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OSTEOARTHRITIS and CARTILAGE

Complexity of IL-1β induced gene expression pattern in human articular chondrocytes

By Daniel Margerie*, Johannes Flechtenmacher†, Frank H. Büttner*, Alfred Karbowski‡, Wolfhart Puhl†, Rudolf Schleyerbach* and Eckart Bartnik*

**Hoechst AG, Kalle-Albert, Biomedical Research, D-65174 Wiesbaden; the †Department of Orthopaedic Surgery, University of Ulm, Ulm; and the ‡Department of Orthopaedic Surgery, University of Mainz, Mainz, Germany*

Summary

The mRNA fingerprinting technique, differential display reverse transcription polymerase chain (DDRT-PCR), was used to detect changes in the overall pattern of gene expression in human articular knee chondrocytes induced by interleukin-1β (IL-1β), the prototypical inducer of catabolic responses in degenerate joint diseases. One hundred different primer combinations generated approximately 10 000 different PCR fragments for IL-1β treated, as well as for untreated human chondrocytes, cultivated in alginate beads. This represented 53% of all expressed chondrocyte genes as based on statistical considerations. Side by side comparisons of differential display patterns originating from two different donor tissues yielded 44 reproducibly, differentially-displayed cDNA fragments, which were subcloned and sequenced. Sequence homology searches revealed sequence identities to the human necrosis factor α (TNF-α) and IL-1 regulated gene TSG-6, fibronectin, osteopontin, calnexin, and the DNA repair enzyme ERCC5. The differential expression was confirmed with Northern and quantitative PCR analyses. The known function of these genes and their known IL-1 responsiveness indicate that the employed model system reflects the pleiotropic effects of IL-1 on the overall gene expression in human articular chondrocytes and identifies genes involved in very different biochemical pathways. Twenty-seven cDNAs lacked sequence homologies to known genes and may represent novel genes.

Key words: Differential display, Chondrocytes, IL-1, Osteoarthritis.

Introduction

The expression of specific genes is changed during the course of degenerative joint diseases like osteo- and rheumatoid arthritis [1]. Those genes include the matrix-degrading matrix metalloproteinases (MMPs), which are upregulated during degradative processes [2] and the structural matrix components like the aggrecan core protein, which are downregulated [3].

The differential expression of various early and late responsive genes including c-fos, c-jun, junB and metallothionin [4] suggests that changes in gene expression affect a multitude of disease regulatory and metabolic pathways.

In our study, we wanted to test the usefulness of the mRNA fingerprinting technique of differential display reverse transcription polymerase chain reaction (DDRT-PCR) to detect changes not only in the expression of specific, single genes but changes in the overall gene expression, reflecting the pleiotropic action of the cytokine interleukin-1 (IL-1) on various metabolic processes. DDRT-PCR was originally described by Liang and Pardee [5] and further refined by Liang *et al.* [6] and Bauer *et al.* [7]. This new tool promises access to low abundant transcripts, which are difficult to detect with conventional subtractive hybridization studies, and it permits the simultaneous identification of both up- and down-regulated genes.

In order to gain an understanding of ethiopathological processes leading to cartilage degradation, the most direct approach would be a comparison between the gene expression pattern in macroscopically intact versus osteoarthritic cartilage. But such studies would also resolve patient specific differences in the expression of genes, which could potentially conceal disease relevant changes in gene expression. This lead us to start differential gene expression studies with a practicable model system comparing the gene expression in IL-1β stimulated and unstimulated, cultured human articular knee chondrocytes obtained from joint replacement surgery.

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Address correspondence and reprint requests to: Dr E. Bartnik, Hoechst Marion Roussel, Kalle-Albert, Biomedical Research, H 528, Rheingaustr. 190, D-65174 Wiesbaden, Germany.

The most important reasons for choosing IL-1β as the mediator were that it acts on many connective tissue cell types, and that IL-1 is the prototypical inducer of catabolic responses in chondrocytes [8]. It stimulates expression of proteases, including MMPs [9], leading to degradation and release of proteoglycans (PGs) [3, 10, 11], induces an increase of the constitutive hsp70 [12], of IL-8 [13], COX-2 and PLA_2 [14] mRNA. In addition, IL-1 suppresses α1 type II procollagen expression [15] and it inhibits synthesis in chondrocytes [3, 10, 16–19]. The net result of IL-1 treatment of cartilage tissue *in vitro* [12, 20], or the injection of IL-1 into the synovial cavity [21, 22], is loss of cartilage matrix by a combination of excessive catabolism and inadequate anabolism of matrix macromolecules for tissue repair. These results and the presence of IL-1, PG fragments and proteolytic enzymes in inflamed joints [23–25], point to an important role for IL-1 in cartilage degradation in rheumatoid arthritis and during inflammatory episodes in osteoarthritis.

Quantitative PCR served to validate our model system by detecting an IL-1β mediated upregulation of MMP-3 message. To optimize reproducibility and sensitivity of our chondrocyte gene expression analysis, we employed in parallel the large number of 100 different primer combinations for DDRT-PCR on chondrocytes originating from two different tissue donors. Thus, we were able to detect 44 reproducibly, differentially-expressed cDNA fragments. Subsequent cloning and sequence analysis revealed the identity of some cDNAs to known genes, including fibronectin and TSG-6, already known to be upregulated by IL-1.

The identification of these genes allows interesting speculations on the specific role of these genes in the IL-1β mediated chondrocyte metabolism. In addition, this approach may eventually enable the identification of new target molecules for drug intervention and candidate genes to monitor chondrocyte metabolism during clinical studies of degenerative joint diseases.

Materials and Methods

EXPERIMENTAL PROCEDURES

Cell culture

u Articular knee cartilage specimens were obtained from four different tissue donors (donor A: male 65-years old, donor B: female 73-years old, donor C: female 60-years old, and donor D: female 63-years old) undergoing total joint replacement surgery for osteoarthritis. None of these individuals had received treatment by radiation or chemotherapy. After surgery, articular cartilage slices were aseptically dissected from both femoral condyles, tibia plateaus and patellae and subjected to sequential enzymatic digestion with pronase (Boehringer Mannheim) and collagenase (Worthington) according to Häuselmann *et al.* [26]. Released chondrocytes were suspended in sterile 0.15 M NaCl containing 1.25% low viscosity alginate (FMC BioProducts) at a concentration of 4×10^6 cells/ml, and squeezed through a 22 gauge needle into 102 mM $CaCl_2$ solution to form cell entrapping spherical beads containing an average number of $44 \pm 2\times10^3$ chondrocytes. A total number of 2×10^7 cells were maintained in 50/50 HAMS' F12/DMEM (Life Technologies), supplemented with 10% heat inactivated fetal calf serum (Sigma), 25 μg/ml ascorbic acid (Sigma) and 50 μg/ml gentamycin (Life Technologies) in a humidified atmosphere of 5% CO_2 at 37°C. Cultures were allowed to recover for 3 days with daily medium changes. Then, the beads were subdivided into two populations for further 3 days of culture in the presence or absence of 50 pg/ml recombinant human IL-1β (Genzyme). To release chondrocytes from alginate, beads were dissolved in dissolution buffer containing 55 mM sodium citrate, 30 mM EDTA and 0.15 M NaCl and placed at room temperature for 10 min. Viability of cells was checked by eosin-red exclusion.

Primer syntheses

Arbitrary oligodecamer primers 1–10 and degenerate anchored oligo-dT primers ($T_{12}MN$) were synthesized using the 392 DNA synthesizer (Applied Biosystems) and purified by denaturing polyacrylamide gel electrophoresis. Some oligodecamer primers, 11–25, were purchased from Biometra. All employed primers are shown in Table I.

RNA isolation and first strand cDNA synthesis

Total RNA from cultured human articular chondrocytes was prepared according to the acid guanidinium–isothiocyanate phenol–chloroform method [27] and incubated with 10 U RNase free DNase I (Life Technologies) in 20 mM Tris-HCl (pH 8.3), 50 mM KCl and 2.5 mM $MgCl_2$ for 30 min at 37°C to remove any residual chromosomal DNA contamination. After extraction with phenol–chloroform (3:1), the supernatant was ethanol precipitated in the presence of 0.3 M NaOAc (pH 5.2), and the RNA was finally redissolved in diethyl pyrocarbonate treated water. Integrity of the RNA

was checked on formaldehyde–agarose gels. Total RNA (0.4 μg) were reverse transcribed using 200 U superscript reverse transcriptase (Life Technologies) in a 40 μl reaction volume containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, dNTP mix (dATP, dTTP, dCTP, dGTP) of 200 μM each, 40 U RNase Inhibitor (Boehringer Mannheim) and 2.5 μM degenerate oligo-dT primer at 37°C for 1 h. Reactions were terminated by heating to 95°C for 5 min. For quantitative PCR, first strand cDNA synthesis was performed from total RNA of tissue donor D using a cDNA synthesis kit (Boehringer Mannheim).

Differential display

cDNAs were amplified in a DNA thermal cycler (Perkin Elmer, model 480) in 20 μl reactions containing 2.5 μM of the original T_{12}MN-primer used in cDNA synthesis in combination with 0.5 μM arbitrary upstream primer, dNTP mix (dGTP, CTP, dTTP) of 0.5 μM each, 10 μCi α-[^{35}S]dATP (1000 Ci/mmol, 10 mCi/ml; Amersham), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin and 2.5 U AmpliTaq DNA polymerase (Perkin Elmer). Light mineral oil was overlaid and thermal cycling was performed as follows: 30 s of denaturation at 94°C, 2 min of annealing at 40°C, 30 s of extension at 72°C for 40 cycles followed by 5 min postextension at 72°C. For gel electrophoresis, each PCR product was mixed with 5 μl dye solution (95% formamide, 20 mM EDTA, 0.05% bromphenolblue and 0.05% xylenecyanol FF), denatured at 95°C for 2 min and quenched on ice. Each sample (6 μl) was loaded on a 6% acrylamide/7 M urea gel (35×43×0.4 cm). For detection of radiolabeled PCR fragments, gels were transferred to blotting paper without acetic acid–methanol fixation; dried gels were exposed to X-ray film (Kodak X-OMAT) for 48 h.

Table I
List of all degenerate 3′ oligo dT-primers [T_{12}MN] and all arbitrary 5′ oligodecamer-primers used for DDRT-PCR

Degenerate 3′ oligo dT-primers [T_{12}MN]	
T_{12}MA	5′-T T T T T T T T T T T T M A
T_{12}MT	5′-T T T T T T T T T T T T M T
T_{12}MG	5′-T T T T T T T T T T T T M G
T_{12}MC	5′-T T T T T T T T T T T T M C
M = dA, dG, dC	
N = dA, dT, dG, dC	
Arbitrary 5′ oligodecamerprimers	
U1	5′-G G T C C C T G A C
U2	5′-G A A A C G G G T G
U3	5′-G T G A C G G G T G
U4	5′-G C G T A A C G C C
U5	5′-G T G A T C G C A G
U6	5′-A G C C A G C G A A
U7	5′-G A C C G C T T G T
U8	5′-A G G T G A C C G T
U9	5′-C A A A C G T C G G
U10	5′-G T T G C G A T C C
U11	5′-T A C A A C G A G G
U12	5′-T G G A T T G G T C
U13	5′-C T T T C T A C C C
U14	5′-T T T T G G C T C C
U15	5′-G G A A C C A A T C
U16	5′-A A A C T C C G T C
U17	5′-T C G A T A C A G G
U18	5′-T G G T A A A G G G
U19	5′-T C G G T C A T A G
U20	5′-G G T A C T A A G G
U21	5′-T A C C T A A G C G
U22	5′-C T G C T T G A T G
U23	5′-G T T T T C G C A G
U24	5′-G A T C A A G T C C
U25	5′-G A T C C A G T A C

Elution and reamplification of cDNAs

Careful side by side comparisons of band patterns identified those PCR fragments that appeared differentially expressed. Those were cut from acrylamide gels, transferred into eppendorf tubes and rehydrated for 10 min with 100 μl 10 mM Tris-HCl (pH 7.5), 1 mM EDTA at room temperature. After boiling the gel slices for 15 min, PCR fragments were recovered by ethanol precipitation in the presence of 0.3 M NaOAc and 20 μg glycogen (Boehringer Mannheim) as a carrier and redissolved in 10 μl sterile water. Five microliters of this volume was used for reamplification by PCR using appropriate primers and conditions as described above except for dNTP concentration at 20 μM and omission of radioisotopes. Reamplified cDNAs were visualized by electrophoresis on a 2% agarose gel, cut out and eluted from the gel matrix by ultrafiltration using spin columns (Millipore). Purified cDNAs were then either used for cloning or as probes for Northern-blot analysis.

Cloning and sequencing of cDNAs

Reamplified cDNA probes were cloned into the pCRII-vector using the TA cloning system (Invitrogen). Plasmid DNA sequencing of subcloned cDNAs with either Sp6 or T7 primer was carried out on both strands using sequenase 2.0 sequencing kit (USB).

DNA sequence analysis

Searching for homology between subcloned cDNAs and sequences already listed in one of the DNA databases (GenBank release 91, DDBJ and EMBL database) was performed using the FASTA

program developed by Pearson and Lipman [28] included in the GCG software package (Genetics Computer Group, Madison, U.S.A.). Alternatively, a newer program for rapid similarity searches was applied [29] using the Basic Local Alignment Search Tool (BLAST) at NCBI (National Center for Biotechnology Information).

Quantitative PCR

For quantitative PCR, a 598 bp internal standard containing gene specific primer sites for MMP-3 (5'-CACTTCAGAACCTTTCCTGGCATC-3' and 5'-GCTTCAGTGTTGGCTGAGTG-3', corresponding to nts 338–361 and 725–744) and a 596 bp internal standard containing gene specific primer sites for TSG-6 (5'-GGTGTGTACCACAGAGAAGCA-3' and 5'-GGGTTGTAGCAATAGGCATCC-3', corresponding to nts 174–194 and 437–457) was constructed using the PCR-MIMIC kit (Clontech). Then, serial dilutions of the internal MMP-3 standard (fivefold dilutions from 1000–1.6 attomol) respectively of the internal TSG-6 standard (twofold dilutions from 0.5–0.016 attomol) were added to the PCR reactions containing constant amounts of cDNA reverse transcribed from 20 ng of total RNA from either stimulated or not stimulated cells as described above. PCR was performed in 50 µl reactions in 1× PCR-buffer (10 mM Tris-HCl at pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatin) with 200 µM dNTP mix, 0.4 µM primer and 2.5 U AmpliTaq DNA polymerase (Perkin Elmer). Thermal cycling was performed as follows: 30 s of denaturation at 94°C, 30 s of annealing at 60°C and 45 s of extension at 72°C for 25 cycles. Ten microliters of each PCR tube was separated on a 2% agarose/ethidium bromide gel and fluorescence was quantified by computer imaging using BioImage software (Millipore) to calculate the relative amounts of target and standard-DNA. The logarithm of the ratios of the two products were then plotted after correcting for the difference in size between them as a function of the log of the initial amount of standard-DNA added to each reaction to calculate the point of equimolarity of standard and target DNA.

Northern-blot analysis

Isolation of RNA from cultured chondrocytes used for Northern-blot analysis was performed exactly as described above. For gel separation, 10 µg of total RNA were denatured by heating at 65°C for 10 min in a solution of 50% formamide, 20 mM MOPS and 2.2 M formaldehyde, separated through 1% agarose containing 2.2 M formaldehyde and transfered to a positively charged nylon membrane (Amersham) by standard capillary blotting procedures [30]. After U.V. cross-linking, the blots were prehybridized for 1 h in rapid-hyb-buffer (Amersham) at 65°C. The following probes, obtained by reverse transcription (RT)-PCR from human articular chondrocytes, were radiolabeled for hybridization with α-[^{32}P]dCTP (3000 Ci/mmol, 10 mCi/ml; Amersham) using random nonamer primers (Amersham): A 330 bp cDNA corresponding to nts 61–390 from human osteopontin cDNA (GenBank J04765), a 340 bp cDNA corresponding to nts 881–1220 from human calnexin (GenBank M94859), a 289 bp cDNA corresponding to nts 4292–4580 from human fibronectin. (GenBank X02761), a 226 bp cDNA corresponding to nts 597–822 from human TSG-6 (GenBank M31165) and a 263 bp cDNA corresponding to nts 3097–3359 from human ERCC5 (GenBank D16305). After hybridization for 2 h at 65°C the blot was subsequently washed in 2×SSC, 0.1% SDS at 37°C for 15 min (1×SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), followed by two sucessive washes with 1×SSC, 0.1% SDS at 65°C for 10 min respectively. If necessary, a final high stringency wash was performed with 0.1×SSC, 0.1% SDS at 65°C for 15 min. The blots were then analyzed by autoradiography using Kodak X-Omat films at –80°C with intensifying screens for 2–7 days and intensity of bands was quantified with a phosphorimager (Biorad). All blots were stripped with boiling 0.5% SDS solution and reprobed with labeled murine β-actin cDNA (Clontech) to demonstrate equal loading of RNA in each lane.

Results and Discussion

THE USED MODEL SYSTEM AND ITS VALIDATION

To detect IL-1β mediated changes in the overall gene expression potentially reflecting processes connected with cartilage degradation, we compared the mRNA profile of human articular knee chondrocytes treated with and without IL-1β. For this, human knee cartilage from joint replacement surgery was enzymatically digested to yield approximately $3–5 \times 10^6$ chondrocytes/g of cartilage tissue. Embedded in alginate, human articular chondrocytes maintain their differentiated phenotype [26] and show almost identical behavior to cartilage slice cultures when treated with IL-1β. We, therefore, chose the alginate embedding and culture conditions as given by Häuselmann *et al.* [31]. For the choice of the employed IL-1β concentration of 50 pg/ml we were guided by the finding that this concentration leads to a 85% inhibition of ^{35}S-sulfate incorporation into PG

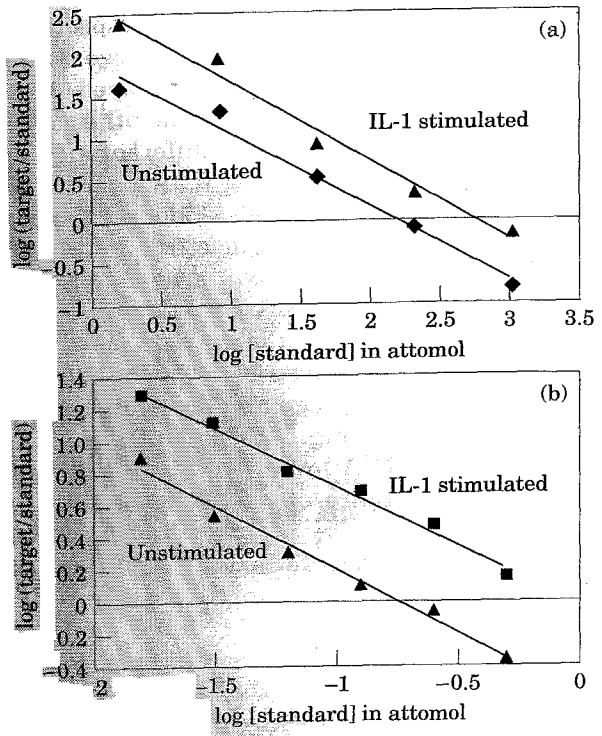

FIG. 1. Analysis of the results of quantitative PCR experiments to determine MMP-3 (a) and TSG-6 (b) mRNA steady state levels from chondrocytes of donor D. The log of the ratio of amplified target to standard products is graphed as a function of the log of the known amount of standard added to the PCR reaction.

macromolecules as compared with untreated control cultures [31] and by the *in vivo* concentrations of IL-1 in the synovial fluids of patients with osteoarthritis, which ranges from 20–128 pg/ml [32].

After 3 days of preculture to 2×10^7 chondrocytes, alginate beads were subdivided into two populations, one was stimulated with 50 pg/ml recombinant human IL-1β for 72 h, while the other was maintained in culture medium without IL-1β, before both populations underwent DDRT-PCR analysis.

To examine the IL-1β responsiveness of alginate embedded chondrocytes in our model system, we determined the relative amount of MMP-3 transcripts in both cell populations. Because IL-1 is known to stimulate the expression of MMP-3 in many connective tissue cells, including synoviocytes and chondrocytes [22, 33], this criterium should suffice to provide the basis for our differential display analysis. To quantify MMP-3 mRNA steady-state level, quantitative, competitive PCR (QPCR) using an internal standard was employed [34]. Fig. 1(a) shows the log of the ratios of the PCR products specific for MMP-3 and the standard-DNA graphed as a function of the initial amount of standard-DNA added to the PCR reactions for the determination of the point of equimolarity. Using this approach, we determined in three independent experiments 4827 ± 792 ($N=3$) MMP-3 mRNA molecules/pg of total RNA for unstimulated chondrocytes, and $17{,}188 \pm 2066$ ($N=3$) mRNA molecules per pg of total RNA for IL-1β stimulated chondrocytes, an upregulation by a factor of 3.6. Thus, chondrocytes cultivated in alginate from patients with osteoarthritis undergoing joint replacement surgery, do indeed respond to IL-1β with an upregulation of MMP-3 message.

DIFFERENTIAL DISPLAY ANALYSIS

A total of 2×10^7 chondrocytes released from the alginate beads yielded 40 μg total RNA, which was subsequently subjected to DDRT-PCR analysis. Because our goal was to detect changes in the overall gene expression, we wanted to resolve as many differentially expressed genes as possible and thus employed the large number of 100 different primer combinations in order to increase our chances to detect weaker expressed genes. The used primer combinations comprised four different degenerate 3'oligo dT-primers and 25 different 5'oligodecamer primers, as given in Table 1. They resolved a total of approximately 10 000 cDNAs on high resolution acrylamide gels for each cell population analyzed. According to theoretical calculations [35] this amount and length of primers should represent 53% of all expressed cellular genes. Fig. 2 shows some examples of differential displays resolving approximately 80–120 cDNA fragments per single lane. The visual side by side comparison between lanes resolving cDNA fragments generated from IL-1β stimulated versus unstimulated chondrocytes identified a total of 123 recognizable differentially displayed cDNA fragments.

To address the reproducibility and the major drawback of DDRT-PCR, its 'noise level' of false positives [6], five primer pairs were used repeatedly in initial experiments on the same cDNA preparation. Although some minor variances were seen, probably due to the noise level of DDRT-PCR, most bands were reproducibly displayed. Primer combinations differed to some extent in their ability to yield similar overall band patterns (see Fig. 3 and note differences in reproducibility between primer pairs I and II). Because we expected that patient specific differences and polymorphisms in gene products would influence DDRT-PCR results more than PCR reproducibility, we validated our differentially displayed fragments stringently by applying the same 100 primer combinations to RNA originating from a second

tissue donor. Fig. 3 compares the DDRT-PCR analysis with two different primer pairs on the RNA from two different tissue donors. All possible combinations of bands can be seen: bands occuring in all lanes, irrespective of both IL-1β stimulation and tissue donor; bands appearing irrespective of stimulation, but specific for one of the two donors; IL-1β dependent differentially displayed bands specific for one donor, but not for the other donor; and finally bands, which were differentially and reproducibly displayed by using RNA from the two different donors. Of the latter, we identified a total of 52 bands eliminating 71 bands from our initial screen. Of the 52 bands, 68% arose from IL-1β stimulated chondrocytes. As seen in Fig. 3 (arrows), candidate bands differ in their signal intensities. The differentially displayed band in Fig. 3(a) and (b) is much stronger upregulated by IL-1β, than the differential band in Fig. 3(c) and (d). This may reflect cellular mRNA levels or it is based on the nonquantitative nature of the PCR amplification process itself. The size of the 52 differentially displayed cDNA fragments varied from 175–600 bp, with an average size of approximately 300 bp. Forty-four of the initial 52 fragments were subcloned into the TA cloning vector pCRII and sequenced. Fig. 4 depicts the differentially displayed bands later identified to correspond to TSG-6, fibronectin, osteopontin and calnexin.

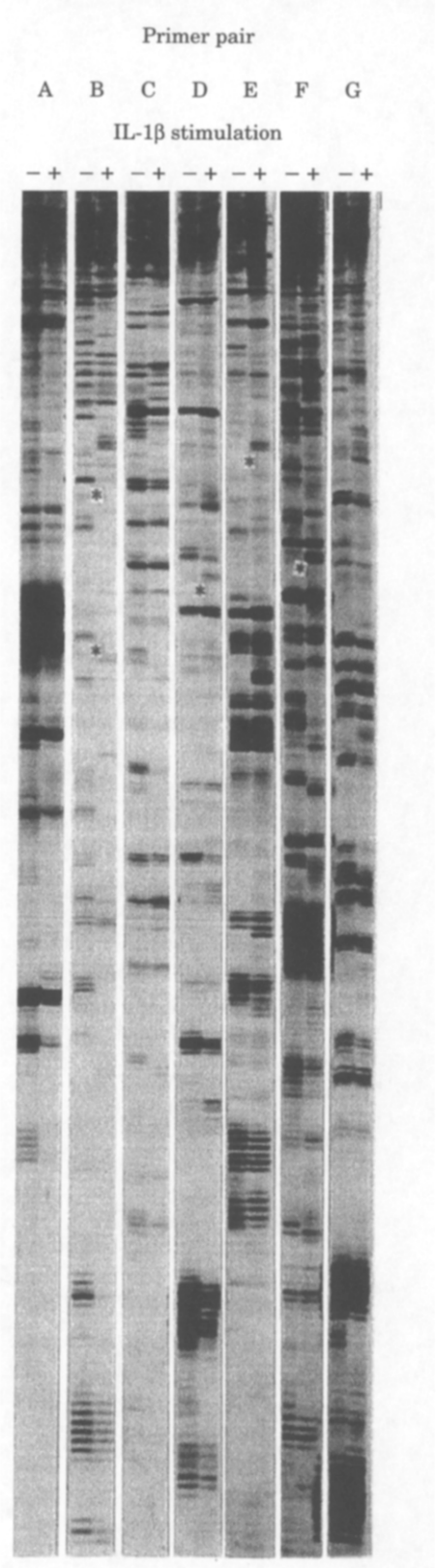

FIG. 2. Examples of differential displays for the detection of IL-1β induced changes in overall gene expression patterns in human articular knee chondrocytes cultivated in alginate beads. Total RNA derived from chondrocytes of donor B stimulated for 72 h with IL-1β (+) and RNA from not stimulated cells (–) was subjected to DDRT-PCR using following primer combinations: A = T_{12}MG and U22; B = T_{12}MG and U23; C = T_{12}MG and U24; D = T_{12}MG and U25; E = T_{12}MC and U22; F = T_{12}MC and U24; G = T_{12}MC and U25. Examples of differentially expressed cDNAs which could be reproduced using RNA from donor A are denoted with asterisks.

GENES IDENTIFIED THROUGH HOMOLOGY SEARCHES AND THEIR POTENTIAL ROLE IN IL-1β MEDIATED PROCESSES

Sequence homology searches revealed that 27 sequences did not match with sequences in the data bases and thus could represent new genes. Nine sequences matched expressed sequence tags (ESTs), gene fragments identified in human genome sequencing projects without functional assignments. Five sequences showed identity with the following known human genes (GenBank accession number and our clone identifier given in brackets): fibronectin (X02761, TTO20/1);). TSG-6: tumour necrosis factor (TNF)-stimulated gene-6 (M31165, TAU7/2); osteopontin (J04765, TAU1/1); calnexin (M94859, TTU2/2) and ERCC5 (D16305, TTO16/2).

The known functions of these genes and their known IL-1 responsivness, reproduced in our

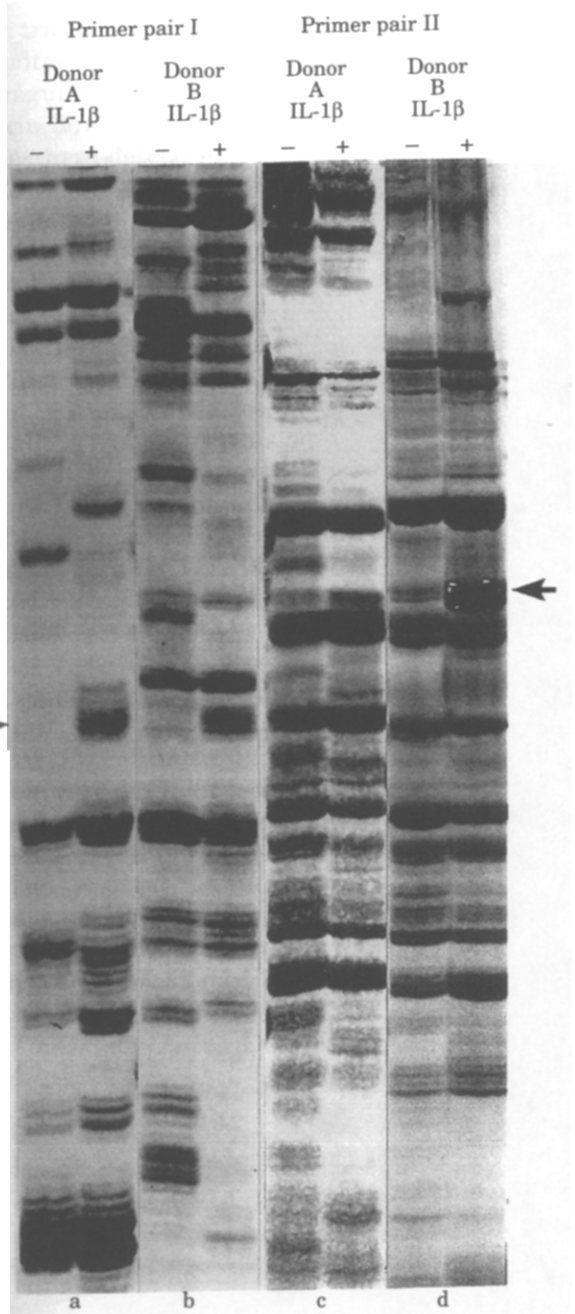

FIG. 3. Differential display analysis as in Fig. 2 using two different primer combinations (I = T_{12}MC and U23; II = T_{12}MC and U24) to reveal individual differences in the IL-1β induced gene expression pattern between two different tissue donors (donor A and donor B). Arrowheads denote reproducibly, differentially displayed cDNA fragments. See results for further details.

model system, argue that our model system identi-fied a multitude of genes directly or indirectly involved in the context of cytokine induced loss of cartilage matrix.

Fibronectin, a glycoprotein involved in cell–matrix interactions, is present at low levels in the extracellular matrix of normal cartilage [36], but gets accumulated in the synovial fluid and the inflamed synovial and pannus surfaces of the knee joints in rheumatoid arthritis, and to a lesser degree in osteoarthritis patients [37, 38]. Intra-articular injection of fibronectin fragments causes a severe depletion of cartilage PGs *in vivo* [39], which is explained by the induced release of several proteinases, including MMP-3 [40]. Therefore, upregulation of fibronectin by IL-1β in our model system can be seen as a positive feedback regulation, enhancing the self-destructive potential of chondrocytes.

TSG-6, a TNF and/or IL-1 induced, secretory 39 kDa glycoprotein with extensive sequence homology with a region implicated in hyaluronate binding [41], was detected by Wisniewski *et al.* [42] in high levels in synovial fluids of patients with various forms of arthritis, while synovial fluid, obtained from persons without known joint disease, did not contain any detectable TSG-6 protein. The local source for TSG-6 in the joint cavity are synoviocytes [42] and articular chondrocytes [43].

Using quantitative PCR, as described above, on chondrocytes from tissue donor D [Fig. 1(b)], we were able to determine 1.56 ± 0.26 ($N = 2$) TSG-6 mRNA molecules/pg of total RNA for unstimulated

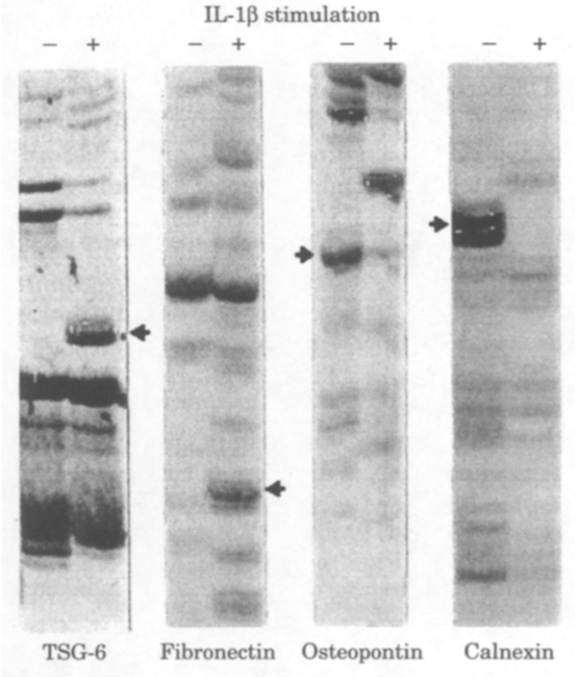

FIG. 4. Parts of differential displays containing bands identified as cDNA fragments coding for TSG-6, fibronectin, osteopontin and calnexin. Arrowheads denote fragments originating from IL-1β treated (+) or untreated (–) human articular knee chondrocytes. Primer pairs used: H = T_{12}MA and U17; I = T_{12}MT and U10; J = T_{12}MA and U11; K = T_{12}MT and U12.

human articular chondrocytes cultured in alginate beads, and 9.29 ± 1.94 ($N = 2$) molecules/pg of total RNA in IL-1β stimulated cells. Thus IL-1β upregulated TSG-6 mRNA steady state level by a factor of 5.9.

TSG-6 forms complexes with the serine protease inhibitor inter-α-inhibitor [44]. This complex inhibits plasmin and could interfere with the activation cascade of proMMPs. This may correspond with the experimental finding, that recombinant human TSG-6 exerts potent anti-inflammatory activity in the murine air pouch model of inflammation elicited by carrageenan or IL-1 [45].

ERCC5, the human homolog to yeast RAD2 [46], is a component of the excision nuclease complex involved in mammalian DNA excision repair [47]. Although we do not yet understand the direct link between the upregulation of ERCC5 and matrix degradation, the finding of this gene may indicate the wide scope of cytokine influenced cellular processes.

For osteopontin, a sialophosphoprotein mediating cell–matrix and cell–cell interactions [48], several reports document a IL-1, TNF and lipopolysaccharide (LPS) inducible expression of osteopontin in epidermal cells, bone cells and osteoblasts [49], whereas constitutive expression was observed in kidney and placenta [50]. Hwang *et al.* [51] reported, that osteopontin suppresses the induction of inducible nitric oxide synthase (iNOS) activity by LPS, probably at the level of induction of iNOS gene expression. This indicates a role for osteopontin as a general modulator of nitric oxide synthesis. Large amounts of NO are produced in human articular chondrocytes in alginate culture in response to IL-1 [31]. NO also plays a regulatory role in the activation of MMPs [52]. Our finding that IL-1β mediates downregulation of osteopontin may thus point to a compensatory mechanism, which would allow upregulation of iNOS, increased production of NO and thus PG degradation.

Calnexin is an endoplasmic reticulum (ER) resident chaperone, which binds to incorrectly or incompletely folded glycoproteins [53] and thus retains them in the ER [54]. IL-1 downregulates the expression of PGs in chondrocytes [3, 17, 18], probably by inhibiting the protein core sythesis [16]. Therefore, we speculate, that the IL-1β induced downregulation of calnexin mRNA steady state level reflects a downregulation of the total chondrocytic PG biosynthesis pathway, including the quality control system of the ER.

The upregulation of TSG-6 and fibronectin and the downregulation of osteopontin and calnexin mRNA steady state level was verified by Northern analyses using total RNA from IL-1β stimulated and unstimulated chondrocytes originating from tissue donor C. As seen in Fig. 5, all messages were found differentially expressed. TSG-6 expression was detected only in IL-1β stimulated chondrocytes. A phosphorimager quantification revealed an IL-1β induced upregulation of fibronectin full

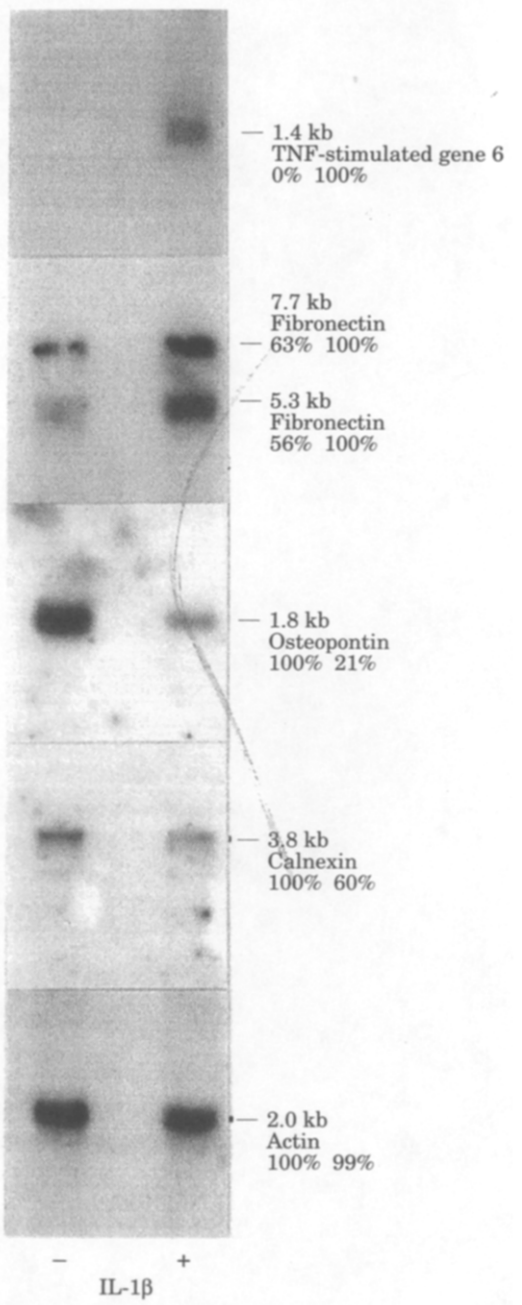

FIG. 5. Northern blot hybridization analysis of total RNA of human articular knee chondrocytes from donor C cultivated in alginate beads with (+) and without IL-1β (–) stimulation, probed with TSG-6, fibronectin, osteopontin, calnexin, and β-actin cDNA. The expected sizes of mRNAs are given. A phosphorimager quantification determined the relative amounts of messages (given in %).

length transcript (7.7 kb) by 37% and upregulation of an alternative splice variant (5.3 kb) by 44%, a downregulation of osteopontin by 79% and a calnexin downregulation by 40%. The sensitivity of a Northern detection was not sufficient to detect ERCC5 message in both IL-1 stimulated and unstimulated chondrocytes (not shown).

In addition to the above mentioned genes we also detected ATP-synthase β-subunit (Genbank M27132; TGU13/2), human 28S rRNA (Genbank M11167; TGU8) and human mitochondrial DNA (GenBank V00662; TTU3) as differentially expressed, but missed to detect for example MMPs, especially stromelysin. This may be due to the still limited number of employed primer combinations or, more likely, to the nonquantitative nature of DDRT-PCR unable to reveal differences in gene expression between very high steady-state levels as detected with quantitative PCR for stromelysin in our model system.

Taken together, our study provides the first direct demonstration of the pleiotropic effects of IL-1β on the overall gene expression in human articular chondrocytes and identifies genes involved in very different biochemical pathways. The function of some genes can be deduced from todays knowledge of cellular processes, while the function of others remains unknown. The identified 27 cDNAs without sequence homologies to known human genes may provide further clues to understand the IL-1β-mediated disease mechanism of degenerative joint diseases.

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