Ontogeny of rat chondrocyte proliferation: studies in embryo, adult and osteoarthritic (OA) cartilage

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ABSTRACT

The aim of this work was to study the ontogeny of chondrocyte cell division using embryo, adult and osteoarthritic (OA) cartilage. We searched for mitosis phases and performed a comparative evaluation of mitotic index, basic fibroblast growth factor b (FGFb), transforming growth factor β1 (TGF-β1) receptors, cyclin dependent kinase (CDK1) and Cyclin-B expression in fetal, neonate, 3, 5, 8 weeks old rats and experimental OA. Our results showed that mitosis phases were observed in all normal cartilage studied, although, we found a decrease in mitotic index in relation to tissue development. No mitosis was detected in OA cartilage. We also found a statistical significant reduction in cell number in OA cartilage, compared with the normal tissue. Furthermore, FGFb and TGF-β1 receptors diminished in relation to tissue development, and were very scarce in experimental OA. Western blot assays showed CDK-1 expression in all cases, including human-OA cartilage. Similar results were observed for Cyclin-B, except for 8 weeks, when it was not expressed. Our results suggest that cell division seems to be scarce, if not absent within the OA cartilage studied. Nevertheless, the existence of factors essential for cell division leaves open the question concerning chondrocyte proliferation in OA cartilage, which is likely to be present in the early stages of the disease.

***Keywords*:** proliferation, chondrocytes, mitotic index, growth factors.

INTRODUCTION

Some aspects of cell biology of the articular cartilage are still poorly understood, especially those related to chondrocyte cell cycle and cell division. In order to broaden this knowledge, it is crucial to deep into normal chondrocyte proliferation and, subsequently, to compare it with OA chondrocytes. Elucidation of the mechanisms underlying the chondrocyte proliferation may shed some light on OA pathogenesis.

Chondrocytes are the only cell type within the cartilage; they are well-differentiated cells. It is known that cell division is very rare, or might even be completely missing in already differentiated cells. However, even though chondrocyte proliferation in OA has been reported by others using different probes [1-5], all of them avoid the observation of mitosis as a direct evidence of cell proliferation. Previous studies on proliferation have been based on the incorporation of H^3 thymidine and/or bromodeoxyuridine [6-8], which indicates that the cells have passed through S phase of cell cycle during DNA replication, but does not conclusively demonstrate cell division. Likewise, flow cytometry studies could quantify DNA in cell cycle, but was unable to differentiate G_2 phase from M phase [9, 10]. Other markers of cell proliferation such as PCNA, which is an S phase marker, can also be detected during DNA replication and repair, and therefore are not specific for cell division. Ki-67, another cell proliferation marker, is present during the entire cell cycle; its concentration and/or localization changes during mitotic phases [4, 5, 11, 12], which is when chromosomes are observed and mitosis can therefore be detected. Consequently, the presence of Ki-67 is not necessarily indicative of cell division if it is not associated with chromosomes. Herein all the proliferation markers classically used, while pointing out DNA rep-

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lication during S phase of cell cycle, do not necessarily indicate that mitosis took place. The fact that different phases of mitosis have never been reported in OA cartilage make us to doubt the existence of chondrocyte cell division (at least in its classical mode) and, consequently, the existence of cell proliferation in OA. We also wondered if OA chondrocytes might remain quiescent at G_2 phase of cell cycle, as previously suggested [13-15]. In order to clarify the lost of mitosis phases within the OA cartilage and in turn to ascertain cell proliferation, new strategies are required and the number of chondrocytes within the OA cartilage, as compared to the normal tissue, should also be assessed. Considering this, we studied the role of growth factors and their receptors regarding to chondrocyte cell division. These growth factors, especially TGF-β1, could promote chondrocyte differentiation and induce/inhibit cell proliferation, both *in vitro* and *in vivo*, depending on their concentrations and the cell cycle phase in which they are present [16-22]. The components of the mitosis-promoting factor (MPF) complex, CDK-1 and Cyclin-B, which are essential for triggering mitosis, are also of interest. It was reported that the phosphorylation and dephosphorylation process of this complex may play a key role in cells shifting from G_2 to M phase, and therefore start cell division.

MATERIALS AND METHODS

Sampling

Seventy Wistar rats were divided into 7 groups of ten animals each: fetal (15-17 and 18-20 d), neonate, 3, 5, and 8 w of age, and the experimental OA-induced model. The latter was accomplished by unilateral knee partial meniscectomy and post-surgery training for 20 d, following the procedures previously reported [23]. Samples from the knees of all animals were removed after they were killed using anesthesia overdose, in accordance with National Research Guidelines (NOM-062-ZOO-1999). Cartilage samples with small portions of subchondral bone were obtained and processed according to the type of study.

Mitotic index

Condyles from 35 rats (5 from each study group) were fixed in 10% formalin and embedded in paraffin. The tissues were cut into sections of approximately 6 μm thickness, and were stained using the conventional hematoxyline-eosine (HE) technique [24]. The mitotic index was determined by counting the number of mitotic figures in a thousand cells, scored in three serial sections, from 5 different animals from each group, following the procedure described by Evans [25]. A total of 105,000 cells were counted and the data were represented as mean ± SEM. Besides of mitotic index determination, in the case of experimental OA cartilage, the chondrocytes were scored from the whole section, excluding the fibrosis zones observed. The number of chondrocytes from OA samples were scored on 23 randomly picked microscopic fields obtained from 5 different slides with 3 serial sections in each one; from five different animals and compared with the number of chondrocytes in normal cartilage, on the same number of microscopic fields, slides, sections and animals. Statistical analysis was performed using the Student's *t*-test. All observations were carried out using a bright field microscope (Leica DMLS; Wetzlar, Germany), 40× objective.

Immunofluorescence

The remaining 35 condyles were fixed in 4% paraformaldehyde in PBS at 4°C for 4-12 h, cryosectioned at 4 μm thickness (Leica cryostat CM1100; Heerbrugg, Switzerland), and mounted on gelatin-coated slides. Indirect immunofluorescence technique for detection of growth factor receptors was performed.

A mouse monoclonal antibody for FGFb-R (40 μg/ml in PBS, Santa Cruz Biotechnology; Santa Cruz, California), and a rabbit polyclonal antibody for TGFβ1-R (60 μg/ml in PBS, Santa Cruz Biotechnology; Santa Cruz, California), were used as primary antibodies. Cryosections were hydrated for 10 min in PBS and treated with 0.2% Triton X-100 in PBS for 10 min at room temperature. Pre-incubation was performed with 0.2% IgG-free bovine serum albumin (Sigma Chemical Co., St Luis, MO) for 20 min at room temperature. Overnight incubation at 4°C with primary antibodies was followed by FITC-tagged anti-mouse and FITC-tagged anti-rabbit, respectively (6 μg/ml in PBS, Santa Cruz Biotechnology; Santa Cruz, California), for 1 h at room temperature [26, 27]. Negative control was achieved by excluding the corresponding primary antibodies from the reaction and Vero cells, which are a fibroblastic cell line, were used as positive control. Nuclei were counterstained with propidium iodide (10 μg/ml, Sigma Chemical Co., St Luis, MO) for 1 min.

Confocal microscopy

The analysis of immunodetection of growth factor receptors was performed using an × 60 (NA 1.4) oil immersion objective on a Nikon microscope attached with confocal system (Bio-Rad MRC-60; Watford, UK). Samples were excited with a Krypton-Argon laser in green (418 nm) and red (514 nm) filters. For each area, 10-15 serial optical sections (0.5-1 μm thick) were collected, using the dual channel image system. Merge images of both channels were assessed.

Western blot

Human-osteoartrhitic (H-OA) cartilage samples were included in addition to the rat samples. The samples were obtained during arthroplasty, performed for therapeutic indications, approved by the appropriate Institutional Committee on Medical Ethics.

Wet articular cartilage from all rat samples, except for fetal, were pulverized in liquid nitrogen, homogenized in lysis buffer (100 μl/20 mg of tissue) containing a mixture of protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 5 mM N-ethylmaleimide, 5 μg/ml leupeptin, 3 mM Nα-*p*-tosyl-L-Lysine chloromethyl ketone, 3 mM iodoacetamide) and 4 M urea, and then clarified by centrifugation for 1 min at 13,000 rpm. In the case of H-OA cartilage, protein extraction was accomplished by the method previously reported by Fryer [29]. Protein concentration was measured using Bradford procedure [30]. Ten μg proteins were loaded to each lane, electrophorised in 10% SDS-PAGE and transferred to a nitrocellulose membrane for 2 h. The membrane was preincubated in PBS with 5% skimmed dry milk for 1 h at RT. Afterward, they were incubated overnight at 4°C with monoclonal mouse antibodies against Cyclin-B (1:500 in PBS, Santa Cruz Biotechnology, CA, USA) and CDK-1 (1:250 in PBS, Oncogene Research Products; Darmstadt, Germany). The immunoreactions

were visualized after incubation for 1 h with horseradish peroxidase labeled antimouse secondary antibody (1:3000 in PBS, Santa Cruz Biotechnology; Santa Cruz, California), using the chimioluminiscence ECL+Plus Western blotting detection system (Amersham Pharmacia Biotech; Buenos Aires, Argentina). The expression of actin in the same tissue lysates was analyzed as internal control. To evaluate this technique, the positive control consisted of HeLa cells cultured in medium supplemented with fetal bovine serum (FBS), and negative control was the HeLa cells cultured in medium without FBS. Both controls were processed with the same technique. Bovine albumin was used as control blot to make sure that equal amounts of protein were loaded (data not shown).

Statistics

The quantification of green labeling intensities was achieved by means of MRC/CAS software used with a confocal microscope. For this purpose, all digital images were obtained under the same parameters of the confocal microscope. The Kruskal-Wallis test (nonparametric ANOVA) was performed [28]. Only the green labels were taken into consideration for this test.

RESULTS

Mitotic index decreased during cartilage development

The morphological analysis of fetal and neonate specimens allowed to identify all phases of mitosis within superficial and middle zones of the cartilage (Fig. 1A-E). In specimens of 3, 5 and 8 w of age, the superficial zone of the cartilage showed a greater amount of mitotic cell figures. The mitotic index evaluation indicated that the cartilage samples which showed that the mitotic index reached the highest at 18-20 d of the fetal development (175.12/1000). As the animal's age progressed, the mitotic index decreased gradually from 19.78 at 3 w to 1.67/1000 at 8 w. No mitotic cells were observed in experimental OA (Fig. 1F). Additionally, a remarkably significant reduction in number of chondrocytes was scored within the experimental OA cartilage (133.70 ± 8.45), compared to the normal tissue (226.30 ± 11.32, P = 0.0001) (Fig. 2), on a total of 23 fields from 3 serial sections of 5 different animals (experimental and control groups).

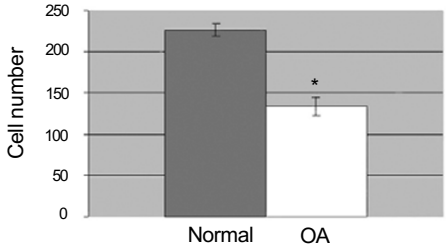

Fig. 2 Quantitative analysis of the number of chondrocytes in rat OA and normal cartilage. * represents P = 0.0001.

Developmental expression of FGFb-R and TGF β-1-R in the chondrocytes

As shown in Fig. 3A, all chondrocytes displayed noticeable labeling of FGFb-R in prenatal and neonate animals. Whereas in 3 and 5 w of age, labeling were clearly limited to the periphery of the articular chondrocytes (Fig. 3B). In 8 w, FGFb-R diminished and was weakly labeled in some cells (Fig. 3C). In experimental OA cartilage, the chondrocytes were arranged in clusters; some were marked, whereas others lacked a mark (Fig. 3D).

TGF β1-R labeling was abundant in fetal and neonate

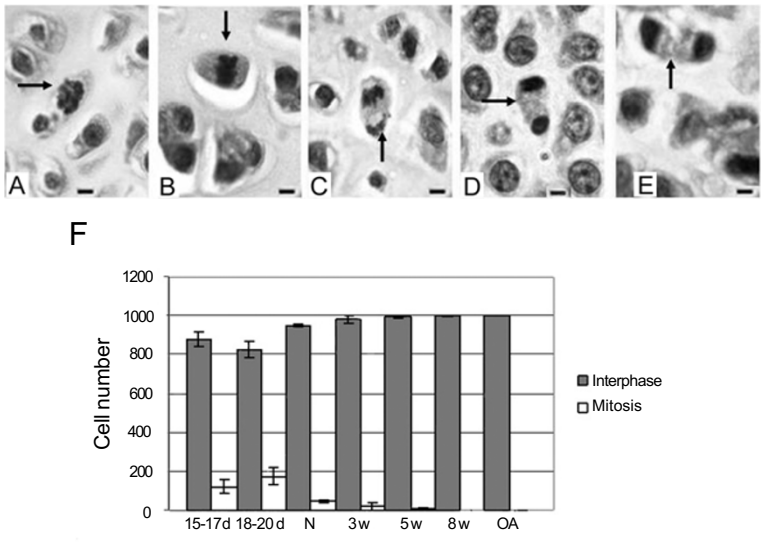

Fig. 1 Mitotic index decreased during cartilage development. (**A**-**E**) HE staining of cartilage specimens in (**A**) prophase, (**B**) metaphase, (**C**) anaphase, (**D**) Telophase, (**E**) cytokinesis were indicated by arrows. scale bar = 10 μm. (**F**) Quantitative analysis of mitotic index in cartilage specimens of 15-17 d, 18-20 d of fetal period, neonate (N), 3 w, 5 w, 8 w old rats and OA.

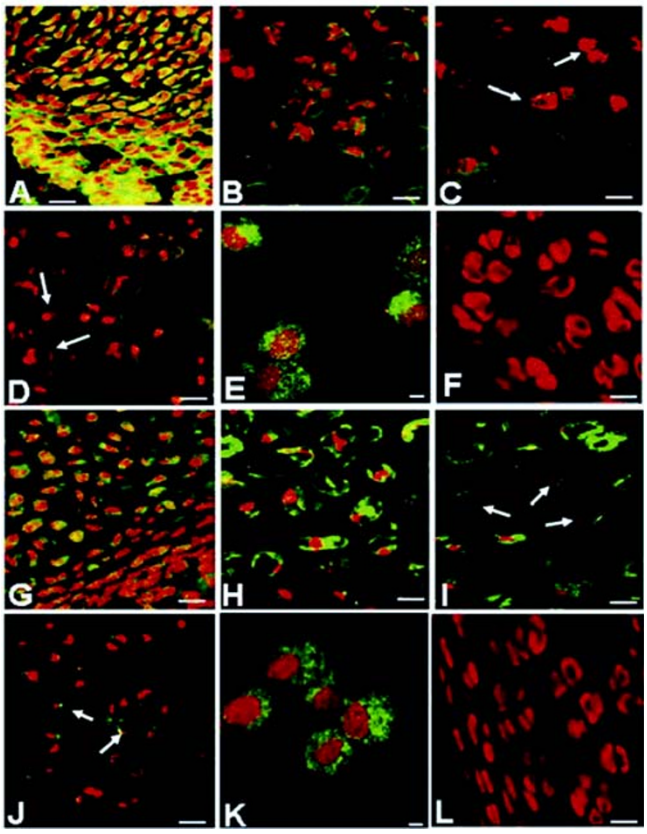

Fig. 3 Immunolabeling images of FGFb-R (**A**-**F**) and TGF β1-R (**G**-**L**) in chondrocytes of different developmental stages. (**A**, **G**) Fetal cartilage; (**B**, **H**) 5 w old rat; (**C**, **I**) 8 w old rat; (**D**, **J**) Rat-OA; (**E**, **K**) Vero cells served as positive control; (**F**, **L**) Negative control. The growth factors were stained in green and the nuclei in red labeling. The images were achieved by confocal microscopy and the scale bar = 10 μm.

cartilages (Fig. 3G). In 3, 5 and 8 weeks of age, the labeling delineated the chondrocyte profile (Fig. 3H). As age increased, labeling progressively diminished (Fig. 3I), whereas in OA cartilage the labeling almost disappeared completely (Fig. 3J).

The scoring of pixel intensity showed that labeling of both receptors (FGFb-R and TGF β1-R), diminished progressively as age increased. In experimental OA, the two receptors displayed the lower values. Statistical analysis revealed a significant difference among the samples of each one of the two growth factor receptors, which varies from $P = 0.05$ to $P = 0.01$ (Fig. 4).

Regulation of Cyclin-B and CDK-1 in chondrocytes

The Western blot analysis showed an evident Cyclin-B expression in neonates and in samples of 3 and 5 w of age, though it was not detectable in 8 w, possibly due to very low mitotic index at that time. However, experimental

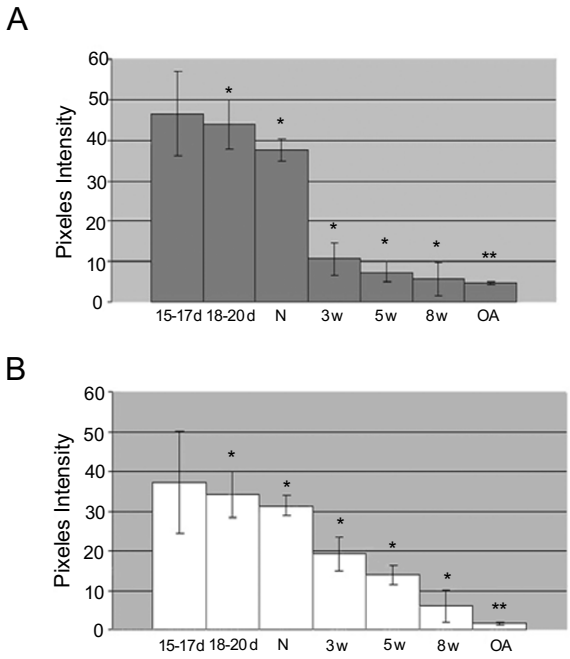

Fig. 4 The pixel labeling intensity of (**A**) FGFb-R and (**B**) TGF β1-R were analysed by Kruskal-Wallis test and represented as mean ± SEM. All the samples were compared with that of the 15-17 d fetal rat. *indicates $P = 0.05$ and **indicates $P = 0.01$.

and human OA samples showed the presence of this protein. The antibody recognized a protein of 60 kD and another one of 58 kD. The protein with greater electrophoretic mobility likely represents a non-phosphorylated form of Cyclin-B, as previously was suggested by Kanatsu-Shinohara [31]. On the other hand, CDK-1 was present in all samples, including human and rat OA, although the band was weak in the 8 w sample (Fig. 5B). The analysis of the bands showed the highest levels for both proteins in the 3 w samples. Whereas in OA samples, Cyclin-B was similar in human and rat, and CDK-1 was higher in human samples (fig. 5).

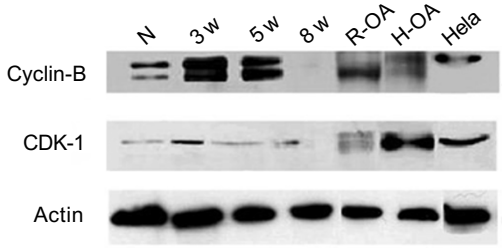

Fig. 5 The expression of Cyclin-B, CDK-1were detected by Western blot. Actins were loaded to serve as internal control. N, neonate; 3 w, three weeks; 5 w, five weeks; 8 w, eight weeks; R-OA, Rat-OA; H-OA, Human-OA; Hela, positive control HeLa Cells.

DISCUSSION

Our results indicated, as expected, that chondrocytes showed a decreased proliferation pattern in relation to rat's development. We did not find similar proliferation pattern in tested OA cartilage. Within the developing cartilage, our results showed a significant decrease in the amount of division-associated factors such as FGFb-R, TGFβ1-R, CDK-1 and Cyclin-B. As we mentioned previously, it is well known that FGFb and TGF-β1 could notably enhance articular chondrocytes proliferation. And special attention has also been paid to IGF-1 as a factor involved in cartilage repair. In this direction, studies have reported that in human cartilage cell culture [32] and in cultured articular chondrocytes from different animals [33-35], the combined use of the three growth factors significantly increased proteoglycans synthesis. However, mitogenic activity was similar to cultures in which the medium contained bovine fetal serum alone [35]. Previous studies by Osborn [33] and Trippel [34] reported the same results. All together, it seems that the main and most important role of IGF-1 in cartilage repair is associated to matrix restoration. We believe that parallel to matrix restoration chondrocyte proliferation is equally important when cartilage repair is on focus, and that the absence of mitotic figures in OA cartilage is a question still open for debate.

Although cell clustering has been frequently observed in human OA and in the experimental OA model used for this work, no mitotic figures were observed in clusters. More so, in the kinetics study of experimental OA cartilage, in the observations (5, 10, 20, 45 and 60 d after rat OA induction), no cell division was found [23]. Here, we reported a dramatic reduction in the number of chondrocytes in the rat-OA cartilage, as compared to the normal controls. Although, as expected, tissue mass of OA cartilage was smaller as compared to normal controls. The validity of our comparison was accomplished because we scored an equal number of microscopic fields using the same magnification for normal control and OA cartilages.

All these results made us believe that cell division is practically absent in OA cartilage, mainly during the advanced stages of the disease. Nevertheless, binucleated and paired chondrocytes were frequently observed in both normal and OA rat cartilage. The possibility that asymmetric mitosis could be present, as described in chick growth-plate cartilage [36], needs to be explored.

Interestingly, we found the existence of Cyclin-B and CDK-1 in OA samples. The latter might lead to the assumption that proliferation is potentially possible; nevertheless, these findings alone are not enough, since they are not the only factors that determine mitosis initiation, at least in the classic way. It is well known that the phosphorylation/dephosphorylation process plays a crucial role in triggering mitosis. Therefore, the induction of cell division in adult and OA cartilage remains an open subject. Recent studies point out the possibility of inducing chondrocyte proliferation by using novel techniques such as tissue engineering [37], mesenchymal stem cells [38], and dedifferentiated articular chondrocytes [39], in an attempt to restore the functional chondrocyte population in OA cartilage. These approaches shed more light on this subject.

According to our results, we can conclude that it is possible to consider that, in late stages of the OA-induced rat model, cell proliferation within the cartilage is extremely diminished, if not absent. However, it is necessary to emphasize that, even though chondrocytes from our OA model show similarities to human-OA, extreme caution has to be taken when extrapolating results between those two species.

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