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Identification of Human Semaphorin E Gene Expression in Rheumatoid Synovial Cells by mRNA Differential Display

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Rheumatoid arthritis (RA) is characterised by chronic inflammation of synovial tissue with aggressive proliferation of synovial cells causing destruction of cartilage and bone. Immunopathological mechanisms, infectious causes and genetic factors have been discussed, but the etiology of the disease has not been understood until now. Especially, the mechanisms driving tumourlike growth and invasive behaviour of fibroblastoid synovial cells have not been identified yet. Our aim is to find cellular factors which are mediators for such pathways. One possibility to approach this, is searching for disease-relevant genes. We applied the mRNA-differential display technique to compare mRNA expression patterns of normal and rheumatic synovial fibroblasts. We identified an upregulation of the human semaphorin E gene in rheumatoid synovial fibroblastoid cells. Interestingly semaphorin E is a member of a protein-family described to play an immunosuppressive role via inhibition cytokines. A relevance of this finding towards the pathogenesis of RA is discussed.

An important feature of the rheumatic disease is chronic inflammation of joints resulting in chondral and subchondral tissue destruction. RA synovial fibroblasts are a major component of this invasive growing tissue, consisting of a vascular and fibrous granulation tissue arising from the joint recessus and extending onto the surface of the cartilage. These fibroblasts are morphologically altered and highly activated, similar to phenotypic characteristics that have been interpreted either as signs of reversible cellular transformation or as the result of strong, but reversible stimulation originating from the surrounding inflammatory microenvironment (1, 2). This tumour-like synovial cell proliferation (3) is responsible for progressing restriction of movement, which in most cases finally results in invalidity. While this disorder is at least in part immunologically mediated, much of its pathogenesis is still unknown. For that reason many investigators used other approaches to find causes for the disease, during the last decades. The search for candidate genes has become of common interest. A suitable strategy to detect such genes is the differential display reverse transcription-method (DDRT) (4). This technique allows the comparison of mRNAs discordantly expressed in different tissue species and cells. Differentially expressed genes have to be confirmed by northern blot analysis. Using this strategy we compared the gene expression patterns of fibroblastoid synovial cell cultures from normal and RA-patients and identified differentially expressed genes that are involved in the rheumatic disease.

MATERIALS AND METHODS

Cell culture. Specimens from synovial tissue were obtained from patients with rheumatoid arthritis who underwent synovectomy or from patients who underwent an amputation of their leg because of a trauma or a malignant tumour (above the joint). Latter ones served as healthy controls. Clinical diagnosis of RA was confirmed by histopathological judgement. Synovial tissue was dissected free of adipose tissue and blood clots. Then minced with a scalpel and incubated for 24 h in a solution of 100 U/ml collagenase II (Gibco) in Dulbecco's modified Eagle's minimal essential medium supplemented with 10 % fetal calf serum. Partially digested tissue was rinsed and placed in 75 cm^2 flasks. Several medium replacements and subcultivations using trypsin/EDTA (Sigma) followed until cells appeared confluent. Fibroblastoid synovial cells were used within 10 passages for subsequent experiments.

RNA preparation and Northern blot analysis. mRNAs to be used for differential display were extracted using the Quick Prep Micro mRNA Purification Kit (Pharmacia Biotech). Therefore the medium was poured out and the cells were scraped off with a sterile cell-rubber. After centrifugation, the pellets were washed twice with PBS, before the extraction buffer was added.

Total RNA for Northern blot analysis was isolated as described by

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Chomczynski and Sacchi (5). From each sample fifteen micrograms of total RNA were electrophoresed in a 1 % agarose gel containing 0.66 M formaldehyde, capillary transferred to nylon membranes (Gene Screen, NEN-DuPont), and hybridized with differential probes. Labeling of probes was performed with 50 μCi [α-32 P] dCTP (3000 Ci/mmol; Amersham) and Random Primer DNA labeling System (Gibco, BRL).

Densitometric quantitation. mRNA northern blot signals were quantitated densitometrically using a UMAX Powerlook scanner and a Power Macintosh computer with Adope Photoshop 3.0, Magic Scan and NIH-Image software by averaging three separate measurements of the left, middle, and right areas of each band. To correct for the uneven amount of RNA loaded in each lane, all samples measurements were calculated as the ratio of the average areas between the specific transcript and the 28s rRNA. The value of 28s RNA level obtained was defined as 100 % and all other intensities were referred to it. Values thus calculated were indicated as % of the respective 28s signal intensity.

mRNA differential display. mRNA differential display was performed as described by Lohmann et al. (6). 250 ng of mRNA was reverse-transcribed using the First-Strand cDNA Synthesis Kit (Pharmacia Biotech) and several 5′-T_{12}VN oligonucleotides (T_{12}AG, T_{12}GC, T_{12}CG, T_{12}AC) at a final concentration of 2 μM as anchor primer. The resulting cDNA was diluted 1:30 and 1:50, and served as template in a 10-μl PCR which contained 2,5 μM T_{12}VN primer in combination with one of 20 different arbitrary decamers (Kit A, Operon Technologies), 0,5 U *Taq* DNA Polymerase (Pharmacia Biotech), 20 μM of each dNTP and 1,5 mM $MgCl_2$. 43 cycles of PCR were performed with cycle times of 30 sec at 94°C, 60 sec at 42°C and 30 sec at 72°C. PCR amplification products were mixed with 5 μl of 90 % formamide/dye solution, heated for 2 min at 85°C, and 8.5 μl were run on a polyacrylamidegel containing 7 M urea (Clean Gel 15 %/ 48 S, ETC Elektrophorese Technik) at 11 mA for 20 min and then for 2 h at 18 mA in an discontinuous buffer system (Disc buffer pH 8.4, ETC Elektrophorese Technik) using a horizontal electrophoresis system (Multiphor II, Pharmacia Biotech). For detection of the amplified cDNA products, the gel was stained with $AgNO_3$ according to Bassam et al. (7).

Reamplification, cloning, and sequencing of cDNAs. Differentially expressed PCR-bands were cut out of the gel and subsequently rehydrated in 5 μl H_2O by boiling for 5 min. The reamplification of the eluted cDNAs was performed with the same primer combination and reagents as the initial PCR, with the except of the dNTP-concentration, which was increased to 200 μM. PCR conditions were as follows: 60 sec at 94°C, 60 sec at 40°C and 60 sec at 72°C (40 cycles). Successfully reamplified cDNAs were eluted out of a 1 % agarose gel with an extraction kit (Qiagen) and radiolabeled as described above. Those reamplification products which differed in their intensities of expression-signals between normal and rheumatic samples in Northern experiments were cloned directly into the pCR TM 2.1-vector using the TA cloning kit (Invitrogen). DNA sequences of the inserts were determined with the ABIPRISM Dye Terminator Cycle Sequencing kit (Perkin–Elmer).

RESULTS

mRNA Differential Display Analysis

For each cell culture sample at least 160 PCR reactions were performed. Seven differentially expressed cDNA-bands were detected on the polyacrylamidegels. Figure 1, shows DDRT-PCR amplifications obtained with three separate primer combinations (T_{12}AG/A1, T_{12}AG/A2 and T_{12}AG/A3). Four representative PCR products that were differentially expressed in rheumatic and normal fibroblastoid cells are indicated. The candidates were isolated and reamplified.

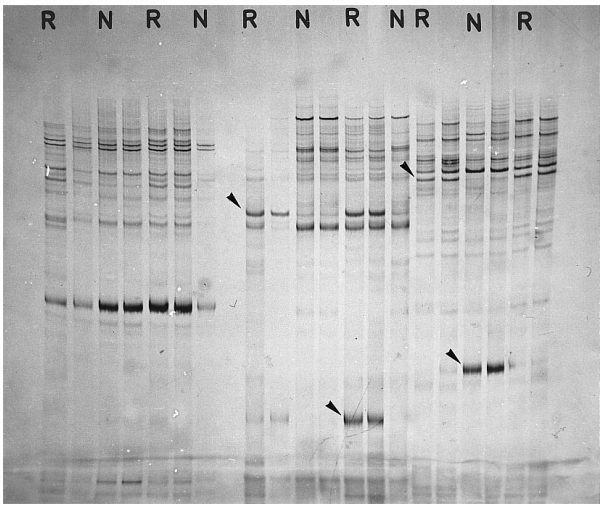

FIG. 1. Differential display gel of normal (N) and rheumatic (R) fibroblastoid synovial cells with three different arbitrary primers (A1-A3). Differentially expressed cDNA fragments are indicated by arrows.

Northern Blot Analysis

Northern blot analyses from RNA of normal and rheumatic fibroblastoid synovial cells were hybridized with each of the radioactively labeled reamplification-products. Only two of the cDNA probes (A22 and A32) resulted in significant differential expression signals (Figure2). The densitometric quantitation of the hybridization signals of cDNA fragment A22 (Figure 2A) showed a 3-4 fold up-regulation in RA-cells. The second probe A32 (Figure 2B) resulted in an 10-fold up-regulation in RA-cells.

DNA Sequence Analysis

The two differentially expressed cDNA-fragments were individually subcloned (clones A22 and A32) and sequenced. Computer search against NCBI Genbank indicated that the inserted sequence of clone A32 (493 bp insert) is completely part of the human semaphorin E gene, and the cDNA sequence of A22 (334 bp insert), is 98 % identical to Transforming growth factor β 1 binding protein.

DISCUSSION

In rheumatoid arthritis the cellular phenotype and the architecture of the synovial membrane is greatly altered (2). It has been shown that fibroblasts derived from inflammatory synovitis are one of the major

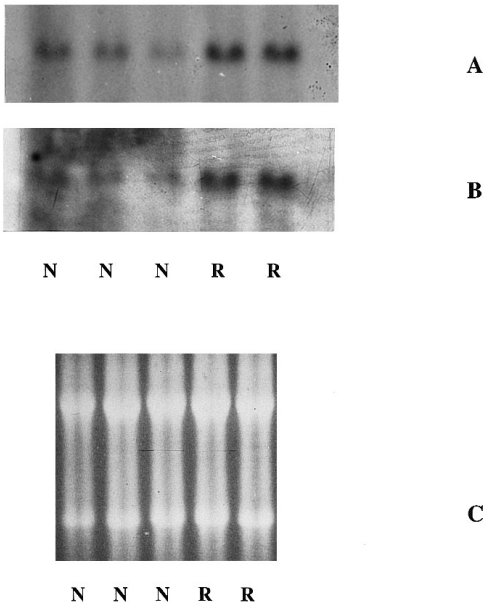

FIG. 2. Northern blot analyses of clones A22 (A) and A32 (B) in normal (N) and rheumatic (R) fibroblastoid synovial cells. RNA loading control by Ethidiumbromid staining (C).

sources of damaging mediators in this disorder. Elevated levels of a number of cytokines such as IL-1 (8), IL-2 (9), IL-6 (10) and TGFβ (11) have been detected there. Abnormal gene programmes are activated that result in altered cell behaviour, leading to proliferation of synovial cells which subsequently, invade into the cartilage and bone (12). Gene expression studies in fibroblastoid synovial lining cells from rheumatoid and other chronic inflammatory synovial tissue led to the identification of a variety of genes (12, 13). A recently developed method to identify new marker genes is the mRNA differential display technique (4). It has been successfully used to identify differentially expressed genes from a number of experimental systems (14-17). We applied the technique to search for new, relevant genes associated with rheumatoid arthritis. By comparing fibroblastoid synovial cells of normal and rheumatic tissue, we detected a 334 bp cDNA fragment, which is up-regulated 3-4 fold in RA-cells (Figure 2 A). Sequence analysis and subsequent genebank database search identified the mRNA TGFβ1 binding protein. The fact that TGFβ1 is discussed to play the most prominent role in RA (18, 19) and that TGFβ1 binding protein is necessary for the activation of TGFβ1 function (20, 21) demonstrates that our method indeed can specifically identify expressed genes relevant for RA-disease.

The mRNA of a second differentially expressed cDNA-fragment (493 bp) was confirmed to be up-regulated 10-fold in RA-cells using northern blot analyses (Figure 2B). Sequence analysis of the cloned fragment showed 100% homology to the recently detected human semaphorin E (Acc.nr.: AB000220, NCBI Genebank). The homologous mouse-semaphorin E is known to be a member of a gene-family encoding transmembrane- and secreted molecules, which are known to create domains inhibitory for axonal extension (22, 23). Mouse-semaphorin E is highly homologous to chicken collapsin (23, 24) and human Sema III (22, 23). Expression studies revealed that mouse-Semaphorin E is widely expressed in neural tissue, but also in connective and mesenchymal tissue, suggesting that it is involved in the histogenesis of tissues other than neural (23). An interesting aspect is the fact that semaphorin genes contain a conserved domain of amino acids which partially has been found in two poxviruses. Poxviruses have been shown to encode proteins that cause infected cells to produce secretory proteins which block the immune response of the host by either binding to cytokines or preventing activation of cytokines (25). In this context Kolodkin and co-workers supposed that semaphorins may function in the immune system as natural immunosuppressants to turn down the immune response (22). This observation represents an interesting point of view to speculate about the function of human semaphorin E in the pathogenesis of RA. The fact that semaphorin E is homologous to viral cytokine inhibiting proteins makes it to a possible regulator of inflammatory processes and implicates a direct involvement in the process of RA. It has to be shown now that semaphorin E has the potential to influence the immune response in RA. Using RA-tissue sections we next want to find out with mRNA in situ hybridization and immunohistochemistry which cells show semaphorin E expression.

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