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Functional genomics in sarcoidosis – reduced or increased apoptosis?[1]

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Summary

Background: A variety of studies have stressed the importance of the control of inflammatory cell longevity and the balance of pro-survival and pro-apoptotic signaling pathways. The aim of the study was to investigate the systemic activation of apoptosis pathways using cDNA array technology in patients with acute onset sarcoidosis.

Method: We have performed a comprehensive genomic analysis, applying high-density human GeneChip® probe arrays (HGU95A, Affymetrix) for RNA expression profiling from peripheral blood mononuclear cells from patients with acute pulmonary sarcoidosis and matched healthy controls. Twelve patients and 12 controls were assessed, mean age 36 ± 12 and 33 ± 10 years respectively. Results focus on apoptosis-related gene products. Group differences were assessed with the Mann-Whitney U-test.

Results: Seven patients had self-limited disease (all type I sarcoidosis) and 5 progressive disease requiring immunosuppression (all type II or III sarcoidosis). We found 53 of 112 (47%) apoptosis-related gene products dysregulated in sarcoidosis compared to controls. Particular growth factors, especially heparin-binding EGF-like GF, EGF, PDEGF, SISPDGF2 and VEGF, were upregulated in patients consistent with a pro-survival profile. The Bcl-2 family of genes also showed a net pro-survival profile in sarcoidosis patients. In contrast, alterations in the TNF-pathway were compatible with increased apoptosis signals in both, type I and type II/III sarcoidosis patients. Other cell death receptors were equally expressed, as were caspases and p53-associated genes. In contrast to patients with type I-sarcoidosis, patients with progressive type II or III disease showed an upregulation of NFKB and a leak of downregulation of inhibitor of apoptosis 1.

Conclusion: Significant differences in the expression of apoptosis-related genes were found in peripheral blood of patients with acute onset sarcoidosis. Gene expression did not show a definite pattern that was suggestive of pro-survival or pro-apoptosis. However, the number of genes whose altered expression would be predicted to favour increased survival exceeded that of genes likely to reduce survival. Protein-based confirmation of the differences in the activity of apoptosis-pathways needs to be done in further studies.

Key words: gene expression; apoptosis; sarcoidosis; tumour necrosis factor; Bcl-2; gene chip

Introduction

Sarcoidosis is a chronic systemic disorder characterised by the presence of non-caseating granulomas and accumulation of T-lymphocytes and macrophages in multiple organs [1]. Although the most important enigma of sarcoidosis, ie, its aetiology, remains an unsolved problem, the past few years have seen remarkable advances in understanding general immunological and molecular aspects of the mechanisms leading to granuloma formation in sarcoidosis. Accumulation of macrophages and, particularly, CD4-positive T-lymphocytes are present at sites of disease activity, later conglomerating to form

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granulomata [2, 3]. Currently known mechanisms contributing to this cellular accumulation include (1) active migration of CD4-positive T-cells and monocytes from blood under the influence of potent chemotactic factors, including MIP-1, MCP-1, RANTES and IL-8 [4–9] and (2) *in situ*-proliferation of lymphocytes (IL-2 mediated) [10–13], and possibly macrophages [14].

However, the mechanism leading to the persistent accumulation of inflammatory cells is not fully understood. Apoptosis, a dynamic process involved in the control of the "tissue load" of immune effecter cells at inflamed sites, limits inflammatory tissue injury and promotes resolution of inflammation [15, 16]. Whether or not reduced apoptosis is involved in the pathogenesis of sarcoidosis is unclear. Studies looking at Fas and/or TNF-receptor 1 found increased levels of expression on T-lymphocytes in BAL-fluid [17, 18] suggesting increased apoptosis and, similarly, Kunitake [19] found increased numbers of cells going into apoptosis in lung tissue of patients with sarcoidosis compared to controls. These findings are in agreement with increased apoptosis in sarcoidosis. On the other hand it has been postulated as early as 1987 by Cree et al. [20] that apoptosis phenomena might correlate with disease activity and, thus, may play an important role in disease outcome in sarcoidosis.

Many different pathways are involved in apoptosis, the main ones being signaling through death receptors (e.g. FAS, TNFR1) [21], the p53 pathway [22], the Bcl-2 family of genes [23] and the cascade of effector proteases - the caspases [24]. These pathways involve multiple components, which can be pro- and anti-apoptotic. Reduced apoptosis can, therefore, be the net result of reduced expression of pro-apoptotic factors or increased expression of pro-survival products. The large number of genes, whose differential expression could affect cell survival in particular pathological situations, creates two major problems in attempting to analyse the basis for altered cell survival by measuring gene expression on an individual basis. First, it is likely that only a small proportion of involved genes will show altered expression, making identification of those genes difficult. Secondly, and more importantly, the identification of differential expression of a single gene will not necessarily reflect the whole balance of changes in pro-apoptotic and pro-survival gene expression.

With cDNA or oligonucleotide arrays several thousand gene products can be assayed in a single experiment opening a new dimension to gene expression studies (fig. 1) [25–28]. With this technique, target RNA can be copied into labeled cDNA with reverse transcriptase so that the relative abundance of individual mRNAs is reflected in the cDNA product. For gene expression studies single-stranded probe cDNA or oligonucleotides [29, 30] derived from sequences of known genes, cDNA libraries or ESTs can be fixed on filter/glass slide arrays and hybridised with the target cDNA.

To date, there have been few studies of apoptosis in sarcoidosis and these have been limited to the examination of a small number of genes. Using high-throughput arrays we have sought to shed further light on apoptosis signals in the peripheral blood of sarcoidosis patients with self-limited and progressive sarcoidosis.

Methods

Twelve consecutive symptomatic patients presenting with acute onset sarcoidosis were included from August 1999 to May 2000 for this prospective controlled trial. Patients and 12 healthy controls, matched for age and sex, were non-smokers, non-atopic, with no prior history of malignancy, chronic inflammatory disorder or treatment with corticosteroids. All included subjects were white Caucasians and gave informed written consent. Non-atopic state was confirmed by history and negative skin prick test to house dust mite, grass pollen, cat and dog dander. The diagnosis of sarcoidosis was confirmed histologically in 9 patients by transbronchial lung biopsy and in 3 patients, presenting with erythema nodosum and bilateral hilar lymphadenopathy, by a bronchoalveolar lavage CD4/CD8 ratio >3.5. Chest radiographs were staged according to the Silzbach classification [31]. All patients had a clinical follow-up at 6 months including chest radiograph and pulmonary function tests. Seven of 7 patients presenting with type I sarcoidosis fully recovered during the 12-month follow-up period, whereas all 5 subjects with type II/III disease had persistent symptoms and all requiring immunosuppressive therapy. Spirometry and diffusion capacity were measured with a SensorMedics V_{max} 22 series.

Peripheral blood mononuclear cells (PBMCs) were separated from 50 mL heparinised whole blood, drawn at the baseline visit, using gradient centrifugation (Ficoll® Paque, Pharmacia, Uppsala Sweden). The buffy layer was carefully recovered and washed 3 times in culture medium (AIM V®, Life Technologies Paisley U.K.). Cell pellets were frozen and stored at –80 °C.

For GeneChip®-experiments extracted RNA from PBL was reverse transcribed using Superscript® Choice System (Life Technologies). The cDNA was then *in vitro* transcribed (BioArray HighYield RNA Transcript Labelling Kit, Enzo) to form biotin labeled cRNA. Probe hybridisation to the GeneChip® probe arrays (HGU95A, Affymetrix), washing, staining and scanning was done according to the instructions of the manufacturer. For validation of specific genes with altered expression validation experiments were done using real-time RT-PCR (TaqMan®).

Power analysis based on assumed expression variability and expectable intergroup differences was done. However, it has to be appreciated that every cytokine has a distinct variability of expression, which for most of them is not yet known. Generally, sample sizes of 12 probes for each phenotype tested are in accordance with published articles and current understanding. The primary end point being the differential expression of all tested gene products. For data analysis and mining we used GeneSpring™ (version 4.0.0, Silicon Genetics) and SPSS® (version

10.02, SPSS® Inc) software packages. Two-group gene expression comparison between sarcoidosis patients and healthy controls was done using the Mann-Whitney U-test. A three-group comparison of healthy controls subjects, type I and type II/III-sarcoidosis patients (Kruskal-Wallis-test), as well as other two-group comparisons between two phenotypes were only performed in those cases with a p-value <0.05 [32, 33]. Due to low n-numbers in the two separated phenotypes of sarcoidosis, the interpretation of significant differential expression in these subanalyses requires prudence. Correlation of lung function parameters (DLCO, FEV_1, FVC) and gene expression was analysed with Spearman' rank correlations. A conventional significance level of 0.05 was taken, but required prudent interpretation due to multiple significance testing. The study was approved by the local ethical committee.

Results

Patient characteristics are given in table 1. Results of apoptosis-related gene expression in sarcoidosis compared to matched healthy controls are summarised in table 2. Figure 1 shows the comparative expression in sarcoidosis patients and healthy controls of all 12 626 genes and sequence tags tested. A selection of 112 genes with known involvement in apoptosis were selected and discussed below.

We were able to identify significantly altered expression of 53 of 112 (47%) apoptosis-related genes in sarcoidosis compared to the healthy subjects. The alteration in gene expression followed one of three patterns: genes whose expression was altered in both types of sarcoidosis (n = 14), genes with altered expression in type I, but not type II/III sarcoidosis (n = 23) and genes with altered expression in type II/III sarcoidosis only (n = 8). Eight gene products only reached significance when testing both sarcoidosis phenotypes together against controls (n = 8). We were not able to find a gene expressed in type I or type II/III sarcoidosis exclusively. The genes fell into different apoptosis pathways, which are presented below.

Pro-survival cytokine and growth factor genes

The most upregulated growth factors comprise heparin-binding EGF-like growth factor, endothelial cell growth factor 1, platelet-derived endothelial cell growth factor, c-sisplatelet-derived growth factor 2 and vascular endothelial growth factor. Early growth response proteins 1 and 2, as well as the early growth response gene alpha infer increased growth factor receptor signaling. Even though significance was not reached in both sarcoidosis subgroups in all cases the relative expression levels showed a very similar profile in patients with type I and type II/III sarcoidosis. Heparin-binding EGF-like growth factor (DLCO, FEV_1) and vascular endothelial growth factor expression (FEV_1, FVC) correlated with the impairment of lung function.

TNF-and other cell-death receptor pathways

Increased levels for TNFA (type II/III only), TNFR1, TNFA-inducible protein A20 and TSG-14 (type I only) in sarcoidosis patients as a whole indicated increased TNF signaling. TNF-receptor signaling leads either to cell activation/proliferation or induces apoptosis. Critical elements are intracellular adaptor molecules. We found diminished inhibitor of apoptosis (IAP)-levels in type I sarcoidosis, but not type II/III sarcoidosis patients and diminished TRAF5 in both sarcoidosis phenotypes indicating that the cell activation-arm of the TNF-signaling is not activated, especially in the self-limiting type I disease. FADD, a molecule essential for the induction of apoptosis, was not differentially expressed. The cell-death adaptor molecules RAIDD and the apoptosis associated protein GADD34 (in type II/III trend only) were both upregulated indicating increased TNF-related apoptosis. This constellation of intracellular adaptor molecules is compatible with increased apoptosis rather then proliferation signals through TNF-pathways in the sarcoidosis patients as a whole. However, and as the main difference in TNF-signals between both sarcoidosis phenotypes, there was higher expression of TNFA and NFKB in type II and III disease, inferring greater ongoing cell activation and proliferation compared to type I disease.

On the other hand, there was no evidence of differential expression/signaling through Apo3L/death receptor 3, Fas (CD95)/FasL, TRAIL/

Table 1

Subject characteristics including age, lung function parameters and outcome at 12 months of clinical and radiological follow-up.

	Healthyh	patients	typ I	typ II/III
	(n = 12)	(n = 12)	(n = 7)	(n = 5)
Age [y]	33.4 ± 9.6	35.5 ± 11.5	33.1 ± 10.5	39.7 ± 13.6
FEV_1 [%]	ND	94 ± 18	98 ± 12	89 ± 25
FVC [%]	ND	97 ± 20	105 ± 10	84 ± 26
TLCO [%]	ND	87 ± 24	98 ± 17	69 ± 26
Persistent at 12 months	NA	5/12	0/7	5/5

Table 2

Summary of the gene expression results of 112 genes involved in apoptosis. Genes are grouped according to function and listed in alphabetical order. The table gives besides the gene expression levels (relative to the expression in controls, RE), the p-values of the significance testing between the different phenotypes and testing of Spearman correlations with disease severity measured by DLCO, FVC and FEV_1. A red overlay indicates up- (arbitrary cut-off value of 1.3), a green overlay downregulation (arbitrary cut-off value of 0.7) in gene expression. The significance level is also colour-coded with a p <0.05 light blue, p <0.01 dark blue. C: controls, S all: all sarcoidosis patients together, S I: type I sarcoidosis patients, S II/III: patients with type II/III sarcoidosis. Acc.No: GeneBank accession numbers, HUGO: human genome nomenclature short names. NA not applicable.

GeneNb	expression relative to C			significance testing between groups					correlations			HUGO	Acc.No.	gene
	S all	S I	S II/III	C vs. S all	3 groups	S I vs. C	S II/III vs. C	S I vs. II/III	DLCO	FVC	FEV1			
Death receptors														
Death receptors and ligands														
937	1.325	1.249	1.440	0.033	0.077	0.151	0.035	0.465	0.761	0.983	0.991	TNFA	X02910	Tumor necrosis factor (TNF-alpha)
642	1.158	1.187	1.118	0.021	0.068	0.052	0.073	0.685	0.845	0.587	0.745	TNFR	M32315	Tumor necrosis factor receptor
12155	1.617	1.545	1.722	0.028	0.088	0.076	0.073	0.808	0.396	0.430	0.264	TNFA-ind. A20	M59465	Tumor necrosis factor alpha inducible protein A20
541	1.615	1.620	1.609	0.019	0.065	0.035	0.095	0.745	0.862	0.549	0.133	TNFTSG-14	M31166	Tumor necrosis factor-inducible (TSG-14)
889	0.943	NA	NA	0.453	NA	NA	NA	NA	NA	NA	NA	Fas	X89101	Fas (Apo-1, CD95)
936	0.935	NA	NA	0.149	NA	NA	NA	NA	NA	NA	NA	FasL	U11821	Fas ligand (FasL)
9671	1.327	NA	NA	0.345	NA	NA	NA	NA	NA	NA	NA	APO3L	AF055872	Apo3DR3 ligand (APO3L)
232	1.000	NA	NA	1.000	NA	NA	NA	NA	NA	NA	NA	DDR3	U83600	Death domain receptor 3
365	0.569	0.509	0.664	0.003	0.007	0.002	0.092	0.372	0.609	0.513	0.556	DDR3, soluble	U83598	Death domain receptor 3 soluble form
5580	1.114	NA	NA	0.453	NA	NA	NA	NA	NA	NA	NA	DcR2	AF029761	Decoy receptor 2
787	0.999	NA	NA	0.773	NA	NA	NA	NA	NA	NA	NA	TRAIL	U37518	TNF-related apoptosis inducing ligand TRAIL
4928	0.936	NA	NA	0.862	NA	NA	NA	NA	NA	NA	NA	TRAIL-R2	AF016266	TRAIL receptor 2
4525	1.322	NA	NA	0.664	NA	NA	NA	NA	NA	NA	NA	TRAIL-R3	AF014794	TNF related TRAIL receptor (TRAIL-R3)
5444	1.263	NA	NA	0.144	NA	NA	NA	NA	NA	NA	NA	DR6	AF068868	TNFR-related death receptor-6 (DR6)
Intracellular adaptor molecules														
10586	0.792	0.794	0.788	0.050	0.146	0.076	0.171	0.808	0.753	0.633	0.534	DED-test	AF043733	Death effector domain-containing testicular molecule
842	1.024	NA	NA	0.659	NA	NA	NA	NA	NA	NA	NA	FADD	U86214	Fas-associated death domain
2984	0.665	0.628	0.719	0.021	0.065	0.043	0.092	0.570	0.948	0.106	0.347	TOSO	AF057557	Anti-Fas-induced apoptosis (TOSO)

Table 2 cont.

789	0.739	0.677	0.836	0.011	0.027	0.009	0.171	0.372	0.511	0.095	0.020	IAP1	U45878	Inhibitor of apoptosis protein 1
6631	0.974	NA	NA	0.686	NA	NA	NA	NA	NA	NA	NA	MIHB	U37547	IAP homolog B (MIHB)
711	0.858	NA	NA	0.495	NA	NA	NA	NA	NA	NA	NA	XIAP	U45880	X-linked inhibitor of apotosis protein XIAP
5626	1.088	NA	NA	0.952	NA	NA	NA	NA	NA	NA	NA	XIAP-assoc. F	X99699	XIAP associated factor-1
12037	0.954	NA	NA	0.908	NA	NA	NA	NA	NA	NA	NA	RIP	U50062	RIP protein kinase
802	0.820	0.731	0.962	0.024	0.024	0.011	0.343	0.062	0.136	0.226	0.477	TRADD	L41690	TNF receptor-1 associated protein
361	0.642	0.662	0.615	0.003	0.013	0.022	0.011	0.869	0.518	0.843	0.886	TRAF5	U69108	TNF receptor associated factor 5
230	1.102	NA	NA	0.429	NA	NA	NA	NA	NA	NA	NA	TRAF6	U78798	TNF receptor associated factor 6
9827	1.145	NA	NA	0.083	NA	NA	NA	NA	NA	NA	NA	I-TRAF	U59863	TRAF-interacting protein
4831	0.949	NA	NA	0.248	NA	NA	NA	NA	NA	NA	NA	TRAMP	X63679	TRAMP protein
515	0.770	0.806	0.722	0.024	0.069	0.176	0.015	0.935	0.563	0.217	0.269	TRAP1	U12595	Tumor necrosis factor type 1 receptor associated protein
4967	1.083	NA	NA	0.564	NA	NA	NA	NA	NA	NA	NA	U95218	U95218	T cell-death associated protein
12415	0.798	NA	NA	0.184	NA	NA	NA	NA	NA	NA	NA	PDCD2	S78085	PDCD2=programmed cell death-2Rp8 homolog
12382	2.109	2.272	1.899	0.002	0.007	0.007	0.020	0.372	0.736	0.297	0.470	RAIDD	U79115	Death adaptor molecule RAIDD
7086	2.329	2.444	2.178	0.002	0.007	0.002	0.073	0.935	0.753	0.681	0.429	GADD34	U83981	Apoptosis associated protein
Antiapoptotic death receptor signalling														
3027	0.678	NA	NA	0.101	NA	NA	NA	NA	NA	NA	NA	MEKK1	AF042838	MEK kinase 1 (MEKK1)
784	0.889	NA	NA	0.602	NA	NA	NA	NA	NA	NA	NA	JNKK1	U17743	JNK activating kinase (JNKK1)
1178	1.057	NA	NA	0.929	NA	NA	NA	NA	NA	NA	NA	JNK1	L26318	Protein kinase (JNK1)
8503	0.846	NA	NA	0.470	NA	NA	NA	NA	NA	NA	NA	JNK2	U09759	Protein kinase (JNK2)
2596	2.579	2.507	2.683	0.000	0.000	0.000	0.002	0.935	0.279	0.633	0.307	JUN	J04111	c-jun proto oncogene (JUN)
12534	0.755	NA	NA	0.233	NA	NA	NA	NA	NA	NA	NA	NIK	Y10256	Serinethreonine protein kinase, NIK
3795	0.956	NA	NA	0.564	NA	NA	NA	NA	NA	NA	NA	IKK alpha	AF009225	IkB kinase alpha subunit (IKK alpha)
6007	0.991	NA	NA	0.815	NA	NA	NA	NA	NA	NA	NA	IKK beta	AF031416	IkB kinase beta subunit
6052	1.065	NA	NA	0.326	NA	NA	NA	NA	NA	NA	NA	IKK gamma	AF074382	IkB kinase gamma subunit (IKK-gamma)
416	0.813	0.814	0.812	0.008	0.029	0.022	0.045	0.808	0.306	0.404	0.416	NFKB	M58603	Nuclear factor kappa-B DNA binding subunit
325	1.187	1.067	1.377	0.015	0.015	0.236	0.003	0.167	0.712	0.863	0.601	NFKB65A	L19067	NF-kappa-B transcription factor p65 subunit

Table 2 cont.

Bcl-2 family														
Anti-apoptotic														
1122	1.000	NA	NA	1.000	NA	NA	NA	NA	NA	NA	NA	BCL1	M73554	Bcl-1
946	0.925	NA	NA	0.773	NA	NA	NA	NA	NA	NA	NA	BCL2	U58334	Bcl2, p53 binding protein Bbp53BP2 (BBP53BP2)
931	0.884	NA	NA	0.514	NA	NA	NA	NA	NA	NA	NA	BCL2A	M13994	B-cell leukemialymphoma 2 (bcl-2) proto-oncogene
1141	1.918	1.683	2.304	0.021	0.059	0.083	0.030	0.567	0.583	0.678	0.241	BLC2B	M13995	B-cell leukemialymphoma 2 (bcl-2)
1001	1.074	NA	NA	0.525	NA	NA	NA	NA	NA	NA	NA	BCL2C	M14745	Bcl-2
9518	1.631	1.857	1.361	0.002	0.005	0.002	0.073	0.167	0.548	0.199	0.762	BCL2E1B	AF079221	Bcl-2adenovirus E1B 19kDa-interacting protein 3a
876	1.001	NA	NA	0.729	NA	NA	NA	NA	NA	NA	NA	BCL3S2	U05681	Proto-oncogene (Bcl-3)
10179	2.341	2.561	2.065	0.000	0.000	0.001	0.004	0.372	0.371	0.602	0.280	BCL6	U00115	Zinc-finger protein (bcl-6)
2858	1.020	NA	NA	0.419	NA	NA	NA	NA	NA	NA	NA	BCL7A	X89984	Bcl-7A protein
10518	1.075	NA	NA	0.166	NA	NA	NA	NA	NA	NA	NA	BCL7B	X89985	Bcl-7B protein
6249	0.618	0.554	0.720	0.011	0.039	0.035	0.045	0.569	0.811	0.712	0.555	BCL9	Y13620	Bcl-9
8947	1.083	NA	NA	0.600	NA	NA	NA	NA	NA	NA	NA	BCL10	AJ006288	Bcl-10 protein
677	1.047	NA	NA	0.317	NA	NA	NA	NA	NA	NA	NA	BCLXL	Z23115	Bcl-xL
1102	2.108	2.105	2.114	0.003	0.011	0.003	0.073	0.808	0.897	0.199	0.064	BFL1	U27467	Bcl-2 related (Bfl-1)
1272	1.094	NA	NA	0.204	NA	NA	NA	NA	NA	NA	NA	MCL1X	L08246	Myeloid cell differentiation protein (MCL1)
7286	1.218	NA	NA	0.184	NA	NA	NA	NA	NA	NA	NA	BNIP1	U15172	Bcl-2adenovirus E1B 19kD-interacting protein 1 (BNIP1)
2068	1.074	NA	NA	0.166	NA	NA	NA	NA	NA	NA	NA	BNIP2	U15173	Bcl-2adenovirus E1B 19kD-interacting protein 2 (BNIP2)
771	1.038	NA	NA	0.857	NA	NA	NA	NA	NA	NA	NA	BBC3	U82987	Bcl-2 binding component 3 (bbc3)
947	1.127	NA	NA	0.480	NA	NA	NA	NA	NA	NA	NA	BBC6	U66879	Bcl-2 binding component 6 (bbc6)
Pro-apoptotic														
12406	1.161	NA	NA	0.288	NA	NA	NA	NA	NA	NA	NA	BAK	U16811	Bak
756	0.940	NA	NA	0.550	NA	NA	NA	NA	NA	NA	NA	BAK	X84213	Bak
1171	1.043	NA	NA	0.954	NA	NA	NA	NA	NA	NA	NA	BAXA	L22473	Bax alpha
1172	0.943	NA	NA	0.685	NA	NA	NA	NA	NA	NA	NA	BAXB	L22474	Bax beta
1096	1.147	1.161	1.128	0.050	0.130	0.035	0.343	0.935	0.511	0.471	0.124	BAXD	U19599	Bax delta

Table 2 cont.

1173	0.803	NA	NA	0.073	NA	NA	NA	NA	NA	NA	NA	BAXG	L22475	Bax gamma
2740	1.106	NA	NA	0.419	NA	NA	NA	NA	NA	NA	NA	BID	AF042083	BH3 interacting domain death agonist (BID)
1112	0.972	NA	NA	0.317	NA	NA	NA	NA	NA	NA	NA	BIK	U34584	Bcl-2 interacting killer (BIK)
12414	0.768	NA	NA	0.260	NA	NA	NA	NA	NA	NA	NA	BLK	S76617	Blk=protein tyrosine kinase [B lymphocytes]
8078	0.804	NA	NA	0.088	NA	NA	NA	NA	NA	NA	NA	BNIP3	AF002697	E1B 19KBcl-2-binding protein Nip3
P53														
949	0.731	0.728	0.734	0.002	0.007	0.028	0.003	0.935	0.079	0.572	0.249	ATM	U67092	Ataxia-telangiectasia locus, exons 1a, 1b, 2, 3 and 4
1101	0.634	0.621	0.653	0.011	0.033	0.076	0.015	0.685	0.761	0.513	0.974	ATM	U26455	Phosphatidylinositol 3-kinase homolog (ATM)
1113	0.793	NA	NA	0.326	NA	NA	NA	NA	NA	NA	NA	DNA-PKcs	U34994	DNA dependent protein kinase catalytic subunit
276	1.056	NA	NA	0.954	NA	NA	NA	NA	NA	NA	NA	DNA-PKcs	U47077	DNA-dependent protein kinase catalytic subunit
1108	1.005	NA	NA	0.317	NA	NA	NA	NA	NA	NA	NA	MDM2A	U33199	Mdm2-A (mdm2)
8582	0.963	NA	NA	0.071	NA	NA	NA	NA	NA	NA	NA	MDM2D	U33202	Mdm2-D (mdm2)
944	0.998	NA	NA	0.699	NA	NA	NA	NA	NA	NA	NA	MDM2E	U33203	Mdm2-E (mdm2)
1028	0.868	NA	NA	0.671	NA	NA	NA	NA	NA	NA	NA	MDMX	AF007111	MDM2-like p53-binding protein (MDMX)
812	1.137	NA	NA	0.074	NA	NA	NA	NA	NA	NA	NA	CDC25A	CDC25A	Cdc25A
148	1.007	NA	NA	0.525	NA	NA	NA	NA	NA	NA	NA	ACK	U47077	Activated p21cdc42Hs kinase
10325	0.988	NA	NA	0.729	NA	NA	NA	NA	NA	NA	NA	ACK	L13738	Activated p21cdc42Hs kinase
Interleukins														
9484	3.345	3.593	3.027	0.002	0.009	0.004	0.045	0.808	0.435	0.443	0.230	IL1B	M15330	Interleukin 1-beta
7667	1.752	1.885	1.582	0.002	0.007	0.005	0.027	0.372	0.462	0.379	0.225	IL1RA	X52015	Interleukin-1 receptor antagonist
10401	1.298	1.464	1.097	0.018	0.032	0.009	0.292	0.291	0.462	0.779	0.609	TIL4	AF051152	Tollinterleukin-1 receptor-like protein 4
6810	0.552	0.553	0.550	0.001	0.006	0.007	0.015	0.869	0.359	0.455	0.036	IL5RA	M75914	Interleukin 5 receptor alpha
3993	0.857	0.802	0.941	0.025	0.055	0.017	0.361	0.237	0.494	0.685	0.495	IL7	M29053	Interleukin 7
408	0.495	0.489	0.504	0.000	0.001	0.001	0.008	0.685	0.616	0.829	0.688	IL7	M29696	Interleukin-7 receptor
406	6.857	6.477	7.427	0.003	0.011	0.004	0.058	0.685	0.578	0.443	0.162	IL8	M28130	Interleukin 8
389	2.088	1.728	2.721	0.021	0.069	0.052	0.073	0.808	0.991	0.217	0.549	IL8R	U11870	Interleukin-8 receptor type A
12189	1.537	1.714	1.319	0.008	0.010	0.005	0.170	0.062	0.010	0.208	0.203	IL9R	L39064	Interleukin 9 receptor precursor

Table 2 cont.

68	0.739	0.808	0.651	0.009	0.023	0.063	0.015	0.291	0.181	0.665	0.632	IL10R	U00672	Interleukin-10 receptor
12058	0.776	0.762	0.796	0.043	0.123	0.035	0.292	0.935	0.497	0.779	0.617	IL11RA	U32324	Interleukin-11 receptor alpha chain
Growth Hormones & growth response proteins														
8105	4.786	6.011	3.479	0.001	0.002	0.001	0.023	0.291	0.023	0.063	0.012	HB-EGF-like GF	M60278	Heparin-binding EGF-like growth factor
732	2.514	3.347	1.684	0.008	0.021	0.014	0.073	0.223	0.490	0.572	0.505	EGF1	EGF1	Endothelial Cell Growth Factor 1
6935	1.903	2.328	1.435	0.002	0.006	0.003	0.058	0.223	0.448	0.557	0.579	PDEGF	M63193	Platelet-derived endothelial cell growth factor
631	1.711	1.906	1.471	0.006	0.016	0.004	0.024	0.386	0.890	0.872	0.317	SISPDGF2	M12783	c-sisplatelet-derived growth factor 2
6149	1.479	1.597	1.327	0.011	0.021	0.005	0.246	0.291	0.253	0.542	0.259	VEGF	AF022375	Vascular endothelial growth factor
1048	1.436	1.609	1.225	0.024	0.041	0.011	0.343	0.291	0.117	0.048	0.004	VEGF	AF024710	Vascular endothelial growth factor
419	0.881	0.881	0.881	0.033	0.103	0.097	0.157	1.000	.	.	.	KGF	M60828	Keratinocyte growth factor
430	0.843	0.782	0.938	0.033	0.038	0.018	0.342	0.088	0.126	0.966	0.737	GRF	L00137	Growth hormone-releasing factor
1086	0.831	0.831	0.831	0.033	0.103	0.097	0.157	1.000	.	.	.	PDGFR	X76079	Platelet derived growth factor alpha receptor
911	0.806	0.829	0.775	0.031	0.086	0.143	0.064	0.398	0.684	0.893	0.333	FGF	M27968	Basic fibroblast growth factor
6839	0.805	0.719	0.944	0.038	0.027	0.007	0.673	0.123	0.253	0.499	0.111	GH1&2	J03071	Growth hormone (GH-1 and GH-2)
1161	0.799	0.774	0.836	0.024	0.053	0.018	0.246	0.372	0.211	0.245	0.097	FGFR1	M34641	Fibroblast growth factor (FGF) receptor-1
4385	0.766	0.831	0.683	0.034	0.072	0.194	0.029	0.385	0.995	0.330	0.867	KGFR	M80634	Keratinocyte growth factor receptor
7686	0.669	0.623	0.738	0.008	0.017	0.002	0.292	0.570	0.745	0.713	0.416	EDGF	AF063020	Lens epithelium-derived growth factor
645	0.634	0.607	0.675	0.026	0.067	0.031	0.238	0.237	0.112	0.114	0.113	ILGFBP3	M35878	Insulin-like growth factor-binding protein-3
7929	2.498	2.225	2.937	0.021	0.064	0.075	0.045	0.685	0.563	0.795	0.307	EGR2	J04076	Early growth response protein 2
12349	2.184	1.834	2.788	0.008	0.023	0.043	0.020	0.372	0.897	0.897	0.640	EGR1	X52541	Early growth response protein 1
1219	1.302	1.233	1.405	0.038	0.100	0.108	0.073	0.465	0.253	0.697	1.000	EGRA	S81439	Early growth response gene alpha

Figure 1

Scanned pseudo-colour image of a hybridised human HUG95A GeneChip® expression array with enlarged probe set of a gene as an insert top right. This specific microarray shown contains probe sets interrogating approximately 12,000 full-length human genes and some EST clusters from the UniGene database (Build 95). Using reverse transcriptase messenger RNA (mRNA) is by copied into a cDNA population reflecting the relative abundance of the individual mRNAs. In a next step labelled cRNA is generated by in vitro transcription, hybridised to the GeneChip® expression array, stained with a fluorecent dye and analysed using a laser scanner. The intensity of the hybridisation signal for a given gene is a result of its relative abundance in the mRNA-derived target cRNA. The method is described as providing excellent specificity and reproducibility. Messenger RNA species comprising 1:10,000-100,000 of the mass of the target mRNA, which corresponds to approximately 1 transcript per 100,000, can readily be detected.

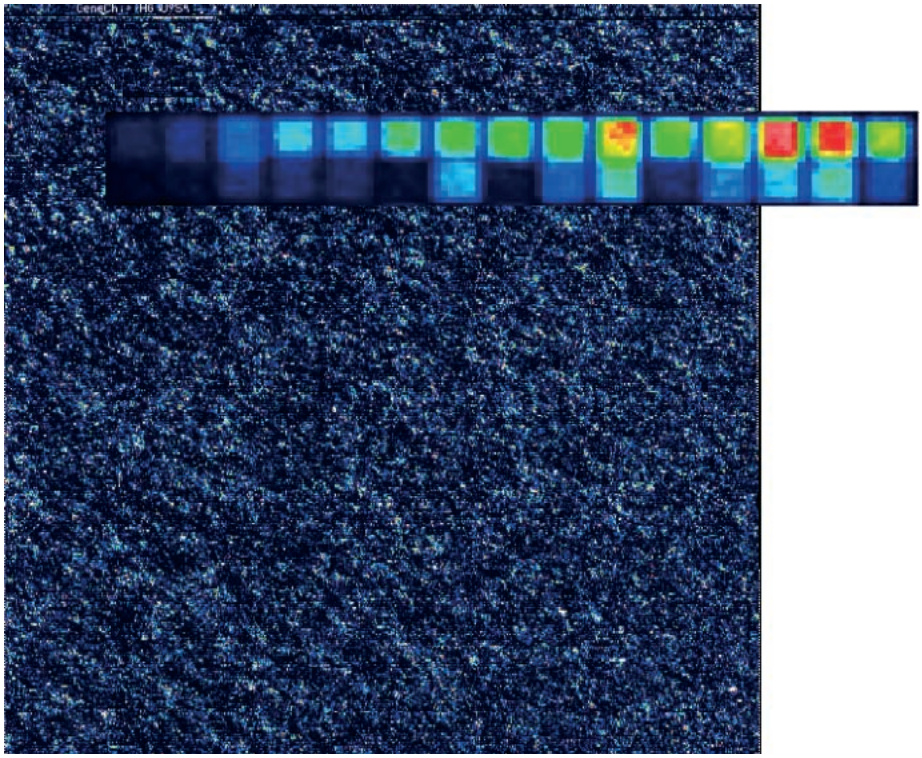
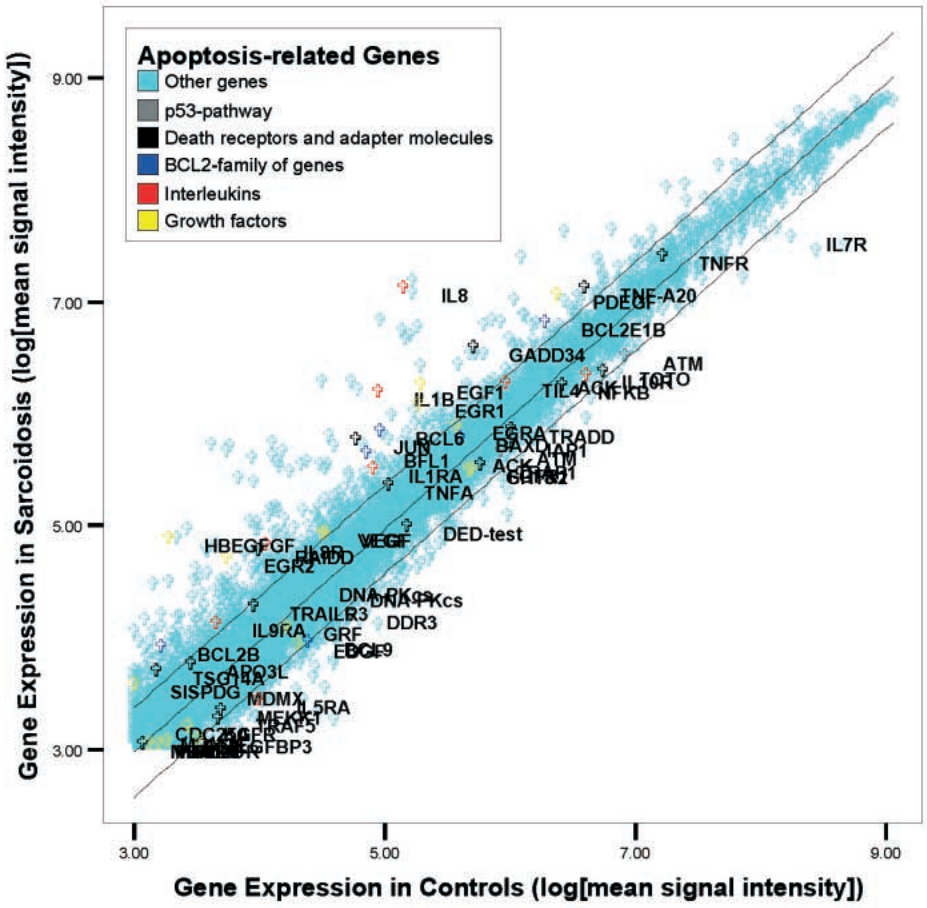


Figure 2

Scatter plot of the mean gene expression in sarcoidosis patients compared to controls. Apoptosis-related genes discussed in this publication are highlighted in colour-codes (for abbreviations see table 2), which allows estimation of the extent of the regulated expression in the context of the overall transcriptional activity. Gene expression in both phenotypes is very similar for most of the genes tested resulting in a R-square of 0.98. Also given are cut-offs of 1.3 and 0.7 expression relative to the other phenotype by additional lines.



TRAIL receptor 2 and 3, decoy receptor 2 and TNF-related death receptor 6. The soluble form of the death domain receptor 3 was significantly downregulated in both phenotypes.

Cascade of caspases

The group of caspases did not show differential expression in sarcoidosis compared to controls.

Bcl-2 family

We measured 19 pro-survival and 10 pro-apoptotic members of the Bcl-2 family. Five of 19 pro-survival Bcl-2 family members were dysregulated, of which 4 (BCL2B, BCL2E1B, BCL6, BFL1) were compatible with reduced apoptosis in sarcoidosis patients. A single pro-apoptotic Bcl-2 gene, BAX delta, was upregulated. Thus, expression of Bcl-2 family genes showed an anti-apoptotic profile.

p53 pathway

There was no evidence for increased responses either to DNA-damage or to oxidative stress in sarcoidosis. The Ataxia Telangiectasia gene ATM, induced by DNA damage, showed reduced expression in sarcoidosis (RE = 0.63). The genes for the p53-neutralising protein MDM2A, D, E and MDMX were equally expressed.

Discussion

Recently, a role for reduced apoptosis has been postulated as contributing towards the accumulation of inflammatory cells in sarcoidosis [34]. However, studies in BAL and lung tissue looking for Fas, TNF-receptor 1, TUNEL and electron microscopy were all indicative of increased apoptosis when compared with specimens from healthy controls in these compartments [17–19]. Nevertheless, it has been suggested but not proven that reduced apoptosis was present in more severe disease when compared to patients with mild forms of sarcoidosis and, therefore, may contribute to disease severity [20]. Applying functional genomics, we were able to detect alterations in the expression of several genes associated with cell survival and/or apoptosis in peripheral blood mononuclear cells of patients with sarcoidosis. These alterations in gene expression could be due to genetic (ie, hereditary) or environmental factors. Although the alterations of the expression of growth factor-related genes showed a concerted pro-survival pattern, the situation regarding apoptosis-related genes was less clear-cut.

The pattern of expression of growth factors and pro-survival related genes was generally balanced in favour of a pro-survival climate in sarcoidosis. Particularly upregulated genes were heparin-binding EGF-like growth factor, endothelial cell growth factor 1, platelet-derived endothelial cell growth factor, c-sisplatelet-derived growth factor 2 and vascular endothelial growth factor. Expression of heparin-binding EGF-like growth factor and vascular endothelial growth factor especially, correlated with the impairment of lung function and could thus serve as markers of disease severity. VEGF, which can be induced by TNFA, IL1 and members of the fibroblast growth factor family [35], was upregulated in both sarcoidosis-phenotypes. VEGF can be inhibited by high doses of dexamethasone [36, 37]. VEGF, expressed by a variety of cells including endothelial cells, is crucial for fibroblast chemotaxis and angioneogenesis. VEGF is involved in lung tissue remodeling as can be seen in patients with chronic inflammatory lung disease and is markedly upregulated under hypoxic conditions [38].

Tumour necrosis factor can either induce cell activation in a NFKB-dependent manner or apoptosis signaling through a cascade of TNF receptor type I, TRADD and the caspases [21, 24]. Different intracellular adaptor molecules seem to play a pivotal role in this switch. The expression of TNF and related signaling genes is compatible with increased apoptosis signals in both sarcoidosis phenotypes. However, and as an important difference compared to patients with self-limited disease, patients with type II and III disease also seem to signal cell activation / proliferation as demonstrated by the significant upregulation of NFKB in these patients only. The role of the lacking downregulation of the inhibitor of apoptosis IAP1 in type II/III-sarcoidosis is unclear, but might be a possible explanation for the differences observed between the sarcoidosis phenotypes. Interestingly, this gene also correlates with lung function tests.

In contrast to the clear dysregulation of the TNF-pathway, all other death receptors tested, namely Apo3L/death receptor 3, Fas (CD95)/FasL, TRAIL/TRAIL receptor 2 and 3, decoy receptor 2 and TNF-related death receptor 6, did not show differential expression. The relevance of the significant downregulation of the soluble form of the death domain receptor 3 is unclear.

Of the 19 measured members of the pro-survival Bcl-2 family, 4 were increased and only BCL9 decreased. This is compatible with a clear net pro-survival effect of the Bcl-2 family of genes. Only 1 of 10 pro-apoptotic Bcl-2 family gene, BAX delta, was slightly upregulated (RE 1.15). However, BAX delta is not able to interact directly with APAF1, which is the usual binding partner of pro-survival Bcl-2 genes, and the downstream regulatory element with inhibitory activity on CASP9. BAX delta exerts its pro-apoptotic effect by binding to pro-survival Bcl-2 proteins, which are then unable to bind APAF1. However, quantitatively the up-

regulation of pro-survival genes was much more prominent. Thus, the Bcl-2 family of genes, clearly had an pro-survival profile.

As far as the p53-pathway is concerned, there was no evidence for increased responses either to DNA-damage or to oxidative stress in sarcoidosis. Apart from the Ataxia Telangiectasia gene ATM, induced by DNA damage, which showed reduced expression, the genes for the p53-neutralising protein MDM2A, D, E and MDMX were all equally expressed.

The finding that the expression of apoptosis effector proteases, the caspases, was not altered in sarcoidosis, is not surprising, given that they are produced in excess well in advance of an apoptotic event and when activated induce apoptosis within minutes without the need for any transcriptional activity.

A potential criticism of our study is analysing peripheral blood when the disease activity predominates in the lung. Indeed, the picture in the circulation might represent a mirror image of the true situation, where "disease-modulating" cells have migrated into tissue. Furthermore, it has been suggested that there is compartmentalisation of the immune process in sarcoidosis [40] and that peripheral blood mononuclear cells are quiescent, although several studies refute this. Sarcoidosis is clearly a systemic disease involving activation of many genes in different organs. It is, thus, likely, and underlined by our results, that sufficient "spill-over" of local inflammatory phenomena are present in peripheral blood (which circulates approximately once per minute through the lungs). We, therefore, hypothesise that a potential primary alteration in gene regulation, either genetically anchored or triggered by environmental factors, would also be present distant from the actual site of inflammation – the lung. Cell sequestration and the contribution of the different cell types to a specific gene expression pattern need to be further investigated in gene expression studies comparing lung tissue, bronchoalveolar lavage and peripheral blood samples. In exclusively looking at gene expression a limitation of our approach is that we cannot give any statement on the activity of the apoptotic pathways. To do so the data should be combined with analysis of the proteins involved in apoptosis including enzyme assays to evaluate the activity of caspases. Such confirmatory protein-based studies need to be done as yet.

In summary, using cDNA technology it was possible to demonstrate that peripheral blood mononuclear cells of patients with acute onset sarcoidosis showed differential expression of a number of genes associated with cell survival and/or apoptosis. Gene expression did not show a definite pattern that was suggestive of pro-survival or pro-apoptosis. Whereas, the TNF-pathway indicated increased apoptosis signaling, the family of Bcl-2 genes and the group of growth hormones clearly had a pro-survival profile. Interestingly, a distinct difference in TNF-signaling between patients with self-limiting type I-sarcoidosis and progressive type II/III-sarcoidosis was identified, which indicates TNF-related proliferation/cell activation signals in the latter. Taken together, the number of genes whose altered expression would be predicted to favour increased survival exceeded that of genes likely to reduce survival. This suggests that the altered gene expression may be determined, at least in part, by systemic factors so that cells are programmed for increased survival before entry into the inflammatory site where local factors may enhance their survival further.

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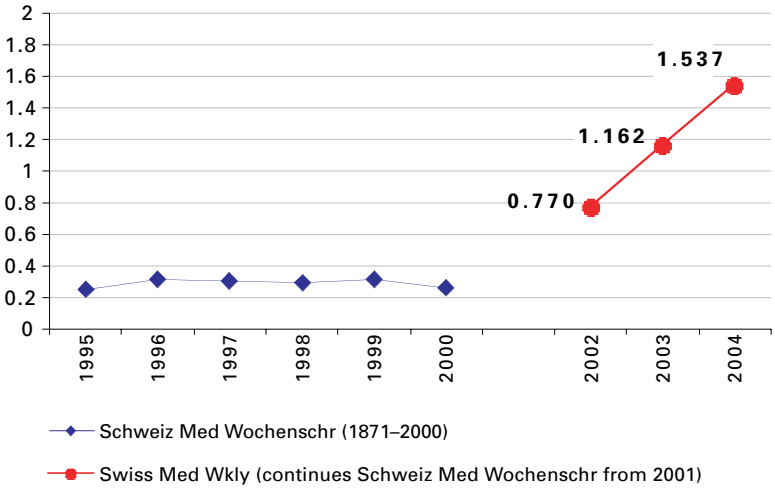

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