cDNA macroarray analysis of gene expression in synoviocytes stimulated with TNFα

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Abstract Gene expression of synoviocytes stimulated with tumor necrosis factor-α (TNFα) was studied by macroarray analysis to elucidate the cellular response and identify new biological functions of known and unknown genes. 10 035 cDNA clones were used to make cDNA macroarrays of representative genes. Synoviocytes expressed large amounts of fibronectin and collagen mRNA. Statistical analysis of the macroarray data revealed 26 genes, including six new genes, which underwent significant alteration of gene expression in response to TNFα stimulation. These findings suggest that the synoviocyte response to TNFα stimulation forms the basis of development of various aspects of the pathophysiology of rheumatoid arthritis.

Key words: Macroarray; Gene expression; Tumor necrosis factor-α; Synoviocyte; Oligo-capping; Rheumatoid arthritis

1. Introduction

Tumor necrosis factor-α (TNFα), a pro-inflammatory cytokine, has been thought to be an important mediator of inflammation such as rheumatoid arthritis (RA) [1]. Synovial tissue inflammation is caused by TNFα secreted by lymphocytes. For example, interleukin 8 (IL8), the level of which increases in synoviocytes in response to TNFα, induces further infiltration by lymphocytes [2]. Thus, TNFα signaling has been looked upon as a therapeutic target molecule of RA. However, the cellular response of synovial cells to TNFα remains to be fully investigated.

cDNA array analysis is a useful method for efficiently exploring the functions of uncharacterized genes in addition to known genes by relating the expression pattern of one gene to those of others. Many types of cDNA arrays have been proposed and applied to expression profiling, e.g. PCR-amplified DNA spots on a slide glass or a nylon membrane and high-density colony grids on a membrane [3–5].

The fundamental features of massive data acquisition, miniaturization, multiplexing and automation of cDNA microarray assays augment the expression profiling data obtained with slide glass-based cDNA microarrays. cDNA microarray analysis has developed a new concept of cellular diagnosis based on the clustering of gene expression patterns [6,7]. Analysis using nylon membrane-based cDNA macroarrays is a conventional method among cDNA array analyzing systems. High-density colony grids are the most conventional cDNA macroarray.

In this study, we used cDNA macroarray analysis to investigate gene expression of synoviocytes with the goal of elucidating the total cellular response to TNFα. The macroarrays were prepared using non-redundant genes, including novel genes [8]. This study has revealed new biological functions of both known and novel genes. These results suggest involvement of a synoviocyte response in the development of various aspects of the pathophysiology of RA.

2. Materials and methods

2.1. Sequencing

Sequencing of the 5′-end of the cDNA insert was performed using a Big Dye Terminator Cycle Sequencing kit and ABI377XL sequencer (Applied Biosystems). The sequencing primer was 5′-TACGGAAGTGTTACTTCTGC-3′. For full-length insert cDNA sequencing, specific primers of the cDNA were designed and used.

2.2. Preparation of macroarrays

A non-redundant gene set was used for the macroarray analysis. 5′ sequences of 55 618 clones were clustered into 17 699 groups using computer programs. This gene set included 2885 known genes and 7150 unknown/homologous genes.

Both high-density colony and PCR-amplified cDNA macroarrays were prepared. High-density colony macroarrays were prepared as described previously [9]. Macroarrays spotted with PCR-amplified insert clones were prepared as follows. Plasmid inserts were amplified using 5′-TACGGAAGTGTTACTTCTGC-3′ and 5′-TGTGGGAGGTTTTTTCTCTA-3′ as sequencing primers. PCR cycles were performed (10 cycles of 20 s at 98°C for denaturation and 6 min at 60°C for annealing and elongation, followed by 20 cycles of 20 s at 98°C for denaturation and 3 min at 60°C for annealing and elongation) using a GeneAmp PCR System 9600 (Perkin Elmer). The amount of each PCR product was evaluated on the basis of the intensity of the band detected by gel electrophoresis. Then, the PCR samples were spotted onto nylon membranes (11×7.5 cm) in a 3×3 format using a Biomek 2000 Robotics Workstation (Beckman) to yield 3456 spots per membrane.

2.3. Probe preparation for macroarray

Synoviocyte total RNA (5 μg) or 1.5 μg of human tissue poly A^+ RNA (Clontech) was combined with 50 pmol oligo(dT) in a total

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volume of 6 μl, heated to 65°C for 5 min and then cooled on ice. 5 μl of dATP (3000 Ci/mmol), 1 μl of 0.1 M DTT, 4 μl of first-strand buffer (Gibco BRL), 2 μl of dNTPs (10 mM each of dTTP, dGTP and dCTP, and 37 μM dATP), 1 μl of 40 U/μl RNASEOUT (Gibco BRL) and 1 μl of Thermoscript RT (Gibco BRL) were then added. Each sample were then incubated at 45°C for 1.5 h to generate ^{33}P-labeled cDNA. 80% of the radioactive label was incorporated into the cDNA probe. The length of the probe ranged from 0.5 to 3.5 kb, as shown by 1% alkaline agarose gel electrophoresis and autoradiography.

2.4. Macroarray hybridizations

The labeled probe was denatured at 98°C for 5 min and then added to 9 ml of hybridization solution pre-warmed to 65°C. Pre-hybridization with 1 pmol/ml oligo(dT) and 10 μg/ml denatured Cot-1 DNA (Gibco BRL) was performed at 65°C for 3 h, followed by hybridization at 65°C for 12 h in the hybridization solution. After hybridization, the membranes were washed twice in 2× SSC, 1% SDS at room temperature for 20 min. The membranes were then washed three times with 0.1× SSC, 1% SDS at 65°C for 20 min.

2.5. Macroarray data analysis

Imaging plates exposed to macroarrays at room temperature for 4 h were scanned with a BAS2000 system (Fuji Photo Film Co., Ltd.). After image acquisition, the scanned images were analyzed using the ArrayGauge software (Fuji Photo Film Co., Ltd.) for quantification of the radioactivity of each spot. Background due to non-specific binding of probes to the membrane was subtracted from the signal intensity of each spot. The data were statistically analyzed.

2.6. Cell culture

Human fibroblast-like synoviocytes (Cell Applications, Inc.) were cultured in Synoviocyte Growth Medium (Cell Applications, Inc.) in an atmosphere of 95% air and 5% CO_2 at 37°C. After the cells had grown confluently, the medium was changed to fresh medium with or without 10 μg/ml human recombinant (*E. coli*) TNFα (Boehringer-Mannheim). The total RNA was isolated using an S.N.A.P.™ total RNA extraction kit (Invitrogen Inc.).

2.7. Northern blot analysis and RNA dot-blot analysis

Alpha cardiac actin, IL8, TFPI2 and FLJ10143 probes were labeled with dCTP (3000 Ci/mmol) using a BcaBEST labeling kit (Takara Shuzo Co., Ltd.).

For RNA dot-blots, 1 μg of human tissue poly A^+ RNA (Clontech) was dotted onto a nylon membrane (Boehringer-Mannheim). For Northern blots, 2 μg of total RNA was fractionated on 1% formaldehyde-agarose gel and blotted onto nylon membranes. The RNA on the membranes was fixed using a Stratalinker UV crosslinker (Stratagene). The membranes were hybridized with the probes at 68°C for 12 h using ExpressHyb™ hybridization solution (Clontech). The membranes were washed twice with 2× SSC, 1% SDS at room temperature for 20 min, followed by three times with 0.1× SSC, 1% SDS at 65°C for 20 min. The membranes were then exposed to autoradiography film with an intensifying screen at −80°C.

3. Results

3.1. Evaluation of macroarray data

First, we compared two kinds of cDNA macroarray systems made with 3456 high-density colony grids and PCR-amplified cDNA spots. In the high-density colony grid macroarray, 10% of the spots showed over three-fold variation between duplicate macroarrays, indicating poor reproducibility (Fig. 1A). In contrast, with the PCR-amplified cDNA macroarrays only 0.3% of the spots showed over three-fold variation between duplicate macroarrays (Fig. 1B). In addition, the signal intensity of each spot between the duplicate PCR-amplified cDNA macroarrays showed less than 1.9-fold variation for 95% of the total spots, showing relatively high-quality reproducibility.

PCR-amplified alpha cardiac actin cDNA spotted on eight nylon membranes was hybridized with probe prepared from

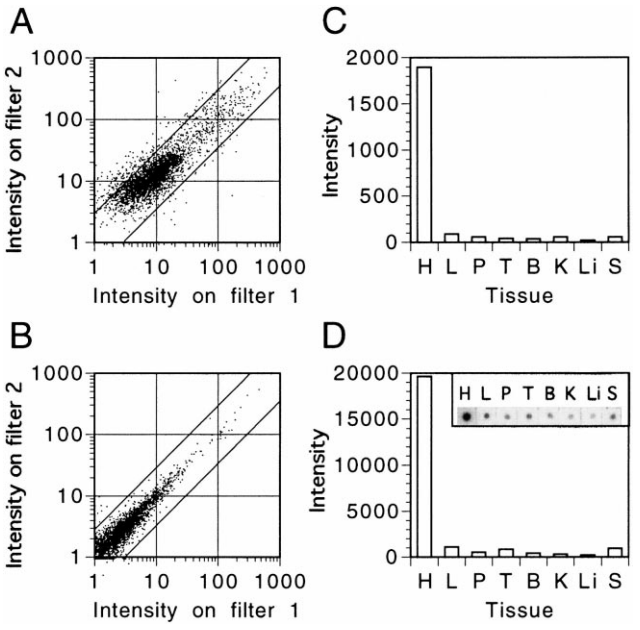

Fig. 1. Macroarrays for the analysis of gene expression. A,B: Duplicate membranes of 3456 spots representing each gene were hybridized with ^{33}P-labeled cDNA probes. The signal intensity of each spot on the filters was plotted. The two lines in the figure show three-fold variation in the signal intensity from each filter. A: High-density colony grid macroarrays. B: PCR-amplified cDNA macroarrays. C: PCR-amplified alpha cardiac actin cDNA was spotted and hybridized with cDNA probes from tissues (H: heart, L: lung, P: pituitary gland, T: thymus, B: brain, K: kidney, Li: liver, S: spleen). Each bar shows the intensity of the spot. D: RNA dot-blot analysis of alpha cardiac actin. The inset is the autoradiogram of the hybridization. Each bar shows the intensity of the spot.

mRNA from eight different tissues (Fig. 1C). These results agreed with the results of RNA dot-blot hybridization with the alpha cardiac actin probe (Fig. 1D), showing the reliability of the macroarray data as a tool for gene expression analysis. The signal intensity of the spot was increased by increasing the amount of probe used (data not shown), indicating a correlation between the signal intensity and amount of probe hybridized. Thus, 10 035 cDNAs were amplified and spotted onto three nylon membranes to make a set of PCR-amplified macroarrays. Each membrane had nine spots of β-actin cDNA as controls.

3.2. Macroarray analysis of the differential expression of genes

Human synoviocytes were cultured for 24 h in serum-containing medium with or without TNFα. The total RNA was used for hybridization with three sets of macroarrays. ANOVA statistical analysis of the control spots showed no differential hybridization among membranes within a set of macroarrays (data not shown), indicating uniform hybridization among membranes. Then, the signal of each spot was normalized based on the assumption that the sum of all spots within a set of macroarrays would be constant between the membranes hybridized with the probe for normal synoviocytes and the probe for TNFα-stimulated synoviocytes. The mean and the standard deviation were calculated for each spot. Three assays was performed to calculate these values. For each sample, spots with poor quality (standard deviation > mean) were not used. As a result, 9655 genes

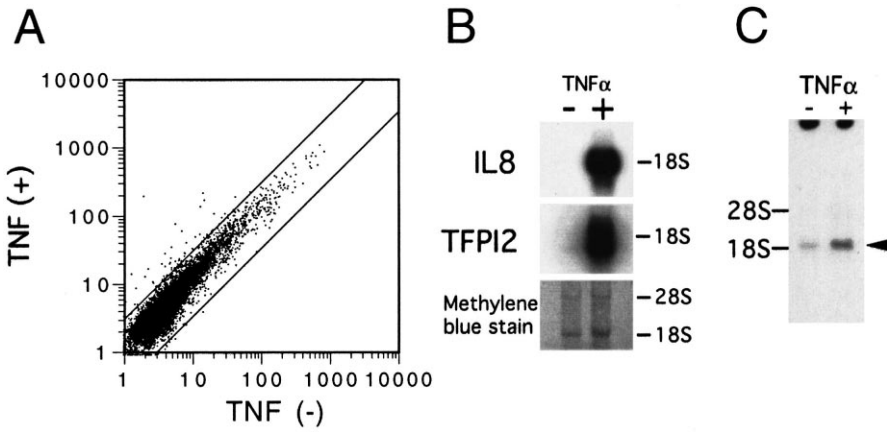

Fig. 2. Macroarray analysis of gene expression in synoviocytes stimulated with TNFα. Synoviocytes were cultured in medium with or without 10 μg/ml TNFα for 24 h. A: The expression of genes was analyzed using PCR-amplified cDNA macroarrays. The mean of the triplicate signal intensity of each gene was plotted. The two lines in the figure show two-fold variation in the signal intensity from each filter. B: Northern blot analysis of IL8 and TFPI2. 2 μg per lane of mRNA were subjected to denaturing-gel electrophoresis and blotted onto a nylon membrane. C: Northern blot analysis of FLJ10143. Arrowhead indicates the bands of FLJ10143.

were selected for further analysis, and 9079 (94% of the analyzed genes) genes showed intensity which was within two-fold variation between the normal and stimulated synoviocytes (Fig. 2A). Genes with over three-fold variation between the normal and stimulated synoviocytes totaled 162 (1.7% of the analyzed genes), suggesting the validity of the above assumption. Twenty-six genes, including six unknown genes, showed over three-fold variation, with statistical significance, between the normal and stimulated synoviocytes (Table 1).

The 26 genes were categorized based on the ontology of their molecular function. Increased expression was observed in 12 of these known genes and in eight unknown genes. Table 1 also lists some examples of genes showing constant expression between the normal and stimulated cells. The top three known genes showing higher signal intensities of 1012, 896 and 758, respectively, were fibronectin 1, vimentin and pre-pro-alpha1(I) collagen in synoviocytes analyzed using the cDNA macroarrays.

3.3. Confirmation of gene expression by Northern blotting

To validate the above results, Northern blotting was performed for two known genes, IL8 and TFPI2, and an uncharacterized gene as examples. Expression of IL8, known to be increased by TNFα [10], was shown by the macroarray analysis to be elevated 65.8-fold in synoviocytes stimulated with TNFα. Northern blot analysis yielded a similar result. IL8 expression was not observed in the normal synoviocytes but was found in the synoviocytes stimulated with TNFα, indicating an almost all-or-nothing phenomenon (Fig. 2B).

The macroarray analysis showed that expression of TFPI2 was elevated 10.2-fold in synoviocytes stimulated with TNFα, and Northern blot analysis gave a similar result (Fig. 2B). The normal synoviocytes expressed TFPI2, and this expression was increased in the synoviocytes stimulated with TNFα.

The macroarray analysis showed that expression of FLJ10143, a new gene, was elevated 9.5-fold in synoviocytes stimulated with TNFα, and this result was confirmed by Northern blot analysis (Fig. 2C). The mRNA size was about 2.1 kb. The normal synoviocytes expressed FLJ10143, and this expression was increased in the synoviocytes stimulated with TNFα.

3.4. Full sequencing analysis of novel clones revealed by macroarray analysis

FLJ10143, whose expression was increased 9.5-fold in the stimulated cells, was a novel gene of 1694 bp encoding 468 aa (GenBank accession No. AK001005) with two immunoglobulin domains in the middle of the sequence and a membrane-spanning region at the C-terminal region (Fig. 3A). The highest similarity (29%) was with poliovirus receptor. Alignment of FLJ10143 with the human genome sequence revealed the presence of an NF-κB binding motif at the −130 base site from the 5′-end of FLJ10143 on the genome sequence (data not shown).

FLJ11467, increased three-fold in the stimulated cells, was a novel gene of 1220 bp encoding 365 aa (GenBank accession No. AK021529) (Fig. 3B). It showed no significant similarity with known proteins. An amino acid sequence motif search revealed the presence of a prenylation motif at the C-terminal position.

FLJ14427, increased 3.1-fold in the stimulated cells, was a novel gene of 1074 bp encoding 208 aa (GenBank accession No. AK027333) (Fig. 3C). There was no significant similarity with known proteins, and no amino acid domain/motif.

FLJ14492, increased 3.5-fold in the stimulated cells, was a novel gene of 2322 bp encoding 619 aa (GenBank accession No. AK027398) with 16 zinc finger C2H2-type domains and an ATP/GTP-binding site motif (Fig. 3D). There was the highest similarity (43%) with zinc finger protein ZNF135.

FLJ14834, decreased 3.3-fold in the stimulated cells, was a novel gene of 1836 bp encoding 150 aa (GenBank accession No. AK027740) (Fig. 3E). There was no significant similarity with known proteins, and no amino acid domain/motif. Alignment of FLJ14834 with the human genome sequence revealed the presence of an NF-κB binding motif at the −156 base site from the 5′-end of FLJ14834 on the genome sequence (data not shown).

FLJ10500, increased 3.3-fold in the stimulated cells, encoded a novel mRNA of 3086 bases (GenBank accession No. AK001362). The amino acid coding region was not able to be determined from this sequence since there was no significant open reading frame.

Fig. 3F summarizes the predicted domains, motifs and membrane-spanning regions of these novel proteins.

Table 1
Genes showing increased or decreased expression in synoviocytes stimulated with TNFα

Accession No.	Gene name	Definition	Molecular function	Synoviocyte[a]	Synoviocyte/TNFα[a]	Fold increase by TNFα	*t*-test[b]	H	L	P	T	B	K	Li	S	Pr
M32578	HLA-DRB1	Major histocompatibility complex, class II, DR beta 1	defense/immunity protein	12.3 ± 7	43.9 ± 6.4	3.6	**	56.6	150.6	46.3	55.5	34.1	51.5	42.7	208.9	751.3
X15414	ALDR1	Aldose reductase	enzyme	13 ± 6.5	39.5 ± 7.5	3	**	54.4	41.2	28.7	13.3	15.2	49.8	21.2	28.2	58.1
AJ271216	DPP3	Dipeptidyl-peptidase III	enzyme	19.7 ± 11.8	58.4 ± 9.3	3	*	65.9	42.1	48.8	24.4	35.1	47.0	32.8	29.7	1787.7
U84573	PLOD2	Pro-collagen lysyl hydroxylase 2	enzyme	22.8 ± 1.3	87.5 ± 24.3	3.8	**	4.8	15.8	9.3	8.6	7.3	10.1	11.4	5.9	10.3
L42452	PDK3	Pyruvate dehydrogenase kinase isoenzyme 3	enzyme	11.5 ± 6.8	50.7 ± 1.3	4.4	**	44.2	40.6	40.3	12.4	27.0	24.3	18.3	20.3	1758.7
M88240	ITIL	Inter-alpha-trypsin inhibitor light chain	enzyme inhibitor	11.1 ± 8	33.7 ± 4.3	3	*	73.3	32.5	71.9	24.8	52.1	44.2	289.9	36.2	633.1
Y00630	PAI2	Plasminogen activator inhibitor, type II	enzyme inhibitor	15.2 ± 1	109 ± 54	7.2	*	26.3	27.5	17.4	10.0	11.7	17.9	14.1	12.5	6.7
L27624	TFPI2	Tissue factor pathway inhibitor-2	enzyme inhibitor	17 ± 4.2	173 ± 75.8	10.2	*	9.6	17.2	8.8	6.1	6.1	9.5	10.0	3.6	11.0
J03191	PFN1	Profilin	ligand binding or carrier	158.6 ± 32.3	55.1 ± 29	0.3	*	45.5	69.1	32.0	39.6	13.9	29.7	33.2	44.1	140.0
M64497	ARP1	Apolipoprotein AI regulatory protein	nucleic acid binding	28.3 ± 4	6.6 ± 1.5	0.2	**	42.3	54.7	26.5	9.2	22.0	63.3	28.5	80.0	39.1
M59465	TNFAIP3	TNFα inducible protein A20	nucleic acid binding	4.8 ± 3.4	22.7 ± 4.9	4.7	**	55.4	70.4	31.0	16.8	24.3	33.2	36.6	23.9	6.8
M24283	ICAM1	Intercellular adhesion molecule 1	signal transducer	5.5 ± 0.7	28.1 ± 4.6	5.1	**	73.8	159.3	75.8	26.7	53.3	51.1	28.2	27.0	21.4
M17017	IL8	IL8	signal transducer	2.4 ± 2	158 ± 40.1	65.8	**	10.3	51.0	6.0	7.7	4.6	4.8	5.2	6.6	6.2
X00734	TUBB5	Beta-tubulin 5	structural protein	47.3 ± 4	155 ± 28.4	3.3	**	135.0	62.4	54.2	42.0	165.7	83.2	79.0	75.1	68.3
AF119662	DKFZP586H2219	E46 protein	unknown	25.1 ± 19	76.4 ± 3.6	3	**	92.3	68.8	75.3	38.8	51.8	55.4	43.7	42.5	2158.7
AK001005	FLJ10143	FLJ10143 fis, weakly similar to poliovirus receptor precursor	unknown	6.5 ± 1.4	61.5 ± 9.8	9.5	**	79.4	37.9	34.8	15.8	30.7	46.3	37.6	21.4	13.9
AK021529	FLJ11467	FLJ11467 fis, clone HEMBA1001647	unknown	23.3 ± 17	70.7 ± 6.8	3	*	66.0	48.8	57.8	28.4	32.3	43.4	42.8	31.3	2029.9

Table 1 *(continued)*

Accession No.	Gene name	Definition	Molecular function	Synoviocyte[a]	Synoviocyte/ TNFα[a]	Fold increase by TNFα	*t*-test[b]	H	L	P	T	B	K	Li	S	Pr
AK027333	FLJ14427	FLJ14427 fis, clone HEMBA1006173	unknown	11.9 ± 9.7	36.5 ± 7.7	3.1	*	55.3	38.6	46.3	20.0	35.7	42.2	26.3	40.6	979.4
AK027398	FLJ14492	FLJ14492 fis, weakly similar to zinc finger protein 135	unknown	12.2 ± 9.5	43.1 ± 10.8	3.5	*	161.2	73.5	69.8	29.9	43.0	98.5	92.0	70.1	1148.1
AK027740	FLJ14834	FLJ14834 fis, clone OVARC1001270	unknown	20.3 ± 4.2	6.9 ± 0.6	0.3	**	21.4	17.5	13.3	7.6	9.3	10.1	10.4	7.2	10.2
U29538	FHL1	Four and a half LIM domains 1	unknown	102.5 ± 26.9	24 ± 5.9	0.2	**	399.9	121.4	61.0	23.1	79.9	25.7	16.1	20.6	102.6
S78825	ID1	Inhibitor of DNA binding 1, dominant negative helix–loop–helix protein	unknown	21 ± 1.8	5.9 ± 1.5	0.3	**	27.3	39.3	29.2	7.0	10.3	15.0	8.2	6.2	82.1
AB018309	KIAA0766	KIAA0766 protein	unknown	8.9 ± 6.4	48.7 ± 6.7	5.5	**	6.6	11.3	15.5	8.6	11.0	7.0	4.3	8.4	17.9
AF380162	FAPP2	Phosphoinositol 4-phosphate adaptor protein-2	unknown	8.8 ± 7.1	29.6 ± 3.7	3.4	*	30.0	22.8	36.2	19.8	24.6	31.1	22.0	24.8	535.4
AF279142	TEM1	Tumor endothelial marker 1 precursor	unknown	52.1 ± 12.6	15.2 ± 3.4	0.3	**	304.4	105.7	138.7	36.8	105.7	212.2	175.3	63.2	10.5
AK001362		FLJ10500 fis, clone NT2RP2000369		9.9 ± 2.3	32.9 ± 1.4	3.3	**	19.8	14.4	13.8	6.4	8.1	9.3	7.9	8.5	17.5
Examples of genes unaffected by TNFα																
AB921288	B2M	Beta 2-microglobulin	defense/ immunity protein	368 ± 29.4	416 ± 93.8	1.1		586.6	1123.0	726.0	388.5	383.8	555.0	341.4	682.6	858.6
X04701	C1R	Complement component C1r	defense/ immunity protein	32 ± 7.2	37.5 ± 10.1	1.2		286.2	1467.0	146.3	335.6	67.1	249.8	218.6	1499.4	1039.7
X60708	DPP4	Dipeptidyl-peptidase IV	enzyme	13 ± 2.3	13.9 ± 2.8	1.1		16.63	16.4	20.27	11.98	10.47	53.44	17.58	11.93	51.67
M28213	RAB2	GTP-binding protein 2	enzyme	28.7 ± 5.4	30.8 ± 2.7	1.1		39.2	187.4	43.7	34.7	25.0	53.0	21.0	68.5	95.5
Y09501	DIA1	NADH-cytochrome b5 reductase	enzyme	45.1 ± 5.4	41.8 ± 7.1	0.9		14.4	31.4	17.0	10.4	9.1	19.2	8.0	9.2	20.9
U43195	ROCK1	Rho-associated, coiled-coil containing protein kinase 1	enzyme	12.8 ± 2.6	13.5 ± 2	1.1		73.9	55.7	58.1	14.5	21.2	29.8	26.4	24.7	26.0

Table 1 (*continued*)

Accession No.	Gene name	Definition	Molecular function	Synoviocyte[a]	Synoviocyte/TNFα[a]	Fold increase by TNFα	*t*-test[b]	H	L	P	T	B	K	Li	S	Pr
AB009010	UbC2	Polyubiquitin UbC	obsolete	483.9 ± 88.1	244 ± 145	0.5		313.6	232.9	1682.3	112.9	316.2	225.7	119.1	240.0	265.9
Z74615	COL1A1	Prepro-alpha1(I) collagen	obsolete	757.8 ± 173	608 ± 100	0.8		146.6	211.1	79.1	52.0	44.5	86.2	376.8	14.7	99.1
X00351	ACTB	β-actin	structural protein	209.8 ± 25.4	157 ± 30.1	0.7		167.0	61.9	80.4	24.4	46.8	66.7	57.0	69.6	58.4

Normalized data were obtained by dividing each spot intensity by the mean of the spot intensity on the filter. Data for the tissues, i.e. heart (H), lung (L), pituitary gland (P), thymus (T), brain (B), kidney (K), liver (Li), spleen (S) and prostate (Pr), are the mean of duplicate spots.

[a]Data are presented as the mean ± S.D. ($n = 3$) after normalization of each spot.

[b]* $P < 0.05$, ** $P < 0.01$.

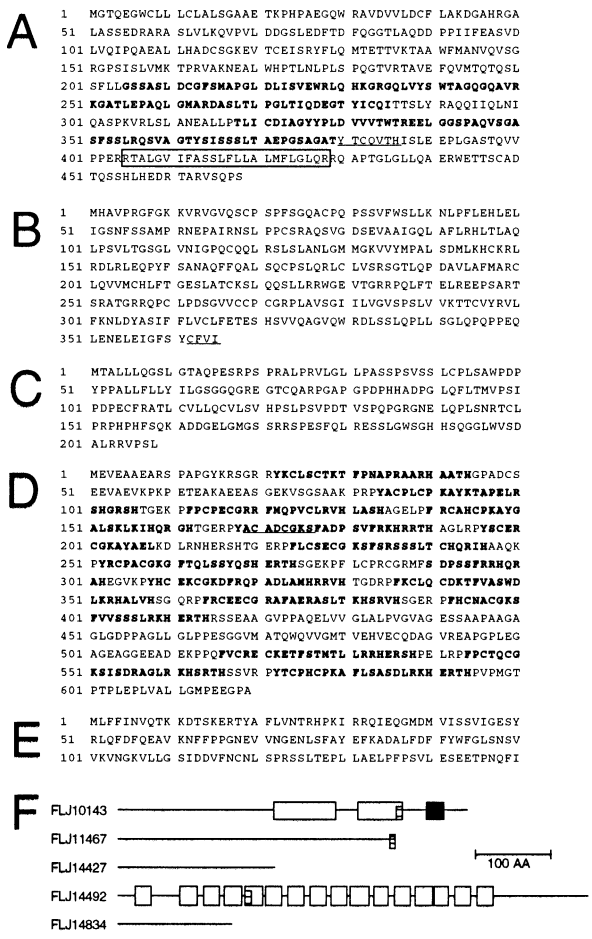

Fig. 3. Amino acid sequences of FLJ clones showing altered expression in synoviocytes stimulated with TNFα. Bold amino acids indicate the probable domain predicted by PFAMA. Underlined amino acids indicate the probable motif predicted by PROSITE. Each motif and domain accession number is shown in the following brackets. Boxed amino acids indicate the membrane-spanning region predicted by SOSUI. A: FLJ10143 protein. The aa locations [205–285] and [318–384] encode the immunoglobulin domains (PF00047). The aa location [380–386] encodes an immunoglobulin major histcompatibility complex motif (PS00290). B: FLJ11467 protein. The aa location [362–365] encodes a prenylation motif (PS00294). C: FLJ14427 protein; D: FLJ14492 protein. The aa locations [22–44], [84–106], [112–134], [140–162], [168–190], [196–218], [224–246], [252–274], [280–302], [308–330], [336–358], [364–386], [392–414], [516–538], [544–566] and [572–594] encode zinc finger C2H2-type domains (PF00096). The aa location [169–176] encodes ATP/GTP-binding site motif A (PS00017). E: FLJ14834 protein; F: illustrations of aa sequences of A–E. Open box: domain; hatched box: motif; closed box: membrane-spanning region.

4. Discussion

Among 9655 analyzed genes, 162 (1.7%) genes showed over three-fold variation between synoviocytes with or without TNFα stimulation. Since 1.7% is larger than 0.3%, the percentage from control analysis for evaluation of this cDNA macroarray analysis, the 162 genes were surmised to include genes with significantly altered expression. Twenty-six such genes were confirmed by statistical analysis of the variation. Northern blot analysis validated these differential expressions. These results suggested that few genes had their expression affected by TNFα stimulation of synoviocyte.

Our expression profiling data for synoviocytes stimulated with TNFα are consistent with the findings of previous reports. It was reported that IL8 was not expressed in normal synoviocytes but was augmented 26-fold in synoviocytes stimulated with TNFα [10,11]. ICAM1, TNFAIP3, ALDR1 and PAI2 were reported to be induced by TNF stimulation of synoviocytes [12], endothelial cells [13], liver cells [14] and melanoma [12], respectively. It was reported that TNFα increased the expression of a PLOD-like gene in the HTB-94 cell line, which is of human origin and maintains a chondrocytic phenotype [15]. These gene expression changes induced by TNFα were detected by our macroarray analysis. In addition to those reported genes, we found altered expression of other known genes (Table 1).

The amount of RNA for each gene expressed in cells could be an important factor in addition to the fold alteration of gene expression for the expression of the cellular functions. It is not easy to compare the amount of RNA between different genes by macroarray analysis. Thus, we roughly compared the amounts of RNA among genes under the assumption that the probe activity and hybridization efficiency were comparable for each gene. Synoviocytes expressed large amounts of fibronectin and collagen in this cDNA macroarray analysis. These results agree with the in vivo study reported by Wolf et al. that showed enhanced deposition of fibronectin and collagen in synovial fluid [16]. Therefore, the main function of synoviocytes seems to be the production of these extracellular matrix (ECM) proteins. Among the analyzed genes, IL8 showed the most significant change in expression by TNFα stimulation. TFPI2 and PAI2 also showed significantly increased expression. The relative amounts of RNA of these three genes were thought to be comparable to that of β-actin in synoviocytes stimulated with TNFα. FLJ10143 showed increased expression, and the amount of its RNA in the cells was thought to be comparable to that of β-actin. IL8 is thought to play a role in the infiltration by lymphoid cells [17]. TFPI2 is thought to suppress ECM degradation in atheroma [18]. In smooth muscle cells, TFPI2 acts as a mitogen [19]. PAI2 is an inhibitor of urokinase-type plasminogen activator and is thought to contribute to local inflammation [20]. Deaminated fibrin, a downstream product of PAI2-related reactions, was the main target of RA-specific autoantibodies [21]. These reported functions of genes are consistent with the pathophysiology of joints in RA. Thus, increased expression of these genes in synoviocytes may play important roles in the early phase of RA.

Other genes for which we demonstrated altered expression also seem to be involved in the pathophysiology of RA. Expression of ICAM1 promoted infiltration by lymphoid cells [22]. The chondroitin sulfate chain of ITIL increased in size proportionally to the severity of the inflammatory response and was postulated to be involved in control of the inflammatory process [23]. PLOD-like protein, an enzyme involved in collagen biosynthesis and its metabolism, was increased in HTB-94 cells [15]. These genes might be involved in promotion of the inflammatory process. It was reported that lipid profiles were altered in patients with RA [24]. The apolipoprotein AI level was significantly higher in patients with RA than in those with degenerative joint disease [25]. ARP1 down-regulated the expression of apolipoprotein AI [26], known to suppress the production of TNFα [27]. TNFAIP3, a nucleic acid binding protein, inhibited NF-κB-dependent gene expression and apoptosis [28]. The decreased and increased expression of ARPI and TNFAIP3, respectively, might be a feedback system suppressing further inflammation. HLA-DRB1, a genotype known to be a risk factor of RA [29], was increased in RA [30]. Its expression might relate to autoantigen presentation. It was reported that DPP2 activity was higher, while DPP4 activity was lower, in synovial fluid of RA patients compared with osteoarthritis patients [31]. We observed a three-fold increase in DPP3 expression without any change in DPP4 expression in the synoviocytes stimulated with TNFα. DPP3 might also be related to the development of pain by the degradation of enkephalin in the joints. These reports represent various aspects of the pathophysiology of RA. It was interesting that we observed all these changes in synoviocytes stimulated with TNFα. Expression of these genes in synoviocytes might be the basis of development of RA.

A discrepancy between our results and those of previous reports concerned the expression of nuclear protein ID1. ID1 was reported to show increased expression in synovial tissues of patients with RA [32]. However, we observed a 0.3-fold decrease in expression of ID1 in the synoviocytes stimulated with TNFα. This difference needs to be investigated by other methods, such as Northern blot analysis. The increased expression of ID1 observed in the in vivo study might reflect the more complicated features of RA compared with the simple model of stimulation of synoviocytes with TNFα.

In conclusion, we used statistical cDNA macroarray analysis to analyze the expression profiles of genes in synoviocytes stimulated with TNFα. Our findings suggest that some of the major functions of synoviocytes are the production of IL8, TFPI2, PAI1 and FLJ10143 upon stimulation by TNFα. The response of synoviocytes to TNFα stimulation may be integral to the development of various aspects of the pathophysiology of RA.

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