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The effect of systemic corticosteroid treatment on the immunolocalisation of Notch-1, Delta, CD105 and CD166 in rat articular cartilage

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Summary

We studied the immunolocalisation of the stem cell-specific markers Notch-1, Delta, CD105 and CD166 in rat articular cartilage and analysed the effect of systemic corticosteroid treatment on the patterns of distribution of cells labelling for these markers. Female Wistar rats were separated randomly into two groups: the control group ($n = 8$) was injected with isotonic salt solution and the corticosteroid group ($n = 8$) was injected with 10 mg/kg intramuscular corticosteroid (methylprednisolone) once a week for a period of 8 weeks. Femoral head specimens from each group were obtained at the end of the treatment and processed for routine histological and immunohistochemical examinations. Quantitative data were obtained by H-SCORE and statistical evaluations were performed. The immunolocalisation of all markers was more apparent in the superficial zone and decreased through the deeper zones in all groups. However, the intensity of labelling was much less obvious in the group treated with corticosteroid compared to control. H-SCORE analysis confirmed that in the group treated with corticosteroid, the intensity of Notch-1, Delta, CD105 and CD166 labelling had decreased significantly compared to control ($p < 0.05$). In conclusion, based on the immunolocalisation of stem cell-specific markers Notch-1, Delta, CD105 and CD166, the data suggest that the stem cells may continue to exist in adult rat articular cartilage. It was also observed that systemic corticosteroid treatment may effect the immunolabelling intensity of these markers, suggesting that corticosteroid treatment may reduce the function and the regenerative capacity of these cells in articular cartilage.
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Introduction

Articular cartilage is a veinless and nerveless connective tissue with high matrix–cell ratio. Articular cartilage chondrocytes are surrounded by hyaline cartilage matrix and secrete matrix proteins that are digested by them. These secretion and digestion processes are mandatory for tissue matrix homeostasis (Tallheden et al., 2003). In adult articular cartilage, chondrocytes are organized anisotropically into distinct vertical columns and horizontal zones as superficial/tangential/surface, transitional, upper and lower radial, and calcifying/calcified/calcification (Ustunel and Demir, 1995; Hayes et al., 2001; Hunziker et al., 2002, 2007; Ustunel et al., 2003; Dowthwaite et al., 2004).

In the postnatal period, when transition from immature isotropic to anisotropic form takes places, all zones except for the uppermost zone are reshaped. The superficial zone contains stem cells that divide slowly (Hunziker et al., 2007) and have regenerative capacity (Dowthwaite et al., 2004). These cells divide by mitosis and produce horizontal (lateral) and vertical (parallel to long axis) new stem cells. Newly formed horizontal (lateral) cells are responsible for increasing the width of articular cartilage. Division in the vertical direction feeds the transit-amplifying cell population, which is located in the transitional and upper radial zones with the capacity for rapid division. At the end of this vertically directed process, longitudinal bone develops (Hunziker et al., 2007). In addition, *in vivo* studies show that the articular cartilage surface zone is responsible for appositional growth of articular cartilage and this growth is facilitated by a chondroprogenitor cell population that is located in the 2–3 cell layer of the surface zone (Archer et al., 1994; Hayes et al., 2001).

Development, reshaping and regeneration of tissue and organs depend on the activity of stem cells. Cartilage and bone tissues develop from mesoderm-originated stem cells. Mesenchymal stem cells (MSCs) are present in different tissues in adult organisms (Arai et al., 2002; Young and Black, 2004).

The Notch genes, first discovered in *Drosophila melanogaster*, appear to play a role in cell fate determination in many processes and are conserved in all organisms. Notch receptors are transmembrane receptors containing extracellular and intracellular domains (Wharton et al., 1985). Four different Notch isoforms (Notch-1, Notch-2, Notch-3 and Notch-4) and three different ligands (Delta, Jagged 1 and Jagged 2) are found in vertebrates. After ligand–Notch interaction, the intracellular domain of Notch passes into the nucleus and participates in transcriptional control (Gridley, 1997; Sherwood and McClay, 1997). Notch signals are defined as a developmental process in cell interaction mechanisms concerned with cell vitality. The Notch pathway regulates cell fate under the control of internal and external developmental signals and is involved in the process of differentiation, proliferation and apoptosis (Sahin et al., 2005).

It has been suggested that the articular cartilage surface zone cells mostly express Notch-1 membrane receptor and its ligand, Delta (Dowthwaite et al., 2004; Archer et al., 2002; Hayes et al., 2003). Richardson et al. (2006) showed superficial zone labelling of Notch-1, Delta, CD105 and CD166 by immunofluorescense in 7-day-old bovine metacarpalphalangeal joints. Furthermore, *in vitro* studies on human articular cartilage suggest that Notch-1, Delta and Jagged-1 are more abundant in colony-forming cells than other non-colony-forming chondrocytes (Ustunel et al., 2008).

CD105 (Endoglin) is a cell-surface glycoprotein that regulates cellular processes including proliferation, cell migration, extracellular matrix production and hematopoiesis. It is a member of the transforming growth factor beta (TGFβ) superfamily and plays a role in wound healing and angiogenesis. CD105 binds to TGFβ1 and TGFβ3 with high affinity. CD105 is mostly present in the vascular system and is involved in connective tissue cell generation (Fonsatti et al., 2001).

Activated cell adhesion molecule (ALCAM, CD166) is a member of the immunoglobulin superfamily and is expressed by T and B cells, thymus epithelial cells, fibroblasts, keratinocytes and neurons. CD166 mediates both heterophilic (CD166-CD6) and homophilic cell–cell connection. It plays a role in cell growth, migration, organ development, hematopoiesis, immune response and tumor growth. Recent data suggest that CD166 provides a role in cell adhesion via cell–extracellular matrix adhesion and in growth and migration processes (Swart, 2002). Alsalameh et al. (2004) suggested that the isolated cells from adult articular cartilage and bone marrow, which express cell-surface markers like CD105 and CD166, have the ability to differentiate towards adipogenesis, chondrogenesis or osteogenesis *in vitro*. They hypothesized that these cells, which represented 4% of the isolated cell population, might be the multipotent progenitor cells. Nevertheless, no data were presented on the zonal distribution of these cells in adult articular cartilage.

Previous studies on the changes in joints after corticosteroid treatment have been related to

articular cartilage damage. In systemic corticosteroid-treated experimental models, there is a reported decrease in protein and proteoglycan synthesis produced by chondrocytes; thus, the matrix structure is broken down, cells lose their vitality and the normal structure of articular cartilage is damaged (Mankin et al., 1972; Shaw and Lacey, 1973; Silbermann and Finkelbrand, 1980; Higuchi et al., 1980; Podbielski and Raiss, 1986; Weiss et al., 1986; Itani et al., 1992; Gogia et al., 1993; Annefeld, 1989). However, the literature that describes the effect of systemic corticosteroid treatment on articular cartilage is still inadequate.

Although there is an extensive research literature on articular cartilage, there remains a lack of detailed information regarding whether cells expressing stem cell-specific markers like Notch-1, Delta, CD105 and CD166 exist in articular cartilage and, if so, what are their zonal distribution *in vivo*. Furthermore, the effects of systemic corticosteroid treatment on the localization and immunolocalisation ratio of these markers *in vivo* have not been assessed. In the study presented here, our aims were to investigate the immunolocalisation and zonal distribution of these markers and to determine the effect of systemic corticosteroid treatment on Notch-1, Delta, CD-105 and CD-166 immunolocalisation in articular cartilage using routine histological and immunohistochemical techniques.

Methods

Animals

In this study, 16 adult female Wistar rats, each weighing 200–250 g, were used. They were allowed free access to standard rat laboratory diet and tap water. In the control group ($n = 8$), animals were injected with intramuscular isotonic salt solution once a week during a period of 8 weeks. In the experimental group ($n = 8$), animals were injected with 10 mg/kg intramuscular methylprednisolone (Prednol-L ampule, Mustafa Nevzat, Turkey) once a week during a period of 8 weeks.

The experimental protocol was approved by the Animal Care and Usage Committee of our university and was in accordance with the declaration of Helsinki and the International Association for the study of pain guidelines.

Tissue processing

At the end of the 8-week period, the rats were killed and their femoral heads were removed. Tissues were processed for embedding in paraffin wax for histochemical and immunohistochemical investigations. Femoral head samples were fixed by immersion in 10% formalin for 1 week. Then, tissues were washed in tap water for 24 h, decalcified in 25% formic acid (Merck KGaA 64271, Darmstadt, Germany) for 3 days, washed in tap water for 24 h and neutralized in 0.35 M sodium sulphate (LACHEMA 304720401, Czech Republic) solution for another 3 days. Tissues were then washed in tap water for 24 h. Then tissues were dehydrated through a graded series of ethanol and embedded in paraffin wax. All samples were serially sectioned at 5-μm thickness and were stained for routine light histological examination after haematoxylin–eosin (HE) staining or for immunohistochemical labelling.

Immunohistochemistry

For immunohistochemical labelling, 5-μm-thick tissue sections were mounted on slides coated with poly-L-lysine (Sigma-Aldrich, St Louis, MO, USA). After de-paraffination in xylene, sections were rehydrated through a decreasing gradient of ethanol. The sections were then treated with proteinase K (DAKO, code: S3004), prepared according to manufacturer's instructions, for 15 min. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in methanol for 15 min. The sections were blocked with a universal blocking reagent (Ultra UV Block, LabVision Corporation, TA-125UB, UK) for 3 min. They were then incubated with primary antisera: rabbit polyclonal anti-CD105 (sc-20632) diluted 1:100, rabbit polyclonal anti-CD166 (sc-25624) diluted 1:200, goat polyclonal anti-Notch-1 (sc-6014) diluted 1:50 and rabbit polyclonal anti-Delta (sc-9102) diluted 1:200 (all from Santa Cruz Biotechnology Inc, Santa Cruz, CA) overnight at 4 °C. Binding was detected by incubating with an avidin–biotin horseradish peroxidase complex (UltraVision Detection System Anti-Polyvalent, Alk-Phos, UK), prepared according to manufacturer's instructions, for 30 min, followed by incubation with diaminobenzidine tetrahydrochloride (DAB) chromogen (Bio-Genex, San Ramon, CA, prepared according to manufacturer's instructions) for 1–2 min. All dilutions and thorough washes between stages were performed using phosphate-buffered saline (PBS; pH 7.4) and all steps were performed at room temperature unless otherwise stated. Sections were counterstained with Mayer's hematoxylin, dehydrated, mounted and examined by light microscopy.

Negative controls were performed by replacing the primary antibody with either normal rabbit IgG

(Santa Cruz Biotechnology, sc: 2051) or normal goat IgG (Santa Cruz Biotechnology, sc: 2053) at the same concentration as the primary antibodies.

Histologic SCORE (H-SCORE) and semiquantitative evaluations

The evaluations of the immunohistochemical labelling in both the control and the experimental groups were accomplished utilizing H-SCORE as in a previous study (Sahin et al., 2005). Briefly, labelled sections were evaluated using an Axioplan microscope (Zeiss, Oberkochen, Germany) with a special ocular scale. From three randomly selected slides, five different fields at $\times 200$ magnification were analysed for immunopositivity. The evaluations were recorded as percentages of positively labelled cells of all types in each of four intensity categories, which were denoted as 0 (no labelling), 1+ (weak labelling, but detectable above control), 2+ (distinct labelling), and 3+ (intense labelling). For each tissue, an H-SCORE value was derived by counting the percentages of cells that labelled positively at each intensity multiplied by the weighted intensity of the labelling [H-SCORE $= SP_i$ (i+1), where i is the intensity score and P_i is the corresponding percentage of the cells]. The H-SCORE values were presented graphically.

Statistical analysis

The data from the H-SCORE were analysed with non-parametric ANOVA on ranks (Kruskal–Wallis test) followed by a *post hoc* Student–Newman–Keuls test. Values are presented as mean $\pm$ SEM. Statistical calculations were performed using SigmaStat for Windows, version 3.0 (Jandel Scientific Corp. San Rafael, CA). Statistical significance was defined as $p < 0.05$.

Results

Routine light microscopic evaluations

When evaluated with HE staining, in both groups the articular cartilage tissue appeared normal and healthy and the order of zonal distribution had not changed in the group treated with corticosteroid (Figure 1b) compared to control (Figure 1a).

Immunohistochemical results

Densities of CD105, CD166, Notch-1 and Delta in articular cartilages of both control and rats treated with corticosteroid were evaluated semi-quantitatively and are summarized in Table 1.

Notch-1

In the control group, the Notch-1 immunopositive cells were mostly located in surface, transitional and upper radial zones and the density of immunolabelling was prolific in these zones. However, in the lower radial and calcification zones the density of Notch-1 immunolabelling was low (Table 1; Figure 2a). In the group treated with corticosteroid, although the Notch-1 immunopositive cell zonal distribution had not changed, the intensity of immunolabelling had (Table 1; Figure 2b).

Delta-1

In controls, the Delta-1 immunopositive cells were only located in the first three zones (surface, transitional and upper radial) with a weaker immunolabelling density compared to Notch-1 immunolabelling. No immunopositivity was observed in lower radial and calcification zones (Table 1, Figure 2c). For the positive zones, the immunolabelling was decreased in the group treated with corticosteroid (Table 1, Figure 2d).

CD105

CD105 immunolabelling showed a similar pattern to that seen for Notch-1. CD105 immunopositive cells were mostly located in surface, transitional and upper zones. Immunolabelling density was very intense within the surface zone and weaker within the transitional and upper radial zones in the control group (Table 1, Figure 2e). In the group treated with corticosteroid, there was a marked decrease in immunolabelling density of CD105, particularly for cells in superficial and transient zones (Table 1, Figure 2f).

CD166

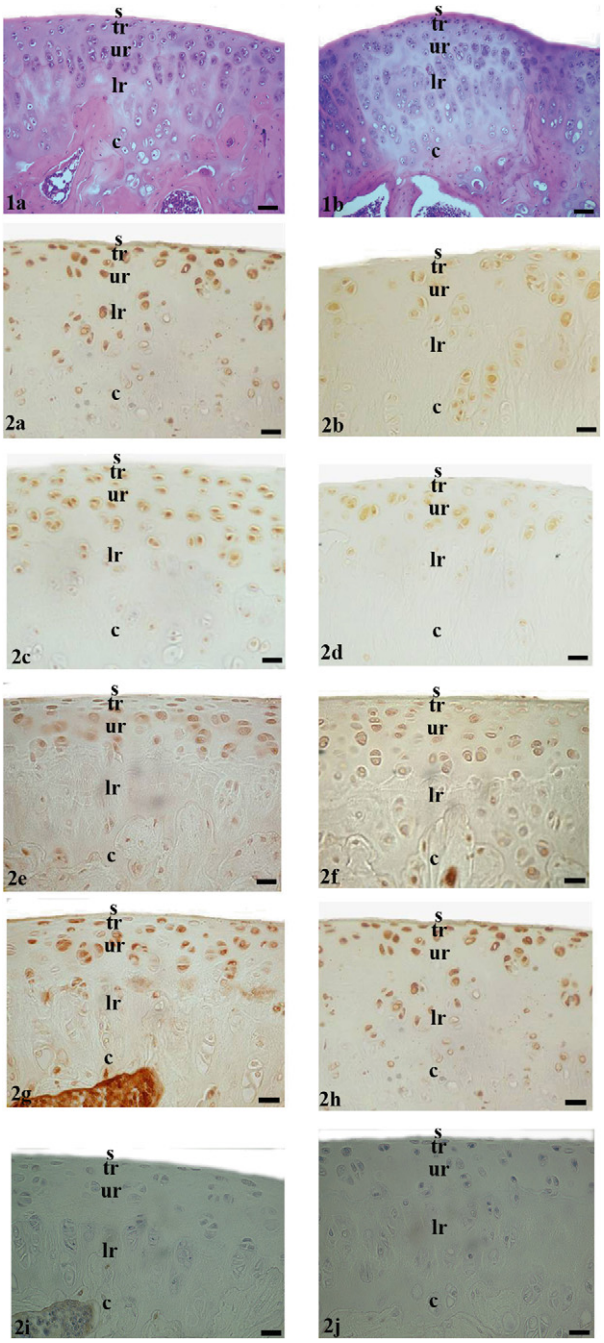
In the group treated with corticosteroid, the CD166 immunopositive cells were very dense in the surface and transitional zones, dense in the upper radial zones and there were no immunopositive cells in the lower radial and calcification zones (Table 1, Figure 2g). In the group treated with corticosteroid, zonal distribution of immunopositive cells did not change but a decrease in their immunolabelling densities was observed (Table 1, Figure 2h).

Controls

No immunolabelling was observed in negative control sections (Figure 2i, j).

H-SCORE results

The intensity of labelling for Notch-1, Delta, CD105 and CD166 immunopositive cells in the animals treated with corticosteroid in comparison to controls is listed in Table 2 and illustrated in Figure 3. In animals treated with corticosteroid, H-SCORE values for immunolabelling with Notch-1, Delta, CD105 and CD166 decreased significantly ($p < 0.05$).



Discussion

Although other studies have been performed to examine the effect of corticosteroids on articular cartilage, none of them have examined the effect of the systemic corticosteroid treatment on the pattern of immunodistribution of stem cell-specific markers. Previous studies suggested that the damage of articular cartilage and the degree of degeneration of extracellular matrix under systemic corticosteroid treatment occur in a dose-dependent manner (Mankin et al., 1972; Shaw and Lacey, 1973; Silbermann and Finkelbrand, 1980; Higuchi et al., 1980; Podbielski and Raiss, 1986; Weiss et al., 1986; Itani et al., 1992; Gogia et al., 1993; Annefeld, 1989). Studies describing the ultrastructural features of chondrocytes after systemic injection of a corticosteroid showed a decreasing volume of rough endoplasmic reticulum and Golgi apparatus, slimming of territorial matrix and increase in glycogen granules and lysosomal content (Higuchi et al., 1980; Podbielski and Raiss, 1986; Weiss et al., 1986; Itani et al., 1992). In the study reported here, the dose of corticosteroid administered did not result in any detectable degenerative changes in articular cartilage morphology.

Notch signalling plays a role in cell fate decision in nerve, vascular, hematopoietic systems and

Figure 1. Histology of articular cartilage tissue architecture, stained sections. (a) Control group. (b) Corticosteroid-treated group shows a similar morphological appearance with control and no deformation in tissue architecture. s: surface zone, tr: transitional zone, ur: upper radial zone, lr: lower radial zone, c: calcified zone. Immunohistochemical labelling of articular cartilage. (a) Notch-1, control group: Notch-1 immunopositive cells in surface and transitional zones. (b) Notch-1, corticosteroid group: marked decrease of Notch-1 immunolabelling in comparison to control group. (c) Delta control group: moderate Delta immunolocalisation in surface, transitional and upper radial zones. (d) Delta, corticosteroid group: immunolabelling was decreased in comparison to control group. (e) CD105 control group: a similar immunolocalisation pattern to that seen with Delta. (f) CD105, corticosteroid group: a dramatic decrease in immunolabelling compared to control, especially in surface and transitional zones. (g) CD166, control group: immunolabelling was very strong in surface and transitional zones; strong in upper radial zone; negative in lower radial and calcification zones. (h) CD166, corticosteroid group: immunolabelling was decreased in comparison to control group. (i) Negative control, incubated with goat polyclonal IgG instead of primary antisera. (j) Negative control, incubated with rabbit polyclonal IgG instead of primary antisera. All scale bars represent 40 μm.

Table 1. Semiquantitative distribution of CD105, CD166, Notch-1 and Delta immunoreactivity in articular cartilage tissue of control and corticosteroid-treated group.

Articular cartilage zones	Control group				Corticosteroid group			
	Notch-1	Delta	CD105	CD166	Notch-1	Delta	CD105	CD166
Surface zone	+++	++	+++	+++	+	+	(+)	++
Transitional zone	++	++	++	+++	+	+	(+)	++
Upper radial zone	+	++	+	++	+	(+)	+	(+)
Lower radial zone	(+)	0	0	0	(+)	0	0	0
Calcified zone	0	0	0	0	(+)	0	0	0

0: negative; (+): weak positive; +: positive; ++: dense positive; and +++: very dense positive.

Table 2. Comparison of Notch-1, Delta, CD105 and CD166 positive cell frequencies in articular cartilage tissue of control and corticosteroid-treated group.

Groups	Notch1	Delta	CD105	CD166
Control	178,700 ± 13,306	142,700 ± 7878	123,600 ± 7339	193,050 ± 2570
Corticosteroid	107,700 ± 7261*	108,800 ± 6935*	90,700 ± 1007*	121,200 ± 5815*

*$p < 0.05$.

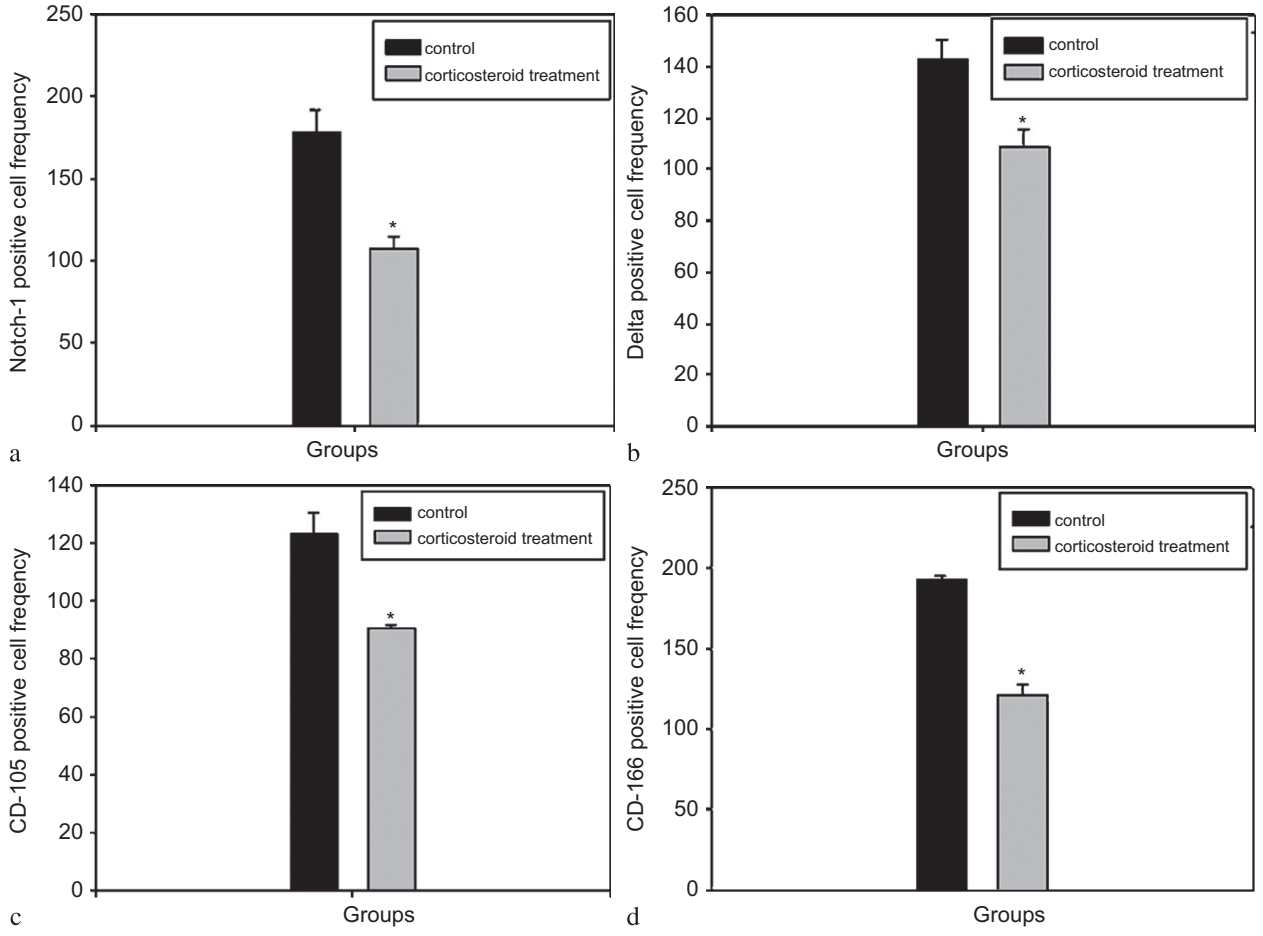


Figure 3. (a) Notch-1, (b) Delta, (c) CD105 and (d) CD166 immunopositive cell frequency in control (black bars) and corticosteroid-treated (grey bars) rat articular cartilage. Significant decrease was observed in corticosteroid-treated group in comparison to control group *$p < 0.05$.

many other tissues like somites, muscle, skin and pancreas. Receptors belonging to the Notch family also play a role in osteoblastic differentiation (Pereira et al., 2002). Blocking cell differentiation maintains cells in an undifferentiated state. Therefore, Notch signalling has been evaluated as a guard against differentiation (Artavanis-Tsakonas et al., 1999). Lowell et al. (2000) showed that the expression of Notch-1 and its ligand Delta-like-1 is more abundant in stem cells of skin than in the more differentiated keratinocytes. They suggested that the high Delta 1 ligand expression triggers stem cell phenotype. In two separate studies it has been suggested that the stem cells are located in the articular cartilage surface zone and these cells are the origin of a transit-amplifying cell pool in transitional and upper radial zones and the growth of articular cartilage depends on the activation of cells in these areas (Dowthwaite et al., 2004; Hunziker et al., 2007). In the study reported here, we observed Notch-1 and Delta immunopositive cells located especially in surface, transitional and upper radial zones, close to the articular cartilage surface in healthy rat articular cartilage. After treatment with corticosteroid, there was a dramatic decrease in immunolabelling of these markers. We suggest that Notch-1 and its interaction with its ligand Delta may be negatively affected after treatment with glucocorticoid, which may disturb the state of articular cartilage cells.

There are no reported studies showing the presence and zonal distribution of CD105- and CD166-expressing cells. CD105 and CD166 are the regulators of cell viability, adhesion, growth, migration and extracellular matrix structure. In the study reported here, we show for the first time that CD105 and CD166 localized in surface, transitional and upper radial zone cells in patterns similar to Notch-1 and Delta immunolocalisation. There was a significant decrease in CD105 and CD166 immunopositivity after treatment with corticosteroid. These results suggest that CD105 and CD166 with Notch-1 and Delta may play roles in cell viability, cell–extracellular matrix interaction, cell migration and overall articular cartilage structure. Thus, decrease of these cell-surface molecules after systemic treatment with corticosteroid may affect the quality of the chondrocytes.

Immunolocalisation of stem cell markers Notch-1, Delta, CD105 and CD166 by articular cartilage cells, located in surface, transitional and upper radial zones, suggests that stem cells may exist in adult articular cartilage. Hence, we hypothesize that these zones are the source of stem cells and their function is necessary for growth in healthy rat articular cartilage. Furthermore, the presence of the supposed stem cells in articular cartilage may indicate roles in cell survival, adhesion to extracellular matrix and homeostasis for these cells in this tissue. Decrease in these markers after systemic corticosteroid treatment may damage articular cartilage and may decrease the pool of stem cells found in this tissue.

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