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

J. Rose †[a], S. Söder ‡[a], C. Skhirtladze ‡, N. Schmitz §, P.M. Gebhard ||, S. Sesselmann ¶, T. Aigner ||*

† Department of Orthopaedic and Trauma Surgery, University of Cologne, Joseph-Stelzmann-Str. 9, 50924 Cologne, Germany
‡ Institute of Pathology, University of Erlangen-Nürnberg, Krankenhausstr. 8-10, 91054 Erlangen, Germany
§ Institute of Pathology, University Hospital Leipzig, Liebigstr. 26, 04103 Leipzig, Germany
|| Institute of Pathology, Medical Center Coburg, Ketschendorfer Str. 33, 96450 Coburg, Germany
¶ Division of Molecular Immunology, Department of Internal Medicine III, Nikolaus-Fiebiger Center, University of Erlangen-Nürnberg, Glückstr. 6, 91054 Erlangen, Germany

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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.
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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.

* Address correspondence and reprint requests to: T. Aigner, Medical Center Coburg, Institute of Pathology, Ketschendorfer Str. 33, D-96450 Coburg, Germany. Tel: 49-9561-226213; Fax: 49-9561-227590.
E-mail addresses: thomas.aigner@klinikum-coburg.de, th.aigner@yahoo.de (T. Aigner).
[a] Both authors contributed equally to the work.

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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.

Table I
Sequences of primers and probes used for quantitative real-time PCR experiments

Gene	Acc.-No.	Primer/Probe	Sequence	[nM]*	$MgCl_2$†
GAPDH	NM_002046	Forward	5′-GAAGGTGAAGGTCGGAGTC-3′	50	5.5
		Reverse	5′-GAAGATGGTGATGGGATTTC-3′	900	
		Probe	5′-CAAGCTTCCCGTTCTCAGCC-3′	100	
MKI67	NM_002417	Forward	5′-CAGTGATCAACGCCGTAGGTC-3′	900	6
		Reverse	5′-TCGGCTGATAGACACTCTCTTTTG-3′	900	
		Probe	5′-CTTCCAGCAGCAAATCTCAGACAGAGGTTC-3′	100	
TERT	NM_198253	Forward	5′-TGCAGAGCGACTACTCCAGCTAT-3′	300	5
		Reverse	5′-AAAGAGTTTGCGACGCATGTT-3′	300	
		Probe	5′-CTCACCTTCAACCGCGGCTTCAA-3′	100	

* Concentration of primer/probe in TAQMAN reaction in nM.
† $MgCl_2$ concentration in TAQMAN reaction in mM.

Chondrocytes from normal [$n = 7$; mean age 62 yrs (41–72 yrs)] and OA [$n = 8$; mean age 68 yrs (57–78 yrs)] cartilage were isolated as described previously[12]. To recover metabolic homeostasis after enzymatic digestion, cells were initially cultured for 2 days in plastic flasks.

The COMET assay was performed using the COMET Assay Reagent Kit from Trevigen (Trevigen Inc, Gaithersburg, USA) according to manufacturer's instructions. Briefly, primary chondrocytes were trypsinized, 2×10^4 cells were mixed with Low Melting Agarose and applied to a COMET slide. After lysis a horizontal electrophoresis at 1 V/cm and 300 mA was performed for 20 min at 4°C under alkaline conditions (pH > 13). Subsequently the samples were dried and stained with ethidiumbromide (2 μg/ml, Sigma–Aldrich) for 2 min. On average, 80 randomly selected cells per sample were captured under an IX70 Microscope (Olympus) and digital fluorescent images were obtained using the analysis software (SIS, Muenster, FRG). Pictures were analyzed and the tail moment was calculated employing the macro driven Scion Image Software (Frederick, Maryland, USA).

Proliferation-assay

Proliferation rates for cells treated with hydrogen peroxide (H_2O_2) or tert-butylhydroperoxide (TBHP) were measured with a chemiluminescence assay based on the measurement of BrdU incorporation during DNA synthesis [Cell Proliferation ELISA, BrdU (chemiluminescent), Roche, Mannheim, FRG] according to manufacturer's instructions. Chemiluminescence was detected with a Multilabel Counter Victor2 (PerkinElmer) and related to the amount of DNA measured in parallel using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Karlsruhe, FRG).

Telomere Southern blotting and FACS analysis

Telomere length was analyzed by the Southern blot based TeloTAGGG Telomere length assay (Roche) following the protocol of the manufacturer using 2 μg genomic DNA isolated directly from articular cartilage sample [normal: $n = 5$; mean age 63 yrs (52–72 yrs) and OA: $n = 15$; mean age 64 yrs (44–78 yrs)]. Additionally, telomere length was analyzed by the "telomere PNA kit/FITC for flow cytometry" (Dako) according to the protocol supplied by the manufacturer and measured with a Becton–Dickinson FACSCalibur cytometer [cartilage samples: normal: $n = 7$; mean age 62 yrs (52–70 yrs) and OA: $n = 7$; mean age 67 yrs (57–78 yrs)].

Cell isolation, culture and challenge with DNA-damaging agent

Chondrocytes of normal knee joints [$n = 4$; mean age 56 yrs (40–70 yrs)] were freshly isolated enzymatically as described previously[12] and cultured in high density monolayer cultures (10^5 cells/cm^2) for 48 h and then challenged with 0.025–0.5 mM H_2O_2, 5–10 μM TBHP, 0.1 μg/ml actinomycin D or 0.2 U/ml bleomycin for 16 h. The resulting DNA damage was evaluated by the COMET assay (pH 13.1). The (dis-)coordinate expression of vimentin and S-100 protein was evaluated by FACS analysis as described above. For statistical analysis Wilcoxon signed rang test for paired probes was used.

Statistical analysis

For statistical analysis the non-parametric Mann–Whitney or Wilcoxon signed rang tests were used (using the SPSS-software). "n" indicates independent experiments form different donors (and not technical replicates). P-values <0.05 were considered significant.

Results

OA chondrocytes show a discoordinated expression of S-100 protein and vimentin compared to normal articular chondrocytes

Heterogeneity in gene expression patterns between different samples of OA cartilage has been shown by many studies convincingly. Microheterogeneity within the same sample has been reported anecdotally[8,14], but was never investigated on a systematic level. Therefore, in this study, we selected two genes, vimentin and S-100 protein, which are expressed by nearly all articular chondrocytes[15]. We performed double-immunofluorescence in order to evaluate co-expression levels of both genes in normal and OA articular cartilage sections [$n_{normal} = 17$; $n_{OA} = 16$, Fig. 1(a), Supplementary Figs. 1 and 2]. Co-expression of both genes was confirmed in the vast majority of normal articular chondrocytes and a significant increase in a dissociated expression of both genes in OA cells [Fig. 1(a), lower two panels, Fig. 1(b): normal vs OA: 96.5% (±2.1%) to 90.4% (±3.6%), $P < 0.001$]. Importantly, the distribution of cells with a discoordinated expression pattern was random and not related to any zone or focal areas. The superficial layer in normal and OA cartilage could not be compared as it is missing in OA cartilage.

In parallel, a similar analysis was performed on isolated chondrocytes from eight normal and seven OA cartilages [Fig. 1(c and d)]: co-immunostaining and subsequent FACS analysis revealed similar results ($P < 0.02$).

OA articular chondrocytes show a senescent phenotype

Previous reports have suggested that OA chondrocytes show a senescent phenotype and thus express e.g. the senescence-associated ß-galactosidase[6,16]. Also, large scale gene expression analysis could identify numerous genes related to cellular

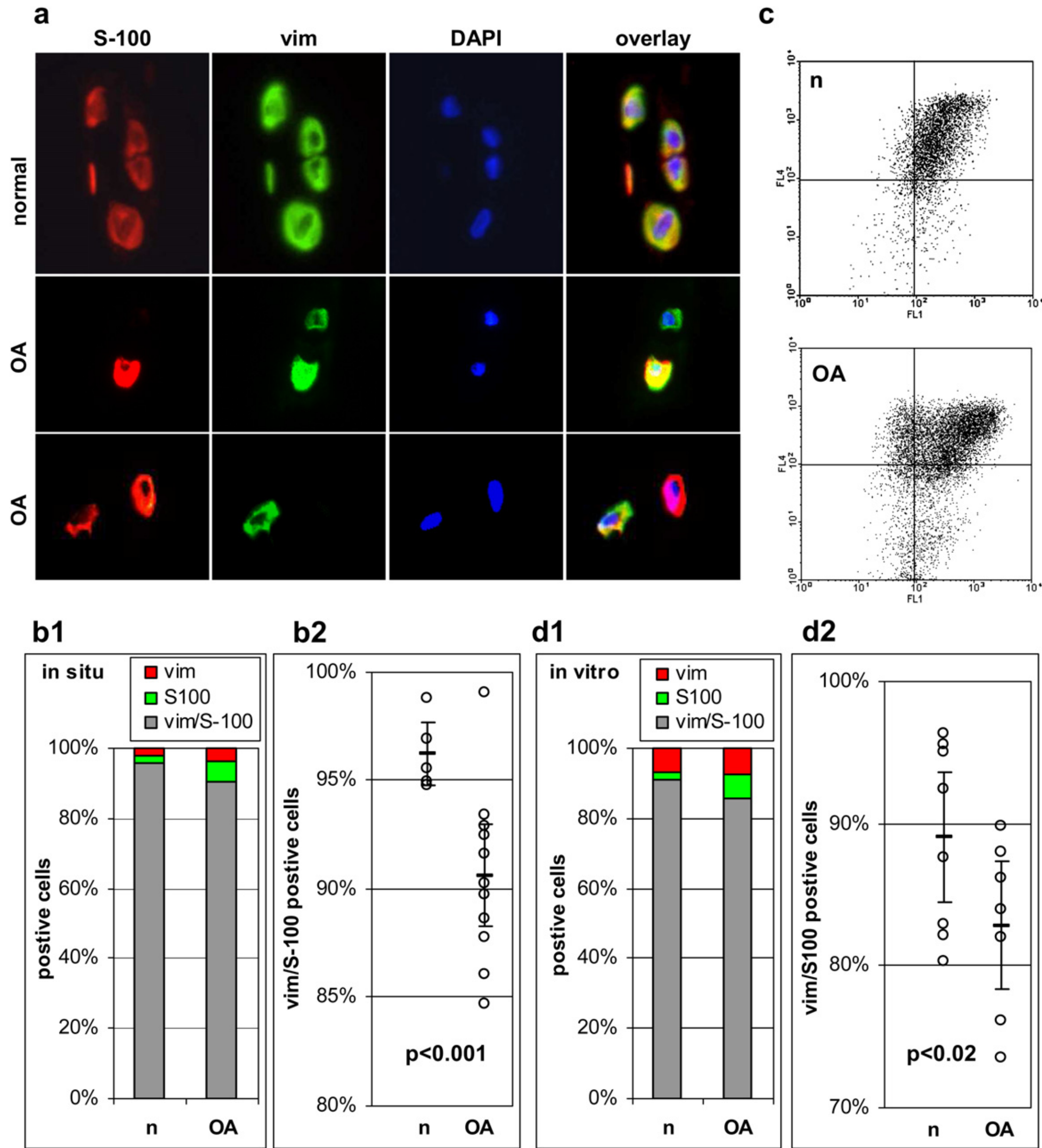

Fig. 1. Analysis of the co-expression of vimentin and S-100 protein by double-immunofluorescence: a: representative samples of normal (n) and OA cartilage tissue (middle panel: one cells show no staining for S-100 protein, lower panel: one cell shows no staining for vimentin); b(1,2): graphical representation of the *in situ* double-immmunofluorescence analysis [b2: representing a dotblot for the single results (S-100-vim-double-positive; the single values, the mean and the 95% confidence-interval are given) represented in b1 as a bar]. c: FACS analysis of the co-expression of vimentin (*Y*-axis) and S-100 protein (*X*-axis) in isolated chondrocytes immediately after isolation. d(1,2): graphical representation of the *in vitro* FACS analysis [d2: representing a dotblot for the single results (S-100-vim-double-positive; the single values, the mean and the 95% confidence-interval are given) represented in d1 as a bar].

senescence and/or integrity[6] (own unpublished data). Therefore, we investigated key characteristics of the two modes of senescence in primary OA *versus* normal articular human chondrocytes: telomere shortening for replicative senescence and accumulating DNA damage for progressive/stress-induced senescence (for review see Shay and Wright[17]).

OA chondrocytes show no critical telomere shortening

First, we investigated the presence of a significant shortening of the telomere length in the OA chondrocytes by Southern analysis. These experiments revealed only a minor shortening of telomere length in OA chondrocytes compared to normal ones [$n_{normal} = 5$; $n_{OA} = 5$; Fig. 2(a and b)]. Of note, no critical telomere shortening [lower than 6–7 kb (for review see Shay and Wright[17])] was detectable. Telomere FACS analysis using the “telomere PNA kit/FITC for flow cytometry” (Dako) confirmed that there is no significant decrease in OA chondrocytes in telomere length (data not shown).

Conventional and real-time PCR revealed no expression of telomerase in any normal and OA cartilage sample (all samples <0.00001/GADPH, data not shown; $n_{normal} = 10$; $n_{OA} = 15$). Also the expression of the Ki67 antigen, which is closely correlated to cell proliferation[18], remained very low, though it was significantly increased compared to the normal [Fig. 2(c); $n_{normal} = 8$; $n_{OA} = 16$; $P < 0.001$].

The genomic integrity is impaired in OA chondrocytes

For determining genomic DNA damage (namely double- and single strand breaks as well as apurinic and apyrimidinic sites)

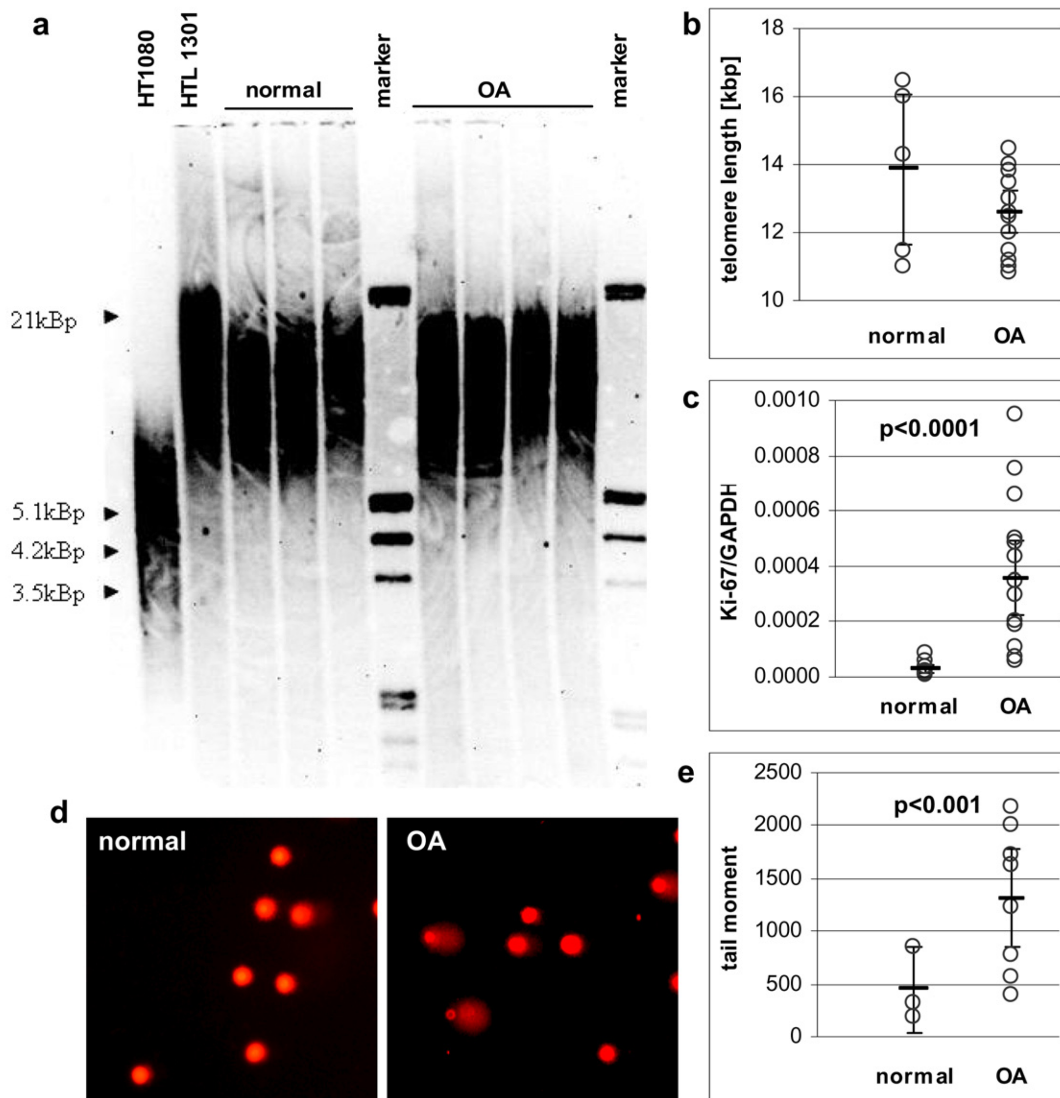

Fig. 2. a, b: Analysis of telomere length by Southern blotting analysis showing no significant loss of telomere length in four analyzed OA *versus* three analyzed normal cases [HT1080: human fibrosarcoma cell line (ATCC: CCL-121 with a known telomere length of 5–7 kb); HTL1301: human T-cell leukemia (HPACC: No 01051619) cell line with known telomere length (>25 kb[34]; b: schematic representation of average telomere length of all experiments). c: Expression analysis of Ki67 mRNA expression by real-time PCR [$n = 10$, mean 64 yrs (45–88 yrs); OA: $n = 15$, mean age 73 yrs (60–85yrs)]. d, e: COMET analysis of chondrocytes directly isolated from normal and OA cartilage (e: schematic representation). b, c, e: the single values, the mean and the 95% confidence-interval are given.

a COMET assay (pH 13.1) with chondrocytes directly isolated from normal ($n = 3$) and OA ($n = 8$) cartilage was performed [Fig. 2(d–e)]. This revealed a significantly higher DNA damage in the OA *versus* the normal articular chondrocytes (tail moment 2.5 fold higher; $P < 0.001$). No correlation to the age of the patients was found, both for normal and OA samples.

Genotoxic stress and genomic DNA damage are able to induce stochastic gene expression in chondrocytes as well as a senescent-like chondrocyte phenotype in vitro

After administration of classical genotoxic agents such as the reactive oxygen species (ROS)-inducer H_2O_2 (0.5 mM) or DNA-damaging agents actinomycin D (0.1 μg/ml) and bleomycin (0.2 U/ml) for 16 h, increased DNA damage in the COMET assay [$n = 4$; Fig. 3(a–e)]. Additionally, treatment with genotoxic agents produced a senescence-like phenotype, observed by the up-regulation of the senescence-associated ß-galactosidase [Fig. 3(f–h)] and the suppression of cell proliferation at higher dosis [Fig. 3(i)], though partly cytotoxic or pro-apoptotic effects cannot be excluded by the technology applied.

Next, we investigated whether random DNA damage is able to induce the heterogeneity of gene expression found within the primary articular chondrocytes. Thus, freshly isolated chondrocytes were subjected to genotoxic (bleomycin, actinomycin D) or oxidative stress (H_2O_2) ($n = 4$; Fig. 4). Both types of stressors led to a strong increase in DNA damage within the cells as confirmed by the COMET assay (pH 13.1) [Fig. 4(b)]. Though we cannot fully exclude an early apoptotic phenotype as reason for the increased DNA fragmentation, no cytomorphological evidence was visible to us in phase contrast microscopy.

FACS analysis after co-immunostaining for vimentin and S-100 protein revealed similar results for all three agents: a significantly ($n = 4$; $P < 0.02$) increased dissociation of gene expression compared to the control (untreated) cells [Fig. 4(c and d)].

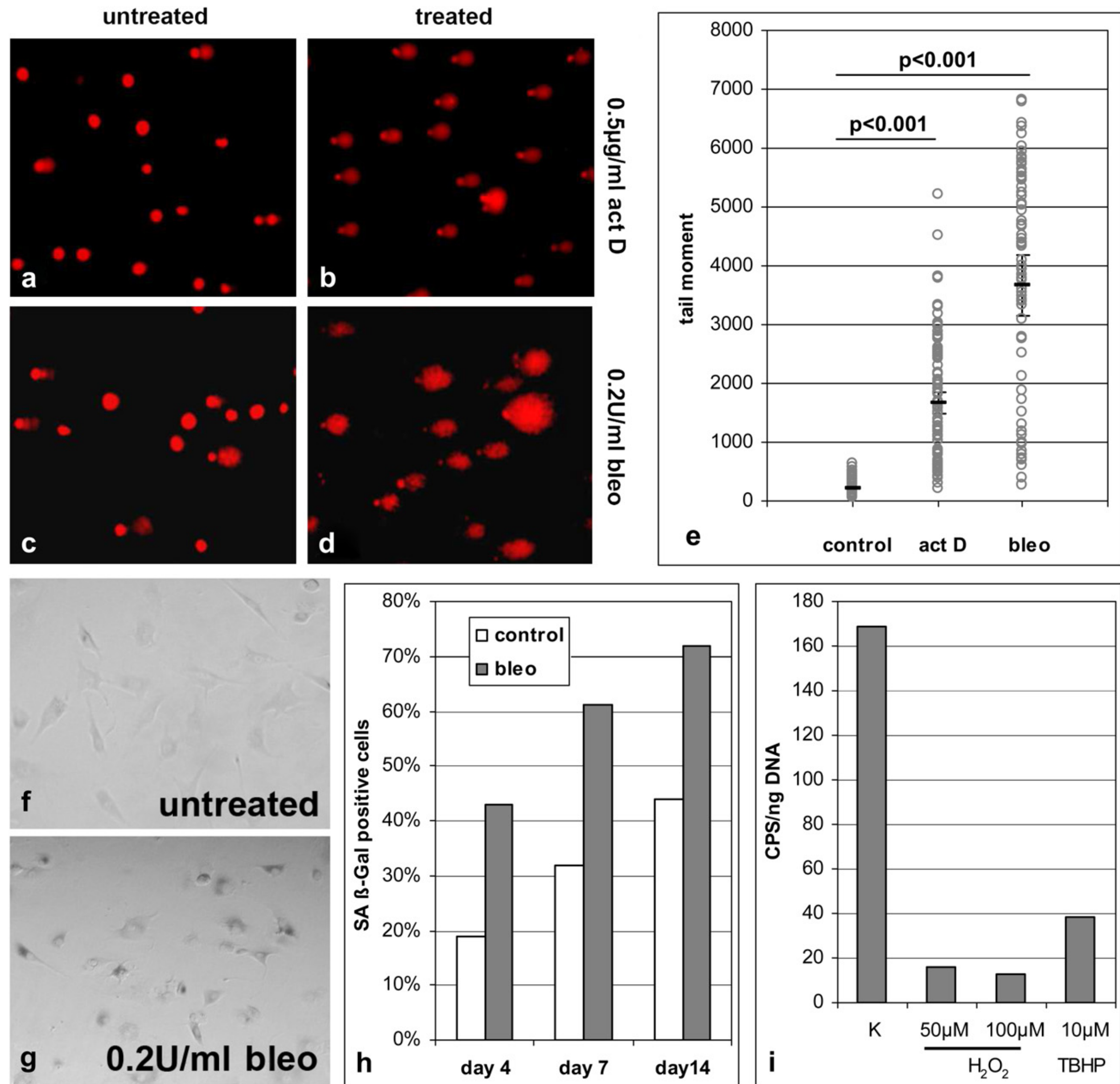

Fig. 3. a–e: COMET assay of normal primary human chondrocytes: a + c: images of control cells, b: image of cells treated with 0.1 μg/ml actinomycin D (act D), d: image of cells treated with 0.2 U/ml bleomycin (bleo), e: evaluation of tail moments in one exemplary case; the single values, the mean and the 95% confidence-interval are given; f–g: β-galactosidase (β-gal) assay of primary human chondrocytes, f: image of control cells, g: image of cells treated with 0.2 U/ml bleomycin, h, i: evaluation of % β-gal(+) cells (h) and proliferative activity (i) of chondrocytes treated with H_2O_2 or TBHP (CPS: counts per second) in one exemplary case.

Discussion

In our study, we present the first systematic experimental evidence using two selected genes that suggests that OA chondrocytes show a significantly increased discoordinated gene expression compared to normal cells. This follows up a general impression of heterogeneous gene expression in OA chondrocytes from previous studies, which however presented only anecdotal evidence involving many genes such as Smad proteins, mediators of the mitogen-activated protein (MAP) kinases signaling cascade etc.[7,8,19]. Though clearly, all these genes can be individually down-regulated by many different mechanisms, the completely patchy distribution of these abnormal cells in our study explicitly argues against any systematic (zonal, focal, etc.) regulation. This is particularly true for molecules such as vimentin and S-100 protein, which are as yet unknown targets of regulators within the cells, but are thought to be constitutively expressed in adult chondrocytes[7]. Obviously, our data ask for extension addressing other genes as well in future studies. Also, the analysis of gene expression *in situ* on the mRNA level, another option which might provide more direct evidence of chaotic gene expression, represents at first sight an appealing technical alternative. However, *in situ* hybridization analysis appears not to be technically feasible, most of all because the sensitivity of the technology is not high enough to pick up gene expression levels of most genes in normal (and OA) articular chondrocytes.

OA chondrocytes display a degenerative or “senescent” cellular phenotype[6,20,21] and are more fragile both in terms of vitality as demonstrated by high rates of cell death after cell isolation *in vitro*[22,23] and their phenotypic instability *in vivo*[7]. They also show increased expression of markers of cellular

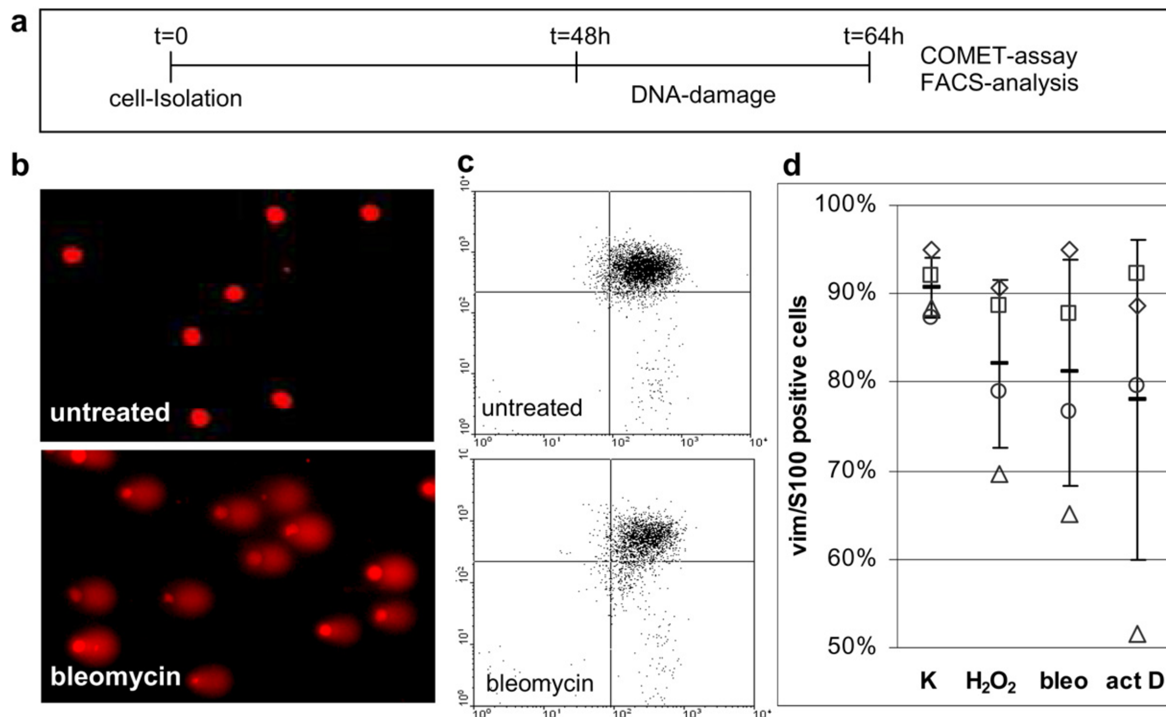

Fig. 4. a: Analysis of the co-expression of vimentin and S-100 protein using FACS analysis after oxidative (500 μM H_2O_2) and genotoxic stress [0.1 μg/ml actinomycin (act D) D, 0.2 U/ml bleomycin (bleo)]: freshly isolated normal primary articular chondrocytes were cultured for 48 h in high density monolayer culture and subsequently exposed to the oxidative and genotoxic stressors for 16 h. b: The resulting DNA damage was evaluated by the COMET assay (pH 13.1). c: The (dis-)coordinate expression of vimentin and S-100 protein was evaluated by FACS analysis (vimentin: *Y*-axis; S-100 protein: *X*-axis). d: graphical evaluation of the FACS analysis [$n = 4$; mean age 56 yrs (40–70 yrs)]; the single values, the mean and the 95% confidence-interval are given.

senescence such as senescence-associated ß-galactosidase[24]. Still, OA chondrocytes show no evidence of classical replicative senescence in general: no critical telomere shortening was observed in our and other studies[25–27]. Despite the absence of telomerase expression in adult articular chondrocytes[25,26] this is not surprising for a largely post-mitotic cell such as the adult chondrocyte. Even in OA cartilage, very low proliferative activity is found[14]. The expression of Ki67, a proliferation specific antigen, is increased, but still very low in OA chondrocytes. This minor increase in proliferative activity might be reflected in the slight reduction in telomere length found in OA cells[14,28], as adult chondrocytes do not express telomerase. Alternatively, this slight reduction might also be mainly related to stress-induced telomere derangement. Obviously, the situation would be very different in childhood and adolescence, during which chondrocytes proliferate and loose some telomere length[24].

Our data suggest that there is a significant increase in DNA damage in OA chondrocytes. A similar observation was recently reported for a pig model of cartilage degeneration[29]. Though the observed DNA damage might not directly lead to mutational protein alterations; it might modulate or even inhibit gene transcription activity e.g., by changing the binding capacity of transcription factors. In most cells, significant DNA damage would induce apoptotic cell death, which is, however, rather rarely encountered in OA chondrocytes[14]: again this supports a senescent phenotype of OA chondrocytes, as one characteristic of senescent cells is their resistance to apoptotic cell death[30].

In this study, we show that DNA damage induces a senescent-like phenotype in chondrocytes *in vitro* with typical core features such as expression of senescence-associated ß-galactosidase and inhibition of proliferative activity. However, future studies have to further characterize the type of senescence induced. Whereas this awaits further studies, we show in this work that H_2O_2 is not only able to induce DNA damage, but also gene expression heterogeneity at least in two model molecules similar to that observed in OA chondrocytes. The basic finding suggested by our data, that increased stochastic DNA damage leads to diversification of gene expression (i.e., a stochastic or chaotic gene expression) is not specific for OA chondrocytes, but has also recently been shown to occur in cardiac myocytes[31]. These results in two different cellular systems, both largely post-mitotic in the adult, support the concept that a gradual increase in stochastic DNA damage and consequently in transcriptional noise introduces phenotypic variation and instability among the aged myocardiocytes and chondrocytes. This would explain many features, particularly the heterogeneity of chondrocyte behavior[32] leading to the progressive decline in joint cartilage function. The study on cardiomyocytes aging and our data stress the notion that the diversification of gene expression due to stochastic DNA damage – besides regular cell functioning – is a core mechanism in cellular aging in general and in OA cartilage degeneration in particular.

The observed rather limited dissociation of gene expression after DNA damage *in vitro* (<10%) as well as *in vivo* (in OA articular cartilage) is to be expected: a higher aberration rate in single genes would not be compatible with the survival of cells in any condition even if one assumes redundancy of cellular pathways vital for the cells. This limited DNA damage might allow the chondrocytes to survive largely in normal conditions in which the cells do not need to fulfill complex activities (except maintaining minimal cellular function to provide low-level matrix turnover). However, these minor alterations might be disastrous in the case of complex cellular activities of the cells such as cartilage restoration after tissue destruction: minor malfunctioning accumulates to major disregulation as soon as a large number of transcription factors, matrix protein genes etc. are involved all being exposed to potential genetic damage.

In summary, we present experimental evidence for an aging cell phenotype of OA chondrocytes, induced by a random genomic DNA damage accumulation over time, most likely due to continuous or even accelerated oxidative stress (for review see Afonso *et al.*[33] and by Henrotin *et al.*[34]) caused by a decline in the oxidative defense of OA chondrocytes[6]. We suggest that the senescent cell phenotype is different from replicative senescence proposed in previous studies[28,35], which might still contribute in special circumstances such as post-traumatic OA. Most likely, OA chondrocytes show progressive/stress-induced senescence, progressing over time due to accumulated DNA damage and subsequent chaotic gene activation pattern (and not telomere shortening). A discoordinated gene expression on the single-cell level as shown for OA chondrocytes in this study is exactly one core characteristic of progressively senescent cells[31].

Altogether, primary OA is certainly based on a genetic background/predisposition and the cells follow *per se* basic biological/biochemical rules. In contrast to chondrodysplastic joint degeneration, which is mainly explained by this, primary non-inherited OA is suggested to be additionally driven by the progressive aging of the cells due to accumulating stochastic DNA damage leading to genetic diversity, heterogeneous gene expression and finally failing of cell and tissue homeostasis.

Author contributions

JR, SSo, CS, NS and SSe contributed to the collection and assembly as well as analysis and interpretation of the data. PMG contributed to the analysis and interpretation of the data and provided statistical expertise. TA was responsible for the conception and design of the study, the interpretation of the data, obtaining of funding.

All authors were involved in drafting or revising the article and approved the final manuscript.

Role of the funding source

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Conflict of interest

The authors declare that they have no competing interests.

Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.joca.2012.05.009.

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