative modifications of synthesis of the intracellular and extracellular proteins involved in these changes can be observed under well-defined culture conditions, by using two-dimensional (2-D) electrophoresis. This method has been used successfully to study the proliferation and differentiation of human keratinocytes (Celis et al., 1992) and cell-specific markers of human skin fibroblasts (Graham et al., 1988). It has also been used to study the alterations in protein phosphorylation in rabbit articular chondrocytes after treatment with interleukin-1 (Hulkower et al., 1989).

In this study, we used a high-density culture model of foetal bovine chondrocytes characterized by absence of cell proliferation and maintenance of the synthesis in large amounts of cartilage-specific collagens for at least 1 month in culture (Ruggiero et al., 1993). Various concentrations of RA were added to the culture medium, and the differentiation state of the chondrocytes was determined by analysing the synthesized collagens. Intracellular protein modifications were analysed by 2-D gel electrophoresis. Interestingly, two of the proteins most extensively down-regulated by the cells after RA treatment were type II collagen and a 78 kDa protein, glucose-regulated protein 78 (GRP 78), identified by micro-sequencing. GRP 78 (also known as immunoglobulin-binding protein) is a protein of the endoplasmic reticulum which recognizes malformed proteins (Lee, 1992) and binds to denatured proteins such as gelatin (Nandan et al., 1989) and to the C-terminal propeptide of type I procollagen in cells from patients with osteogenesis imperfecta (Chessler and Byers, 1993).

Abbreviations used: RA, retinoic acid; 2-D, two-dimensional; GRP 78, glucose-regulated protein of 78 kDa.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Cell cultures

Chondrocytes were isolated from the inner cartilage layers of the tibio-femoral joint of 4-month-old bovine foetuses after removal of the superficial layer. The minced tissue was subjected to enzymic digestion with a mixture of 0.2% hyaluronidase and 0.25% collagenase/Dispase (Boehringer) in PBS at 37 °C for 4 h under gentle magnetic stirring. The isolated cells were washed three times in RPMI/NCTC medium (Seromed/Sigma) containing 10% (v/v) foetal-calf serum (Seromed) and seeded at high density, 0.6×10^6 cells/cm². They were grown at 37 °C in 25 cm² closed flasks (Corning) in RPMI/NCTC (1:1, v/v) medium supplemented with 10% foetal-calf serum and penicillin (50 units/ml), streptomycin (50 µg/ml) and amphotericin B (1 ng/ml), with a change of medium every 2 days. No ascorbate was added to the culture media during the study, since ascorbate alters the phenotype of chondrocytes (Daniel et al., 1984).

After 2 days of culture, all-*trans*-retinoic acid (Sigma) dissolved at 4 mM in 95% ethanol was added to the freshly changed medium. The same volume of ethanol was added to control cultures. The cells were then cultured in the presence or absence of 0.5, 1 or 10 µM RA for 72 h with a change of medium every 3 days. RA stock solutions were kept at -20 °C, protected from light.

The DNA content of cell cultures was measured by a fluorimetric method (Lipman, 1989). Calf thymus DNA (Sigma) was used as standard.

Cell metabolic labelling and protein analysis

After 5 days in culture, the media of RA-treated and control cultures were replaced with fresh RPMI lacking methionine and foetal-calf serum for 2 h before the cells were rinsed with PBS. The cells were then labelled for 14 h in 2.5 ml of methionine-free RPMI supplemented with 20 or 50 µCi/ml [³⁵S]methionine (1000 Ci/mmol; SJ 204, Amersham) for intracellular protein analysis and for collagen analysis, respectively, in the presence or absence of RA (1 or 10 µM).

Collagen analysis

For analysis of collagen, the culture-medium proteins were first precipitated with 240 mg/ml (NH₄)₂SO₄ overnight at 4 °C. The precipitates were dissolved in 0.5 M acetic acid and submitted to limited digestion with 0.1 mg/ml pepsin (Sigma) at 4 °C for 6 h. The cell layer was submitted to limited pepsin digestion for 16 h. After neutralization, the pepsin-digested material was precipitated with 240 mg/ml (NH₄)₂SO₄ overnight at 4 °C and suspended in Laemmli electrophoresis buffer. Samples of culture medium and of cell layer were assayed by scintillation counting. Samples were kept at -20 °C until assay. The pepsin-digested samples were fractionated by SDS/PAGE in a 6% separating gel under reducing and non-reducing conditions, as described by Laemmli (1970). Volumes containing the same radioactivity (c.p.m.) were loaded on each lane of the gel. The gels were fixed with 50% methanol/10% acetic acid, treated with Amplify (Amersham), dried on Whatman 3 MM paper and either exposed to Amersham X-ray film at -70 °C or subjected to direct determination of radioactivity by using the Instant Imager (Packard) or the digital autoradiograph LB 287 (EG-G Berthold). With these instruments, gel radioactivity is counted directly, with precise linearity and true nuclear counting statistics. The proportion of major collagens was either determined and quantified densitometrically on the fluorographs or

calculated by direct quantification of the radioactivity of each lane of the gels. In each case, the relative proportions of type II, IX and XI collagens were calculated on the basis of molecular composition and relative number of methionine residues present in the analysed chains and in the intact molecule. Co-migration of the α1(II) chain with the α3(XI) chain is taken into account for these collagens.

Intracellular protein analysis and computerized analysis of 2-D PAGE

After [³⁵S]methionine labelling, the medium was removed and the cell layer was washed twice with PBS. The cells were detached after a brief incubation in 0.1% trypsin/EDTA in PBS and suspended by repeating pipetting to give a single-cell suspension. The pellet obtained after centrifugation was washed three times in PBS and resuspended in 75 µl of lysis solution [9.8 M urea, 2% (w/v) Nonidet P-40, carrier ampholytes (1.7% pH 5-7 and 0.3% pH 3-10) and 100 mM dithiothreitol]. Portions of the cell lysates were assayed by scintillation counting. Samples were kept at -70 °C until assay.

The proteins of the cell lysate samples were separated by 2-D PAGE. In the first dimension, isoelectrofocusing was carried out as described by Bravo et al. (1982) in long (145 mm × 1 mm) or short (75 mm × 1 mm) polyacrylamide gels containing 8 M urea, 2% Nonidet P-40 and 2% carrier ampholytes (1.7% Serva pH 5-7, and 0.3% Bio-Rad pH 3-10). Isotopically labelled samples were focused at 10 500 V·h for the long gel and at 2500 V·h for the short gel, by using NaOH (20 mM) and H₃PO₄ (10 mM) as electrolytes. The loaded volumes (10 µl) of ³⁵S-labelled intracellular proteins contained 1×10^6 ethanol-precipitable d.p.m. Equilibration buffer contained 0.5 M Tris/HCl, 2% SDS and 5 mM dithiothreitol. The second-dimension SDS/PAGE was performed on 10%-polyacrylamide gel.

Coomassie Blue-stained gels were dried and autoradiographed for 48 h with Hyperfilm β max (Amersham). After the fluorograms had been scanned with a CCD camera, the 2-D gels were analysed by using the Bio Image Vision 110 computerized imaging system (Millipore).

'In-gel' protein digestion and microsequencing

The Coomassie Blue-stained protein spots were excised from the dried gels and digested 'in-gel' (Rosenfeld et al., 1992) with a modified pig trypsin (Promega). The resulting tryptic peptides were separated by reversed-phase h.p.l.c. on a C₁₈ Aquapore column (2.1 mm × 220 mm; Applied Biosystems) in the presence of 9 mM trifluoroacetic acid with an aqueous gradient of acetonitrile (0-60%) for 60 min. The eluate was monitored by the A₂₁₈ and A₂₈₀ with an Applied Biosystems 1000 S Diode detector. Peptides were collected in Microfuge tubes and concentrated to 20 µl in a SpeedVac (Jouan). Micro-sequencing of the peptides was performed in a 473 A gas-phase sequencer (Applied Biosystems). The amino acid sequences were compared by using the Antheprot computer program (Geourjon et al., 1991; Geourjon and Deleage, 1993). The protein database was from SwissProt.

RESULTS

Morphology of cultured chondrocytes

When chondrocytes were isolated from foetal bovine cartilage and plated at high density (0.6×10^6 cells/cm²), most of the cells adhered within 2 h and rapidly formed multi-layers of rounded cells. Typical polygonal chondrocytes were observed after 24 h of culture and for the remaining 5 days of culture.

Figure 1 Effects of RA on bovine chondrocytes cultured at high density

Phase-contrast micrographs of foetal bovine chondrocytes after 5 days of culture: (a) control culture; (b) RA-treated culture ($10 \mu\text{M}$ RA for the last 3 days). Similar effects were obtained with $1 \mu\text{M}$ RA. Magnification $\times 48$.

The effect of RA was determined by culturing the cells in the presence of 0.5, 1 and $10 \mu\text{M}$ RA from day 2 to day 5. The cells began to lose their characteristic polygonal shape within 24 h of RA treatment with the two higher concentrations and became more elongated and flattened after 48 h (Figure 1). But 3 days were required for similar changes to be induced by the $0.5 \mu\text{M}$ RA dose. These morphological changes were associated with a decrease in extracellular-matrix deposition in comparison with the control cultures.

DNA content and protein synthesis

No variation in DNA content or cell number was observed during RA treatment, even at the highest concentration (Table 1).

After 5 days of exposure to $10 \mu\text{M}$ retinoic acid (from day 2 to day 5 of culture) and radiolabelling during the last 14 h with [^{35}S]methionine, chondrocytes exhibited a 48 % decrease in total protein synthesis, corresponding to a 50 % decrease in synthesized protein in the cell layer and a 34 % decrease in culture medium (Table 1). The largest decrease in labelling (60 %) was noted for pepsin-resistant protein in the cell layer; the decrease in intracellular protein synthesis determined by comparison in cell lysates was only 35 %. The percentages of [^{35}S]methionine-labelled protein recovered in the cell layer were similar in control and RA-treated cells (88 and 85 % respectively).

Collagen synthesis

The effect of RA on the collagen phenotype was examined by SDS/PAGE analysis of [^{35}S]methionine-labelled pepsin-resistant proteins in the culture medium and cell layer (Figure 2). Type II and XI collagens were the most abundant proteins in both untreated and RA-treated cultures. Type IX collagen was detected by the presence of its COL 2 domain (band X3, according to Ricard-Blum et al., 1982) in the cell layer, but not in the cell medium. Type I collagen was absent from all fractions, as indicated by the complete absence of the $\alpha 2(\text{I})$ chain, even after over-exposure of the gels or immunoblotting with anti-(type I collagen) antibodies (results not shown). Type III collagen was synthesized by both treated and untreated cells, as indicated by the presence of a high-molecular-mass component that migrated like the $\alpha 1(\text{I})$ chain after reduction.

Synthesis of the different collagen types (II, IX and XI) was analysed proportionally by direct autoradiography of the gels, corrected for the methionine content of each chain and the molecular composition of the collagens (Table 2). RA inhibited the overall synthesis of type II collagen by 86 % (80 % in the cell layer and 89 % in the culture medium), which was greater than the change in total protein synthesis. The rate of synthesis of type XI and IX collagens decreased less than did that of type II collagen: 25 % and 31 %, respectively, in the cell layer, and 17.4 % in the culture medium for type XI collagen. The relative proportions of the three collagens were therefore highly modified in both the cell layer and the culture medium, varying the cell layer from II:IX:XI proportions near 100:10.4:15 in the control

Table 1 Effects of RA on cell number, DNA content and incorporation of [^{35}S]methionine into total and pepsin-resistant proteins by bovine foetal chondrocytes cultured at high density

At day 5, untreated and RA-treated ($10 \mu\text{M}$ RA for the last 3 days) chondrocytes were cultured for 14 h with [^{35}S]methionine [$50 \mu\text{Ci}/\text{ml}$ for cell layer and culture medium in the first set of experiments ($n = 2$) and $20 \mu\text{Ci}/\text{ml}$ for cell lysate in the second set of experiments ($n = 5$)]. Total radioactive proteins were quantified in samples of the cell lysate and cell layer and, after dialysis, in the culture medium. Radioactive pepsin-resistant material was quantified after pepsin digestion and salt precipitation of the cell layer and culture medium. Results for cell number, DNA content and cell lysate are means $\pm$ S.D. of five separate experiments: * for differences from values for RA-treated cells and control cells, $P < 0.001$.

Culture	$10^{-6} \times$ No. of cells ($n = 5$)	$10^{-6} \times$ DNA ($\mu\text{g}/\text{flask}$) ($n = 5$)	$10^{-6} \times$ Radioactivity (d.p.m.) in:				
			Cell layer ($n = 2$)		Culture medium ($n = 2$)		Cell lysate ($n = 5$) Total
			Total	Pepsin-resistant	Total	Pepsin-resistant	
Control	17.8 ± 4	224 ± 20	108	18.2	14.5	6	35.6 ± 6
RA-treated	16.3 ± 3	211 ± 22	54.3	7.3	9.6	5.8	$23.2 \pm 7.2^*$

Figure 2 SDS/PAGE analysis of protein biosynthesis in the media and cell layer of foetal bovine chondrocytes cultured with RA

At day 5, untreated and RA-treated ($10 \mu\text{M}$ RA for the last 3 days) chondrocytes were cultured for 14 h with [^{35}S]methionine as described in the Materials and methods section. A fluorograph is shown of [^{35}S]methionine-labelled proteins recovered from the cell layers (lanes 1 and 2) and the culture medium (lanes 3 and 4) by ammonium sulphate precipitation in control (lanes 1 and 3) and RA-treated cultures (lanes 2 and 4). Samples were submitted to limited pepsin digestion before SDS/PAGE on 6% acrylamide gel without reduction. Loaded samples contained identical amounts of ethanol-precipitable radioactivity (c.p.m.). However, in lane 4 a larger amount of radioactivity was present in low-molecular-mass (< 20 kDa) peptides compared with the other lanes (result not shown). The exact composition of the high-molecular-mass (200–300 kDa) components in lane 3 is as yet unknown. The collagens detected are indicated.

cells to 100:62:87 in RA-treated cells. The absence of type IX collagen from the culture media of both treated and untreated cultures is noteworthy. Similar relative variations in collagen types were obtained in other series of cultures labelled with [^3H]proline or [^{14}C]proline (results not shown).

Intracellular protein analysis by 2-D PAGE

Chondrocytes from three different bovine foetuses were cultured for 5 days, in the absence or presence of $10 \mu\text{M}$ RA during the last 3 days, and labelled with [^{35}S]methionine for the last 14 h of culture. The control cell lysates and RA-treated cell lysates were then subjected to 2-D PAGE, and the corresponding autoradiographs were analysed with the computerized imaging system Bio Image. Reproducible differences were obtained by analysis of the sets of gels corresponding to three separate experiments. Some 20 of the 200 spots analysed per gel were up-regulated 2-fold or more, and 15 spots were down-regulated 2-fold or more.

Figure 3 Separation by 2-D PAGE of [^{35}S]methionine-labelled polypeptides extracted from foetal bovine chondrocyte lysates

Labelled polypeptides were extracted at day 5 after 14 h of labelling with [^{35}S]methionine from (a) control chondrocyte lysates obtained as described in the Materials and methods section and (b) RA-treated chondrocyte lysates ($10 \mu\text{M}$ retinoic acid for the last 3 days of culture). Ethanol-precipitable material containing 1×10^6 c.p.m. was loaded on to isoelectric-focusing (IEF) (first dimension) gels. After separation by SDS/PAGE (second dimension), autoradiographs were generated and analysed for spot position and intensity. For comparison, polypeptides with major changes are numbered on both gels.

Among these spots, six of them were regularly modified and specifically analysed. Two were down-regulated [spot 1 (78 kDa; pI 4.8) by 75% and spot 5 (50.4 kDa; pI 6.6) by 50%] and three were up-regulated [spot 4b (56.9 kDa; pI 5.8) by 75%, spot 7 (38.1 kDa; pI 5.4) by 78% and spot 8 (38.1 kDa; pI 5.7) by 62%].

Table 2 Effects of RA on the relative proportions of type II, IX and XI collagens synthesized in chondrocyte cultures

Values were obtained by direct quantification of the radioactivity of each collagen-specific band from SDS/PAGE fluorographs, as described for Figure 2, in the cell layer and in the culture medium. In each case, the levels of newly synthesized type II, IX and XI collagens were calculated on the basis of the molecular composition and the number of methionine residues in each molecule. The data are presented as two separate experiments: Expt. 1 and Expt. 2. ND, not detected.

Collagen type	$10^{-3} \times$ Radioactivity (d.p.m.) in:							
	Cell layer				Culture medium			
	Control		RA-treated		Control		RA-treated	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
II	192	271	40	40	143	172	27	6
IX	57	30	16	19	ND	ND	ND	ND
XI	40	53	18	29	33	26	35	14

(Figure 3). Changes associated with the action of RA ($10 \mu\text{M}$) on days 2–5 were also investigated in Coomassie Blue-stained 2-D gels. The same variations seen between control and treated cultures on the fluorograms (newly synthesized proteins) were also noted after Coomassie Blue staining for spots 1 and 4, but spots 7 and 8 were undetectable (result not shown).

As spot 1, corresponding to an abundant protein detectable on Coomassie-Blue-stained gels, differed widely for control and RA-treated cultures, five such no. 1 spots were submitted to micro-sequencing after 'in-gel' tryptic digestion. One tryptic peptide, T15, separated by h.p.l.c. (results not shown) was sequenced after concentration under vacuum. Comparison of the sequence of this peptide (NTVVPTK) with sequences stored in the Swiss Prot databank indicated that it corresponds to GRP 78. This conclusion is supported by the molecular mass and pI found for this protein in human cells (Celis et al., 1992).

DISCUSSION

We have examined changes in morphology and protein synthesis in foetal bovine chondrocytes in high-density culture after treatment with high concentrations of all-*trans*-RA. In the presence of $10 \mu\text{M}$, the cells began to lose their polygonal shape, and by 48 h they were elongated and fibroblastic. Such morphological changes, induced by alterations in the chondrocyte cytoskeletal architecture, are usually associated with cell de-differentiation (Benya and Padilla, 1986; Brown and Benya, 1988; Horton et al., 1987). RA had been reported to have inhibitory, stimulatory or no effects on chondrocyte proliferation (Hein et al., 1984; Enomoto et al., 1990), depending mostly on the culture conditions and cell origin. We found that 3 days of treatment with a high concentration of RA did not affect the proliferation of chondrocytes plated at high density.

The chondrocyte-specific collagen pattern was not modified qualitatively by RA treatment: type II, IX and XI collagens were present, and type I collagen was absent; notably type III collagen was present in both control and RA-treated cultures. Under control conditions, synthesis of type III collagen was observed as soon as 24 h after cell plating, independently of the presence or absence of ascorbate (M.-C. Ronzière, A.-M. Freyria, J. Farjanel, D. Hartmann and D. Herbage, unpublished work). Type I collagen was detected in control cultures after 13 days of culture and only in the presence of ascorbate (Ruggiero et al., 1993; M.-C. Ronzière, A.-M. Freyria, J. Farjanel, D. Hartmann and D. Herbage, unpublished work). Thus, as previously reported by Kuettner et al. (1982) in the same high-density culture model, bovine chondrocytes grown in the absence of ascorbate exhibit a stable differentiation state (at least between days 1 and 13 of culture), characterized by synthesis of cartilage-specific collagens (types II, IX and XI) and type III collagen. Human chondrocytes from osteoarthritic cartilage appear to have a similar pattern of synthesis (Aigner et al., 1993).

Direct measurement of the radioactivity in each band of gels for the cell layer and culture medium showed a 86 % decrease in synthesis of type II collagen by chondrocytes after 3 days of exposure to RA. Such down-regulation of collagen type II synthesis was shown to be the result of the activation of both positive and negative regulatory elements of the gene (Yamada et al., 1991). The inhibition was greater than the decrease in total protein synthesis, confirming a specific effect of RA on one differentiated function of bovine chondrocytes. The decrease in type IX and XI collagen synthesis was smaller (a maximum of 50 %). RA thus induced the changes in morphology and in synthesis of type II, IX and XI collagens that have been described

in most previous studies, but did not trigger the switch from synthesis of type II to type I collagen (Hein et al., 1984; Benya and Padilla, 1986; Yasui et al., 1986; Horton et al., 1987; Sanchez et al., 1991; Pearson and Sasse, 1992; Dietz et al., 1993). The specific cell type (foetal bovine chondrocytes) and culture conditions used in this study, high cell density and ascorbate-free medium, seem to have hampered, or at least slowed, the de-differentiation process observed in chondrocytes in monolayer cultures after treatment with RA at a similar doses and for similar periods (Hein et al., 1984).

Qualitative and quantitative changes in the synthesis of intracellular proteins induced by RA, analysed by 2-D PAGE and image analysis, demonstrated that one of the most highly modified spot (75 % decrease) corresponded to a protein of 78 kDa and pI 4.8. This protein was characterized as GRP 78, also known as the immunoglobulin-heavy-chain-binding protein (Munro and Pelham, 1986; Hendershot et al., 1988). It is a protein of the endoplasmic reticulum which belongs to a super-family of stress-inducible proteins and is involved in many processes, including translocation, and folding and assembly of oligomeric proteins (Lee, 1992). Our results indicate that it is very likely that the uncharacterized 80 kDa protein reported to have been down-regulated by treatment of chick chondrocytes with RA (Horton and Hassell, 1986) is GRP 78. The decrease in GRP 78 synthesis observed in our study may be the result of interaction between RA and cytoplasmic or nuclear elements of the chondrocytes. Binding of RA to the nuclear receptors RAR α and γ (Dolle et al., 1989) is one mechanism by which specific gene expression may be controlled; however, it is not yet known if the GRP 78 gene has binding sites for RAR, or whether GRP 78 synthesis during RA treatment is controlled at the transcriptional or the post-transcriptional level.

The decreases in the rates of synthesis of GRP 78 and type II collagen are clearly greater than those of overall protein synthesis. It is now well recognized that a 47 kDa heat-shock protein (Hsp 47, also called 46 kDa glycoprotein, J6 or colligin) (Nakai et al., 1992) acts as a molecular chaperone for procollagens I and IV. Recent data (Sauk et al. 1994) revealed that Hsp 47 is associated with $\alpha 1(\text{I})$ procollagen at a very early point during translocation of the nascent procollagen chain, favouring a specialized specific function relative to chain selection or completion of stable folding in type I procollagen. Since contradictory results have been obtained with regard to expression of Hsp 47 in regions of type II collagen production (Miyaishi et al., 1992; Shroff et al., 1993), it is not known whether the molecular-chaperone requirement for type II collagen is satisfied by Hsp 47 or by another endoplasmic-reticulum protein, such as GRP-78. Both GRP 78 and Hsp 47 bind denatured collagen, but their functions seem to be different (Nandan et al., 1989). Recently it was shown that GRP 78 is induced by and bound to type I procollagen molecules with mutations (osteogenesis imperfecta) that interfere with, but do not prevent, pro- α -chain association (Chessler and Byers, 1993). These data support the hypothesis that GRP 78 functions to moderate the effects of mis-folded proteins and more generally constitutes a response to certain physiological conditions (Lee, 1992). More work is obviously needed to clarify the mechanisms underlying the decrease in the synthesis of both GRP 78 and type II collagen in RA-treated chondrocytes and to demonstrate whether a co-ordinated regulation exists between these two events.

We thank Professor J. Remacle and his group (Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium) for their helpful collaboration. This study was supported in part by an INSERM grant (CRE 930308) and funds from a co-operation action between CGRI-FNRS (Belgium) and CNRS (France).

REFERENCES

- Adolphe, M. and Benya, P. (1992) in *Biological Regulation of the Chondrocytes* (Adolphe, M., ed.), pp. 105–139, CRC Press, Boca Raton, FL.
- Aigner, T., Bertling, W., Stöb, H., Weseloh, G. and von der Mark, K. (1993) *J. Clin. Invest.* **91**, 829–837.
- Askew, G. R., Wang, S. and Lukens, L. N. (1991) *J. Biol. Chem.* **266**, 16834–16841.
- Benya, P. D. and Padilla, S. R. (1986) *Dev. Biol.* **118**, 296–305.
- Bravo, R., Small, J. V., Fey, S. J., Mose Larsen, P. and Celis, J. E. (1982) *J. Mol. Biol.* **159**, 121–143.
- Brown, P. D. and Benya, P. D. (1988) *J. Cell Biol.* **106**, 171–179.
- Bruckner, P., Hörler, I., Mandler, M., Houze, Y., Winterhalter, K. H., Eich-Bender, S. G. and Spycher, M. A. (1989) *J. Cell Biol.* **109**, 2537–2545.
- Celis, J. E., Rasmussen, H. H., Madsen, P., Leffers, H., Honore, B., Dejgaard, K., Gesser, B., Olsen, E., Gromov, P., Hoffmann, H. J., Nielsen, M., Celis, A., Basse, B., Lauridsen, J. B., Ratz, G. P., Nielsen, H., Andersen, A. H., Walbum, E., Kjærgaard, I., Puype, M., Van Damme, J. and Vandekerckhove, J. (1992) *Electrophoresis* **13**, 893–959.
- Chessler, S. D. and Byers, P. H. (1993) *J. Biol. Chem.* **268**, 18226–18233.
- Daniel, J. C., Pauli, B. W. and Kuettnner, K. E. (1984) *J. Cell Biol.* **99**, 1960–1969.
- Descalzi Cancedda, F., Gentili, C., Manduca, P. and Cancedda, R. (1992) *J. Cell Biol.* **117**, 427–435.
- Dietz, U., Aigner, T., Bertling, W. M. and von der Mark, K. (1993) *J. Cell Biochem.* **52**, 57–68.
- Dolle, P., Ruberte, E., Kastener, P., Petkovitch, M., Stoner, C. M., Gudas, L. and Chambon, P. (1989) *Nature (London)* **342**, 702–705.
- Enomoto, M., Pan, H., Suzuki, F. and Takigawa, M. (1990) *J. Biochem. (Tokyo)* **107**, 743–748.
- Geourjon, C. and Deleage, G. (1993) *Comput. Applic. Biosci.* **9**, 87–91.
- Geourjon, C., Deleage, G. and Roux, B. (1991) *J. Mol. Graphics* **9**, 188–190.
- Graham, C. A., McLean, W. H. I., Hughes, A. E. and Nevin, N. C. (1988) *Electrophoresis* **9**, 343–351.
- Grant, W. T., Wang, G. J. and Balian, G. (1987) *J. Biol. Chem.* **262**, 9844–9849.
- Hein, R., Krieg, T., Mueller, P. K. and Braun-Falco, O. (1984) *Biochem. Pharmacol.* **33**, 3263–3267.
- Hendershot, L. M., Ting, J. and Lee, A. S. (1988) *Mol. Cell Biol.* **8**, 4250–4256.
- Horton, W. E. and Hassell, J. R. (1986) *Dev. Biol.* **115**, 392–397.
- Horton, W. E., Yamada, Y. and Hassell, J. R. (1987) *Dev. Biol.* **123**, 508–516.
- Hulkower, K. I., Georgescu, H. I. and Evans, C. H. (1989) *FEBS Lett.* **257**, 228–232.
- Iwamoto, M., Golden, E. B., Adams, S. L., Noji, S. and Pacifici, M. (1993) *Exp. Cell Res.* **205**, 213–224.
- Kuettnner, K. E., Memoli, V. A., Pauli, B. U., Wrobel, N. C., Thonar, E. J.-M. A. and Daniel, J. C. (1982) *J. Cell Biol.* **93**, 751–757.
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
- Lee, A. S. (1992) *Curr. Opin. Cell Biol.* **4**, 267–273.
- Lipman, J. M. (1989) *Anal. Biochem.* **176**, 128–131.
- Mayne, R. and Brewton, R. G. (1993) in *Extracellular Matrix of Cartilage: Collagen* (Woessner, J. F., Jr. and Howell, D. S., eds.), pp. 81–108, Marcel Dekker, New York.
- Miyaishi, O., Sakata, K., Matsuyama, M. and Saga, S. (1992) *J. Histochem. Cytochem.* **40**, 1021–1029.
- Munro, S. and Pelham, H. B. (1986) *Cell* **46**, 291–300.
- Nakai, A., Satoh, M., Hirayoshi, K. and Nagata, K. (1992) *J. Cell Biol.* **117**, 903–914.
- Nandan, D., Ball, E. H. and Sanwal, B. D. (1989) *Biochem. Cell Biol.* **68**, 1057–1061.
- Pacifici, M., Golden, E. B., Iwamoto, M. and Adams, S. L. (1991) *Exp. Cell Res.* **195**, 38–46.
- Pearson, D. and Sasse, J. (1992) *J. Biol. Chem.* **267**, 25364–25370.
- Petit, B., Freyria, A.-M., van der Rest, M. and Herbage, D. (1992) in *Biological Regulation of the Chondrocytes* (Adolphe, M., ed.), pp. 33–84, CRC Press, Boca Raton, FL.
- Ricard-Blum, S., Hartmann, D., Herbage, D., Payen-Meyran, C. and Ville, G. (1982) *FEBS Lett.* **146**, 343–347.
- Ronzière, M.-C., Ricard-Blum, S., Tiollier, J., Hartmann, D. J., Garrone, R. and Herbage, D. (1990) *Biochim. Biophys. Acta* **1038**, 222–230.
- Rosenfeld, J., Capdevielle, J., Guillemot, J.-C. and Ferrara, P. (1992) *Anal. Biochem.* **203**, 173–179.
- Ruggiero, F., Petit, B., Ronzière, M. C., Farjanel, J., Hartmann, D. and Herbage, D. (1993) *J. Histochem. Cytochem.* **41**, 867–875.
- Sanchez, M., Gionti, E., Pontarelli, G., Arcella, A. and De Lorenzo, F. (1991) *Biochem. J.* **276**, 183–187.
- Sauk, J. J., Smith, T., Norris, K. and Ferreira, L. (1994) *J. Biol. Chem.* **269**, 3941–3946.
- Shroff, B., Smith, T., Norris, K., Pileggi, R. and Sauk, J. J. (1993) *Connect. Tissue Res.* **29**, 273–286.
- von der Mark, K. (1986) *Rheumatology* **10**, 272–315.
- von der Mark, K., Kirsch, T., Aigner, T., Reichenberger, E., Nerlich, A., Weseloh, G. and Stöb, H. (1992) in *Articular Cartilage and Osteoarthritis* (Kuettnner, K., Hascall, V. and Schleyerbach, R., eds.), pp. 221–234, Raven Press, New York.
- Yamada, Y., Horton, W., Miyashita, T., Savagner, P., Hassell, J. and Doege, K. (1991) *J. Craniofacial Genet. Dev. Biol.* **11**, 350–356.
- Yasui, N., Benya, P. D. and Nimni, M. E. (1986) *J. Biol. Chem.* **261**, 7997–8001.