Physiological levels of hydrocortisone maintain an optimal chondrocyte extracellular matrix metabolism

J Wang, D Elewaut, I Hoffman, E M Veys, G Verbruggen

Objective: To investigate the effects of physiological doses of hydrocortisone on synthesis and turnover of cell associated matrix (CAM) by human chondrocytes obtained from normal articular cartilage.

Methods: Human articular cartilage cells were obtained from visually intact cartilage of the femoral condyles of five donors and maintained in culture for one week to reach equilibrium in accumulated CAM compounds. 0, 0.05, 0.20, and 1.0 µg/ml hydrocortisone was added to the nutrient media during the entire culture period. Cells were liberated and levels of CAM aggrecan, type II collagen, and fibronectin, of intracellular IGF-1, IL1α and β, and of their respective plasma membrane bound receptors IGFRI, IL1RI, and the decoy receptor IL1RII, were assayed by flow cytometry.

Results: In comparison with controls, hydrocortisone treated chondrocytes, at all concentrations, expressed significantly higher plasma membrane bound IGFRI. Intracellular IGF-1 levels remained unchanged. Together with these changes, reflecting an increased ability to synthesise extracellular matrix (ECM) macromolecules, hydrocortisone treated cells expressed significantly higher amounts of the plasma membrane bound decoy IL1RII. Concurrently, intracellular IL1α and β levels and membrane bound IL1RI were down regulated. Levels of CAM aggrecan, type II collagen, and fibronectin were significantly up regulated in the chondrocytes treated with hydrocortisone.

Conclusion: 0.05 µg/ml hydrocortisone treated chondrocytes had decreased catabolic signalling pathways and showed an enhanced ability to synthesise ECM macromolecules. Because IL1 activity was decreased and the expression of IL1RII decoy receptor enhanced, more of the ECM macromolecules produced remained accumulated in the CAM of the chondrocytes. The effects were obtained at doses comparable with physiological plasma levels of hydrocortisone in humans.

Aside from the up regulation of local tissue “inflammatory” pathways, a variable degree of inflammation can occur in the synovium of osteoarthritic joints. This tissue is then invaded by lymphomyeloid cells, some of which produce the catabolic cytokines that are extremely harmful for the joint tissues. Intra-articular injections of corticosteroids effectively alleviate the pain caused by local inflammation in synovial joints. Therefore, in osteoarthritis (OA), mainly during episodes of inflammatory exacerbations, local administration of glucocorticoid hormones is common practice.

Disagreement among doctors has long existed about the physiological and/or toxic effects of corticosteroids on articular cartilage. Most early in vivo experiments showed that corticosteroids, especially after intra-articular administration, may provoke or worsen cartilage lesions. However, a systematic review of these reports shows that degeneration of articular cartilage, resulting from steroid administration, occurred after the administration of unrealistically high doses of this hormone. Similarly, Charcot-like arthropathy has been reported in man after a series of about 20 intra-articular injections of steroids. The suppression, by supraphysiologically doses of glucocorticoids, of aggrecan and type II collagen synthesis rates in cartilage explant cultures from different origin is a possible explanation for the failure of the cartilage cell repair function after administration of steroids. The deleterious effects on articular cartilage as previously reported may, however, have been provoked by local inflammatory reactions after repeated administration of insoluble crystalline preparations with prolonged action.

More recently, and in contrast with the older observations, single or intermittent local or systemic administration of physiological doses of corticosteroids in different models of experimentally induced OA showed that these drugs had a protective effect on osteoarthritic cartilage—for example, in the meniscectomised rabbit model, in the chemically induced cartilage damage in the guinea pig, and in the Pound-Nuki dog model of OA. Despite the presence of possible species differences, these drugs had similar protective effects on the osteoarthritic cartilage of human patients. Different mechanisms can underlie the chondroprotective effects of corticosteroids in vivo. Glucocorticoids inhibit the infiltration of lymphomyeloid cells in the synovium, and thus reduce the release of cytokines and suppress their effects on the neighbouring joint tissues. The protective effect of the treatment was also ascribed to the ability of glucocorticoids and other steroid hormones, at physiological doses, to stimulate the synthesis and/or to inhibit the degradation of the extracellular matrix (ECM) of articular cartilage, as shown in cartilage explant cultures. Probably, inhibition of the degradation of the ECM was due to the ability of these hormones to reduce pathological neutral protease activity in both cartilage and synovium.

These anabolic and anti-catabolic effects of glucocorticosteroids on cartilage might be the consequence of an effect of steroid hormones on some of the important chondrocyte

Abbreviations: CAM, cell associated matrix; DMEM, Dulbecco’s modified Eagle’s medium; ECM, extracellular matrix; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HBSS, Hank’s balanced salt solution; IGF-1, insulin-like growth factor 1; IGFRI, insulin-like growth factor receptor 1; IL, interleukin; IL1RI, interleukin 1 receptor; mAb, monoclonal antibodies; MFI, mean fluorescence intensity; OA, osteoarthritis; PBS, phosphate buffered saline; PE, phycoerythrin
autocrine/paracrine metabolic pathways. Hydrocortisone has been reported to act synergistically with different essential growth and differentiation factors to affect the synthesis of the ECM ground substance. In addition, glucocorticosteroids might directly affect the synthesis of the ECM because the in vitro effects of these hormones have been shown to occur in the absence of serum.

Our study was undertaken to investigate the metabolic effects of physiological doses of hydrocortisone on ECM metabolism of human chondrocytes isolated from normal articular cartilage.

**MATERIAL AND METHODS**

**Isolation of articular chondrocytes**

Human articular chondrocytes were isolated as described elsewhere, with a few modifications. Briefly, human articular cartilage was obtained at necropsy from femoral condyles of five different donors (four men, one woman, age range 18–80 years) within 24 hours post mortem. All donors had died after a short illness. None of them had received corticosteroids or cytostatic drugs. Visually intact cartilage was harvested and prepared for culture. Cartilage removed from the femoral condyles was diced into small fragments and the chondrocytes were isolated by sequential enzymatic digestion (hyaluronidase, pronase, and collagenase) of the ECM as described in detail. Isolated cells were then centrifuged for 10 minutes at 1500 rpm, washed three times in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS), tested for viability (trypan blue exclusion test), and counted. More than 95% of the cells were usually viable after isolation.

**Cultures in alginate gel**

Chondrocyte cultures in alginate beads were prepared as described elsewhere, with some modifications. Chondrocytes suspended in one volume double concentrated Hanks’s balanced salt solution without calcium and magnesium (HBSS; Gibco) were carefully mixed with an equal volume of 2% alginate (low viscosity alginate from Macrocystis pyrifera; Sigma) in HBSS, and autoclaved for 15 minutes. The final cell concentration was 5×10⁶ chondrocytes per ml in 1% alginate. The chondrocyte/alginate suspension was then slowly dripped through a 23 gauge needle into a 102 mM calcium chloride solution. The beads were allowed to polymerise for 10 minutes at room temperature. The calcium chloride was then removed, the beads were washed three times with 0.15 M sodium chloride and, finally, maintained in a six well plate with 1×10⁶ cells per culture (each well containing 20 alginate beads; ±50 000 chondrocytes per bead) in 4 ml of DMEM with 10% FCS and 50 μg ascorbate per ml in an incubator at 37°C under 5% CO₂. The nutrient medium was replaced twice weekly for seven days. Recently, we showed that ECM metabolism by chondrocytes reached steady state after one week in this alginate culture system.

**Effect of hydrocortisone on the expression of ECM molecules and on intracellular cytokine and growth factor levels**

The chondrocytes were cultured in DMEM supplemented with 10% FCS and increasing amounts (0, 0.05, 0.20, and 1.0 μg/ml) of hydrocortisone during the entire seven day culture period. Hydrocortisone was used because it is the physiologically occurring glucocorticoid and was used as a solution of hydrocortisone sodium succinate in phosphate buffered saline (PBS) with benzyl alcohol q.s. (Solucortef, Upjohn). The contents of the vials were diluted to the desired concentrations of 0.05 to 1.0 μg/ml.

**Preparation of chondrocytes for flow cytometry**

After seven days of culture the nutrient media of the chondrocyte cultures were removed. The alginate beads were washed and dissolved by incubation for 10 minutes with 3 ml of 55 mM trisodium citrate dihydrate pH 6.8 and 0.15 M NaCl at 25°C. The resulting suspension was centrifuged at 1500 rpm for 10 minutes to separate cells with their cell associated matrix (CAM) from the constituents of the interterritorial matrix. The pellet with the chondrocytes was recovered to assess the CAM macromolecule contents, the plasma membrane bound receptors, and the intracellular levels of cytokines and growth factors.

Insulin-like growth factor receptor 1 (IGFR1), interleukin 1 receptors I and II (IL1RI and –II) on the cell membrane, and aggrecan, type II collagen, and fibronectin in the CAM were tested directly after incubation with the appropriate antibodies for 30 minutes in the dark at 4°C. 20 μl of 50 μg/ml fluorescein isothiocyanate (FITC) labelled antibodies was used to react with 2×10⁶ cells resuspended in 100 μl PBS. To evaluate the expression of IGFR-I, IL1α and β inside the cells, chondrocytes were permeabilised with a Cytofix/Cytoperm Plus Kit (PharMingen, San Diego, CA, USA) according to the manufacturer’s instruction. Briefly, cells in culture were incubated with monensin (GolgiStop, 4 μl/6 ml medium) for five hours to block the protein transport from Golgi apparatus. Cells isolated from alginate were then permeabilised with Cytofix/Cytoperm solution for 20 minutes. After a wash in 1× Perm/Wash solution, the cells were incubated with monoclonal antibodies (mAb).

**Antibodies used for flow cytometry**

Mouse antihuman mAb (subclass: IgG1) against IGFR1, IL1RI, IL1RII, IL1β, and the mouse IgG1 negative control were purchased from R&D systems (Abingdon, United Kingdom; IGFR1: clone 33255.111; IL1RI: clone 35730.111; IL1RII: clone 34141.11; IL1β: clone 8516.311). Mouse antihuman IGF-1 and IL1α mAb were purchased from Biosource Europe (Nivelles, Belgium; IGF-1: clone AHC0014; IL1α: clone 624B3F2). Polyclonal antifibronectin rabbit IgG were obtained from Chemicon International (Harrow, UK). Mouse antihuman chondrocyte-specific aggrecan mAb (clone 4D11-2A9; Biosource Europe) were reacted with the G1 domain of the invariable hyaluronan binding region of the human aggrecan molecule, and were used to detect the aggrecan in the chondrocyte CAM. Mouse antihuman type II collagen mAb (clone II-4C11; ICN Biochemicals, Ohio, USA) were chosen to detect type II collagen. All the antibodies (except aggrecan and type II collagen) were conjugated with FITC (isomer I; Sigma-Aldrich, Belgium) as previously described. The anti-aggrecan and type II collagen mAb were conjugated with phycoerythrin (PE; Sigma-Aldrich) as described. The conjugated mAb were used in a direct immunofluorescent staining protocol for flow cytometry. Appropriate FITC or PE labelled isotype matched mouse or rabbit IgG1 (Becton Dickinson: clone x40) were used as a negative control.

**Flow cytometric analysis**

Stained cells were analysed on a flow cytometer (FACSort; Becton Dickinson, San Jose, CA, USA) with CELLQuest software. From each sample, 15 000 events were analysed. Cells were gated on forward and side scatter to exclude dead cells, debris, and aggregates. Propidium iodide was additionally used to exclude dead cells when the epitopes outside the cells—that is, IGFR1, IL1RI and –II, and ECM molecules, were analysed. The mean fluorescence intensity (MFI) of the positive cell population, which is due to the binding of the conjugated antibodies, was used to quantify the presence of IGFR1, IL1RI and –II on the plasma membrane, the presence
of the ECM molecules in the CAM, and the accumulation of IGF-1, IL1α and β inside the cells. MFI values were obtained by subtraction of the MFI of the negative control population from the MFI of the positive stained population. For comparison between experiments the Quantum Simply Cellular Microbead Kit (Sigma) was used to calibrate the fluorescence scale of the flow cytometer. The product contains a mixture of four uniform microbead populations, which differ by their incremental abilities to bind mouse IgG. The microbeads were stained and processed in parallel with the cell samples using the same amount of FITC labelled antibodies and incubation time. The fluorescence scale of the cytometer was adapted before every experiment in order to keep identical MFI's for the four peaks of the calibration beads. The MFI of cell samples was then analysed without changing any instrument settings.

Statistics
MFI values for the different parameters in each of the donor chondrocytes were calculated from triplicate cell cultures. These MFI values were used to generate median values for each variable in the group of five donors. The Wilcoxon rank sum test for paired samples was used to analyse the changes in these groups. The MFI values in the untreated controls were then normalised to 100 to allow percentage changes after the exposure to different concentrations of hydrocortisone to be presented. Paired Student’s t tests were used to test significance and to study the effect sizes of the changes in the chondrocyte cultures after hydrocortisone treatment. Significance levels for all tests were set at p = 0.05.

RESULTS
Table 1 presents the real MFI values for CAM macromolecules, the plasma membrane bound receptors, and the intracellular levels of cytokines and growth factors in controls and in hydrocortisone treated chondrocyte cultures. Percentage changes in chondrocyte fluorescence intensity with hydrocortisone are given in the figures.

Factors promoting synthesis and accumulation of ECM: IGF-1/IGFR1 autocrine pathway and IL1RII
In comparison with controls, hydrocortisone treated chondrocytes expressed significant and dose related increases of plasma membrane bound IGFR1 (fig 1A). On average, the IGFR1 levels increased by 25.6%, 30.7%, and 37.0% at hydrocortisone doses of 0.05, 0.2, and 1.0 μg/ml of nutrient medium. The response to hydrocortisone did not level off at higher doses because each increase in the concentration of the test drug resulted in additional significant increases of the plasma membrane receptor levels. Intracellular IGF-1 levels remained unchanged after exposure to the steroid (fig 1B).

<table>
<thead>
<tr>
<th>Hydrocortisone (μg/ml)</th>
<th>0.00</th>
<th>0.05</th>
<th>0.20</th>
<th>1.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1</td>
<td>55.2 (35.4–61.9)</td>
<td>55.2 (35.4–61.1)</td>
<td>55.5 (35.1–61.3)</td>
<td>55.3 (35.7–61.6)*</td>
</tr>
<tr>
<td>IGFR1</td>
<td>11.4 (8.2–12.9)</td>
<td>14.7 (9.6–16.7)*</td>
<td>15.6 (10.0–17.3)*</td>
<td>15.8 (10.6–18.3)*</td>
</tr>
<tr>
<td>IL1RII</td>
<td>11.1 (8.5–17.5)</td>
<td>13.2 (10.9–18.7)*</td>
<td>14.9 (11.4–19.9)*</td>
<td>15.6 (12.4–20.1)*</td>
</tr>
<tr>
<td>IL1α</td>
<td>34.9 (23.6–72.6)</td>
<td>24.2 (21.4–63.9)*</td>
<td>21.8 (20.3–62.9)*</td>
<td>20.4 (19.9–61.8)*</td>
</tr>
<tr>
<td>IL1β</td>
<td>78.5 (45.1–81.9)</td>
<td>65.1 (38.4–86.7)*</td>
<td>54.0 (35.5–64.5)*</td>
<td>53.1 (36.3–61.7)*</td>
</tr>
<tr>
<td>IL1RI</td>
<td>11.7 (9.4–15.4)</td>
<td>10.9 (9.2–15.0)*</td>
<td>10.8 (8.5–14.7)*</td>
<td>10.2 (8.0–14.1)*</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>41.8 (28.3–57.9)</td>
<td>96.3 (41.8–118.8)*</td>
<td>114.9 (53.0–180.1)*</td>
<td>118.8 (61.1–187.9)*</td>
</tr>
<tr>
<td>Collagen</td>
<td>15.7 (12.0–24.6)</td>
<td>19.8 (15.3–30.0)*</td>
<td>20.0 (17.4–32.9)*</td>
<td>26.9 (17.9–34.6)*</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>221.5 (193.8–202.2)</td>
<td>363.7 (252.3–631.7)*</td>
<td>454.1 (261.9–849.9)*</td>
<td>545.8 (304.8–793.1)*</td>
</tr>
</tbody>
</table>

Median MFI values, lower and upper ranges are given for the different variables under the influence of 0.00, 0.05, 0.20, and 1.00 μg of hydrocortisone per ml of nutrient medium.

*Wilcoxon rank sum test for paired samples, significant change compared with control, p<0.05.
Along with these changes that reflected an increased capability of synthesising the appropriate ECM macromolecules, hydrocortisone treated cells expressed significantly higher amounts of plasma membrane bound decoy IL1RII. Percentage changes induced for this receptor were 19.9%, 29.3% and 35.3%, on average, at hydrocortisone culture medium levels of 0.05, 0.2, and 1.0 µg/ml, respectively. Once more, each increase in the concentration of hydrocortisone in the culture medium resulted in an additional and significant increase of the expression of IL1RII (fig 1C).

Factors promoting degradation of ECM: IL1/IL1RI autocrine pathway

Concurrently, intracellular IL1α and β levels and membrane bound IL1RII were found to be down regulated after exposure of the chondrocytes to hydrocortisone.

Hydrocortisone treated chondrocytes showed significant and dose related decreases of intracellular IL1α and β. IL1α levels inside the cells on average decreased by 19.1%, 23.7%, and 25.6%, and IL1β levels were reduced by 14.3%, 23.3%, and 25.9% at hydrocortisone doses of 0.05, 0.2, and 1.0 µg/ml of nutrient medium, respectively (figs 2A and B). The response of IL1α to hydrocortisone did not level off at higher doses. Each increase in the concentration of the test drug resulted in a further significant decrease of this cytokine inside the chondrocyte. In contrast, the response of IL1β to hydrocortisone levelled off at 0.2 µg/ml of this steroid. Together with both isoforms of IL1, the expression of the signalling receptor of these catabolic cytokines, IL1RI, was down regulated on average by 3.8% (p = 0.017), 6.4% (p = 0.009), and 10.5% (p = 0.003) of the baseline values at increasing doses of hydrocortisone (fig 2C).

Cell associated matrix content

The levels of CAM aggrecan, type II collagen, and fibronectin were significantly up regulated in the chondrocytes treated with hydrocortisone.

In comparison with controls, hydrocortisone treated chondrocytes expressed significant and dramatic dose related increases of CAM aggrecan. Aggrecan levels on average increased by 90.2%, 161.1%, and 184.4% at 0.05, 0.2, and 1.0 µg/ml of hydrocortisone in the culture medium. Each increase in the culture medium levels of the drug provoked an additional significant increase in CAM aggrecan (fig 3A). Equally, hydrocortisone treated chondrocytes expressed significant and dose related increases of CAM type II collagen. These collagen levels on average increased by 27.0%, 37.8%, and 59.4% at 0.05, 0.2, and 1.0 µg/ml of hydrocortisone in the culture medium. The response to hydrocortisone levelled off at 0.2 mg/ml of the drug in the medium, and a further increase in the concentration of the steroid did not cause an additional increase in CAM collagen (fig 3B). A similar response of the chondrocytes to the steroid was observed for CAM fibronectin. Increases of baseline values by 31.7%, 47.2%, and 70.7% at the test doses used were seen (fig 3C).

DISCUSSION

Contrasting results in previous experiments on cartilage explants may in part be due to the varying conditions of the existing ECM, the heterogeneity of chondrocyte populations in the different samples, and their different viability. These problems are avoided by the use of homogeneous cell populations of isolated chondrocytes in suspension culture. Chondrocyte culture in alginate gel is considered to be a valid system for studying both anabolism and catabolism of chondrocytes. Articular cartilage cells in this culture condition maintain their phenotype and synthesise cartilage-specific collagens and ECM aggrecans. The culture model allowed in vivo chondrocyte phenotypes to be studied in laboratory conditions. Topographically dependent phenotypic expression of ECM compounds in joints, as well as the age related decline in the chondrocyte response to growth factors, was preserved when the chondrocytes were cultured in alginate beads. The newly synthesised ECM compounds are assembled into a well ordered matrix as present in hyaline articular cartilage. The advantage of alginate is the reversibility of the gelled condition of this matrix, allowing the study of the CAM surrounding the isolated chondrocytes.

Our studies have shown that hydrocortisone treated chondrocytes, even at the lowest 0.05 µg/ml concentration, showed a significantly increased accumulation of CAM aggrecan, type II collagen, and fibronectin. This increased accumulation of CAM macromolecules obviously resulted from a decrease in the activity of the catabolic pathways.

Figure 2  Effects of increasing doses of hydrocortisone on the IL1/IL1RI autocrine pathway. Percentage changes in chondrocyte MFI due to the binding of monoclonal antibodies specific for IL1α (A), IL1β (B), and IL1RI (C) are shown for varying doses of hydrocortisone in the culture medium. Each dot represents the mean value of the three results obtained in a single donor. Median values are indicated for each dose of hydrocortisone. Results of paired samples t test (p values) are given in the figure.
because the intracellular levels of both IL1 isoforms were depressed in chondrocytes after the exposure to hydrocortisone. The intracellular growth factors and cytokines represent a reservoir of biologically active agents, of which variable amounts are secreted in response to various stimuli. It can be expected that the intracellular amounts of these bioactive molecules predict their extracellular function. Further suppression of the IL1 catabolic pathway was seen as the expression of the signalling IL1RI receptor decreased, and as the plasma membrane IL1RII decoy receptor levels increased concomitantly, on the cells exposed to hydrocortisone. The decreased activity of the IL1 mediated catabolic pathways in hydrocortisone treated chondrocytes—as reported by others—explains the well documented reduction of neutral protease activities in steroid treated osteoarthritic cartilage samples.21 22 27 29 These studies, as well the results presented here, are in line with the observation that steroid hormones prevented the enzymatic degradation and depletion of ECM proteoglycan in a model of endotoxin mediated cartilage degradation46 and retarded the degradation and clearance of sulphated proteoglycans from condylar cartilage of newborn mice treated with steroid hormone.48

A functional IGF-1/IGFRI pathway is critical for the synthesis of a normal ECM41 and seems also to be essential for preservation of the components of this matrix. Too small an amount of IGF-1 can result in a suboptimal expression of the IGFRII decoy receptor and an uncontrolled activity of the catabolic IL1 pathway.49 50 As the experiments conducted here showed that hydrocortisone enhanced the expression of IGFRI on the cell membrane, it can be suggested that the chondrocytes treated with hydrocortisone are more susceptible to their own autocrine IGF stimuli, and therefore, synthesise more and higher molecular weight aggrecans.

As a result of both the enhanced ability to synthesise ECM macromolecules, and the decreased activity of the catabolic pathways, hydrocortisone treated chondrocytes showed significantly up regulated levels of CAM aggrecan, type II collagen, and fibronectin. Glucocorticoids thus play a part in the maintenance of homoeostasis and the integrity of normal and diseased articular cartilage. The effects were obtained at doses comparable with the physiological plasma levels of hydrocortisone in the human species. The evening and morning hydrocortisone plasma concentrations in humans are 0.05 and 0.2 µg/ml, respectively. As reported earlier,51 higher doses failed to provoke additional effects. The results presented here show a fundamental role for physiological doses (that is, endogenous production) of corticoids in maintaining normal human chondrocyte metabolism.

It is as yet speculative to defend the use of steroids to protect cartilage in clinical conditions without obvious inflammation. It has been recognised that the same cytokines that are produced during inflammation also control ECM homoeostasis in normal and osteoarthritic cartilage and are produced by the chondrocytes themselves.52 53 In osteoarthritic chondrocytes or cartilage, the up regulation of the catabolic IL1α and β pathways has been reported57 58 and a correlation with the occurrence and the degree of OA pathology was noted for IL1β.59 60 The increased catabolic cytokine levels are embodied by well documented increases in metalloproteinase activities, which are higher in osteoarthritic cartilage than in morphologically normal cartilage from the same joint.54–57 If cartilage cells from osteoarthritic tissues needed higher steroid levels than the physiological levels to adequately repress to the anabolic condition, the exogenous administration of corticoids might be useful to protect cartilage in clinical conditions without obvious inflammation. The sensitivity of osteoarthritic cartilage cells to the effects of hydrocortisone has not yet been tested.

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Figure 3  Effects of increasing doses of hydrocortisone on the levels of CAM aggrecan, type II collagen, and fibronectin. Percentage changes in chondrocyte MFI due to the binding of monoclonal antibodies specific for aggrecan (A), type II collagen (B), and fibronectin (C) are shown for varying doses of hydrocortisone in the culture medium. Each dot represents the mean value of the three results obtained in a single donor. Median values are indicated for each dose of hydrocortisone. Results of paired samples t test (p values) are given in the figure.


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