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DNA damage, discoordinated gene expression and cellular senescence in osteoarthritic chondrocytes

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SUMMARY

Objective: The initiation/progression factors of osteoarthritic (OA) cartilage degeneration and the involved biological mechanisms remain rather enigmatic. One core reason for this might be a cellular senescence-like phenotype of OA chondrocytes, which might show a fundamentally different behavior pattern unexpected from the biological mechanism established in young cells.
Design: This study was designed to investigate one core property of senescent cells, the heterogeneity of gene expression, in OA chondrocytes by double-labeling immunolocalization using two genes (vimentin, S-100 protein) as surrogates, which are constitutively expressed by (normal) chondrocytes. The level of genomic DNA damage in OA chondrocytes was compared to normal chondrocytes and *in vitro* experiments designed to demonstrate that stochastic genomic DNA damage is able to induce heterogeneity of gene expression in chondrocytes.
Results: We show a significantly increased heterogeneity of gene expression for vimentin and S-100 protein as well as a significantly increased genomic DNA damage in the OA compared to normal chondrocytes, whereas no evidence of critical telomere shortening was found. *In vitro* experiments demonstrated that stochastic genomic DNA damage induced by increased oxidative or genotoxic stress is able to induce the heterogeneity in gene expression found in the OA cells *in situ*.
Conclusions: Our results suggest that OA chondrocytes show a special form of age-related cell degeneration, "progressive/stress-induced senescence", progressing over time due to accumulated DNA damage and subsequent chaotic gene activation pattern. This promotes increased malfunctioning of the cells and finally the loss of their capacity to keep up cell and tissue homeostasis, i.e., prevent OA.

Introduction

Osteoarthritis (OA) is a very common severe disabling disease, particularly in the aging Western societies. Doubtless, biomechanical factors and perpetuated use of joints during life contribute to their degeneration; however the homeostasis of anabolic and catabolic events on the molecular level also plays a crucial role for maintaining the tissue integrity of the articular cartilage. This delicate balance of anabolic and catabolic gene regulation within articular chondrocytes, which is needed for maintaining functional cartilage matrix, is mainly regulated by a balanced interplay between anabolic growth factors such as bone morphogenetic proteins (BMPs) and insulin-like growth factors (IGFs), and catabolic cytokines such as interleukin 1ß (IL-1ß) (for review see Aigner *et al.*[1]). All of these complex interactions are based on genetic predisposition (for review see Ikegawa[2]) and epigenetic modulation of the genome (for review see Roach and Aigner[3]).

Many data regarding these factors have accumulated over the last decades, but still the initiation and progression of the disease process and the involved biological mechanisms remain rather enigmatic. One core reason for this was recently suggested to be that very old cells are present within the (aged and) OA articular tissue[4–8]; in fact, these cells seem to have largely survived since the end of adolescent growth, i.e., more than 30–40 years in most individuals suffering from OA. These old chondrocytes appear to have a senescent-like phenotype and might show a fundamentally different behavior pattern unexpected from behavior known in young (non-senescent) cells.

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One important feature of senescent cells in general, which represents a major hindrance to these cells to react in a bio-"logical" manner, is a rather stochastic derangement of gene expression, i.e., highly increased expression of a wide array of genes. This is particularly true if the senescent phenotype is directly linked to DNA damage, as in the case of progressive/stress-induced senescence (in contrast to replicative senescence, which is mainly caused by chromosomal instability due to telomere shortening) after repetitive cell duplication[9,10]. A chaotic gene expression pattern on the cellular level appears to be one potentially important facet of chondrocyte behavior in OA cartilage[7,8], though this has never been investigated systematically in the tissue.

In the present study, we analyzed the heterogeneity of gene expression within OA compared to normal cartilage using double-immunofluorescence as well as immuno double labeling fluorescence activated cell sorting (FACS) analysis. We also investigated the senescent phenotype of the OA chondrocytes in particular by demonstrating the increased cellular DNA damage without any evidence of critical telomere shortening. We show that stochastic DNA damage in articular chondrocytes can induce the senescent phenotype as well as the discoordinated gene expression pattern, which is typical for OA chondrocytes.

Material and methods

Cartilage samples

For the study of mRNA expression levels, cartilage from human femoral condyles was processed as described previously[11]. Normal articular cartilage (numbers are given in the respective sections) was obtained from autopsies, within 48 h of death. OA cartilage was obtained from total knee replacement (numbers are given in the respective sections). Cartilage was considered to be normal if it showed no significant surface fibrillation. Cases of rheumatoid arthritis were excluded from the study. Only primary degenerated and not regenerative cartilage (osteophytic tissue) was used.

The study was approved by the ethics committee of the university.

Histology and immunohistochemistry (double-immunofluorescence)

Histologic studies and conventional immunohistochemical studies were performed on paraformaldehyde fixed and paraffin embedded specimens of normal [$n = 17$; mean age 58 yrs (41–83 yrs)] and OA [$n = 16$; mean age 67 yrs (54–78 yrs)] articular cartilage. For histologic evaluation, tissue sections were stained with Hematoxylin and Eosin (HE) staining and toluidine blue for sulfated proteoglycans (PGs). Immunohistochemical studies were done with antibodies for vimentin [mouse monoclonal clone V9.1., Dako (Hamburg, FRG), dilution 1:200 no pretreatment] and S-100 [rabbit polyclonal, labeling mostly S-100B, Dako, dilution 1:1000; predigestion pronase (2 mg/ml, phosphate buffered saline (PBS), pH 7.3, 60 min at 37°C; Boehringer Mannheim; FRG)]. Vimentin was detected using a tyramide-amplification protocol (PerkinElmer, Rodgau – Jügesheim, FRG) and Cy3-labeled streptavidin complexes (dilution 1:1000; BioGenex, San Ramon, USA). S-100 protein was detected using Cy5-labeled secondary antibodies (anti-rabbit; 1:100; Jackson Inc., Baltimore, USA). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (1:10,000). For control, primary or secondary antibodies were replaced by PBS or non-immune serum (BioGenex) and the samples processed as described above. None of the control samples showed any significant staining. Fluorescence micrographs were taken with an AX70 Olympus microscope and a DT5 Olympus digital camera (Olympus, Tokyo, Japan). Pictures were systematically evaluated by double-immunofluorescence microscopy for positivity of vimentin and S-100 protein of preserved cells within chondrocyte lacunae (as proven by positivity of DAPI). For statistical comparison the Mann–Whitney test was used.

Flow cytometry for vimentin/S-100 double expression

Chondrocytes were freshly isolated enzymatically as described previously[12] and directly used for FACS analysis. For these studies, cells obtained from normal [$n = 8$, mean age 64 yrs (52–72 yrs)] and OA donors [$n = 7$, mean age 69 yrs (57–78 yrs)] were used.

For each experiment 4.5×10^5 cells were fixed and permeabilized in 200 μl 1% paraformaldehyd (PFA) (in PBS pH 7.2; Merck, Darmstadt, FRG) for 10 min at 4°C. The cells were washed three times in PBS and resuspended in the antibody solution. The anti vimentin (1:200) and anti S-100 (1:10,000; both Dako) antibodies were incubated in 0.1% saponin (in PBS pH 7.4; Sigma–Aldrich, Taufkirchen, FRG) for 30 min at 37°C. After pelleting the cells were washed three times in PBS supplemented with 3% fetal bovine serum (FBS) (Biochrom, Berlin, FRG). Then the sample was incubated with a mixture of fluoresceinisothiocyanat (FITC)-labeled anti-rabbit (1:100; Jackson Inc.) and Cy5-labeled anti mouse antibodies (1:200; Jackson Inc.) in 0.1% saponin PBS for 30 min at 37°C. The cells were washed twice, resuspended in PBS pH 7.2 containing 1% PFA and immediately measured with a Becton–Dickinson FACSCalibur cytometer (San Jose, USA).

For each sample, gates were applied to the forward scatter/side scatter (FSC/SSC) to exclude cell debris (particles size $<<5$ μm) and cell clusters (particles size $>>15$ μm). In each group 10^4 cells were measured. Cells were considered to be S-100/vimentin double positive when they were located in the upper right quadrant of the FL-1 (S-100)/FL-4 (vimentin) diagram. The borders of the quadrants were calibrated using measurements of single stained cells. For statistical comparison the Mann–Whitney test was used.

mRNA expression analysis – RNA isolation and cDNA synthesis – real-time polymerase chain reaction (PCR)

Normal [$n = 10$; mean age 64 yrs (42–72 yrs)] and OA articular cartilage [$n = 15$; mean age 68 yrs (57–78 yrs)] was obtained as described above. Cartilage was frozen in liquid nitrogen immediately after removal and stored at −80°C until required for RNA isolation. Total RNA from cartilage tissue was isolated as described previously[13]. First strand cDNA was synthesized using the First Strand Synthesis Kit from Boehringer (Boehringer Mannheim, FRG), using 2 μg RNA. Real-time PCR (TAQMAN, Applied Biosystems, Foster City, USA) was utilized to detect proliferation-associated gene Ki67 (MKI67) as well as telomerase (TERT) (Table I). Primers (MWG Biotech, FRG) and TAQMAN probes (Eurogentec, Seraing, Belgium) were designed using PRIMER EXPRESS™ software (Applied Biosystems) and used as described previously[12]. For all genes specific standard curves were performed in parallel using sequence specific control probes to obtain quantifiable results. All experiments were performed in triplicates. For standardization of the gene expression levels as determined by TAQMAN analysis mRNA ratios relative to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were calculated by dividing the mRNA copy number of the respective gene by the copy number obtained for GAPDH. Statistical evaluation of significant differences in expression levels was done by Mann–Whitney test.

COMET assay

In order to evaluate DNA damage within the cells, the so-called COMET assay was performed.