Constitutive expression of c-fos and c-jun, overexpression of ets-2, and reduced expression of metastasis suppressor gene nm23-H1 in rheumatoid arthritis

Steven Dooley, Ivanna Herlitzka, Rainer Hanselmann, Ayhan Ermis, Wolfram Henn, Klaus Remberger, Thomas Hopf, Cornelius Welter

Abstract

Objectives—To identify genes that are involved in the development and progression of rheumatoid arthritis (RA).

Methods—We used a multiple gene analysis system and a set of available genes participating in processes such as proliferation, differentiation, tumour progression, and metastasis, to identify their RA related expression. Synovial tissues from 22 patients with RA were evaluated in comparison with those from six patients with osteoarthritis and two patients with non-inflamed joints as controls, using northern blot and reverse transcriptase polymerase chain reaction experiments.

Results—Our data confirm the role of c-fos and c-jun as constitutive signal transmitters in solid RA tissues, thus demonstrating the potential of the approach. Activation of both genes persisted through multiple passages of the cells in tissue cultures derived from the synovial lining of RA tissues. There was an increased expression of ets-2 in 30% of RA samples and an up to 30-fold decreased expression of the potential metastasis suppressor gene nm23-H1 in 90% of RA tissues, compared with control tissues.

Conclusions—The data presented show for the first time a significant decrease of nm23-H1 expression in RA, which is possibly involved in local invasiveness, and a strong activation of the ets-2 nuclear oncogene in about one third of RA tissues, which may also be part of a pathway leading to advanced disease stages. The constitutive expression of c-fos and c-jun in RA tissue most probably results from a continuing inflammatory stimulus. These findings with cell cultures suggest an intrinsic activation mechanism of these early response genes in RA.

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Rheumatoid arthritis (RA) is an inflammatory disorder characterised by infiltration of immunocompetent cells into the synovial tissue and synovial fluid of joints. Distinctive alterations in patterns of growth and cell-cell interactions characterise the rheumatoid synovium.

Most striking is the conversion of the lining cell layer from a predominantly single cell architecture into a multilayered proliferative cellular aggregation associated with changes in the cytomorphology of the synovocyte.¹ RA synovial tissue, which is rich in blood vessels, invades the periarthritic cartilage and bone, destroying the joint. RA synovial fibroblasts are a major component of this invasive pannus, consisting of a vascular and fibrous granulation tissue arising from the joint recessus and extending onto the surface of the cartilage. A number of studies indicate that these fibroblasts are morphologically altered and highly activated, similar to phenotypic features 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 micro-environment.^{2–3} On the basis of these findings, a tumour-like expansion of the rheumatoid pannus tissue was suggested.⁴ In contrast, it has been proposed^{5–7} that the transitional fibroblastic zone frequently present in the cartilage-pannus junction in rheumatoid joints is derived from the overlying cartilage. The origin of the destructive features of the rheumatoid synovium is not understood, and remains to be studied in detail. Using cytogenetic analysis, we have previously shown in primary cell cultures and higher passages of rheumatoid arthritis synovial tissue that trisomy 7 as a clonal change occurred in up to 20% of the cells analysed.⁸ Interestingly, the proportion of cells having +7 increased with the duration of the in vitro culture.

We have now used a molecular approach to identify genetic parameters of the invasive and destructive behaviour of solid RA tissue. We investigated a set of genes involved in processes such as proliferation, differentiation, tumour progression, and metastasis, with respect to mRNA expression using a multigene dot and slot blot assay as described previously.^{9–10} Tissues obtained from patients diagnosed as having RA according to the American College of Rheumatology criteria¹¹ were compared with disease controls (biopsy samples of patients with osteoarthritis (OA)). The data obtained by labelling reverse transcribed complementary DNA (cDNA) of a limited number of samples were enlarged in reverse transcriptase polymerase chain reaction (RT-PCR) and northern

Institut für
Humangenetik,
Universität des
Saarlandes, 66421
Homburg, Germany
S Dooley
A Ermis
W Henn
C Welter

Pathologisches Institut
I Herlitzka
K Remberger

Unfallchirurgie
R Hanselmann

Orthopädische Klinik
T Hopf

Correspondence to:
Steven Dooley.

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blot analysis to demonstrate significance. We also used the multigene analysis system to compare the pattern of expression of the above mentioned genes in cultures with low and high numbers of trisomy 7 cells.

Materials and methods

PATIENTS AND CELL CULTURING

Tissues were obtained from patients diagnosed as having RA according to the criteria of the American College of Rheumatology.¹¹ As disease controls, tissues from six patients with OA were examined. Synovia (containing synovium infiltrating cells) were obtained either by synovectomy or during joint replacement surgery. Synovial fibroblast cell culturing was performed as described previously.⁸

RNA PURIFICATION AND NORTHERN BLOT ANALYSIS

Total RNA was isolated from rheumatoid or healthy tissues by the guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski and Sacchi.¹² Samples of 20 µg total RNA were resolved by electrophoresis on 1% agarose-formaldehyde gels and transferred to GeneScreen nylon membranes according to the instructions of the manufacturer (NEN Du Pont, Boston, USA). After ultraviolet cross linking, the blots were hybridised at 65°C in 0.5 mol/l sodium phosphate (pH 7.2), 7% sodium dodecyl sulphate (SDS), and 1 mmol/l sodium EDTA for 16 hours as described by Church and Gilbert.¹³ After hybridisation, the membranes were washed once in 250 mmol/l sodium phosphate (pH 7.2)/1% SDS for 15 minutes and twice in 100 mmol/l sodium phosphate (pH 7.2)/1% SDS for 15 minutes. Autoradiography was performed at -70°C using intensifying screens. For multiple hybridisations, the bound probe was removed by incubating the filters twice for five minutes in 0.1 × standard saline-citrate (SSC) (3.0 mol/l sodium chloride/0.3 mol/l sodium citrate, pH 7.0) and 1% SDS at 95°C. To ensure that removal was complete, the blots were exposed to radiographic film for two days.

ANALYSIS OF GENE EXPRESSION PATTERNS

RNA of five different RA tissues were used as substrate to prepare a first strand cDNA, which was labelled to high specific activity using random priming technology. In parallel, we tested direct labelling of the first strand cDNA with reverse transcriptase and α -³²P-desoxy ATP (dATP), which resulted in lower labelling efficiency. As controls, we hybridised the same filters with total RNA probes from two healthy synovia samples and three samples of osteoarthritis tissues.

Total RNA (1–10 µg) was added to a tube containing 0.5 µg (100 nmol/l) oligo desoxythymine in a total volume of 11 µl, heated to 70°C for 10 minutes, and then chilled on ice for five minutes. Six microlitres of magnesium chloride solution (25 mmol/l), 3 µl of

10 × PCR buffer II (Perkin Elmer), 2 µl of each desoxynucleotide triphosphate (dNTP) (10 mmol/l), and 1 µl of RNase inhibitor (20 U/µl) were added and the mixture placed on ice for two minutes before the addition of 1 µl murine leukaemia virus reverse transcriptase (50 U/µl) and incubation for one to two hours at 42°C. To verify the presence of first strand cDNA and to determine the length of the DNA fragments, 1 µl of α -³²P-dATP (Amersham, 3000 Ci/mmol/l) was added and the reverse transcriptase reaction checked by electrophoresis of 1 µl of the mixture through an agarose gel and exposure to radiographic film for one hour. Radioactive DNA probe was produced from the cDNA reaction by random priming in a total volume of 50 µl according to the manufacturers' procedures (Amersham, BRL, for example). The reaction was allowed to proceed at 37°C for two to four hours or overnight and then stopped by the addition of 1 µl EDTA (0.5 mol/l). A Stratagene push column was used to separate probe from unincorporated radioactive nucleotides. The probe was used within one week. GeneScreen membrane (NEN/Dupont) and BioRad slot blot manifold or positively charged nylon membrane (Eppendorf), together with the Eppendorf-Event dot blot manifold, were used for binding of the various gene probes. Membranes were soaked with 0.4 mol/l Tris, pH 7.5, for five minutes. The plasmid DNAs were denatured for 10 minutes at room temperature in 0.25 mol/l sodium hydroxide (NaOH)/0.5 mol/l sodium chloride (NaCl). The DNA probes were diluted in 0.1 × SSC/0.125 mol/l NaOH and bound to the membrane as one single slot of 1 µg or in three different dilutions (250 ng, 25 ng, and 2.5 ng). The DNA samples were loaded into the manifold and left for 30 minutes before suction was applied. The membranes were removed from the manifold, neutralised by rinsing in 0.5 mol/l NaCl/0.5 mol/l Tris, pH 7.5, dried in air, and then fixed by auto-ultraviolet cross linking using a Stratalinker (Stratagene). Hybridisation was performed as described for northern blot analysis.

SEMIQUANTITATIVE RT-PCR ANALYSIS

Using total RNA preparations purified from different RA samples and several controls, we performed semiquantitative RT-PCR reactions to investigate the expression of c-fos, c-jun, mad, max, c-myc, mx1, ets-2, nm23-H1, nm23-H2, and β -actin. Five hundred nanograms of total RNA was reverse transcribed using the kit from Perkin Elmer as described in the manufacturer's procedure. Subsequent PCR was performed with 1 µl of the reverse transcriptase reaction using 15 pmol of each primer, 5 µl 4 mmol/l dNTP mixture (Stratagene, 1:25 dilution of stock), 10 µl Pharmacia Taq Polymerase buffer, and 2.5 U (= 0.5 µl) Taq Polymerase (Pharmacia) in a 100 µl reaction using a two step PCR as described in the Perkin Elmer RT-PCR kit manual. The number of cycles was decreased to 25 to ensure that exponential amplification was maintained. A 10 µl aliquot was analysed on 1–1.5%

agarose gels, depending on the size of the resulting fragments.

DENSITOMETRIC ANALYSIS

RNA-PCR products and mRNA northern blot signals were quantitated densitometrically using a UMAX Powerlook scanner and a Power Macintosh computer with Adobe 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 varying amounts of RNA and PCR product loaded in each lane, all sample measurements were calculated as the ratio of the average areas between the specific transcript and the 28s ribosomal RNA (rRNA) or PCR product and β -actin. The value of 28s rRNA obtained was defined as 100% and all other intensities were referred to it. Values thus calculated were indicated as % of the respective 28s or β -actin signal intensity.

PLASMIDS AND PROBES

Protein coding gene probes used for northern blot analysis were labelled to high specific activity using random priming technology using hexanucleotide primers (c-fos, c-jun, ets-2, p53) and gene specific primers (nm23-H1). The probe used in control hybridisations was a synthetic 26 bp oligonucleotide selected from the human 28s rRNA sequence.¹⁴ Phosphorus-32 labelling in this case was achieved by T4 polynucleotide kinase (Bio-labs). Probes dotted onto membranes were either created by PCR using gene specific primers, or used as recombinant plasmids. The primers were: **junB** (JunB (55); 1.5 kb human cDNA in Bluescript KS+); **junD** (junD; 1.5 kb human cDNA in Bluescribe M13+); **c-jun** (5'-GCCTACAGATGAACTCTTTCTGGC-3'upstream (up) and 5'-CTGAAACATCGC-ACTAGCCTTTGG-3'reverse (rev) primers from human cDNA sequence generating a 527 bp fragment); **c-fos** (5'-GGATAGCCTCTC-TTACTACCAC-3'up and 5'-TCCTGTCTAT-GGTCTTCACAACG-3'rev primers from human cDNA sequence generating a 280 bp PCR fragment); **fra1** (pSP fra1; 1.5 kb human fra1cDNA in pSP64); **fosB** (pTZ fosB; 1.7 kb human cDNA in pTZ18); **L-myc** (pLmyc 10; 1.8 kb human DNA in pJB327); **N-myc** (pNb1; 1 kb human DNA in pBR322); **c-myc** (5'-CTCAACGTTAGCTTCACCAACAGG-3'up and 5'-CTCCACATACAGTCCTGG-ATGATG-3'rev primers from human c-myc DNA sequence creating a 400 bp PCR fragment); **max** (5'-GGGCTCATCATAATG-CACTGGAAC-3'up and 5'-TCTTCAGGC-TCAGACTCTGAGCTG-3'rev primers from human max cDNA creating a 386 bp PCR fragment); **mad** (5'-GAAGAATAGACG-GGCTCATCTTCG-3'up and 5'-TCTTAG-AGACCAAGACACGCCTTG-3'rev primers creating a 474 bp PCR fragment); **mxil** (5'-AGCACTGCCAACAGATCTACACAC-3'up and 5'-TGGCCAGTGAATATTTT-GCCCTG-3'rev primers, generating a 541 bp PCR fragment); **c-myb** (pHM2-6; 2.6 kb

human c-myb DNA in pKH47); **erbB** (pheB; 2.5 kb human erbB in pBR322); **c-Ha-ras** (pEJ6-6; 6.6 kb c-Ha-ras DNA in pBR322); **c-Ki-ras** (pSVE SW4A-9; 0.5 kb human c-Ki-ras DNA in M13 mp8); **ets-2** (5'-ATGT-CCAGGAAAGTGGCCAAGAAG-3'up and 5'-TGACATGGTTGTTTCTTCCACC-3'rev primers creating a 336 bp PCR fragment); **p53** (pR4-2; 1.7 kb human p53cDNA in pUC8); **RB** (p0-9R; 0.9 kb human RBcDNA in pSP65); **CD44** (5'-GACAGAC-ACCTCAGTTTTTCTGGA-3'up and 5'-TT-CCTTCGTGTGTGGGTAATGAGA-3'rev primers from human CD44cDNA creating a 400 bp PCR fragment); **nm23-H1** (5'-GGCAGACCACATTGCTTTTC-3'up and 5'-GCTGGGAGGAAGCATTTTAATC-3'rev primers creating a 185 bp PCR fragment); **nm23-H2** (5'-GAATAAGAGGTGGACAC-AACAGC-3'up and 5'-CCGGCTTCCAAA-AGCTTTATTGGC-3'rev primers creating a 145 bp PCR fragment); **pS2** (ppS2; 0.3 kb human pS2cDNA in pT7T3); **β -actin** (5'-GTCCTCTCCCAAGTCCACACA-3'up and 5'-CTGGTCTCAAGTCAGTGATACA-GGTAA-3'rev primers from human cDNA creating a 300 bp PCR fragment); **GAPDH** (pBLUGAP; 1.4 kb rat GAPDHcDNA in pBluescript KS+); **pBR322** (Boehringer Mannheim); **pUC18** (Boehringer Mannheim).

Results

MULTIPLE GENE EXPRESSION ANALYSIS

Figure 1 shows a representative blot, and table 1 summarises the results. c-fos was active in all of five RA samples, whereas no or only weak signals were detected in controls. The ets-2 nuclear oncogene was positive in two of five RA tissues tested, and in one of three OA samples. c-myb gave a very strong signal in two RA tissues and nm23 signals were decreased in all five RA samples compared with control hybridisations. In addition, occasional differences were found concerning the genes p53, fra1, c-myc, mad, fosB, max, c-Ki-ras, and the housekeeping gene GAPDH. A relatively constant amount of mRNA was found in all samples for mxi, RB, and β -actin. These preliminary data were used as basis for the investigation of a greater number of samples by RT-PCR and northern blot analysis.

Figure 1 Representative slot blot autoradiography after filter hybridisation with rheumatoid arthritis specific (RA) and osteoarthritis specific (OA) whole cDNA probes. Positions of the target gene sequences are indicated. (Table 1 summarises the results of all the slot and dot blot experiments.)

Table 1 Summary of the results of slot and dot blot experiments: number of samples among the five rheumatoid arthritis (RA) tissues and five osteoarthritis (OA) tissues expressing the different probes. Positions were evaluated as positive (different signal intensities not discriminated) or negative

	RA (n = 5)	OA (n = 5)
c-fos	5	1
c-jun	3	2
c-myc	1	3
nm23-H1	0	3
nm23-H2	2	2
p53	1	0
c-myc	3	2
ets-2	3	3
mxil	1	2
L-myc	0	0
CD44	1	0
N-myc	0	0
Ha-ras	1	0
junD	1	1
RB1	1	1
fra1	3	3
junB	2	3
pS2	1	1
erbB-2	0	0
N-ras	0	0
mad	2	2
c-Ki-ras	1	0
max	4	3
fosB	2	1
GAPDH	5	5
β -actin	5	5
pUC18	0	0
pBR322	0	0

RT-PCR AND NORTHERN BLOT ANALYSIS

Table 2 summarises the results and figure 2 shows representative RT-PCR experiments. c-fos and c-jun analysis revealed strong PCR product signals in all the RA samples tested (fig 2B), while they were rarely obtained in controls (not shown). In contrast, c-myc was negative in most RA samples, but was expressed in OA samples (not shown). Max and mxi (not shown) were present at constitutive but very weak levels in all samples, and mad was expressed in single samples (fig 2A). The amount of nm23-H1 gene product (fig 2C), but not that of nm23-H2, was significantly reduced in RA compared with OA control tissue. The data obtained by RT-PCR were partially verified in subsequent northern blot analysis (fig 3).

c-fos, c-jun, ets-2, nm23-H1, and p53 sequences were used as hybridisation probes. As has been reported previously by several other groups, a significant expression was found for c-fos, which was activated in 90% of RA samples (18 of 20); this underlines both the

importance of c-fos in rheumatoid arthritis signal transduction and the potential of our approach. Control tissues were negative for c-fos (two of five) or displayed a low level of transcripts (three of five). c-jun mRNA also was increased in about 50% of RA samples investigated (10 of 19), but some control tissues also were positive (two of five). We identified two different transcripts of 3.4 and 2.6 kb in RA and in control samples. It remains to be determined if this was the result of sequence homology of our human c-jun probe with other members of the jun family of transcription factors, or if there exist different splicing products. p53 transcripts were not detected in northern blot analysis (not shown), but ets-2 transcripts were found in all samples (RA and controls) displaying at least a weak signal after one week of exposure time. In about one third of RA samples (six of 18), however, the expression of ets-2 as demonstrated by densitometric analysis was significantly increased, by six to 20-fold (figs 2A, 3B, tables 2, 3). Densitometric analysis also revealed the expression of nm23-H1 to be significantly decreased (two to 30-fold) (tables 2, 3) in 90% of RA tissues (18 of 20) compared

Figure 2 Reverse transcriptase polymerase chain reaction analysis using total RNA from different rheumatoid arthritis (RA) samples and some controls (underlined numbers) and primer pairs amplifying regions of the indicated genes. **A:** Five different RA samples displaying enhanced expression of ets-2 and mad in three of five lanes; the signal intensities were referred to the β -actin product. **B:** Seven different RA samples showing strong expression of both c-fos and c-jun genes. **C:** Nineteen different RA and osteoarthritis (OA) (underlined) samples showing reduced amplification rate of nm23-H1 in RA. The β -actin control PCR reaction is shown for samples 12–19. **D:** Comparison of the transcript level of several genes in early and late passage RA cell cultures with different percentages of trisomy or polysomy 7 cells (passage 3: 5% trisomy 7; passage 25: 54% trisomy or polysomy 7). f = c-fos; j = c-jun; a = β -actin; n = nm23-H1; e = ets-2.

Table 2 Summary of the results obtained in reverse transcriptase polymerase chain reaction analysis: target genes of the primer pairs and the rheumatoid arthritis and osteoarthritis samples used. The β -actin signal of each sample was taken as 1.0 and the densitometric data of each signal were correlated to it

	Tissue	ets-2	mad	c-fos	c-jun	nm23-H1	nm23-H2	mxi	β -actin
KA	RA	5.33	3.23	8.73	8.93	0.42	1.43	0.43	1.0
AG	RA	0.46	3.62	8.75	8.88	0.12	1.52	0.12	1.0
LP	RA	0.43	—	8.32	9.03	0.23	1.33	0.23	1.0
LA	RA	0.81	1.22	7.99	9.66	0.16	1.43	0.41	1.0
AL	RA	8.24	0.92	8.43	9.43	0.13	1.81	0.61	1.0
MR	RA	7.44	0.43	8.56	8.65	0.05	1.21	0.88	1.0
SH	RA	0.46	3.66	8.67	9.32	—	0.63	0.31	1.0
KJ	RA	0.33	0.33	8.56	8.42	0.20	0.34	0.12	1.0
GI	RA	4.66	0.35	9.13	8.13	—	0.98	0.13	1.0
WI	RA	5.39	1.33	8.33	9.43	0.16	0.32	0.33	1.0
DE	RA	0.62	4.12	7.54	9.34	0.13	1.43	0.41	1.0
MB	RA	0.58	1.23	8.65	9.55	—	1.36	0.94	1.0
KM	RA	0.26	0.23	8.67	8.63	0.06	1.88	0.32	1.0
BJ	RA	4.88	0.41	7.92	8.71	0.14	1.62	0.29	1.0
SP	RA	0.32	3.32	8.96	8.08	3.62	1.54	0.26	1.0
WE	OA	0.24	0.24	0.81	0.77	2.89	1.62	0.36	1.0
FE3	OA	0.41	0.33	—	0.73	4.12	1.55	0.43	1.0
SA	OA	0.30	—	0.83	0.61	2.64	1.48	0.82	1.0
HM	OA	0.31	0.32	—	0.62	3.43	1.32	0.32	1.0

with OA samples. The RT-PCR experiments revealed no differences for nm23-H2 between RA samples and controls (not shown).

CELL CULTURE EXPERIMENTS

Comparison of primary fibroblastoid synovial cell cultures with a low number of cells displaying trisomy 7 and higher passages with more than 50% trisomy or polysomy 7 cells did not yield different signal patterns with the gene probes tested. Transcripts for c-fos, c-jun, ets-2, nm23-H1, nm23-H2, β -actin, and GAPDH were present, and no significant qualitative or quantitative differences could be detected in either dot blots or semiquantitative RT-PCR screening (fig 2D). This indicates that genes other than those analysed are affected by the increase of chromosome number 7 in RA cell cultures. It was very interesting that c-fos and c-jun expression, in common with findings for

Table 3 Summary of the results obtained in northern blot analysis. The 28s ribosomal RNA hybridisation signal value was taken as 1.0 in each sample and the densitometric measurements of the different probe signals were referred to it

	Tissue	c-fos	c-jun	ets-2	nm23-H1
KA 12112	RA	0.88	0.29	0.92	0.14
AG 15102	RA	0.76	0.65	0.18	0.13
LP 12033	RA	0.85	0.54	0.15	0.13
LA 13074	RA	0.80	0.33	0.10	0.13
AL 19044	RA	0.68	0.59	0.81	0.16
MR 17064	RA	0.82	0.54	0.76	0.14
SH 14094	RA	0.71	0.32	0.14	0.12
KJ 04054	RA	0.73	0.26	0.12	0.13
ST 24114	RA	0.58	0.70	0.78	0.13
AA 24114	RA	0.81	0.65	0.14	0.14
DC 24114	RA	0.64	0.61	0.12	0.15
RH 03122	RA	0.85	0.33	0.13	0.32
GI 25084	RA	0.78	0.66	0.83	0.12
WI 21114	RA	0.60	0.65	0.77	0.14
DE 23033	RA	0.81	0.64	0.12	0.13
DM 27035	RA	0.72	0.56	0.12	0.14
MB 27035	RA	0.73	0.61	0.13	0.14
KM 03045	RA	0.69	0.72	0.12	0.13
BJ 31035	RA	0.36	0.33	0.79	0.12
SP 24035	RA	0.54	0.36	0.13	0.30
SE 02035	RA	0.67	0.63	0.11	0.34
TI 02035	RA	0.32	0.54	0.63	0.26
WE 12073	OA	0.26	0.34	0.13	0.39
FE 08055	OA	0.68	0.29	0.12	0.32
FE 30055	OA	—	0.18	0.12	0.48
SA 31055	OA	0.22	0.23	0.13	0.36
WI 16073	OA	0.24	0.20	0.12	0.13
HM 11014	OA	—	0.18	0.13	0.36

Figure 3 Northern blot analysis using total RNA of different rheumatoid arthritis (RA) and osteoarthritis (OA) (underlined) samples. Hybridisation probes and the length of the corresponding RNA molecules are indicated. **A:** Significant activation of c-fos and c-jun, and decreased expression of nm23-H1 in RA tissue; 28s ribosomal RNA band of the corresponding RNA blots as reference. Variation of RNA loading as a result of limited amounts of tissue from some samples. Differences were determined densitometrically (see table 3). With c-jun as probe, a 3.4 kb band not described previously was detected in RA and control tissue. **B:** Four of six different RA samples displaying strong expression of ets-2. Two signals, 4.7 and 3.2 kb, also demonstrated in several other tissues. A 28s specific oligonucleotide probe used as reference hybridisation.

solid probes, were also constitutively increased in cell cultures of fibroblastoid cells, which are not permanently under the influence of cells that are mediators of inflammation (fig 2B).

Discussion

In the rheumatoid synovium, abnormal gene programmes are activated that result in altered rates of proliferation and enable cells to invade the periarticular cartilage and bone, thus destroying the joint. A wide variety of mediators, factors, and possibly cell-cell interactions target the cell and activate distinct and separately regulated processes. It has been shown that fibroblasts derived from inflammatory synovitis displayed relative increases in the steady state concentrations of mRNA for the proinflammatory cytokine interleukin-6, the enzymes cathepsin D and stromelysin (which are potentially capable of mediating tissue destruction and matrix remodelling), the cytoskeletal component vimentin, and major histocompatibility complex class II molecules, but diminished amounts of c-myc.⁹ We have used a recently described approach for further analysis of patterns of gene expression in rheumatoid arthritis. Because many genes are analysed simultaneously with relatively small amounts of RNA, the method is ideally suited for measurement of gene expression when tissue is scarce or the sample size is small. The preliminary results obtained with this technique were then investigated in more detail in RT-PCR and northern blot analysis.

First we found that c-fos was significantly activated in 90% of the solid RA tissues investigated. Similar data have been reported previously by several other groups and have already been discussed extensively.¹⁵ It is very interesting that a gene that physiologically undergoes transient activation as an early response gene is constitutively switched on in a disease model. One explanation is that this

may represent a response to cytokines permanently released by immune and mesenchymal cells in the synovial lining and subsynovial layer. In contrast to this is the finding that c-fos is also constitutively activated in fibroblastoid cell cultures derived from the synovial lining of joints affected with rheumatoid arthritis. Its activity thus appears to be an intrinsic characteristic of these fibroblastoid cells, at least in cell culture, insofar as it was found to persist through multiple passages of the cells in the absence of an additional continuing inflammatory stimulus. In addition to c-fos, its partner c-jun is also induced in about 50% of RA samples, to a minor extent. In common with c-fos, c-jun is activated constitutively in cell culture. It remains to be clarified whether these data imply that there may be a long term response of these normally transiently activated genes, or that an exogenous stimulus is needed in RA tissues; a detailed analysis of the regulatory pathways involved in steady state activation of c-fos and c-jun, both of which have been significant markers for RA, will be very interesting. The immunohistochemical localisation of the gene products and a functional analysis at the protein level to clarify if the activities of both genes are mediated via heterodimerisation and AP1 motif binding are under investigation in our laboratory.

A further important finding was the detection of two different hybridisation signals in northern blot analysis with c-jun as probe (2.6 kb regular, and a novel 3.4 kb band not described previously). We have begun to investigate if there are homologies between our PCR probe and another transcript (using Genbank computer search programs), if the two bands are specific for synovial samples (by investigating serum induced tumour cell lines with our c-jun probe), and if there are perhaps different transcription start sites in the gene (using reverse primer extension analysis of the transcripts).

ets-2 gene activity was found in RA samples and in controls (normal synovia and OA samples), at least yielding a very weak mRNA hybridisation signal. Using RT-PCR analysis, which is a more sensitive system, it was possible to demonstrate this more impressively. In 33% of RA tissues (six of 18), however, the transcription of ets-2 was increased six to 20-fold (densitometric analysis). ets-2 transcripts were also detected in RA cell culture in early passages (small numbers of trisomy 7 cells) and in higher passages with more than 50% of trisomy or polysomy 7 cells. The members of the ets family are nuclear proteins^{16 17} that can bind to specific DNA sequences located mainly in purine rich motifs.^{18 19} ets-2 mRNA can be detected in all tissues tested, but in highly variable amounts. Its expression has been associated with a proliferative role in many tissues,²⁰ and therefore an enhanced activity in several RA samples may reflect disease progression. In this context, it is of importance that several known genes involved in the degradation of the extracellular matrix, such as those encoding stromelysin 1, collagenase, and urokinase plasminogen activator,²¹ contain ets

binding sites in their regulatory flanking sequences. In this way, ets-2 may play a part in coordinating events that lead to the degradation of cartilage—an important process in invasive growth. Why an increased expression of ets-2 was found only in about 30% of RA samples remains unclear. It may be hypothesised that other, ets-2 independent, pathways exist that have the same effect as ets-2 overexpression. The same problem is encountered with the analysis of oncogene expression in human cancer, in which again only a proportion of tumours of the same histological type display changes in activity characteristic of distinct oncogenes. Nevertheless, it would be of value to investigate the specific role of the ets-2 gene product in rheumatoid synovial cells.

In view of the fact that a basic feature of joint damage in RA is the progressive erosion and destruction of articular cartilage and adjacent connective tissues, our data on nm23 are very important. The nm23 gene was originally identified on the basis of its reduced mRNA and protein levels in highly metastatic melanoma cell lines.²² Two nm23 gene isoforms, nm23-H1 and -H2, both encode 17 kDa proteins.²³ Generally, the published data indicate that a reduced concentration of nm23, or its structural alteration, result in a loss of the suppressor function. In agreement with this hypothesis, we detected a reduced nm23-H1 transcript level in 90% of the RA samples investigated, compared with OA or normal synovial tissue. This finding provides evidence that nm23 may be involved in the invasive behaviour of rheumatoid synovial cells, as in the process of metastatic spread of specific tumours. As is the case for the metastatic process in human and animal tumours, at present we do not know the underlying function of the nm23 proteins in RA tissues. Some of the identified gene activities we describe have not yet been investigated in detail (c-myc, for example) and will be the subject of future study; others did not change significantly in comparison with controls, or occurred rarely in the sample pool as a whole, and were therefore disregarded.

Because use of this assay was limited to the gene probes available in our laboratory, we are now aiming to establish a differential mRNA extraction cloning strategy that allows us to identify new gene markers of RA in comparison with OA samples.

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