

# Effects of dexamethasone on the growth of cultured rabbit articular chondrocytes: relation with the nuclear glucocorticoid-receptor complex

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**SUMMARY** This study reports that dexamethasone at a high dose ( $10^{-4}$  mol/l) induced slowing of the *in vitro* proliferation of rabbit articular chondrocytes in both monolayer and clonal culture. This effect is consistent with an inhibition of DNA and RNA synthesis and was characterised by an accumulation of cells in the  $G_0G_1$  phase of the cell cycle, as shown by flow cytometric analysis. Therefore we determined the extent of nuclear localisation of dexamethasone-receptor complexes. The results showed a discrepancy between 50% growth inhibitory dose ( $10^{-4}$  mol/l) and the apparent affinity,  $K_D$  ( $1.4$  (SD  $0.2$ )  $\times 10^{-9}$  mol/l). Thus the growth inhibition of rabbit articular chondrocytes by dexamethasone did not seem to be related exclusively to an interaction with the glucocorticoid-receptor complexes.

**Key words:** cartilaginous cell culture, cell proliferation, flow cytometry, anti-inflammatory drug, binding.

The extensive use of glucocorticoids in the therapy of articular inflammatory diseases is due, in a large part, to their anti-inflammatory, immunosuppressive and antiallergic properties. The activity of these molecules is unfortunately associated with adverse effects, sometimes toxic, which result from these same pharmacological properties. Clinical trials have shown that locally injected glucocorticoids may have a beneficial effect on the course of rheumatoid arthritis. Repeated injections of corticosteroids, however, seem to lead to progressive joint destruction.<sup>1</sup> During the inflammatory process many complex interactions have been observed with different immunocompetent cells containing various targets for the glucocorticoids. Their catabolic effect generally results in an inhibition of cellular growth, which might even induce cellular lysis. It appears that the glucocorticoids act directly at the level of regulatory mechanisms for cellular proliferation. Two opposite effects have been shown: firstly, an inhibitory effect on various cell types<sup>2-11</sup> and

secondly, a stimulating effect for a limited number of other cell lines.<sup>12-15</sup> The present work concerns the effects of dexamethasone on the *in vitro* proliferation of rabbit articular chondrocytes. Chondrocytes, which maintain the regeneration of cartilaginous tissue by repopulation and reconstruction of the extracellular matrix, are exposed to the anti-inflammatory steroids during therapy, as well as the cells implicated in the inflammation process. This study describes the effects of dexamethasone on chondrocyte growth assessed by analysis of proliferation kinetics, including growth curves, cloning efficiency, incorporation of labelled precursors into DNA or RNA, and DNA flow cytometric analysis. Comparisons were made with the fibroblast cell line L 929 sensitive to dexamethasone. Since glucocorticoid specific receptors have been demonstrated for the chondrocyte in a previous study,<sup>16</sup> we investigated a possible relation between the presence of receptors localised at the nuclear level and the effect of dexamethasone on proliferation.

## Materials and methods

### CELL CULTURE

Cartilage was taken from the shoulder and knee joints of rabbits (Fauve de Bourgogne) aged 1 to 2

Accepted for publication 11 July 1986.

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months. Chondrocytes were enzymatically released from cartilage slices by Green's method.<sup>17</sup> Isolated cells were then cultured in HAM F 12 medium supplemented with 10% fetal calf serum (IBF) containing antibiotics (penicillin 10 IU/ml, streptomycin 10 µg/ml), and maintained at 37°C in an atmosphere of 5% CO<sub>2</sub> in air. When monolayers reached confluence cells were trypsinised in order to obtain the first subculture. Chondrocytes were seeded as described in the text. Fibroblasts L 929 were obtained from the American type culture collection (CCL 1, NCTC clone L 929) and were cultured in minimum essential medium supplemented with 5% calf serum and the above mentioned antibiotics.

#### TREATMENT

One day after seeding the culture medium for each assay was changed and replaced by new medium containing different concentrations of dexamethasone (Sigma).

#### GROWTH CURVES

Plastic Petri dishes (35 mm) were seeded with  $7 \times 10^4$  cells. After incubation for two, three, four, or six days the cells were removed by trypsinisation, and growth in the absence or presence of dexamethasone was measured at each stage by counting the cells in three dishes using an haemocytometer.

#### CLONAL GROWTH

Chondrocyte cultures were trypsinised and single cell suspensions prepared; 200 cells were plated into 35 mm Petri dishes containing 2 ml of complete medium. After incubation for 24 h cultures were treated with dexamethasone at the appropriate concentrations, and the clonal plates were incubated undisturbed for eight days at 37°C in 5% CO<sub>2</sub>/95% air. The cells were then fixed with absolute methanol and stained with Giemsa (Merck). Plating efficiency was measured in triplicate. In the control and treated Petri dishes the number of clones containing more than 50 cells and the number of clusters containing less than 50 cells were counted separately.

#### <sup>3</sup>H PRECURSOR INCORPORATION STUDIES

Cells were plated in 75 cm<sup>2</sup> flasks ( $5 \times 10^5$  cells/flask). Twenty four or 48 hours after treatment the cells were incubated for 90 min in medium containing [<sup>3</sup>H]thymidine (2 µCi/ml (74 kBq/ml) – 10 Ci/mmol (370 GBq/mmol); CEA 91191 Gif-sur-Yvette Cedex, France) or [<sup>3</sup>H]uridine (2 µCi/ml (74 kBq/ml) – 10 Ci/mmol (370 GBq/mmol); CEA), the medium was removed, and each flask washed twice in phosphate buffered saline (PBS). The cells

of the substratum were trypsinised, centrifuged, and the supernatant fraction discarded. Cold 0.3 M perchloric acid (2 ml) was added to the cell pellet and maintained in ice for 30 min. After centrifugation and aspiration 0.25 ml of the supernatant (perchloric acid soluble extract) was added to 10 ml of scintillation fluid (Lumagel) for the measurement of the amount of [<sup>3</sup>H]uridine incorporated into RNA. The DNA was then extracted from the pellet (acid insoluble fraction) by 1.2 ml of 1 M perchloric acid (30 min at 70°C); 0.25 ml of the supernatant after centrifugation was collected and treated as above for the measurement of the amount of [<sup>3</sup>H]thymidine incorporated into DNA.

For each assay a separate aliquot was used for the measurement of DNA content from each flask by Burton's method.<sup>18</sup>

#### FLOW CYTOMETRIC ANALYSIS

Cells seeded at  $2.5 \times 10^5/25$  cm<sup>2</sup> plastic flask were harvested by trypsinisation after various periods of treatment with dexamethasone. The cells were fixed in 70% ethanol, treated with ribonuclease, and stained with propidium iodide (Sigma) (0.05 mg/ml) as described by Crissman and Steinkamp.<sup>19</sup> Cells (about  $5 \times 10^4$ ) remaining in the staining solution were analysed in a cytofluorograf (model FC 200/4800 A, Ortho Instruments). The relative percentage of cells in G<sub>0</sub>G<sub>1</sub>, S, and G<sub>2</sub>+M were estimated according to the method of Fried and Mandel<sup>20</sup> based on DNA per cell distribution.

#### NUCLEAR LOCALISATION OF GLUCOCORTICOID-RECEPTOR COMPLEXES

Cells were seeded in 150 cm<sup>2</sup> tissue culture flasks at  $3 \times 10^6$  cells/flask in 30 ml of complete medium, changed twice weekly.

#### Labelling

Experiments were performed after six days of incubation. Four hours before labelling the complete medium was replaced by a serum free medium. All the flasks were treated with various concentrations of [<sup>3</sup>H]dexamethasone (40 Ci/mmol (1.48 TBq/mmol), Amersham), and half also received a 1000-fold excess of non-radioactive dexamethasone (Sigma). At the end of the incubation period (45 min at 37°C) medium was removed to determine free [<sup>3</sup>H]dexamethasone.

#### Cell and nuclei harvesting

To each flask was added either 10 ml of PBS free from Ca<sup>2+</sup> or Mg<sup>2+</sup> or 10 ml of this buffer supplemented with methyl methanethiolsulphonate (MMTS;Sigma), an inhibitor of thiol proteases. After scraping the cells with a rubber policeman,

flasks were immediately cooled to 0–4°C. The cells were suspended by dispersion and transferred into cold centrifugation tubes. After centrifugation for five minutes at 800 g the supernatants were removed, the pellets washed with 10 ml of either PBS or PBS-MMTS, and the suspensions sedimented a second time. The pellets were finally resuspended with 3 ml of PBS or PBS-MMTS. A 1 ml aliquot of these suspensions was used to measure the receptors in the whole cells, and the remaining 2 ml used to measure the nuclear receptors. The following procedure was used for the analysis of whole cells: after centrifugation the supernatants were discarded and 0.55 ml of distilled water was added to the pellets. After 15 min at 0°C (to cell disruption), 250 µl of the suspension was used for the measurement of cell bound radioactivity and another 250 µl for DNA<sup>18</sup> and protein<sup>21</sup> determinations. For nuclei determina-

tions, after centrifugation, the pellets were mixed with 2 ml of STM (sucrose 250 mmol/l; TRIS (trometamol) 50 mmol/l; Mg<sup>2+</sup> 5 mmol/l, pH 7.4 at 4°C) with or without MMTS (20 mmol/l), then with 2 ml of the same medium containing 2% Triton X-100 (Sigma). The mixture was suspended by 30 strokes in a Dounce homogeniser, and cellular lysis was checked by trypan blue exclusion. After centrifugation the crude nuclear pellets were washed with 4 ml of STM or STM-MMTS. Finally, 0.55 ml of distilled water was added to the nuclear pellet and it was treated as described above.

## Results

### CELL PROLIFERATION

The effects of dexamethasone at different concentrations after various lengths of exposure are indicated in Fig. 1a. The maximal inhibition (45 (SD 6)%) was obtained after three days of treatment at 10<sup>-4</sup> mol/l. After this period we observed a plateau phase in the control chondrocytes, whereas the treated cells continued to grow at a slower rate, overtaking that of the control cultures at the sixth day (Fig. 2a). Thus as soon as the control chondrocytes left the exponential growth phase the inhibition of proliferation of treated chondrocytes apparently disappeared. In contrast, when the same protocol was used with the transformed glucocorticoid sensitive L 929 fibroblasts<sup>22–25</sup> there was a significant difference in cell behaviour (Figs 1b and 2b): after three days of exposure to the steroid (10<sup>-8</sup> mol/l) the inhibition of fibroblasts (30 (1.75)%) was greater than that of chondrocytes (10 (2.5)%) (Figs 1a and 1b). After two days of treatment there was an inhibition of more than 25% at a dose of 10<sup>-6</sup> mol/l for the fibroblasts, whereas the same inhibition was obtained at 10<sup>-4</sup> mol/l with the chondrocytes. A similar difference in sensitivity was also recorded after three days of treatment. Thus 100-fold less dexamethasone was necessary to obtain the same inhibition with the fibroblasts as with the chondrocytes. Fig. 2b also shows that after three days the control fibroblasts continued to grow and did not reach a plateau phase as did the chondrocytes; this could explain the observed apparent increase in inhibition.

### CLONAL GROWTH

Treatment for eight days with different concentrations of dexamethasone showed that at 10<sup>-4</sup> mol/l and 10<sup>-6</sup> mol/l it reduced the number of clones (Table 1). The lower concentrations of dexamethasone had no significant effect, a result consistent with the observed growth inhibition using the monolayer culture.

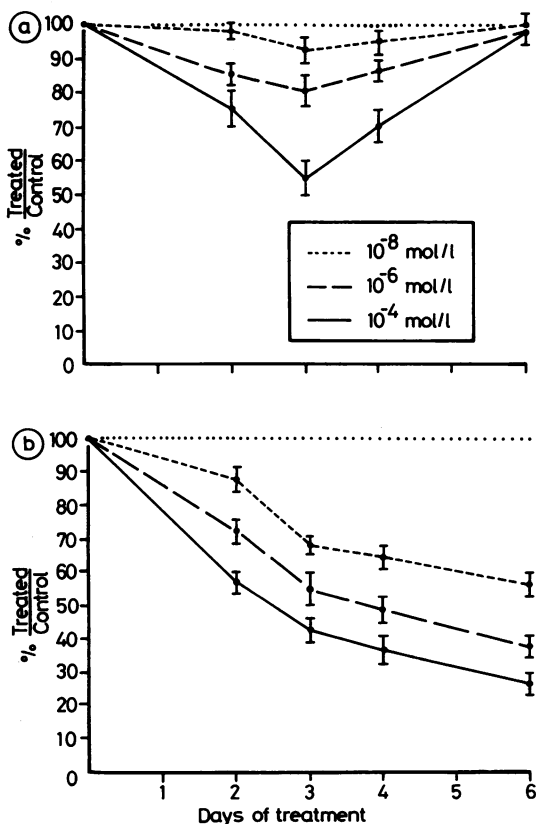


Fig. 1 Time course proliferation of (a) chondrocytes and (b) fibroblasts during treatment for six days with different concentrations of dexamethasone. Numbers of cells are given as percentage values of control cultures. Values are means (SD) of triplicate dishes from three experiments.

Table 1 Effect of dexamethasone on colony formation

Dexa-methasone concentration (moll)	Number of clones*	Number of clusters	Plating efficiency (%)	Inhibition (%)
0	61 (4)	7	30.5	—
10 <sup>-4</sup>	35 (2.5)	16	20	42.5
10 <sup>-6</sup>	43 (3)	13	22	29.5
10 <sup>-8</sup>	59 (2.5)	7	29.5	—
10 <sup>-10</sup>	60 (4)	8	30	—

\*The results show the mean (SD) number of colonies for five flasks.

Table 2 Effect of 10<sup>-4</sup> moll dexamethasone on the incorporation of [<sup>3</sup>H]thymidine and [<sup>3</sup>H]uridine into chondrocytes after treatment for 24 or 48 hours

Precursor	Incubation period	
	24 h	48 h
[ <sup>3</sup> H]Thymidine	70 (3.5)*	77 (1.5)
[ <sup>3</sup> H]Uridine	67 (5)	78 (3)

\*Results are the average (SD) of six flasks/group expressed as a percentage of the control.

DNA AND RNA SYNTHESIS

Cells incubated for 24 hours with 10<sup>-4</sup> mol/l dexamethasone showed a 30% decrease in both the amount of [<sup>3</sup>H]thymidine incorporated into DNA and [<sup>3</sup>H]uridine incorporated into RNA, decreasing to 20% after treatment for 48 hours (Table 2). This was in keeping with the results obtained with the growth curves, except that the onset of inhibition of chondrocyte proliferation was seen after 24 h rather than between days 2 and 3.

FLOW CYTOMETRIC ANALYSIS

These experiments showed significant differences in the phase fractions between control and treated cells after treatment for 24 hours (Table 3). With both

Table 3 Cell cycle analysis based on DNA distribution

Cellular type	Dexamethasone concentration (moll)	24 h			48 h		
		G <sub>0</sub> G <sub>1</sub>	S	G <sub>2</sub> +M	G <sub>0</sub> G <sub>1</sub>	S	G <sub>2</sub> +M
Chondrocytes	0	46 (0.5)*	35 (2)	19 (2.5)	71 (1.5)	12 (4)	17 (3)
	10 <sup>-4</sup>	58.5 (2)	24 (0.5)	17.5 (2)	75.5 (3)	10 (3)	14.5 (2.5)
Fibroblasts	0	40.5 (2)	37 (4)	22.5 (6)	53 (3)	29 (5)	18 (2)
	10 <sup>-4</sup>	56 (1.5)	21 (6)	23 (6)	65.5 (1)	18 (3)	16.5 (1.5)

Computed percentages of cells in G<sub>0</sub>G<sub>1</sub>, S, and G<sub>2</sub>+M were obtained according to the method of Fried and Mandel.<sup>20</sup> The model consisted of a phase fraction estimation in which the sum of normal distribution is fitted to the DNA histogram. One distribution approximates the G<sub>0</sub>G<sub>1</sub> compartment, one distribution approximates the G<sub>2</sub>+M compartment, and the remainder approximates the S compartment.

\*Each value represents the mean (SD) of four measurements and is representative of two separate experiments.

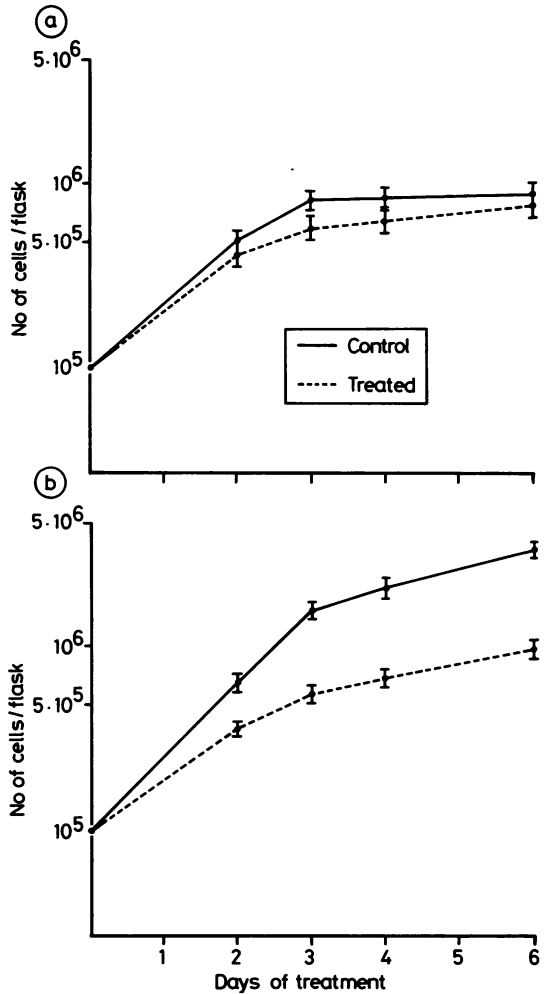


Fig. 2 Growth curve of (a) chondrocytes or (b) fibroblasts cultured for seven days in the presence or absence of 10<sup>-4</sup> moll dexamethasone. Values are means (SD) of triplicate dishes from three separate experiments.

cell types the treatment caused a diminution in the relative percentage of cells in the S phase and an accumulation of cells in G<sub>0</sub>G<sub>1</sub>, whereas the G<sub>2</sub>+M phase was not altered. This accumulation of cells in G<sub>0</sub>G<sub>1</sub> was maintained for the fibroblasts after exposure to dexamethasone for 48 h, though the control distribution showed a decrease of the S phase in association with an enhancement of G<sub>0</sub>G<sub>1</sub> phase. This phenomenon was probably the basis for the slowing of control culture proliferation observed in the growth curves after three days. In contrast with the fibroblasts, the control population of chondrocytes showed a significant arrest in the G<sub>0</sub>G<sub>1</sub> phase at day 2. Because the cells were in plateau phase at day 3 it was not possible to detect a dexamethasone induced slowing of the culture proliferation. Thus the inhibitory effects on the proliferation of the chondrocytes and fibroblasts were different.

#### LOCALISATION OF GLUCOCORTICOID-RECEPTOR COMPLEXES IN RABBIT ARTICULAR CHONDROCYTES

Specific glucocorticoid receptors have been demonstrated in these two cellular types<sup>16 25 26</sup>; we have investigated the possibility that the relative lack of sensitivity of the chondrocytes is associated with a low number of nuclear glucocorticoid-receptor complexes. Preliminary experiments using cells lysed with hypotonic buffer proved difficult to interpret because they produced too many aggregates or nuclei surrounded by cell fragments. Therefore Triton X-100, a neutral detergent, was used. This produced good cell lysis and particularly 'clean' nuclei. Kaufmann *et al* reported that, owing to the lability of nuclear complexes during cellular fractionation and nuclear isolation, techniques using detergent need to be performed under conditions which included the presence of sulphhydryl blocking reagents, such as MMTS.<sup>27</sup> Using medium contain-

Table 4 Influence of MMTS on the stability of the nuclear dexamethasone-receptor complex in chondrocytes

Sample	No treatment		With MMTS	
	cpm/ $\mu$ g DNA	Nuclear sites (%)	cpm/ $\mu$ g DNA	Nuclear sites (%)
Whole cells	76 (7)*	} 25	84 (8)	} 69
Nuclei	19 (5)		58 (5)	

Tissue culture flasks (150 cm<sup>2</sup>) were incubated for 45 min at 37°C in serum free medium (HAM F 12) containing 20 nmol/l [<sup>3</sup>H]dexamethasone. For further details see 'Materials and methods'.

\*Values are the mean (SD) from three results.

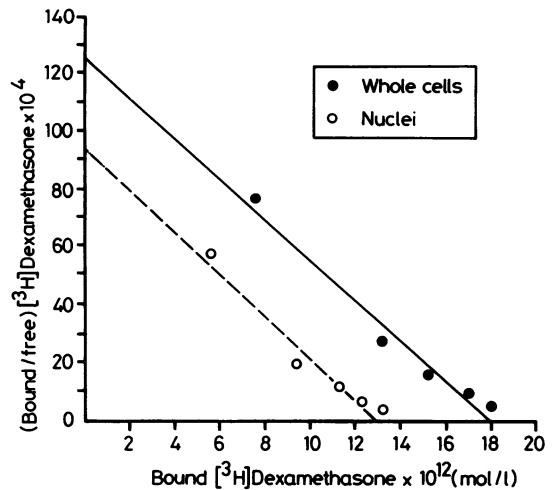


Fig. 3 Scatchard analysis of specific [<sup>3</sup>H]dexamethasone binding to whole cells and nuclei at various glucocorticoid concentrations in a typical experiment using MMTS. The apparent affinity,  $K_D=1.4 \times 10^{-9}$  mol/l.  $B_{max}$  (cellular)=4.1 fmol/ $\mu$ g DNA=26 500 binding sites/cell ( $r=-0.98$ );  $B_{max}$  (nuclear)=2.9 fmol/ $\mu$ g DNA=19 100 binding sites/cell ( $r=-0.97$ ); nuclear sites=72%.

ing MMTS we demonstrated (Table 4) the localisation of the activated complex in the nucleus of rabbit articular chondrocyte (69% v 25% nuclear sites). The number of sites ( $B_{max}$ ) and the apparent affinity ( $K_D$ ), both for the whole cells and the nuclei (Fig. 3), were calculated by Scatchard analysis.<sup>28</sup> The  $K_D$  values were identical for whole cells and nuclei.

#### Discussion

These results show that concentrations of dexamethasone in the range of  $10^{-6}$  to  $10^{-4}$  mol/l inhibited cell growth of chondrocytes in a dose and time dependent manner. This inhibition was also observed using a DNA precursor or determination of the percentage of S cells by flow cytometry. The inhibition of RNA synthesis is in accordance with other reports that dexamethasone acts also on metabolic systems other than DNA.<sup>29-31</sup> In our experimental conditions, however, the chondrocytes were not particularly sensitive to this glucocorticoid since significant growth inhibition was only observed at  $10^{-4}$  mol/l. This was previously described by Ronot *et al* with another glucocorticoid drug: methylprednisolone.<sup>32</sup> Comparison of these inhibitory effects on chondrocytes with those observed on L 929 fibroblasts or other cell types<sup>5 6 9 33 34</sup> indicates that treatment with dexamethasone for iden-

tical times and doses was less effective on the cartilaginous cell. Since chondrocytes have been shown to exhibit specific functions and good proliferation capacity,<sup>35</sup> the lack of sensitivity of these cells to glucocorticoids compared with other cell types involved in the inflammatory process is probably not due to the culture conditions.

We have confirmed the observation of Blondel *et al*<sup>16</sup> that rabbit articular chondrocytes in culture are a target cell for glucocorticoids, i.e., subject to hormonal control. Indeed the glucocorticoid-receptor complexes were able to undergo activation and transformation resulting in an effect at the genome level because the majority became localised in the nucleus. In view of the discrepancy between the dose of dexamethasone responsible for 50% inhibition ( $10^{-4}$  mol/l) and the  $K_D$  value ( $1.4$  ( $SD 0.2$ ) $\times 10^{-9}$  mol/l) it seems that inhibitory action on proliferation cannot be explained exclusively by binding to glucocorticoid receptors. These results are therefore in contrast with many studies that have shown a correlation between the presence of specific glucocorticoid binding of these molecules and growth inhibition for various cell types.<sup>2 4 9 36-38</sup> On the other hand, it has been shown that resistance to glucocorticoids is often associated with a decrease in receptor activity<sup>22 33 39</sup> or sometimes with a defect in the translocation process.<sup>40</sup> The data presented here indicate that the dexamethasone induced growth inhibition of chondrocytes does not involve solely a glucocorticoid effect mediated by specific receptors. This result is in accordance with that of Keller *et al*, who described evidence for more than one mechanism of action of glucocorticoids.<sup>41</sup> Our work suggests that there are at least two different mechanisms involved in the inhibition of chondrocyte proliferation by glucocorticoids. In addition to a direct control of a small amplitude, in which the receptors are involved, there is also a strong inhibitory effect at high concentrations, probably the consequence of several metabolic inhibitions (metabolism of sugars, amino acids, etc) due to the glucocorticoids, and which results in a decrease of cellular activity.

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