Identification of mechanically induced genes in human monocytic cells by DNA microarrays

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Background Hypertension is a risk factor for coronary heart disease. Macrophages are critically involved in both atherogenesis and plaque instability. Although macrophages may be subjected to excess mechanical stress in these diseases, the way in which biomechanical forces affect macrophage function remains incompletely defined.

Objective To investigate the molecular response to mechanical force in macrophages.

Design and methods We used a DNA microarray with 1056 genes to describe the transcriptional profile of mechanically induced genes in human monocytic THP-1 cells. Mechanical deformation was applied to a thin and transparent membrane on which cells were cultured. After THP-1 cells were pre-incubated in the presence of phorbol 12-myristate 13-acetate (0.2 μmol/l) for 24 h, THP-1 cells attached to the membrane were subjected to biaxial mechanical strain. Interleukin-8 concentrations were determined using an enzyme-linked immunosorbent assay.

Results In DNA microarray analysis, cyclic mechanical strain at 1 Hz induced only three genes more than 2.5-fold at 3 and 6 h in THP-1 cells: prostate apoptosis response-4 (3.0-fold at 3 h, 6.7-fold at 6 h), interleukin-8 (4.3-fold at 6 h) and the immediate-early response gene, *IEX-1* (2.6-fold at 6 h). Real-time reverse transcriptase polymerase chain reaction analysis confirmed the amplitude-dependent induction of these three genes. In addition, mechanical strain increased interleukin-8 protein expression.

Conclusion The present study demonstrates that human monocytic cells respond to mechanical deformation with induction of immediate-early and inflammatory genes. These findings suggest that mechanical stress *in vivo*, such as that associated with hypertension, may play an important part in atherogenesis and instability of coronary artery plaques, through biomechanical effects on vascular macrophages. *J Hypertens* 20:685–691 © 2002 Lippincott Williams & Wilkins.

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Introduction

The interactions of mechanical forces and cells of the vasculature are relevant to many cardiovascular diseases. Hypertension is a risk factor for atherosclerotic cardiovascular diseases, including coronary artery disease, stroke, heart failure, and peripheral artery disease [1,2]. In addition, plaque rupture may have a critical role in the pathogenesis of unstable angina and in the progression of coronary lesions [3]. Macrophages participate in atherogenesis and commonly localize at sites of fatal human coronary plaque rupture [4,5]. Matrix metalloproteinases are constitutively expressed by the macrophage foam cells within atheroma of hypercholesterolemic rabbits [6]. Aikawa *et al.* [7] reported that macrophages in atheroma of rabbit aortas strongly expressed tissue factor. These data suggest that macrophage-related proteolysis and promotion of thrombosis may contribute to the propensity of those plaques to rupture and trigger thrombosis. Although macrophages in atherosclerotic lesions may be subjected to mechanical stress in these diseases, the way in which mechanical stress affects macrophage function remains incompletely defined.

In contrast to differential display, DNA microarray technology allows expression monitoring of hundreds or thousands of genes simultaneously, and provides a format for parallel gene expression studies [8,9]. High-density arrays of oligonucleotides or complementary DNAs (cDNAs) are placed on glass slides by high-

speed robotic printing. Each printed oligonucleotide or cDNA on the microarray is suitable for molecular hybridization, thus allowing rapid assessment of mRNA expression of all arrayed genes in cells or tissues of interest. In addition to identifying large clusters of genes that respond to a given stimulus, DNA microarray technology may be used to identify a few genes that comprise a highly specific molecular response [10,11]. Recently, microarray analysis of gene expression has been applied to tissue from myocardial infarction [12], cardiac hypertrophy [13], and failing human hearts [14].

Using a DNA microarray and a mechanical deformation device that applies a highly uniform biaxial strain field to a culture substrate, we investigated the transcriptional profile of mechanically induced genes in THP-1 cells (a human monocytic cell line). We found that human monocytes/macrophages respond to mechanical deformation with induction of immediate-early and inflammatory genes. These results suggest that mechanical stress *in vivo*, such as that associated with hypertension, may have an important role in atherogenesis and instability of coronary-artery plaques.

Methods

Materials

Phorbol 12-myristate 13-acetate (PMA) and RPMI 1640 medium were purchased from Sigma Chemical Co. (St Louis, Missouri, USA). Fibronectin, bovine fetal calf serum (FCS) and Hanks' balanced salt solution (HBSS) were purchased from Life Technologies, Inc. (Rockville, Maryland, USA). All other chemicals used were of the highest grade commercially available.

Cell culture

THP-1 cells (human acute monocytic leukemia cells) were obtained from American Type Culture Collection (Rockville, Maryland, USA), cultured at 37°C, 5% carbon dioxide in RPMI 1640 medium supplemented with 10% FCS, and 50 μmol/l 2-mercaptoethanol, and maintained at a cellular density of $2 \times 10^5 - 10^6$ cells/ml, as described previously [15].

Mechanical strain device and preparation of cells

Mechanical deformation was applied to a thin and transparent membrane on which cells were cultured – an approach that produces controlled cellular strain. The device used in this study provides a nearly homogeneous and uniform biaxial strain profile; that is, strains that are equal at all locations on the membrane and in all directions [16]. The membrane undergoes cyclic deformation as the platen assembly moves sinusoidally, with a frequency and amplitude derived by the motor speed and the cam size, respectively. We have previously documented membrane strains with high-resolution video [17].

For the preparation of THP-1 cells to be subjected to mechanical strain, autoclaved membrane dishes were coated with 2 μg/ml of fibronectin in 13 ml of HBSS (138 mmol/l NaCl, 5.3 mmo/l KCl, 4.0 mmol/l $NaHCO_3$, 1.3 mmol/l $CaCl_2$, 0.5 mmol/l $MgCl_2$, 0.4 mmol/l $MgSO_4$, 0.4 mmol/l KH_2PO_4, 0.3 mmol/l Na_2HPO_4, 5.6 mmol/l glucose) for 12 h at 4°C, because macrophages adhere on the RGD site of fibronectin [18]. The membrane dishes were then washed twice with 10 ml of phosphate buffered saline (10 mmol/l sodium phosphate, 140 mmol/l NaCl, pH 7.2). THP-1 cells were plated on the coated membrane dish at a density of 10 000 000 cells/dish in 13 ml of RPMI 1640 medium and exposed to 0.2 μmol/l PMA for 24 h. This treatment has been reported to provoke THP-1 cells to acquire macrophage-like characteristics, such as cell adhesion [19,20]. Before applying the mechanical strain, we replaced the medium with 13 ml of fresh RPMI 1640 medium. Mechanical strain was then applied at 1 Hz. Control dishes were treated identically, but received no mechanical strain for the required times.

Transcriptional profiling

THP-1 cells cultured on fibronectin-coated membranes were harvested immediately after 1, 3 and 6 h of cyclic deformation (1 Hz) or no deformation, and total RNA was isolated by the guanidinium thiocyanate–phenol–chloroform method [21]. Preparation of biotin-labeled cRNA probes was performed with the following steps: conversion of RNA to single-strand cDNA by reverse transcriptase reactions and synthesis of double-strand cDNA template using SuperScript II kit (Life Technologies, Inc.), purification of double-strand cDNA template using a QIAquick PCR purification kit (Qiagen Inc., Valencia, California, USA), T7 amplification and transcription of biotin-labeled cRNA (AmpliScribe kit; Epicentre Technologies, Madison, Wisconsin, USA) according to the manufacturer's instructions. The DNA microarray hybridization of biotin-labeled cRNA was performed using ExpressChip HO2 array (Mergen Ltd, San Leandro, California, USA) according to the manufacturer's instructions. The ExpressChip HO2 array has 1056 well-characterized genes with putative functions. A complete listing of genes contained within ExpressChip HO2 can be found at http://www.mergen-ltd.com/HO2.htm. The chips were subjected to laser scanning and signal detection by the GMS418 Array Scanner (Takara Biomedicals, Shiga, Japan). The intensity of emission signals in each oligonucleotide hybridization was normalized to that of the glyceraldehyde-3 phosphate dehydrogenase (GAPDH) signal and analyzed using the ImaGene software package (BioDiscovery Inc., Los Angeles, California, USA).

Real-time reverse transcriptase polymerase chain reaction analysis

For reverse transcriptase polymerase chain reaction, RNA was reverse transcribed using T7-dT primer (5'-TCT AGT CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG GCG TTT TTT TTT TTT TTT TTT TTT-3') and Superscript II reverse transcriptase (Life Technologies, Inc.). Real-time quantitative PCR was performed in a 96-well microtiter plate (Perkin-Elmer/Applied Biosystems, Foster City, California, USA) with an ABI Prism 7700 Sequence Detector Systems (Perkin-Elmer/Applied Biosystems) according to the manufacturer's instructions. By using the SYBR Green PCR Core Reagents Kit (Perkin-Elmer/Applied Biosystems, P/N 4304886), fluorescence signals were generated during each PCR cycle via the 5'- to 3'-endonuclease activity of Taq Gold [22] to provide real-time quantitative PCR information. The oligonucleotide primers used for real-time PCR analysis are shown in Table 1. No-template controls and the samples were added in a total volume of 50 µl/reaction. Potential PCR product contamination was digested by uracil-*N*-glycosylase, because dTTP is substituted by dUTP [22]. All PCR experiments were performed with the hot-start method. In the reaction system, uracil-*N*-glycosylase and Taq Gold (Perkin-Elmer/Applied Biosystems) were applied according to the manufacturer's instructions [22,23]. Denaturing and annealing reactions were performed 40 times at 95°C for 15 s, and 62°C for prostate apoptosis response-4 (*PAR-4*) and interleukin-8 (*IL-8*) and 54°C for the immediate-early response gene, *IEX-1*, for 1 min, respectively. The increase in the fluorescence signal is proportional to the amount of specific product [24]. The intensity of emission signals in each sample was normalized to that of GAPDH as an internal control.

Measurements of IL-8

IL-8 concentrations in the culture supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacture's instructions (Amersham Pharmacia Biotech, Amersham, Buckinghamshire, UK). The absorbance at 450 nm was measured and concentrations were determined by interpolation of a standard calibration curve. The lower limit of detection of IL-8 was 5 pg/ml.

Statistical analysis

Data are expressed as means ± SEM. Differences were analyzed by the Kruskal–Wallis test, and subsequent pairwise comparisons were made by the Mann–Whitney *U* test; values of $P < 0.05$ were considered statistically significant.

Results

DNA microarray analysis of mechanically induced genes in THP-1 cells

Results are presented in Figure 1. For the present study, to minimize false-positive elements, we used a threshold value of 2.5-fold to define differential gene expression. Transcriptional profiles of genes induced by 1% biaxial mechanical strain at a frequency of 1 Hz in THP-1 cells at 3 and 6 h were remarkably similar (data not shown). Among the 1056 well-characterized genes with putative functions, only three genes were induced more than 2.5-fold at 3 and 6 h (Table 2), and no genes were mechanically induced at 1 h. Thus, although the 3 and 6 h microarray hybridizations were performed only twice, the results of these hybridizations regarding induced genes were nearly identical. Among the 1056 genes, 157 elements were therefore called positive at 6 h, and 154 genes were called positive but did not change more than 2.5-fold (available in an online-only Data Supplement at http://www.jhypertension.com).

Mechanically induced genes: real-time RT-PCR analysis

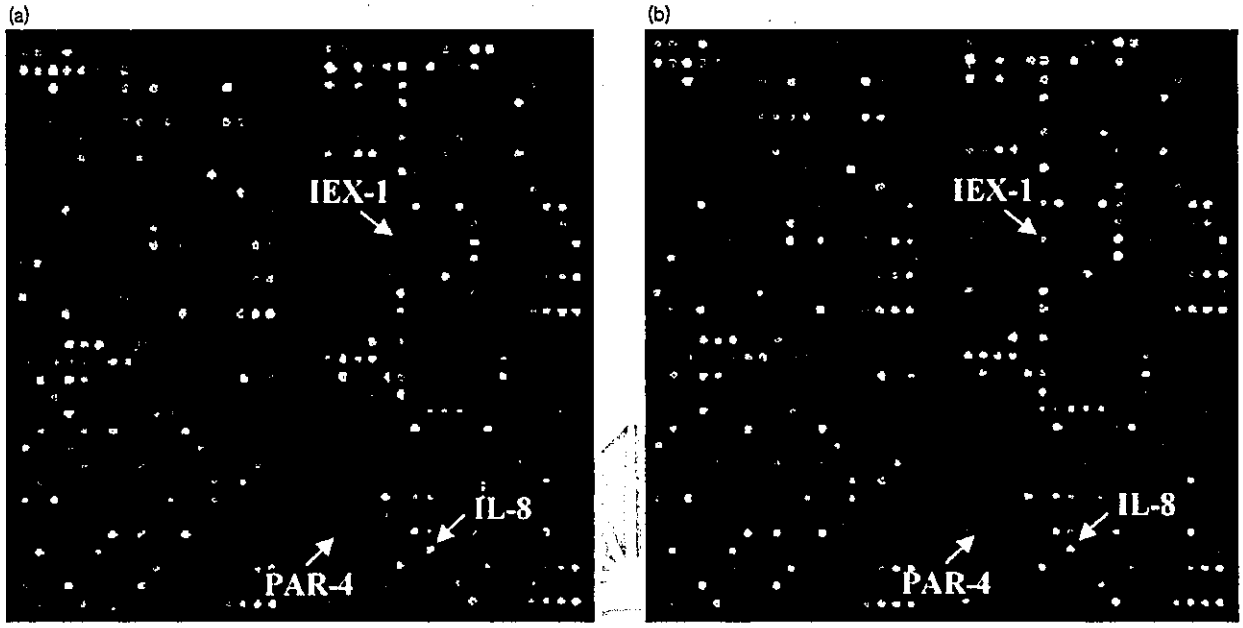
Genes induced more than 2.5-fold in the oligonucleotide microarray were confirmed by real-time quantitative RT-PCR analysis in two independent experiments using THP-1 cells. As shown in Figure 2, all three genes were induced more than 2.5-fold at 6 h: *PAR-4* 3.2-fold, *IL-8* 3.7-fold and *IEX-1* 3.4-fold, whereas these three genes did not change more than 2.5-fold at 3 h: *PAR-4* 0.6-fold, *IL-8* 1.3-fold and *IEX-1* 1.8-fold. Thus these findings demonstrated that 1% biaxial mechanical strain at a frequency of 1 Hz caused the induction of these three genes, *PAR-4*, *IL-8* and *IEX-1*, at least at 6 h in THP-1 cells. In addition, when THP-1

Table 1 Design of primers for real-time reverse transcriptase-polymerase chain reaction (PCR)

Gene		Primer sequences (5'–3')	PCR product (bp)
PAR-4	Sense	5'-GCAGATATAAAAGCACAACCAGTG-3'	134
	Antisense	5'-CAGTGTGCTACTTGAAACCAGAGT-3'	
IL-8	Sense	5'-GAACTGAGAGTGATTGAGAGTGGA-3'	134
	Antisense	5'-CTCTTCAAAAACTTCTCCACAACC-3'	
IEX-1	Sense	5'-TCTACCCTCGAGTGGTGAGTATC-3'	126
	Antisense	5'-ACTAAGGGGAGACAAAACAGGAG-3'	

PAR-4, prostate apoptosis response-4; *IL-8*, interleukin-8; *IEX-1*, immediate-early response.

Fig. 1



Gene expression monitored with the use of DNA microarrays. (a) THP-1 cells subjected to no mechanical strain (control). (b) THP-1 cells subjected to 1% cyclic mechanical strain for 6 h. THP-1 cells stimulated by 0.2 μmol/l were incubated in the presence or absence of 1% mechanical strain for 6 h. Each microarray contained elements representing 1056 different genes. mRNA from THP-1 cells was used to prepare cDNA labeled with [Cy^3]-deoxyuridine triphosphate. The cDNA probes were hybridized to the microarray. In the image of the subsequent scan, genes for which the mRNAs are more abundant in the stretched THP-1 cells appear as white spots. The arrows indicate the spots representing the following genes: prostate apoptosis response-4 (*PAR-4*); interleukin-8 (*IL-8*), and immediate-early response (*IEX-1*).

Table 2 **Genes differentially expressed by more than 2.5-fold, among 1056 genes with putative functions, in THP-1 cells**

	Fold change	
Gene	3 h	6 h
PAR-4	3.0	6.7
IL-8	1.8	4.3
IEX-1	2.2	2.6

PAR-4, prostate apoptosis response-4; *IL-8*, interleukin-8; *IEX-1*, immediate-early response.

cells were subjected to cyclic strains of 1, 2 and 3% at 1 Hz for 6 h, the effects of mechanical strain on *PAR-4*, *IL-8*, and *IEX-1* mRNA expressions were amplitude-dependent (Fig. 3).

Effects of mechanical strain on IL-8 protein production

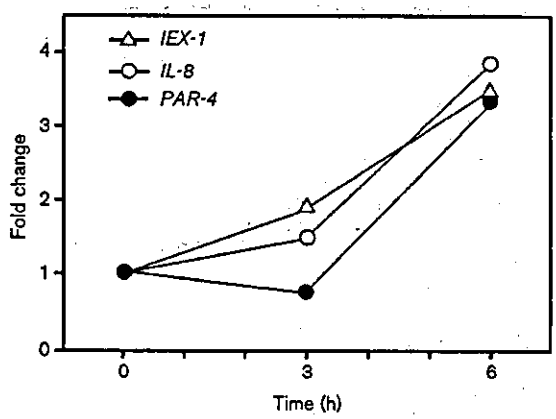
Next, we investigated whether IL-8 protein was induced by mechanical strain in THP-1 cells. The expression of IL-8 protein was analyzed by ELISA. As shown in Figure 4, IL-8 protein production by strain-stimulated THP-1 cells increased in a time-dependent manner.

Discussion

The present study using oligonucleotide microarray and real-time RT-PCR analyses demonstrates that human monocytes/macrophages THP-1 cells respond to mechanical deformation with amplitude-dependent induction of *PAR-4*, *IL-8* and *IEX-1* mRNA. These findings suggest that mechanical stress *in vivo*, such as that associated with hypertension, may have an important role in atherogenesis and instability of coronary artery plaques.

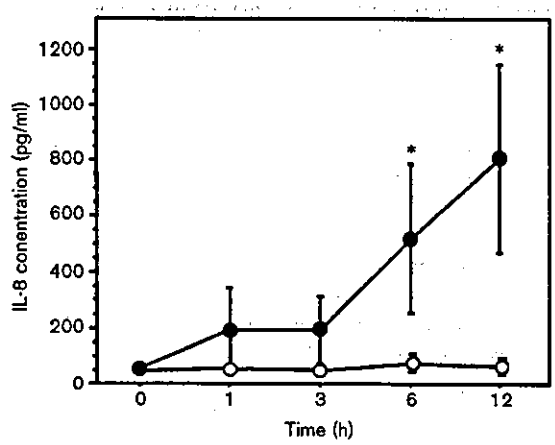
IL-8, a multifunctional molecule that belongs to a CXC chemokine supergene family, is produced by smooth muscle cells [25], endothelial cells [26], epithelial cells [27], and monocytes and macrophages [28,29]. The predominant producer cells for IL-8 are monocytes. IL-8 has chemotactic activity for neutrophils and T lymphocytes and is involved in the pathogenesis of a variety of diseases [30,31]. Apostolopoulos *et al.* [32] reported that IL-8 is produced by macrophages from human atheromatous plaques. IL-8 secretion by macrophages in atherosclerotic plaques may be important in the initiation and amplification of inflammation in atherosclerosis. Yue *et al.* [33] reported that IL-8 is chemotactic for vascular smooth muscle cells and may play a part in the migration of smooth muscle cells from

Fig. 2



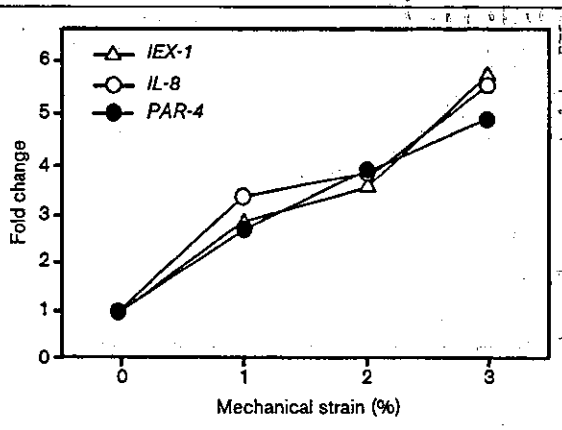
Results of quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. THP-1 cells stimulated by 0.2 μmol/l phorbol 12-myristate 13-acetate were incubated in the presence or absence of 1% mechanical strain for 6 h. RNA was isolated by the guanidinium thiocyanate–phenol–chloroform method and then analyzed by quantitative real-time RT-PCR as described in Methods. The amount of mRNA expression was expressed as relative change standardized to control cells in each experiment. Each value represents the mean of two experiments.

Fig. 4



Effects of mechanical strain on interleukin-8 (IL-8) protein production. THP-1 cells stimulated by 0.2 μmol/l phorbol 12-myristate 13-acetate were exposed for 12 h to 0% (○) or 1% (●) cyclic mechanical strain (1 Hz). IL-8 concentrations in the culture supernatants were determined using an enzyme-linked immunosorbent assay. Data are means ± SEM of three samples. $^*P < 0.05$ compared with control samples not exposed to strain.

Fig. 3



Dependence of gene expression in THP-1 cells on amplitude of mechanical strain. THP-1 cells stimulated by 0.2 μmol/l phorbol 12-myristate 13-acetate were exposed for 6 h to 0, 1, 2 and 3% cyclic mechanical strain (1 Hz). RNA was isolated by the guanidinium thiocyanate–phenol–chloroform method and then analyzed by quantitative real-time reverse transciptase-polymerase chain reaction as described in Methods. The amount of mRNA expression was expressed as relative change standardized to control cells in each experiment. Each value represents the mean of two experiments.

media into intima of vessel walls. IL-8 is a powerful trigger for firm adhesion of monocytes to vascular endothelium [34]. In addition, *IL-8* has been shown to be induced by mechanical stretch in endothelial cells [35] and epithelial cells [36]. Okada *et al.* [35] demonstrated that cyclic stretch increased the concentrations of IL-8 and monocyte chemoattractant protein-1, but not those of IL-1β, tumor necrosis factor-α, IL-6, granulocyte-colony stimulating factor or macrophage-colony stimulating factor. Furthermore, in the present study, cyclic mechanical strain induced the expression of IL-8 mRNA and proteins in THP-1 cells. Thus IL-8 may play a critical role in the atherosclerotic effects of mechanical stress in vascular walls.

IEX-1 is a nuclear factor (NF)-κB-inducible immediate-early gene that is induced by radiation, 12-*O*-tetradecanoylphorbol-13-acetate, Fas, and tumor necrosis factor-α [37]. Wu *et al.* [38] demonstrated the existence of two *IEX-1* splice variants, *IEX-1*S and *IEX-1*L, and showed that *IEX-1*L is an apoptosis inhibitor involved in NF-κB-mediated cell survival. The presence of functional binding sites for NF-κB and p53 in the *IEX-1* promoter supports its role in regulation of growth and differentiation and the stress response [39]. In this study, mechanical stress induced *IEX-1* gene expression in THP-1 cells. Macrophages are subjected to mechanical stress immediately after monocytes transmigrate into the lesion of vessel wall. Therefore, the induction of *IEX-1* by mechanical deformation may participate in differentiation of monocytes/macrophages and promotion of atherogenesis.

The *PAR-4* gene was isolated in a screen for genes transcriptionally induced by apoptotic signals in rat ventral prostate [40]. PAR-4 is a widely expressed

leucine zipper protein that confers sensitization to apoptosis induced by exogenous insults. Johnstone *et al.* [41] reported that PAR-4 interacted with WT1, the Wilms' tumor suppressor protein. We found that mRNA of PAR-4, a pro-apoptotic molecule, was also mechanically induced in THP-1 cells. Further studies are required to clarify whether mechanical deformation acts as a pro-apoptosis or anti-apoptosis factor in macrophages.

Study limitations

The number of genes on the microarray chip used in this study was limited. We focused on the genes related to growth factors, cytokines and apoptosis, because macrophages in atherosclerotic lesions have the potential for secretion of growth factors and cytokines, and for modulating apoptosis. In the present study, there was a difference in the fold change in expression between the microarray and RT-PCR experiments. The results of microarray experiments should be confirmed by the RT-PCR experiments or other methods, although DNA microarray is a powerful method for screening genes. In addition, *IEX-1* and *PAR-4* are related to cellular growth and apoptosis. In this in-vitro system, about 60% of cells adhered to the membrane 12 h after even 1% mechanical strain, whereas more than 90% of cells adhered 6 h after 1–3% mechanical strain. It is difficult, therefore, to investigate whether mechanical stretch itself induces apoptosis or proliferation in macrophages.

Macrophages can secrete numerous cytokines and matrix metalloproteinases. Mechanical stress is greatest in the shoulder region of the atheroma [42], therefore macrophages in these regions are considered to be involved critically in inflammation and matrix degradation. Three mechanically induced genes, *IL-8*, *IEX-1*, and *PAR-4* may be important in the pathogenesis caused by macrophages in cardiovascular diseases. These findings suggest that mechanical stress *in vivo*, such as that associated with hypertension, may have an important role in atherogenesis and instability of coronary artery plaques.

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DNA microarray analysis of T cell-type lymphoproliferative disease of granular lymphocytes

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Summary. Lymphoproliferative disease of granular lymphocytes (LDGL) is characterized by the clonal proliferation of large granular lymphocytes of either T- or natural killer cell origin. To better understand the nature of T cell-type LDGL, we purified the $CD4^-CD8^+$ proliferative fractions from LDGL patients ($n = 4$) and the surface marker-matched T cells isolated from healthy volunteers ($n = 4$), and compared the expression profiles of 3456 genes using DNA microarray. Through this analysis, we identified a total of six genes whose expression was active in the LDGL T cells, but silent in the normal ones. Interestingly, expression of the gene for interleukin (IL) 1β was specific to LDGL T cells, which was further confirmed by the examination of the serum level of IL-1β protein. Given its important role in inflammatory reactions, the disease-specific expression of IL-1β may have a causative relationship with the LDGL-associated rheumatoid arthritis. Spectratyping analysis of the T-cell receptor repertoire also proved the monoclonal or oligoclonal nature of LDGL cells. These data have shown that microarray analysis with a purified T-cell subset is an efficient approach to investigate the pathological condition of T cell-type LDGL.

Keywords: DNA microarray, LDGL, interleukin 1β.

Sustained outgrowth of large granular lymphocytes (LGL) is currently recognized as a clinical entity distinct from other leukaemia/lymphomas; designated as lymphoproliferative disease of granular lymphocytes (LDGL), granular lymphocyte-proliferative disorder (GLPD) or LGL leukaemia (LGLL) (Lamy & Loughran, 1998, 1999). The monoclonal or oligoclonal expansion of LGL often exceeds 2×10^9/l in the peripheral blood (PB) of patients, and the origin of these cells is either $CD3^+$ T cell or $CD3^-$ natural killer (NK) cell. Although LDGL is usually a chronic and indolent disease, its clinical course is often complicated by manifestation of neutropenia/anaemia and/or rheumatoid arthritis (Semenzato *et al*, 1997).

The aetiology of LDGL is poorly understood. Although LDGL cells express high levels of both of Fas and Fas ligand (FasL), reports have indicated that LDGL cells are resistant to the Fas/FasL-mediated cell death pathway (Lamy *et al*, 1998). Interestingly, Epling-Burnette *et al* (2001) have found that STAT3 (signal transducers and activators of transcription, 3) was constitutively activated in LGL cells by an as yet unidentified mechanism, which may partially account for the Fas-resistance in these cells. However, activation of the STAT family of signalling proteins can be found in a wide range of human leukaemias (Coffer *et al*, 2000), and therefore such STAT3 activation is not likely to be the principal cause of the LGL expansion.

Given the fact that oligoclonal expansion of T cells can be found in non-malignant cells (Fitzgerald *et al*, 1995), it has not been fully determined as to whether the chronic elevation of LGL counts found in LDGL is merely a sustained growth of antigen-stimulated T cells or of malignant nature. We do not therefore have reliable criteria on which diagnosis of T cell-type LDGL can be made.

To define the useful molecular markers characteristic of LDGL and to better understand the pathogenesis of this condition, microarray analysis would be a suitable tool in the identification of LDGL-specific genes. However, a simple

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Table I. Laboratory data of the patients with T-LDGL and clonality of proliferating T cells.

Patient number	WBC (10^9/l)	LGL (10^9/l)	Neutrophil (10^9/l)	Hb (g/dl)	Surface phenotype of LGL						Clonality
					CD2	CD3	CD4	CD8	TCRαβ	TCRγδ	
1	5·40	4·54	0·27	3·2	+	+	−	+	+	−	Oligo
2	5·72	4·58	0·34	10·0	+	+	−	+	+	−	Oligo
3	6·77	6·30	0·32	8·3	+	+	−	+	+	−	Oligo
4	2·10	1·64	0·13	14·9	+	+	−	+	+	−	Oligo
5	7·21	4·61	1·51	10·4	+	+	−	+	+	−	Mono
6	11·60	8·70	1·51	7·2	+	+	−	+	+	−	Oligo
7	17·25	14·15	2·76	12·7	+	+	−	+	+	−	Oligo
8	4·70	3·62	1·02	3·5	+	+	−	+	+	−	Oligo
9	12·97	7·10	2·33	12·3	+	+	−	+	+	−	Oligo
10	8·65	6·82	1·45	12·8	+	+	+	−	+	−	Mono
11	8·08	5·69	1·37	12·4	+	+	+	+	+	−	Mono
12	8·10	4·70	0·73	9·8	+	+	−	+	−	+	Oligo
13	5·70	1·85	2·15	3·8	+	+	−	+	−	+	Oligo
14	7·60	3·16	1·06	7·7	+	+	−	−	−	+	Mono

WBC, white blood cell; LGL, large granular lymphocyte; Hb, haemoglobin; CD, cluster differentiation; TCR, T-cell receptor; oligo, oligoclonal; mono, monoclonal.

Clonality was determined by using PCR-based spectratyping for T-cell receptor Vβ or Vδ genes.

comparison of mononuclear cells (MNC) among T cell or NK cell-type LDGL patients would mistakenly lead to pseudo-positive results, which mainly represent T-cell subset-specific or NK cell-specific genes. To minimize such ambiguity, a surface marker-matched population should be purified from patients as well as healthy volunteers before microarray analysis, and the transcriptomes should be directly compared among these background-matched populations (Miyazato *et al*, 2001; Ohmine *et al*, 2001).

We isolated $CD4^-CD8^+$ LGL fractions from patients with T cell-type LDGL ($n = 4$) and healthy volunteers ($n = 4$), and analysed the expression profiles of 3456 human genes of these samples using DNA microarray. A total of six genes were shown to be only active in the LDGL-derived $CD8^+$ cells, including those for interleukin 1β (IL-1β), Titin and α-thalassaemia/mental retardation syndrome X-linked (ATRX). The disease-specific induction of these genes was also confirmed by the quantitative TaqMan™ real-time polymerase chain reaction (PCR) method among 14 patients and 13 healthy volunteers. Furthermore, we demonstrated a significant elevation in the serum level of IL-1β only in the patient group, which may have a causative relationship with rheumatoid arthritis, one of the most frequent complications of LDGL. Taken together, our microarray analysis with a background-matched T-cell subset was highly useful, not only in the identification of possible candidates for the novel diagnostic markers of T cell-type LDGL, but also in providing a clue for the molecular basis of the rheumatoid arthritis associated with this condition.

PATIENTS AND METHODS

Patient samples. A total of 14 individuals with T-cell LDGL were registered into this study: 11 patients were positive for T-cell receptor (TCR) αβ chain expression, while the other three were positive for TCR γδ chains (Table I). Southern blot analysis of the TCR β and/or γ genes confirmed the clonal expansion of the affected T cells in all nine cases examined. Peripheral blood (PB) mononuclear cells (MNC) and sera of age- and sex-matched healthy volunteers ($n = 13$) were also used in this study. All samples were obtained under written informed consent. For the microarray analysis, the $CD4^-CD8^+$ LDGL fractions (cases 1–4 in Table I) were purified from PB by either magnetic bead cell separation system (DYNAL, Oslo, Norway) or flow cytometry (FACSVantage SE, BD Biosciences, San Jose, CA, USA). The same $CD4^-CD8^+$ fractions were also purified from age- and sex-matched healthy volunteers ($n = 4$). The purity of $CD8^+$ cells was evaluated by staining with Wright–Giemsa solutions as well as by flow cytometric analysis with antibodies to CD3, CD4, CD8, TCR αβ, and TCR γδ (BD Biosciences).

RNA preparation and microarray analysis. Total RNA was extracted from $CD4^-CD8^+$ samples by the acid guanidinium method and was subjected to amplification as described previously (Van Gelder *et al*, 1990). The correlation coefficient of DNA microarray data with RNA before and after one round of amplification was calculated to be 0·93. Then, 2 μg of the amplified complementary RNA (cRNA) was converted to double-stranded cDNA which was then used to prepare biotin-labelled cRNA with the use of the Express-Chip labelling system (Mergen, San Leandro, CA, USA), and was allowed to hybridize with microarrays (HO-1–3; Mergen) that contain oligonucleotides corresponding to a total of 3456 human genes (the gene list of the arrays is available at http://www.mergen-ltd.com/). The microarrays were then incubated consecutively with streptavidin, with antibodies to streptavidin, and with Cy3-conjugated secondary

antibodies (all from Mergen). Detection of hybridization signals and statistical analysis of the digitized data were performed with the 418 array scanner (Affymetrix, Santa Clara, CA, USA) and GENESPRING 4.0.7 software (Silicon Genetics, Redwood, CA, USA) respectively. The expression level of each spot on the array was normalized to the median expression value for all genes in each array hybridization. In the hierarchical clustering analysis, similarity was measured by the standard correlation with a separation ratio of 0·5.

Real-time TaqMan™ PCR analysis. Portions of unamplified cDNA were subjected to quantitative real-time PCR with ABI Prism 7700 (PE Applied Biosystems, Foster City, CA, USA) using TaqMan™ fluorogenic oligonucleotide probes. The oligonucleotide primers for IL-1β cDNA were 5′-GATGCACCTGTACGATCACTGAA-3′ and 5′-TGGACCAGACATCACCAAGCT-3′ for PCR and 5′-ACGCTCCGGGACTCACAGCAAA-3′ for the TaqMan probe; those for aldo-keto reductase 1C3 (AKR1C3) cDNA were 5′-CAGCTGGAGATGATCCTCAACA-3′ and 5′-ACTCCGGTTGAAATA CGGATGA-3′ for PCR and 5′-CCAGGACTCAAGTACAAGCCTGTCTGCA-3′ for the TaqMan probe; those for ATRX were 5′-CCAGTGGCTGGTGGTATGC-3′ and 5′-CCTTGGGAAGGTCCTGGATT-3′ for PCR and 5′-CCACCACCATTACAGCGTGCACCAC-3′ for the TaqMan probe. All reactions were carried out using the TaqMan Universal PCR Master Mix (PE Applied Biosystems) at 50°C for 2 min, 94°C for 10 min, and followed by 50 cycles of 94°C for 15 s and 60°C for 90 s. The expression level of target genes was normalized based on that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, using the TaqMan GAPDH control reagents (PE Applied Biosystems).

Enzyme-linked immunosorbent assay (ELISA) of serum IL-1β. The serum level of IL-1β was assayed with the Human IL-1 beta Quantikine HS ELISA Kit (R & D systems, Minneapolis, MN, USA) and monitored by a microplate reader (Bio-Rad, Hercules, CA, USA) at an absorbency of 490 nm. Analyses were performed in duplicate.

Spectratyping of the TCR genes. The double-stranded cDNAs prepared from the purified T cells were subjected to PCR with the primers specific to the 26 Vβ (Gorski *et al*, 1994; Maslanka *et al*, 1995) or three Vδ (Shen *et al*, 1998; Signorini *et al*, 1999) gene loci of TCR as well as to their corresponding constant region. The resultant products were separated through 2% agarose gel, and stained with ethidium bromide. The fluorescent intensities of the corresponding bands were monitored by a CCD video camera (Sony, Tokyo, Japan) and quantified by QUANTITY ONE software (Bio-Rad). The relative intensities of the bands were normalized to those of GAPDH bands. For size spectratyping of the TCR β and δ genes, aliquots of the PCR products were further amplified with a fluorophore-labelled primer specific to Cβ or Cδ gene locus, separated through 6% polyacrylamide gel in an ABI 377 automated sequencer (PE Applied Biosystems), and their sizes were calculated by GENESCAN 672 software (PE Applied Biosystems). The overall complexity of the TCR Vβ gene region (spectratype complexity score, SCS) (Wu *et al*, 2000) in the samples was calculated as the summation of the number of discrete peaks per TCR Vβ subfamily. Reverse transcription (RT)–PCR products of the TCR Vβ or Vδ genes were also purified and inserted into pCR2.1 plasmid vector (Invitrogen, Carlsbad, CA, USA), and subjected to nucleotide sequencing by using an ABI377 automated sequencer.

Statistical analysis. The difference in Vβ complexity or gene expression level between the patients and healthy volunteers was statistically analysed by Student's *t*-test or Mann–Whitney *U*-test with STATVIEW 5.0 software (Abacus Concepts, Berkeley, CA, USA).

RESULTS

Patient profiles

The clinical findings of the individuals with T cell-type LDGL are shown in Table I. The detailed characterization of cases 6 and 7 was previously described by Shimodaira *et al* (1995) and that of case 12 by Ichikawa *et al* (1999). Among the 14 patients, expanding LGL cells of 11 were positive for the expression of TCR αβ chains (nine were $CD4^-CD8^+$, one was $CD4^+CD8^-$ and the other was $CD4^+CD8^+$) whereas three were positive for TCR γδ. The median age at diagnosis was 60 years old (range: 18–78). As evident from Table I, neutropenia and anaemia were often found in the patients. In addition, case 8 suffered from rheumatoid arthritis. Interestingly, case 1 was the mother of case 2.

The clinical courses of most patients were indolent; seven did not receive any treatment and five were symptom-free. However, case 6 died of progressive disease, and cases 7 and 8 died of infection. A total of six patients had been treated with prednisolone, cyclophosphamide, antithymocyte globulin or cyclosporin. Unfortunately, none of these treatments resulted in complete remission.

Cases 1–4 underwent microarray analysis. $CD4^-CD8^+$ LGL fraction was purified from PB MNC, and the purity was assessed to be > 99% as judged by flow cytometry (data not shown). The cytospin preparation of each sample was also stained with Wright–Giemsa solutions to demonstrate the phenotype of purified LGL (Fig 1, top panel). Similarly, the $CD4^-CD8^+$ fractions were purified from age- and sex-matched healthy individuals ($n = 4$) (bottom panel).

DNA microarray analysis

The expression levels of 3456 human genes were studied with DNA microarray among the $CD4^-CD8^+$ fractions obtained from four healthy volunteers and four individuals with LDGL. To visualize these expression profiles, we first conducted a hierarchical clustering analysis on the data, resulting in generation of a dendrogram, or 'gene tree', in which genes with similar expression profiles are positioned near each other (Fig 2A). The expression level of each gene was assigned a pseudocolour, with high expression indicated by red and low expression by green. The gene tree revealed that almost half of the genes studied were transcriptionally silent in the purified CD8-positive T cells. It should be noted that several clusters of genes were active only in the T cells from healthy or patient group.

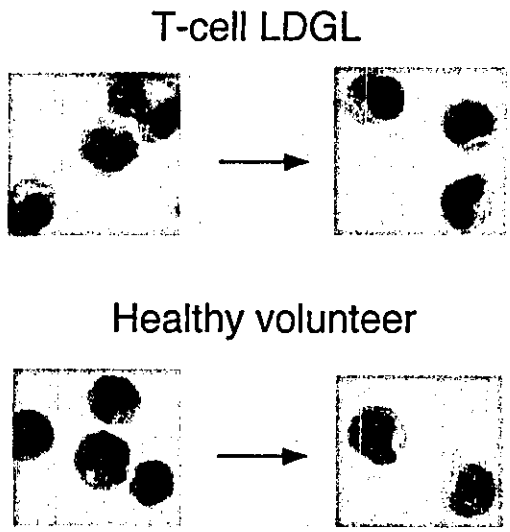

Fig 1. $CD4^-CD8^+$ large granular lymphocytes (LGL) purified from the study subjects. Mononuclear cells (MNCs) (left panel) isolated from the peripheral blood (PB) of a patient with T cell-type lymphoproliferative disease of granular lymphocytes (LDGL) (upper panel) or a healthy volunteer (lower panel) were used to purify $CD4^-CD8^+$ fractions (right panel). The samples are stained with the Wright–Giemsa solutions. Original magnification ×400.

To identify novel molecular markers of LDGL and to screen for the genes involved in the pathogenesis of this condition, we tried to isolate a gene set, the expression of which was specific to LDGL T cells, but not to normal ones. A total of six such genes were isolated from the gene tree in Fig 2A, and the detailed expression profiles of these genes are shown in Fig 2B. The most notable genes isolated were those for IL-1β (GenBank accession no. M15330), ATRX (U72938) and Titin (X90568).

We also isolated another set of genes which were transcriptionally active in normal CD8-positive T cells, but were silent in the LDGL counterparts. Expression profiles in all samples of these six genes are shown in Fig 2C.

Confirmation of disease-specific expression

The LDGL-specific genes may be of clinical importance as they could be potential molecular markers for this disorder. Among the genes shown in Fig 2B, we first focused on that for IL-1β. An imbalance between proinflammatory cytokines and cytokine antagonists is frequently observed in the rheumatoid synovium (Arend, 2001) and IL-1β is a representative of the former group. Therefore, the high expression of IL-1β in LDGL cells might play a role in the tissue damage observed in LDGL-associated rheumatoid arthritis.

To confirm the LDGL-specific expression of IL-1β, we conducted a real-time TaqMan™ PCR to quantify the mRNA copy number of IL-1β. For this purpose, the LDGL T-cells ($n = 14$) were purified from the patients with either TCR αβ-positive or γδ-positive phenotype. Similarly, surface marker-matched T cells were purified from healthy volunteers ($n = 13$). The mRNA was prepared from each sample,

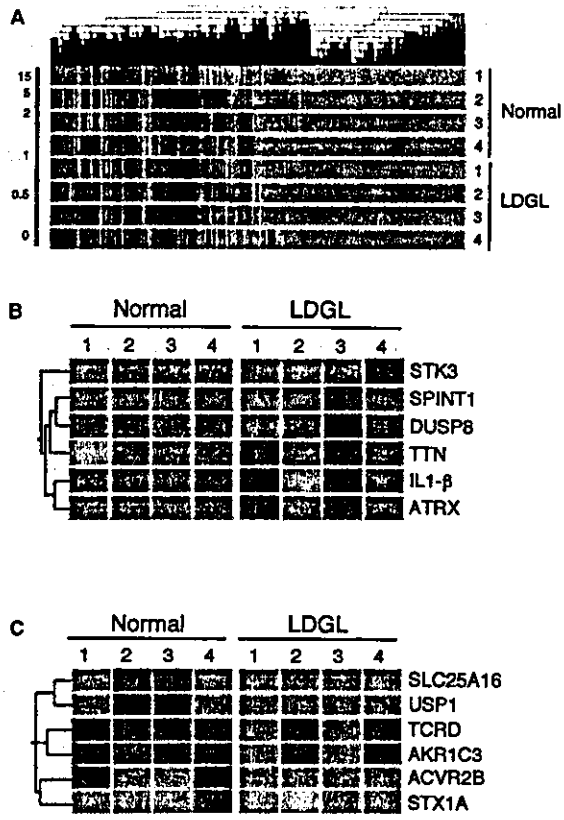

Fig 2. Expression profiles of 3456 genes in purified T cells. (A) Hierarchical clustering of 3456 genes based on their expression profiles in the $CD4^-CD8^+$ cells derived from four healthy volunteers (Normal) and four T-cell LDGL patients (LDGL). Each column represents a single gene on the microarray, and each row corresponds to a different patient (or normal) sample. The normalized fluorescence intensity for each gene is shown colour-coded as indicated on the left. (B) Expression profiles of LDGL-specific genes. Each row corresponds to a single gene, with the columns indicating the corresponding expression level in different samples. On the left side a gene tree is shown, based on the similarity of the expression profile of each gene across the samples. (C) Profiles of genes whose expression level is reduced in the $CD4^-CD8^+$ T cells from LDGL individuals. The dendrogram of such genes is shown on the left. Gene names and accession numbers as well as expression intensity data in (B) and (C) are available upon request.

converted to cDNA, and subjected to TaqMan PCR to amplify the cDNA for IL-1β or GAPDH. The calculated mRNA copy number of IL-1β relative to that of GAPDH was significantly increased in the LDGL T cells compared with that in normal T cells ($P < 0.01$) (Fig 3A). Interestingly, enhanced expression of IL-1β was notable irrespective of the surface marker of LGL cells (either αβ- or γδ-positive). In contrast, expression of the IL-1β gene was very low or absent in T cell-depleted MNC of the LDGL patients, T cells obtained from a patient with adult T-cell leukaemia and in a T-cell line (CEM) established from a patient with acute lymphoid leukaemia (data not shown), further confirming the disease-specificity of IL-1β expression.

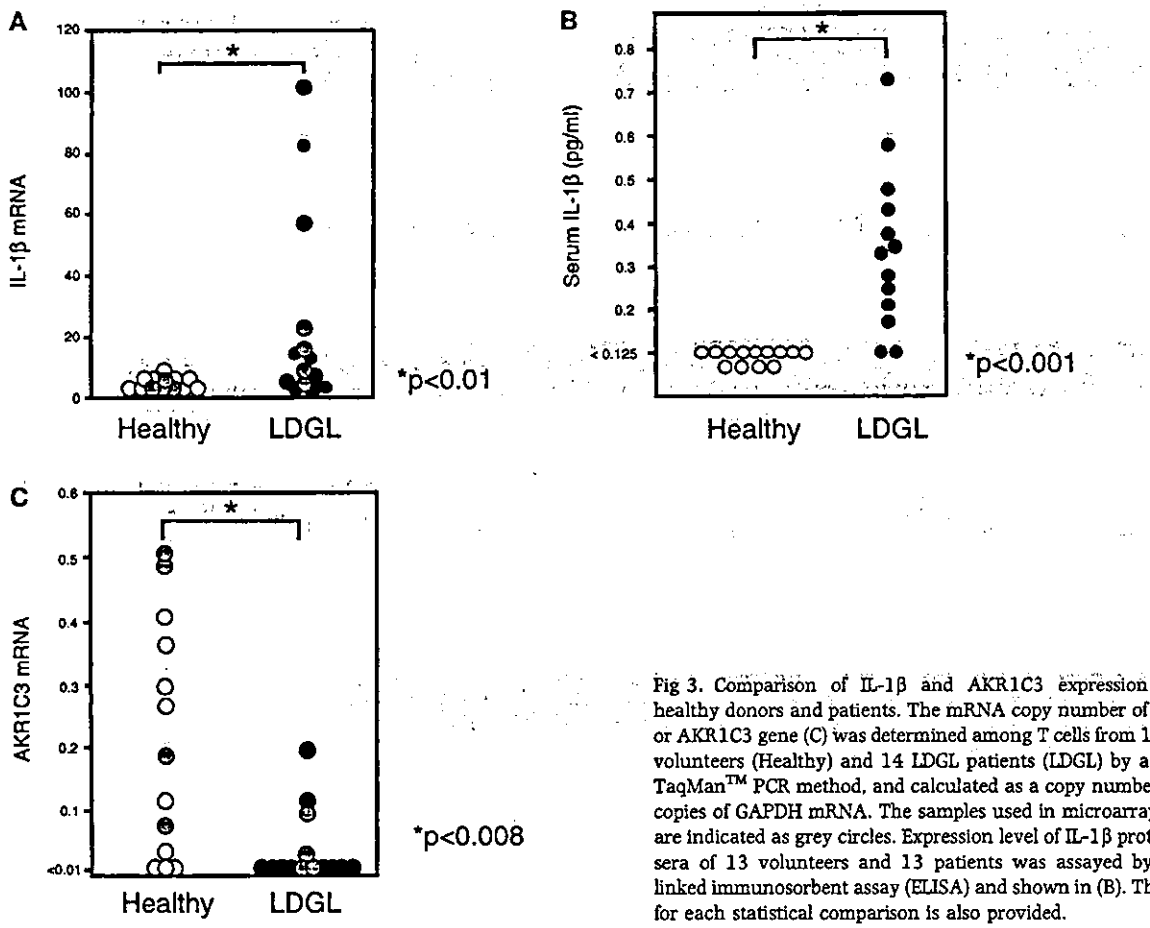

Fig 3. Comparison of IL-1β and AKR1C3 expression between healthy donors and patients. The mRNA copy number of IL-1β (A) or AKR1C3 gene (C) was determined among T cells from 13 healthy volunteers (Healthy) and 14 LDGL patients (LDGL) by a real-time TaqMan™ PCR method, and calculated as a copy number per 100 copies of GAPDH mRNA. The samples used in microarray analysis are indicated as grey circles. Expression level of IL-1β protein in the sera of 13 volunteers and 13 patients was assayed by enzyme-linked immunosorbent assay (ELISA) and shown in (B). The *P*-value for each statistical comparison is also provided.

If the induction of IL-1β in LGL cells really contributes to the systemic damage in the articular synovium, then the patients' sera should have an elevated level of IL-1β protein. To examine this hypothesis, ELISA was used to quantify the serum concentration of IL-1β in the patients ($n = 13$) as well as healthy volunteers ($n = 13$). Intriguingly, as shown in Fig 3B, the protein concentration of IL-1β was markedly elevated only in the LDGL group ($P < 0{\cdot}001$). Of further interest was the finding that, among the samples examined, the serum IL-1β level was highest in case 8 who had rheumatoid arthritis.

To confirm the reliability of our microarray data, we also measured the mRNA level of a normal T cell-specific gene indicated in Fig 2C, AKR1C3 (GenBank accession no. D17793). The relative expression level of AKR1C3 quantified by TaqMan™ PCR was significantly suppressed in the LDGL cells ($P < 0{\cdot}008$), again in a good correlation with the results of microarray analysis (Fig 3C).

The repertoire analysis of TCR Vβ and Vδ in purified T cells

To evaluate the clonality of LGL cells, we have also studied the TCR Vβ and Vδ repertoire for every sample by size-spectratyping and nucleotide sequencing of the RT-PCR products for the corresponding regions.

As controls, the Vβ or Vδ regions of TCR cDNAs were PCR-amplified from the TCR αβ-positive or γδ-positive T cells, respectively, obtained from individual 13 healthy volunteers, and subjected to gel electrophoresis with ethidium bromide staining. The result from one such volunteer is demonstrated in Fig 4A. PCR products were found in most of the 26 subfamilies of the Vβ region and in one subfamily of the Vδ region (data not shown), indicating the polyclonal nature of the T cells in the healthy volunteer. Spectratyping of the products also confirmed the polyclonality within each subfamily of TCR (right inset).

In contrast, purified T cells obtained from case 4 (positive for TCR αβ) gave rise to five main Vβ subfamilies (6·2, 17, 19, 21 and 22), whose relative expression level exceeded 10% (Fig 4B). In support of the oligoclonal expansion of TCR αβ-positive cells in this patient, spectratyping of the PCR products yielded skewed patterns (right inset), and nucleotide sequencing of them demonstrated a presence of predominant Vβ amino acid sequences (Table II). Interestingly, PCR amplification of the Vβ subfamilies only generated a single band for case 5 (Fig 4C), strongly indicating a monoclonal expansion of TCR αβ-positive cells in this individual. Among the 11 TCR αβ-positive LDGL patients, eight cases exhibited an oligoclonal expansion of T cells,

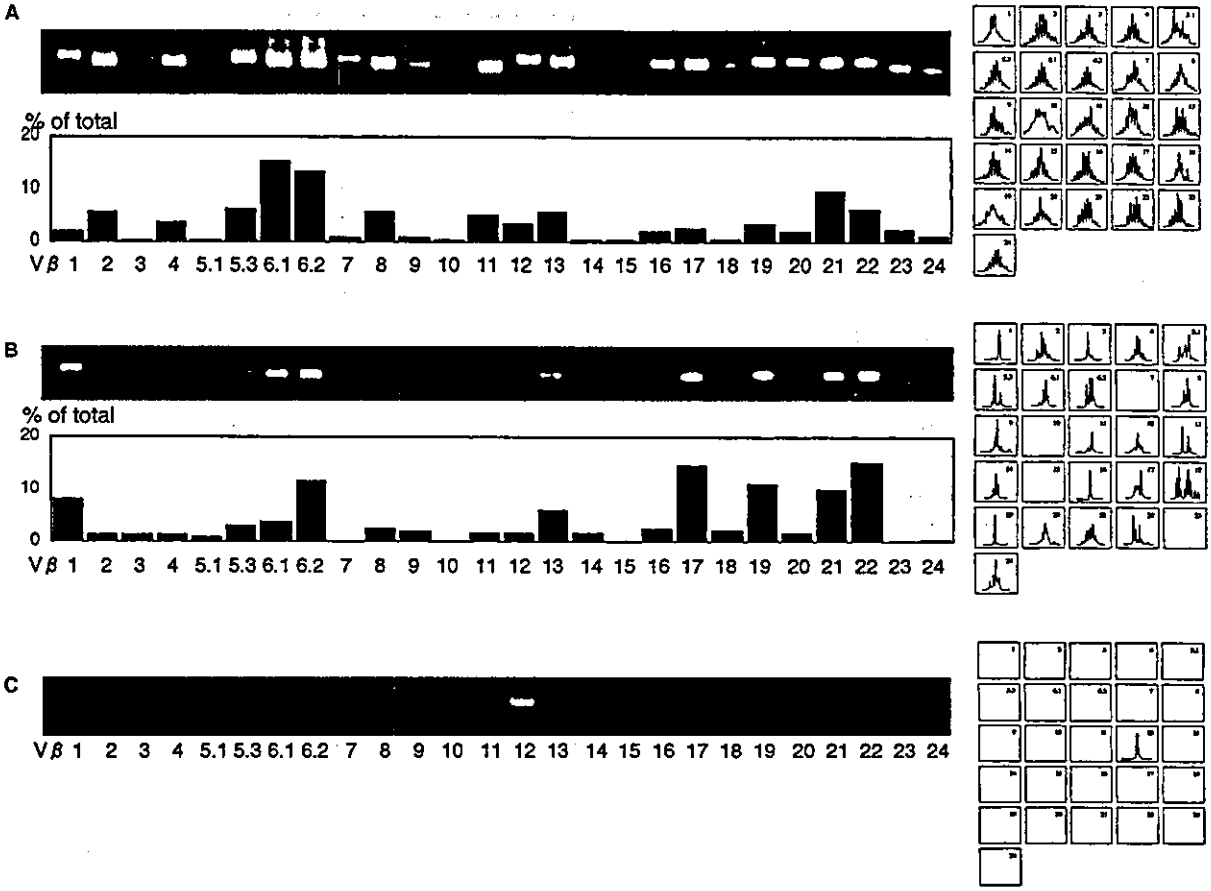

Fig 4. The clonality in the T-cell subsets. The clonality of purified $CD4^-CD8^+$ T cells was assessed by the semiquantitative PCR (gel images) and spectratyping analysis (right insets) of the TCR Vβ region in the samples from a healthy volunteer (A). LDGL case 4 (B) and case 11 (C). The relative percentage for the amount of each PCR product within the total Vβ products is also demonstrated in (A) and (B).

Table II. Junctional amino acid sequences of the VDJ region for TCR β subunit gene.

Patient number	Vβ subfamily	nDn	Jβ subfamily	Frequency
4	Vβ1	GPTTGEGN	Jβ2·1	7/11
		GTGGT	Jβ1·6	2/11
	Vβ3	AN	Jβ2·1	10/10
	Vβ9	DFQGRG	Jβ2·7	6/12
		ASGGATG	Jβ2·1	2/12
		DPTGF	Jβ1·2	2/12
	Vβ16	RLVD	Jβ2·7	10/12
	Vβ19	QDEVGGP	Jβ2·7	7/10
		QSHGQEG	Jβ2·2	2/10
	Vβ22	VSSAI	Jβ2·5	4/12
		SDLAG	Jβ1·1	3/12
11	Vβ12	DV	Jβ2·7	11/11

V, variable; D, diversity; J, joining.

Sequence analysis of Vβ-nDn-Jβ rearrangements in the purified LDGL T cells.

whereas three showed a monoclonal expansion, as indicated in Table I.

We also analysed the TCR Vδ repertoire in the three LDGL patients whose LGL was positive for TCR γδ expression (cases 12–14). Spectratyping of the RT–PCR products revealed a single band (case 14) or skewed (cases 12,13) patterns in the T cells (data not shown). Nucleotide sequencing of the PCR products also supported the monoclonal or oligoclonal nature of the TCR γδ-positive cells in their PB (Table I).

To statistically compare the TCR complexity between the healthy volunteers and LDGL patients, SCS was calculated among 11 each of the volunteers and the patients. The mean value ± SD of SCS was 143·0 ± 5·0 in the control group compared with 26·0 ± 9·0 in the LDGL group. Therefore, as expected, the complexity of TCR was significantly decreased in the patients than in normal individuals ($P < 0·0001$).

Quantification of the ATRX mRNA by real-time Taq-Man™ PCR revealed a preferential expression of this gene in the patients compared with the control group (not

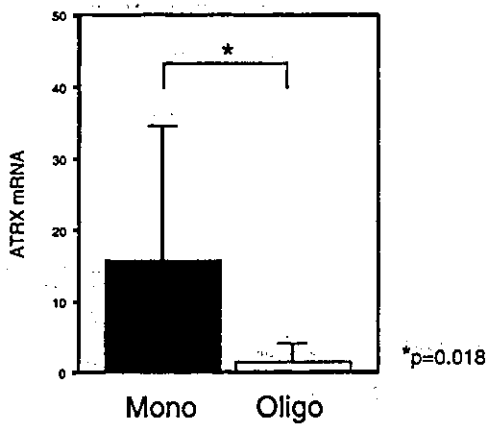

Fig 5. Quantification of the ATRX gene expression in LDGL T cells. Expression levels of the ATRX gene relative to that of GAPDH were determined as in Fig 3, and were compared between the LDGL T cells in the monoclonal expansion (Mono) and those in the oligoclonal one (Oligo).

statistically significant, $P = 0{\cdot}075$) (data not shown). However, as shown in Fig 5, comparison of the ATRX expression between the four patients with monoclonal LGL expansion and the 10 patients with oligoclonal expansion has indicated that the ATRX gene was significantly induced in the former group ($P = 0{\cdot}018$).

DISCUSSION

Through the background-matched population (BAMP) screening (Miyazato *et al*, 2001; Ohmine *et al*, 2001) with DNA microarray, we identified sets of genes, the expression of which were specific to either LDGL cells or surface marker-matched control cells. We also confirmed the mono- or oligoclonal expansion of the LDGL cells by using the spectratyping and nucleotide sequencing of TCR Vβ and Vδ regions.

Among the six LDGL-specific and six control-specific genes identified, our attention was drawn to those for IL-1β and ATRX. IL-1β is one of the most potent proinflammatory cytokines, and, together with tumour necrosis factor (TNF)-α, is known to have a central role in the tissue damage observed in rheumatoid arthritis (Arend, 2001). There is a naturally occurring inhibitory molecule against IL-1β, i.e. IL-1 receptor antagonist (IL-1 Ra). Although IL-1 Ra counteracts the inflammatory activities of the IL-1 family of cytokines, the balance between them is known to lean towards the latter in the rheumatoid synovium. Given the fact that rheumatoid arthritis is one of the most frequent complications found in LDGL, the LGL-specific expression of IL-1β in this disorder may indicate a mechanism through which rheumatoid arthritis is induced in LDGL. It even suggests a possible treatment of rheumatoid arthritis associated with LDGL. If the imbalance plays an essential role in the tissue damage, the restoration of the balance by supplying IL-1Ra would be effective in the treatment of arthritis (Dayer *et al*, 2001).

We also examined whether cytokine-stimulated, normal LGL can express IL-1β. Culturing the $CD4^-CD8^+$ T-cells purified from healthy volunteers in the presence of IL-2 and IL-12 yielded an oligoclonal expansion of the T cells, as judged by the spectratyping analysis. Although such proliferating cells exhibited the phenotype of LGL, they did not significantly express IL-1β (data not shown). Therefore, induction of IL-1β in our study was not simply a result of T-cell activation. Considering the high expression of IL-1β in both TCR αβ- and γδ-positive cells, it may rather be a useful marker to clinically define LDGL.

In addition to tissue damage, IL-1β was demonstrated to have a growth promoting activity on leukaemic cells, probably through an autocrine or paracrine mechanism (Estrov *et al*, 1993). Thus, activation of the IL-1β gene may be directly linked to the pathogenesis of LDGL.

Mutations in the ATRX gene cause X-linked α-thalassemia/mental retardation syndrome or Juberg–Marsidi syndrome (Gibbons *et al*, 1995). Although the *in vivo* function of ATRX is still unclear, it is believed to possess an ATP-dependent helicase activity (Stayton *et al*, 1994), and is involved in the chromosome segregation (Berube *et al*, 2000), DNA methylation (Gibbons *et al*, 2000), and transcription regulation (Picketts *et al*, 1996). Our present study has suggested that the transcriptional activation of the ATRX gene may be correlated to the progression of T cell-type LDGL from the polyclonal stage to the monoclonal one. In our study, there was one LDGL case whose TCR repertoire complexity decreased along with stage progression. In other words, in LDGL, monoclonal selection of malignant cells may take place at an advanced stage. Therefore, high expression of ATRX may be a novel type of prognostic factor for LDGL. It would be an intriguing question as to whether induction of ATRX may directly confer growth advantage toward LGL cells *in vivo*.

In conclusion, our comparison with surface marker-matched population has provided interesting information regarding T-cell LDGL. Further increases in the number of both patients and genes for analysis would shed new light on this rather uncharacterized disorder.

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平成14年11月12日 変更許可申請, 平成15年1月8日 変更許可再申請, 平成15年2月3日 変更許可決定通知, 平成15年12月8日 変更許可申請)

研究計画書

課題名：薬物による肝障害の予防に関する研究

研究責任者の所属・職・氏名：自治医科大学臨床薬理学講座　助手　大島康雄

(1)　試料等提供者の選定方針（合理的に選択していることがわかる具体的な方法。試料等提供者が疾病や薬剤反応性異常を有する場合等にあっては、病名又はそれに相当する状態像の告知方法等。）

目標解析数は 120 で，研究参加に同意された順に研究に参加していただくことを基本方針とする．対象は肝臓の腫瘍性疾患など（肝臓原発の腫瘍・他臓器原発の腫瘍性疾患で肝臓への転移などが考えられるがこれらに限定はしない）のために，医学的に治療上肝臓の切除の適応があると判断された 18 才以上の患者で，書面で確認されたインフォームドコンセント（(7) インフォームドコンセント説明文書および同意文書）を得ることができた患者でかつ以下の除外項目を有しない患者．
除外項目は，薬物性の肝障害を起こしている疑いが強い場合，遺伝性の疾患またはその疑いが濃厚である場合，またはその他担当者が不適切と認める場合．

(2)　研究の目的、意義、方法（対象とする疾患、分析方法等。将来の追加、変更が予想される場合はその旨。第一群試料等提供者の場合には研究の必要性、不利益を防止するための措置等。）、期間、予測される結果、予測される試料等提供者に対する危険及び不利益並びに個人に関する情報の保護の方法（匿名化しない場合の取扱いを含む。）

① 目的

化学物質のヒト肝細胞障害時の遺伝子発現を明らかにすることにある．化学物質を肝臓の細胞に in vitro で作用させた場合の遺伝子発現の変化を肝障害が懸念される化学物質とそうでない化学物質などで GeneChip® を用いて比較検討する．従来の前臨床試験としての毒性試験において，齧歯類などの小動物や細胞株を用いた検討では肝障害（細胞障害）を引き起こさないが，個体としてのヒトでは肝障害を引き起こす化学物質がある．このような現在の前臨床研究では見過ごされるような場合でも，遺伝子レベルでは何らかの変化が生じている可能性は高いと考えられる．そこで，本研究ではこの遺伝子レベルでの変化を検出することを目的とする．

② 意義

遺伝子発現情報を基に肝障害を来しうる化学物質を，開発段階で検出するための基礎データの構築

③ 方法

様々な化学物質を in vitro で肝臓の細胞に作用させた場合の遺伝子発現の変化を GeneChip® を用いて検討する

④ 期間

研究許可を得てから平成19年6月30日まで

⑤ 予測される結果

当研究班でのデータとしては，非腫瘍性（または非罹患部）の肝臓組織を用いて，様々な化学物質を作用させた場合の遺伝子発現プロファイルが得られる．肝障害が懸念される化学物質とそうでない化学物質の遺伝子発現プロファイルを比較することにより肝障害を来たす可能性の高い化学物質に特異的な遺伝子発現プロファイルデータベースを構築することが予定されている．中期的には，これらの情報と齧歯類や細胞株での遺伝子プロファイリングの相関を検討し，ヒトにおける化学物質性肝障害の発現を齧歯類などの小動物での研究や細胞株を利用した *in vitro* 実験で予測する方法を探る

⑥ 予測される試料等提供者に対する危険及び不利益

手術は全て臨床上適応がある場合のみであり，切除範囲も臨床上必要な範囲のみに限定する．手術の臨床上適応・切除範囲および術式は，外科の治療方針に則り，最終的には術前カンファランスの出席者の合意をもとに選択される．本研究では予定切除範囲内の非腫瘍部分（または非罹患部）を分離して使用する．術者や助手など手術に直接関わるスタッフは検体を切除し，これを控えている検体処理要員へ渡すのみであるので，手術時間が著しく延長する可能性は低いと考えられる．これまでも病理検査目的での試料の処理は当院で安全に行われてきており，本研究のための試料提供により手術上の危険性および不利益はないと考える

⑦ 個人に関する情報の保護の方法

本学の個人識別情報管理者により連結不可能匿名化が行われる

(3) 試料等の種類及び量

試料等の種類： 肝臓切除領域のうち非罹患部分の組織 量：1-10ｇ程度．予定人数：120 人

(4) 共同研究機関の名称（あらかじめ共同研究機関を特定できない場合にはその理由及び将来参加が予測される共同機関の類型）。共同研究者の職・氏名

厚生労働省（国立医薬品衛生研究所），毒性部室長 菅野純 ら との共同研究となる予定である

(5) 研究責任者、インフォームド・コンセントのための説明者その他研究実施担当者の所属・職名及び氏名

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自治医科大学臨床薬理学講座 助手 大島康雄

(6) インフォームド・コンセントのための手続及び方法

別紙説明書により研究責任者または説明者が説明する．同意が得られた場合には書面として記録

に残す

(7)　インフォームド・コンセントを受けるための説明文書及び同意文書

別紙のとおり

(8)　代諾者等を必要とする試料等提供者が予定されている場合には、その試料等が研究のために必須である理由及び代諾者等の選定に関する基本的な考え方

試料等提供者が未成年である場合は本研究においては本人の同意および代諾者の同意を必要とする．痴呆などのため代諾が必要となる場合は代諾者の同意を必要とする．代諾者の選定は，試料提供者の意志を尊重することができ、試料提供者について十分理解をしている成人であって，任意後見人・親権者・後見人もしくは保証人が定まっているときはその人または本人の配偶者・成人の子・父母・成人の兄弟姉妹・孫・祖父母・同居の親族またはそれらに準ずると考えられるひとを優先的に代諾者として考慮する．

(9)　遺伝情報の開示に関する考え方

連結不可能匿名化のため開示しない

(10)　研究実施前提供試料等を使用する場合には、その試料等の提供の時期、提供を受けたときの同意の有無、同意を得ている場合にはその内容、同意がない又は不充分な場合には研究対象として使用する必要性

本研究において研究実施前提供試料等を使用する予定はない

(11)　他の研究実施機関から試料等又は遺伝情報の提供を受ける場合には、他の研究実施機関が受けるインフォームド・コンセントの内容

本研究において国立衛生研究所を含む基礎研究を行っている他の研究機関から動物実験などの基礎的遺伝情報の提供を受ける可能性がある．本研究では発現解析が目的であり，ヒトゲノム遺伝子情報の提供を受ける可能性はない．

(12)　試料等又は遺伝情報を国内外の公的研究機関、営利を目的としない団体の研究機関又は他の大学に対して提供する場合には、次の事項
ア　提供の必要性
イ　提供先の機関名
ウ　大学において行われる匿名化の方法
エ　匿名化しない場合には、その理由及び個人識別情報を含む情報の保護の方法
オ　試料等を提供する機関において、提供する試料等の遺伝子解析研究を行うか否か
カ　反復、継続して提供するか否か

本研究において収集された試料を国内外の公的研究機関／営利を目的としない団体の研究機関または他の大学に対して提供する予定はない

(13)　試料等若しくは遺伝情報を国内外の営利を目的とする団体の研究実施機関に提供する場合又は国内外の民間の機関に遺伝子解析の一部の作業若しくは研究用資材の作成を委託する場合には、次の事項
ア　提供の必要性
イ　提供先の機関名
ウ　大学において行われる匿名化の方法
エ　提供先における責任者の氏名、責任体制及び予定する契約の内容

本研究において試料等若しくは遺伝情報を国内外の営利を目的とする団体の研究実施機関に提供する場合又は国内外の民間の機関に遺伝子解析の一部の作業若しくは研究用資材の作成を委託する予定はない

(14)　研究期間の終了後に研究遂行者が試料等を大学で保存する場合には、保存の方法及び必要性（他の研究への利用の可能性及び予測される研究内容を含む。）

試料は研究期間終了後まではフリーザーに凍結保存される．基本的には研究期間終了後は廃棄処分される．しかし，他の研究への利用について同意が得られた検体については，研究期間終了後に保存されている検体を使用して追加解析を行うことがある．これは学会で発表した時または論文を投稿した時に他の科学者から，研究の学術的価値をより高める目的で追加実験をすすめられることが希でないし，また，より学術的価値の高い研究へと発展させることは全ての研究について求められていることである．一方，本研究と明らかに趣旨の異なる研究への利用に関しては再度遺伝子解析研究の許可を申請してから行う．

(15)　ヒト細胞・遺伝子・組織バンクに試料等を提供する場合には、当該バンクを運営する機関の名称、当該バンクの名称及び責任者の氏名並びに試料等の匿名化の方法

本研究においてヒト細胞・遺伝子・組織バンクに試料等を提供する予定はない

(16)　試料等を廃棄する場合には、廃棄の方法及びその際の匿名化の方法

試料を廃棄する時はオートクレーブした後に廃棄される．この時点では試料には，個人情報とは連結不可能な ID コードが付されているのみであるため，またオートクレーブ後は試料から遺伝情報を抽出するのが困難となるため通常の廃棄物として処分される

(17)　第二群、第三群又は第四群試料等提供者から試料等の提供を受ける場合には、遺伝カウンセリングの必要性の有無（第一群試料等提供者から試料等の提供を受ける場合には、試料等提供者等からの求めに応じ、遺伝カウンセリングを実施するものとする。）

本研究は第三群試料等提供者からの試料提供となる．連結不可能匿名化を実施しての解析なので遺伝カウンセリングは必要ない

(18)　研究資金の調達方法

厚生労働省・文部科学省・経済産業省などの公的グラント（研究資金）及び民間の研究助成金などをもって研究を実施する

遺伝子解析研究（研究題目薬物による肝障害の予防に関する研究）への協力のお願いと説明文書

これから、あなたにこの遺伝子解析研究への協力をお願いするため、研究の内容や研究協力に同意していただくための手続などについて説明します。

この説明を十分に理解し、研究に協力しても良いと考えられた場合には、「遺伝子解析研究への協力についての同意書」に署名又は記名・押印し、同意したということをはっきり示してくださるようお願いします。

1　遺伝子と病気

「遺伝」とは、「親の体質が子に伝わること」です。「体質」には、顔かたち、体つきのほか、病気にかかりやすいことなどが含まれます。人の体の状態は、遺伝とともに、生まれ育った環境によって決まりますが、遺伝は基本的な部分で人の体や性格の形成に重要な役割を果たしています。「遺伝」に「子」という字が付き「遺伝子」となると、「遺伝を決定する小単位」という科学的な言葉になります。遺伝子の本体は「ＤＮＡ」という物質です。「ＤＮＡ」はＡ、Ｔ、Ｇ、Ｃという四つの塩基の連続した鎖です。塩基がいくつもつながって遺伝子になります。

１つの細胞の中には数万種類の遺伝子が散らばって存在しています。全ての遺伝情報を総称して「ゲノム」といいます。人体は約６０兆個の細胞から成り立っていて、細胞の一つ一つに全ての遺伝子が含まれています。

遺伝子には二つの重要な働きがあります。一つは、精密な「体の設計図」です。受精した一つの細胞は分裂を繰り返して増え、一個一個の細胞が「これは目の細胞」、「これは腸の細胞」と決まりながら、最終的には約６０兆個まで増えて人体を形作ります。二つ目は、「種の保存」です。先祖から現在まで「人間」という種が保存されてきたのも、遺伝子の働きによります。

ほとんど全ての病気は、その人の生れながらの体質（遺伝素因）と病原体、生活習慣などの影響（環境因子）の両者が組合わさって起こります。遺伝素因と環境因子のいずれか一方が病気の発症に強く影響しているものもあれば、がんや動脈硬化などのように両者が複雑に絡み合っているものもあります。遺伝素因は遺伝子の違いに基づくものですが、遺伝子の違いがあればいつも病気になるわけではなく、環境因子との組合せも重要です。

2　研究に協力するかどうかを考えるために

この研究は、現在解析可能な全ての遺伝子発現について、その発現を解析し、化学物質の有害反応を予測することが可能かどうかを調べることを目的としています。

あなたは、何らかの病気のために肝臓の摘出術が必要です．あなたの摘出肝臓組織を診療記録とともに、この研究に使用させていただきたいのです。

次に、あなたが、この研究に協力するかどうかを決めるために理解していただきたい事項について、順次説明します。

(1)　研究協力の任意性と撤回の自由

研究協力に同意するかどうかは任意です。あなたの自由意志で決めてください。協力に同意されてもされなくても、当院では同じように最善の医療を提供いたします。

いったん同意された場合でも、不利益を受けることなく、いつでも一方的に文書により同意を撤回することができます。その場合は提供いただいた肝臓組織や遺伝子解析の結果は破棄され、診療記録もそれ以降は本研究のために用いられることはありません。ただし、同意を撤回したとき既に研究結果が論文などで公表されていた場合や試料等が誰のものか完全に分からないようにする連結不可能匿名化されていた場合など、肝臓組織や遺伝子解析の結果を破棄できないことがあります。

(2)　あなたが選ばれた理由

この研究では、肝臓組織について調べますので、肝臓の摘出術が必要と診断された方全てに研究のへの協力をお願いしています。あなたは、肝臓の摘出術が必要と診断されましたので、研究への協力をお願いすることにしました。