Intestinal Gene Expression in TNBS Treated Mice Using GeneChip and Subtractive cDNA Analysis: Implications for Crohn's Disease

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So far it has proven difficult to identify a causative gene(s) or gene product initiating the events that lead to inflammation of the intestinal mucosa and, ultimately, progression to Crohn's disease (CD), an inflammatory bowel disease. However, gene transcripts identified in the intestine of trinitrobenzene sulfonic acid (TNBS)-treated mice might suggest a clue, and even represent candidate genes leading to inflammation and mucosal damage, and to subsequent fibrosis. In the present study, DNA microarray (13000 transcripts) methodology was applied to mucosal RNA extracted from TNBS-treated mice, some transcripts of which were validated *via* cDNA subtraction and RT-PCR analyses. Intestinal biopsy samples from CD patients were then analyzed using cDNA mini-array (1300 cDNAs), focusing on gene transcripts associated with cancer and immunity. Mini-array results revealed transcript changes similar and also dissimilar to those found from the DNA microarray analysis. These changes, previously known or newly identified, possibly occurring during the initial and progressive stages of inflammatory conditions may provide a clue to identify marker transcripts and/or targets for the development of future gene therapy.

Key words trinitrobenzene sulfonic acid (TNBS); Crohn's disease; DNA microarray; cDNA subtraction; cDNA membrane

In healthy subjects, gut-associated lymphoid tissues function to protect the host by neutralizing infectious agents or injurious substances.[1)] This protective mechanism is well balanced and usually does not lead to mucosal damage in the intestine. In a state of disease such as inflammatory bowel disease, various immune responses of the gut-associated lymphoid tissues are exaggerated, resulting in the development of a prolonged and severe inflammation of the intestinal mucosa.[2,3)] Human inflammatory bowel disease is a chronic and idiopathic inflammation of the mucosa of the distal small intestine, the etiology of which is as yet unknown. This inflammatory disease is clinically characterized by two overlapping phenotypes, Crohn's disease (CD) and ulcerative colitis. CD is a chronic and incurable condition marked by periods of exacerbation and remission. CD is also characterized by transmural inflammation resulting in abdominal pain, diarrhea and weight loss.[4)] In addition, recent findings suggest that CD is the result of an excessive Th1 response.[5,6)] Therefore, the use of agents that block the Th1 response or promote a Th2 profile might prove to be beneficial in this disease. However, at this point in time treatment is still mostly symptomatic, aimed at reducing inflammation[7,8)] because neither a specific cause nor factors that determine the degree of disease progression have been identified.

Chronic intestinal inflammation induced in mice by intrarectal administration of trinitrobenzene sulfonic acid (TNBS) produces many clinical, histopathologic and immune characteristics that are similar to those of CD in humans.[5,9,10)] In fact, treatment with TNBS induces chronic colitis characterized by severe transmural inflammation associated with diarrhea, rectal prolapse and weight loss. This inflammation is characterized by a massive infiltration of neutrophils and macrophages, producing high levels of proinflammatory cytokines such as tumor necrosis factor (TNF)α, interleukin (IL)-1β, or IL-6 in early stages of the disease. This is followed by T-cell infiltration, particularly of the CD4+ phenotype, resulting in the production of high levels of interferon (IFN)-γ and reduced amounts of IL-4.[11)]

DNA microarray analysis has been applied for the identification of gene transcripts possibly controlling CD.[12,13)] However, it has not been possible to identify a causative gene(s) or gene product that triggers the initial event(s) leading to mucosal inflammation and its progression to CD. It is possible that gene transcripts identified in tissue samples from TNBS-treated mice could represent transcript changes during the initial stage of inflammatory diseases. Upon careful evaluation with those from CD patients, transcripts identified might provide a clue to potential intervention or treatments including gene therapy. The objectives of the present investigation were to identify transcript changes in mucosal samples from TNBS-treated mice and those from CD patients, and to relate these changes for the formulation of transcript changes from the initial and progressive stages to CD development.

MATERIALS AND METHODS

Animal Treatment with TNBS and Tissue Collection Six-week old male mice (C57B6) were purchased from SLC (Shizuoka, Japan). During the 1—2 week acclimatization period, the mice were housed with controlled lighting (14L : 10D), and food and water were given *ad libitum*. Colitis was induced by intracolonic administration of TNBS (Tokyo Kasei, Tokyo, Japan) as previously described.[5,9,10,14)]

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In brief, the mice were lightly anesthetized, an approximately 1 cm long incision was made, and the large intestine with the appendix was extracted from the peritoneal cavity. One hundred microliters of a solution with 1 or 3 mg TNBS dissolved in 50% ethanol ($n=3$ each), respectively, was slowly administered through a 27-gauge needle into the large intestine approximately 1 cm from the appendix toward the descending colon. The infusion was performed over 60 s. The needle was then carefully removed and the large intestine and appendix were placed back into the peritoneal cavity. Control mice ($n=3$) received 100 μl of 50% ethanol solution without TNBS (vehicle) in the same way. Intestinal tissues were removed 48 h later, washed in physiological saline, and approximately 1 cm of mucosa was removed under a microscope.

RNA Isolation, cRNA Production and Microarray Analysis Cellular RNA was extracted from the intestinal mucosa with Isogen reagent (Nippon Gene, Toyama, Japan) according to the protocol suggested by the manufacturer. Total RNA from three mice was pooled and first-strand cDNA synthesis was carried out at 42 °C for 60 min. Complementary DNA synthesis was primed with a T7-primer consisting of [5′-AAGCAGTGGTAACAACGCAGAGTACTAATACGACTCACTATAGGGAGCGG-$(T)_{24}$NN′-3′ (N= A, G, or C; N′=A, G, C, or T)], where NN′ serves to anchor the beginning of the poly(A) tail, which avoids copying of long poly(A) tails by the Moloney murine leukemia virus (MMLV) reverse transcriptase (40 units of Superscript, Invitrogen, Carlsbad, CA, U.S.A.). After copying mRNA sequences, the enzyme's terminal transferase activity adds three to five deoxycytidine (dC) nucleotides to the 3′-end of the first-strand cDNA, to which the T3-primer (SMART oligo, 5′-AAGCAGTGGTAACAACGCAGAGTACAATTAACCCTCACTAAAGGGAACAAAGCTGCGGG-3′) with three deoxyguanine (dG) nucleotides annealed at the 3′-end. This served as an extended template for the reverse transcription (RT) reaction after which templates were switched from the mRNA molecule to the SMART oligo-attached cDNA molecules. When the 5′-end of the synthesized cDNA was tagged with the SMART sequence, double-stranded cDNA amplification was initiated using polymerase chain reaction (PCR) with *Taq* DNA polymerase (Ex Taq, Takara, Shiga, Japan) and the primers specific for each end of these cDNA molecules. PCR amplification consisted of an initial denaturation step at 95 °C for 1 min, followed by 22 cycles of 95 °C for 15 s, 65 °C for 30 s, and 68 °C for 5 min. One microgram of the amplified double stranded cDNA was used as a template for *in vitro* transcription with T7 RNA polymerase and the RNA transcript labeling kit (Enzo BioArray High-Yield RNA Transcript Labeling Kit, Farmingdale, NY, U.S.A.), resulting in the preparation of biotin-labeled cRNAs for DNA microarray analysis.

DNA microarray analysis was performed with TNBS treated or untreated animals ($n=3$ for each treatment) and mouse high-density oligonucleotide arrays (HDAs, 13000 genes, Affymetrix, Santa Clara, CA, U.S.A.). Biotin-labeled cRNAs (15 μg) were hybridized to the array, washed, stained, washed again, and scanned according to the manufacturer's instructions. For the second analysis, RNA was obtained from a new set of animals and DNA array analysis by a different investigator was performed. For each microarray analysis, additional internal standards were established by hybridizing predetermined amounts of biotinylated cRNA to the microarray[15]; BioB (1.5 pM), BioC (5 pM), BioD (25 pM) and CreX (100 pM). These standards gave a linear relationship ($y=0.0223x-5.65$, $r^2=0.989$), and levels of mRNA from vehicle-treated as well as TNBS-induced colitis animals fell within this range.

Complementary DNA Subtraction Studies To confirm the data obtained from the microarray analysis and to identify unique transcripts elicited by TNBS-induced colitis, cDNA subtraction studies were executed using the PCR-select cDNA subtraction kit (Clontech) according to the manufacturer's instructions. Total RNA extracted from the colonic mucosa of mice treated with 3 mg TNBS or vehicle ($n=3$ each) was subjected to double-stranded cDNA synthesis. In this system, cDNA fragments (driver cDNAs) were eliminated from the cDNA fragments (tester cDNAs), to which adaptor sequences had been ligated to their 5′-end, resulting in the selection of cDNAs specific for the tester group. These cDNAs with adaptors were then amplified through PCR using primers specific for the adaptors.[16,17] After PCR amplification, PCR products, a group of cDNAs specific for tester cDNA fragments, were ligated to pGEM T-easy vector (Promega, Madison, WI, U.S.A.), which were used to transform *E. coli* DH5a. A total of 500 colonies was picked and propagated, from which plasmid DNA was extracted and subjected to cDNA sequencing analysis.[17]

RT-PCR Assays to Validate GeneChip and cDNA Subtraction Data RNAs extracted with the Isogen reagent (Nippon Gene) were reverse transcribed using the oligo$(dT)_{12-18}$ primer and SuperScript II (Gibco BRL) according to the protocol suggested by the manufacturer. Transcripts of zinc finger protein 147 (Efp), ephrin A1, amine *N*-sulfotransferase (SLUT-N), ADP-ribosyl cyclase (CD38) and phytanoyl-CoA hydroxylase (Phyh) that had been identified in the microarray and cDNA subtraction procedures were chosen for further analysis. Primers were: Efp, F 5′-cctcaagagtgaactgaagc-3′, R 5′-tgcctacgccgcagaagttg-3′, 572 bp; ephrin A1, F 5′-acgtgcagctgaatgactac-3′, R 5′-acctttttgaggcttcatggtg-3′, 611 bp; SLUT-N, F 5′-ctgttcagctgcttccttcctc-3′, R 5′-catactgggctacagtgaac-3′, 492 bp; CD38, F 5′-acgctgcctcatctacactcag-3′, R 5′-atcacttggaccacaccacag-3′, 429 bp; Phyh, F 5′-tcaacgttttcgagcagag-3′, R 5′-ggtcatagtcttggatgccatg-3′, 488 bp; glyceraldehyde 3-phosphate dehydrogenase (G3PDH), F 5′-accacagtccatgccatcac-3′, R 5′-tccaccaccctgttgctgta-3′, 452 bp. PCR reactions consisted of a mixture of 1 μl RT product, 2.5 μl 10× PCR buffer, 1.0 μl each forward and reverse primer, 0.5 μl dNTP mixture, 19.85 μl ddH_2O, and 0.05 μl *Taq* DNA polymerase (Ex Taq, Takara, Shiga, Japan) and were amplified under the following conditions: 94 °C for 5 min, followed by 32 PCR cycles at 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min. All PCR products were cloned into a pGEM T-easy vector (Promega) and nucleotides were determined using a DNA sequencing kit (Abiprism, Foster, CA, U.S.A.).[15,17]

Analysis of Transcripts in Mucosal Samples from Patients Suffering from Crohn's Disease After careful consultation and informed consent, 10 patients suffering from CD were enrolled into the study at Fujita Health University, Aichi, Japan. Biopsy specimens (approximately 5×5×2 mm) from transmural granulomatous inflammation-fibrostenotic lesions (GFL) and pathologically normal mucosa (PNM) ad-

jacent to GFL were obtained from five CD patients (ages between 19 and 28). Samples from the five remaining CD patients who had been treated with TNFα antibody (REMICADE, infliximab, Tanabe Seiyaku, Co., Ltd., Johnson & Johnson' Japanese affiliate) were not included in this study due to potential variations relative to timing and duration of the TNFα antibody treatment. These biopsy samples subjected to this study represent those obtained before (Group 1, two patients) or on the day of TNFα antibody injection (Group 2, three patients). Tissue samples were rinsed in physiological saline, blotted and immediately frozen in liquid nitrogen. Using the aforementioned Isogen method, total RNA was extracted from each mucosal sample, and subjected to poly(A)+ RNA selection (Toyobo, Osaka, Japan) according to the manufacturer's instructions. In addition, colon RNA from a healthy subject was purchased (Cat. #7986, Ambion, Austin, TX, U.S.A.). First strand cDNA was synthesized from 1 μg of poly(A)+ RNA using reverse transcriptase (Toyobo) with the oligo (dT) primer, following which a poly(dC) tail was added to the 3′-end of the cDNA using terminal deoxytransferase. Second strand cDNA was generated by PCR reaction using an anchored oligo (dG) primer. The probes were labeled with biotin-16-dUTP during PCR reaction using the Gene Navigator cDNA Amplification System (Toyobo). Free biotin-16-dUTP in the reaction mixture, which had not been incorporated into the probes, was removed by ethanol precipitation.

The cDNA mini-array membranes used in this study, GLAB 1300 array possessing 1300 unique human cDNAs associated with various forms of cancer and immunological responses, were prepared in-house by spotting 1 ng each of 500 bp cDNA fragments (GeneticLab, Sapporo, Japan). For an internal control, predetermined amounts of housekeeping gene cDNAs were spotted; β-actin, α-tubulin, ubiquitin, ribosomal protein 9, and G3PDH. The membranes were preincubated in 20 ml of hybridization solution (Toyobo) at 68 °C for 20 min. The denatured biotin-labeled probes were added to 20 ml of fresh hybridization solution, which was placed onto each membrane, and kept at 68 °C overnight. The membranes were then washed three times with 2×SSC/0.1% SDS at 68 °C for 10 min, and three times with 0.1×SSC/0.1% SDS at 68 °C for 10 min.

Specific signals on the filters were detected using a chemiluminescence detection kit (Imaging High, Toyobo) according to the manufacturer's instructions. CDP-Star (Roche Diagnostics, Mannheim, Germany) was used as substrate in the chemiluminescent detection system. A chemiluminescence image for each filter was captured using the Fluor-S Multi-Imager System (Bio-Rad Laboratories, Tokyo, Japan) and gene expression images were quantified with ImaGene (BioDiscovery, El Segundo, CA, U.S.A.). Chemiluminescence intensity was converted to relative numbers using housekeeping genes to which predetermined levels of cRNA standards had been applied (r^2=0.978). The intensities of these signals were also used to compare and adjust signals between filters using the E-gene navigator analysis system (GeneticLab).

RESULTS

Transcript Changes Found in TNBS-Treated Mice Using GeneChip Analysis A time span of 48 h following TNBS treatment before animal sacrifice had been determined based on pathological examination and known changes in inflammatory cytokine expressions. Treatment of mice with 3 mg TNBS induced more severe colitis than the treatment with 1 mg, and resulted in more pronounced changes in transcript expression (unpublished observations).

Table 1 lists the genes whose mRNA levels changed with TNBS treatment. Out of 13000 genes, transcript changes were found in 1179 genes, of which 1094 were decreased, and only 85 gene transcripts were increased. These transcripts were arranged into 12 groups: cell adhesion (2 gene transcripts increased and 10 gene transcripts decreased), signal transduction (4 gene transcripts increased and 76 gene transcripts decreased), cell motility (1 gene transcript increased and 3 gene transcripts decreased), cell death (2 gene transcripts increased and 18 gene transcripts decreased), cell proliferation/growth (0 gene transcripts increased and 46 gene transcripts decreased), intracellular transport (2 gene transcripts increased and 117 gene transcripts decreased), cell organization and biogenesis (6 gene transcripts increased and 34 gene transcripts decreased), cell differentiation (1 gene transcript increased and 12 gene transcripts decreased), DNA repair (0 gene transcripts increased and 6 gene transcripts decreased), RNA processing (0 gene transcripts increased and 16 gene transcripts decreased), response to stress (3 gene transcripts increased and 24 gene transcripts decreased), and immune response (3 gene transcripts increased and 18 gene transcripts decreased) (supplemental data available at www.vm.a.u-tokyo.ac.jp/ikushu/implantation or upon request).

Transcript Changes Found in TNBS-Treated Mice Using GeneChip and cDNA Subtraction Studies Initially, our cDNA subtraction[17] studies were designed to identify genes that were up-regulated by TNBS treatment. However, this was not successful because adaptor sequences could not be ligated to cDNA fragments from TNBS-treated animals. Therefore, adaptor sequences were ligated to cDNA fragments from RNAs of vehicle treated animals, allowing us to identify genes down-regulated by the TNBS treatment. Among 500 colonies picked and their cDNA sequences determined, 24 transcripts identified in the cDNA subtraction study existed in the DNA microarray analysis (Table 1, italicized letters). Among 24 transcripts identified, 4 transcripts found in the DNA microarray analysis increased whereas 20 transcript levels decreased (Table 2).

Validation of Transcripts Detected by GeneChip and cDNA Subtraction Analyses Using RT-PCR Gene transcripts of SLUT-N, Efp, Phyh, CD38 and ephrin A1, identified by both DNA microarray and cDNA subtraction analysis (Table 2), were further validated through RT-PCR. Relative changes in the amounts of transcripts between TNBS untreated-healthy mucosa and regions of TNBS-induced colitis are shown in Fig. 1. SLUT-N, Phyh and Efp exhibited similar changes found in both microarray and cDNA subtraction analyses. However, decreases in CD38 or ephrin A1 transcript, which had been determined by DNA microarray and cDNA subtraction studies, were not seen in the RT-PCR analysis.

Comparison of Transcript Changes Found in TNBS Treated Mice and in CD Patients Transcript changes

Table 1. Transcripts Identified from TNBS Treated Mouse Sample Using DNA Microarray

Function	Change	Accession number	Gene
Cell adhesion	I	NM_011926	CEA-related cell adhesion molecule 1
	I	*XM_129745*	*Procollagen, type III, alpha 1*
	D	X15202	Integrin beta 1
	D	AV238648	Laminin, beta 3
Signal transduction	I	NM_009917	Chemokine (C-C motif) receptor 5
	I	NM_008142	Guanine nucleotide binding protein, beta 1
	I	NM_008859	Protein kinase C, theta
	D	NM_021475	ADAM-like, decysin 1
	D	*M14044*	*Calpactin I heavy chain (p36)*
	D	*L11332*	*CD38 antigen*
	D	*BC002046*	*Ephrin A1*
	D	U53696	Interleukin 10 receptor, beta
	D	L04966	RAB18, member RAS oncogene family
	D	U59864	TRAF family member-associated Nf-kappa B activator
Cell motility	I	NM_019824	Actin related protein 2/3 complex, subunit 3
	D	AI462105	Vinculin
Cell death	D	AJ224738	Death adaptor molecule (Raidd/Cradd),
	D	NM_009807	Caspase 1
	D	*AF242432*	*Neuronal apoptosis inhibitory protein (Naip)*
Cell proliferation	I	*BC003290*	*Cyclin I*
	I	*L12447*	*Insulin-like growth factor binding protein 5*
	D	L49507	Cyclin G1
	D	U35141	Retinoblastoma-binding protein (mRbAp48)
Transport	I	U49393	ATPase, Ca^{++} transporting, ubiquitous
	I	NM_025841	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2
	D	BC008129	3-Monooxgenase/tryptophan 5-monooxgenase activation protein, gamma polypeptide
	D	AK075779	ATPase, H+ transporting, lysosomal interacting protein 1
	D	*BC030837*	*Coatomer protein complex, subunit beta 1*
	D	NM_022997	Vacuolar protein sorting 35
Cell organization and biogenesis	I	AK007633	Small proline-rich protein 2A
	I	*M21495*	*Cytoskeletal gamma-actin*
	I	NM_008471	Keratin complex 1, acidic, gene 19
	I	M29015	Ribosomal protein L7
	I	NM_025284	Thymosin, beta 10
	D	*XM_124563*	*Amine N-sulfotransferase (SLUT-N)*
	D	*L01062*	*ATP synthase alpha subunit*
	D	U16741	Capping protein alpha 2 subunit
	D	*NM_009801*	*Carbonic anhydrase 2 (Car2)*
	D	*U53514*	*Guanylate kinase*
	D	U31758	Histone deacetylase 2
	D	*BC008119*	*Ornithine aminotransferase*
	D	*J03928*	*Phosphofructokinase, liver, B type*
	D	*NM_010726*	*Phytanoyl-CoA hydroxylase (Phyh)*
	D	NM_024209	Protein phosphatase 6, catalytic subunit
	D	*M76727*	*Pyruvate dehydrogenase E1 alpha subunit*
	D	BC021344	Ribosomal protein L22
	D	NM_053124	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5
	D	BC003475	Tubulin, beta 2
	D	*AF061017*	*UDP-glucose dehydrogenase*
	D	*XM_126545*	*Zinc finger protein 147 (estrogen responsive finger protein, Efp)*
Cell differentiation	D	NM_021099	Kit oncogene
	D	*BC030297*	*Lectin, galactose binding soluble 4*
DNA repair	D	AK032331	Myeloid/lymphoid or mixed-lineage leukemia
	D	*AF069519*	*T-G mismatch-specific thymine-DNA glycosylase*
	D	NM_009460	Ubiquitin-like 1
	D	*U55041*	*Uracil-DNA glycosylase (urg)*
RNA processing	D	NM_133196	Cleavage stimulation factor, 3′ pre-RNA subunit 2
	D	NM_010447	Heterogeneous nuclear ribonucleoprotein A1
	D	NM_019721	Methyltransferase-like 3
	D	NM_031179	Splicing factor 3b, subunit 1
Responses to stress	I	M35725	Cu–Zn superoxide dismutase
	I	NM_011260	Regenerating islet-derived 3 gamma
	D	NM_008298	DnaJ (Hsp40) homolog, subfamily A, member 1
	D	*X91864*	*Glutathione peroxidase 2*
	D	U00937	Growth arrest and DNA-damage-inducible 45 alpha

Table 1. Continued.

Function	Change	Accession number	Gene
Immune response	I	NM_011036	Pancreatitis-associated protein
	I	NM_009402	Peptidoglycan recognition protein
	I	NM_011260	Regenerating islet-derived 3 gamma
	D	NM_010382	Histocompatibility 2, class II antigen E beta
	D	*AF109905*	*Histocompatibility locus class III regions Hsc70t*
	D	U51992	Interferon dependent positive acting transcription factor 3 gamma
	D	X04699	T-cell receptor gamma, variable 2

Seventy-two gene transcripts are presented in this Table, in which 24 transcripts italicized were also found in the cDNA subtraction study. Mice were treated with vehicle or TNBS (3 mg) and changes in transcript levels of TNBS treated mouse are expressed relative to those of vehicle treated mouse (control). Transcripts also found in the cDNA subtraction study are italicized. D; decreased, I; increased.

Table 2. Gene Transcripts Found from the DNA Microarray and cDNA Subtraction Studies

Function	Change	Accession number	Gene
Cell adhesion	I	XM_129745	Procollagen, type III, alpha 1
Signal transduction	D	M14044	Calpactin I heavy chain (p36)
	D	**L11332**	**CD38 antigen**
	D	**BC002046**	**Ephrin A1**
Cell death	D	AF242432	Neuronal apoptosis inhibitory protein (Naip)
Cell proliferation	I	BC003290	Cyclin I
	I	L12447	Insulin-like growth factor binding protein 5
Transport	D	BC030837	Coatomer protein complex, subunit beta 1
Cell organization and biogenesis	I	M21495	Cytoskeletal gamma-actin
	D	**XM_124563**	**Amine *N*-sulfotransferase (SLUT-N)**
	D	L01062	ATP synthase alpha subunit
	D	NM_009801	Carbonic anhydrase 2 (Car2)
	D	U53514	Guanylate kinase
	D	BC008119	Ornithine aminotransferase
	D	J03928	Phosphofructokinase, liver, B type
	D	**NM_010726**	**Phytanoyl-CoA hydroxylase (Phyh)**
	D	M76727	Pyruvate dehydrogenase E1 alpha subunit
	D	AF061017	UDP-glucose dehydrogenase
	D	**XM_126545**	**Zinc finger protein 147 (estrogen responsive finger protein, Efp)**
Cell	D	BC030297	Lectin, galactose binding soluble 4
DNA repair	D	AF069519	T-G mismatch-specific thymine-DNA glycosylase
	D	U55041	Uracil-DNA glycosylase (urg)
Stress response	D	X91864	Glutathione peroxidase 2
Immune response	D	AF109905	Histocompatibility locus class III regions Hsc70t

Changes in transcript levels identified in both DNA microarray and cDNA subtraction studies are presented. Bold letters indicate transcripts that were also analyzed through RT-PCR.

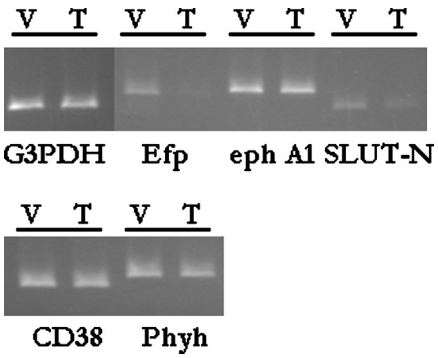

Fig. 1. RT-PCR Analysis of Transcripts Identified by DNA Microarray and cDNA Subtraction Analyses

Transcripts analyzed were control (G3PDH), zinc finger protein 147 (Efp), ephrin A1 (eph A1), amine *N*-sulfotransferase (SULT-N), ADP-ribosyl cyclase (CD38) and phytanoyl-CoA hydroxylase (Phyh) that had been identified in the microarray and cDNA subtraction procedures. V: RNA isolated from vehicle treated intestines ($n=3$, pooled), T: RNA from TNBS treated mouse intestine ($n=3$, pooled).

found *via* mini-array analysis of 1300 genes are listed in Table 3. Among intestinal mucosal samples from 5 patients subjected to the study at Fujita Health University, internal controls showed that intra- and interassay variations among mini-array blots were less than 10%, indicating that signal intensities in each spot represented amounts of mRNAs, which can be compared between blots (patients). Because the TNFα antibody treatment was applied to three patients on the day of biopsy sample collection, data from five patients were divided into two groups; Group 1 received no TNFα antibody treatment, and Group 2 received the TNFα antibody treatment on the day of sample collection. In addition, if transcript levels for particular genes in CD patients were similar to those in a healthy subject, these transcripts were excluded from the subsequent analysis, resulting in transcript changes associated with CD. Among a total of 152 transcript changes in the colitis parts (transmural granulomatous inflammation-fibrostenotic lesions, GFL) of CD patients, 47 were selected

Table 3. Transcripts Identified from Human Colitis Samples Using the 1300 Human cDNA Mini-Array

Accession No.	Gene name	Group 1 Control (B1)	Group 1 Colitis (B2)	Change (B2/B1)	Group 2 Control (A1)	Group 2 Colitis (A2)	Change (A2/A1)
U13697	Caspase-1 (ICE)	106.4	146.7	1.4	186	199.4	1.1
U28015	Caspase-5 (TY, ICErelIII)	159.8	219.8	1.4	125.6	167.6	1.3
X66364	Cdk5 (PSSALRE)	114.2	132.5	1.2	132.8	117.2	0.9
U04815	PITSLRE	152.8	137.9	0.9	160.6	189	1.2
X66363	PCTAIRE-1	148.1	169.4	1.1	131.1	166.1	1.3
D50310	***Cyclin I***	***366.7***	***733.4***	***2***	***810.7***	***1241.3***	***1.5***
M92287	Cyclin D3 (CYL3)	115.5	151.2	1.3	80.6	96.3	1.2
AF164598	Cdc16	140.7	212.8	1.5	50.8	73.3	1.4
X74795	Cdc46 (MCM5)	159	168	1.1	145.7	216.5	1.5
U85962	CBP (CREB-binding protein)	242.6	149.4	0.6	169	150	0.9
U01877	p300	124.7	93.9	0.8	78.1	40	0.5
M62399	NκB p65	108.6	138.3	1.3	100.6	65.7	0.7
M37492	NFκB p50	119.3	161.5	1.4	116.1	128.7	1.1
U91616	IκB-epsilon	143.5	240.4	1.7	111.2	138.3	1.2
M34461	CD38	254.7	561.6	2.2	189.9	275.5	1.5
AF080157	IKKalpha (CHUK)	129.8	101.6	0.8	80.3	49.1	0.6
X02812	***TGF-beta1***	***90.3***	***97.8***	***1.1***	***86.9***	***133.7***	***1.5***
X01394	TNF-alpha	94.6	73.8	0.8	42.9	70.6	1.6
D12614	TNF-beta	88.9	84	0.9	72.7	55.5	0.8
M15329	IL-1alpha	91.6	67.8	0.7	42.8	34.6	0.8
X70508	***IGFBP5***	***245.9***	***375.1***	***1.5***	***241.2***	***441.2***	***1.8***
X13274	Interferon-gamma	207	86.3	0.4	333.5	297.2	0.9
L11695	TGFbeta-RI	86.8	93.8	1.1	102.5	43.4	0.4
M85079	TGFbeta-RII	74.6	90.7	1.2	44.1	46.7	1.1
X55313	TNF-R1	135.4	113.8	0.8	117.1	136.1	1.2
J03143	IFN-gammaR	131.9	113.2	0.9	65.6	74.8	1.1
X01057	IL-2Ralpha	96.7	99.4	1	58.3	46.1	0.8
D11086	IL-2Rgamma	120.8	122.7	1	90.9	160.8	1.8
X61598	HSP47 (colligin)	148.1	158.7	1.1	142.3	215.5	1.5
Y00371	HSC70	186.9	206.8	1.1	415	440.3	1.1
J03209	MMP-3	121.7	240.2	2	65.2	80.2	1.2
M57730	Ephrin A1	387.9	604	1.6	216.7	386.7	1.8
M11233	Cathepsin D	219.9	147.7	0.7	1060.5	828.2	0.8
X03124	TIMP1	215	815	3.8	267.6	558.3	2.1
L23808	MMP-12	103.7	146.8	1.4	106.1	123	1.2
U88878	Toll-like-receptor (TLR) 2	145.8	85.1	0.6	71.1	68.3	1
U88880	Toll-like-receptor (TLR) 4	109.1	103.6	1	69.4	63.9	0.9
U22431	Hypoxia-inducible factor 1alpha	61.9	74.1	1.2	45.1	51.8	1.1
M28210	Rab 3	69.1	46.3	0.7	66.2	30.5	0.5
D31784	K-cadherin (cadherin-6)	93.6	106.2	1.1	60.3	82.8	1.4
Y00796	***Integrin-alpha L (CD11a)***	***152.2***	***241.5***	***1.6***	***115.2***	***133.3***	***1.2***
M74027	MUC-2 (Mucin 2)	194.6	155.1	0.8	104.2	91.1	0.9
M24736	E-Selectin	169.7	129.7	0.8	46.3	21.9	0.5
X58531	***Laminin alpha-1***	***114.9***	***98.4***	***0.9***	***59.6***	***31.5***	***0.5***
M55210	Laminin gamma-1	179.1	140.2	0.8	90.6	71.6	0.8
M84379	HLA-A	729.5	651.2	0.9	2749.8	2843.6	1
U95989	HLA-DR (beta1)	408.9	382.8	0.9	1030.7	1389.3	1.3

Group 1: average of two patients who never received the TNFα antibody treatment, Group 2: average of three patients who received the TNFα antibody on the day of biopsy sampling. Pooled transcript changes between control (pathologically normal mucosa) *vs*. colitis regions (granulomatous inflammation-fibrostenotic lesions) are shown as a simple ratio (colitis/control). Similar changes in transcript levels identified in DNA microarray are shown in italicized-bold letters. Ratios of more than 1 or less than one indicate transcripts that were increased or decreased, respectively, in the colitis regions when compared to those in the controls.

because they have the same or similar functions as the genes represented and transcript changes identified in the DNA microarray, allowing thorough comparisons of transcript changes between TNBS treated mice and CD patients. In Table 3, similar changes in transcript levels identified in DNA microarray are shown in italicized-bold letters; cyclin I, TGFβ1, insulin like growth factor binding protein 5 (IGFBP5), integrin-αL, and laminin α1. Of these, cyclin I, TGFβ1, IGFBP5, and integrin-αL increased in the GFL parts of CD patients.

Comparison of data from both DNA microarray of mouse samples and cDNA mini-array of human biopsy samples is shown in Table 4. There are some similarities between the two analyses, but many changes in transcripts levels found from CD patients were not detected in TNBS treated mouse samples. A definitive conclusion could not be made in CD patients between the TNFα antibody treated and untreated groups due in part to the small sample sizes. It is interesting to note that the amounts of HLA-A and HLA-DR transcripts found in both GFL and control (pathologically normal mucosa, PNM) regions of CD samples were much higher than those of TNBS treated mice or normal human colon; Patients in group 2 (after the TNFα treatment) had even higher HL-A mRNAs than those of group 1 (before the treatment). This is in agreement with previous reports where overexpression of MHC and/or HLA genes was found in intestinal tissue sam-

Table 4. Transcripts Identified from Human Colitis Samples *vs*. TNBS Treated Mouse Samples

Accession No.	Gene name	TNBS treated mouse	Human colitis	
			Before	After
U13697	Caspase-1 (ICE)	−	+	+
X66364	Cdk5 (PSSALRE)	−	+	−
D50310	**Cyclin I**	++	++	+
M92287	Cyclin D3 (CYL3)	CNF	+	+
M62399	NFκB p65	CNF	+	−
M34461	CD38	−	++	+
X02812	**TGF-beta1**	++	+	+
X01394	TNF-alpha	CNF	−	++
X70508	**IGFBP5**	++	+	++
X13274	Interferon-gamma	CNF	−−	−
L11695	TGFbeta-RI	CNF	+	−−
X55313	TNF-R1	+	−	+
J03143	IFN-gammaR	CNF	−	+
X61598	HSP47 (colligin)	CNF	+	+
Y00371	HSC70	CNF	+	+
M57730	Ephrin A1	−	++	++
M11233	Cathepsin D	CNF	−	−
X03124	TIMP1	CNF	++	++
L23808	MMP-12	CNF	+	+
U88878	Toll-like-receptor (TLR) 2	CNF	−	+/−
U88880	Toll-like-receptor (TLR) 4	CNF	+/−	−
U22431	Hypoxia-inducible factor 1 alpha	CNF	+	+
M28210	Rab 3	CNF	−	−
D31784	K-cadherin (cadherin-6)	−	+	+
Y00796	**Integrin-alpha L (CD11a)**	++	++	+
X58531	**Laminin alpha-1**	−−	−	−
M84379	HLA-A	CNF	−	+/−
U95989	HLA-DR (beta1)	CNF	−	+

Transcript changes in colitis samples are relative to own control. Similar changes found in TNBS treated mouse and human colitis samples are shown in bold letters. Changes: ++: more than 1.5-fold increase, +: more than 1.0 fold-increase, +/−: minimal-no change, −: changes between 0.9 and 0.5 (minor changes), −−: changes of 0.5 or less (severe decrease), CNF: changes not found. Before: before the TNF-α antibody treatment, After: after the treatment.

ples from CD patients.[18,19] In addition to the increase in CDK inhibitor mRNAs, transcripts for heat shock protein (HSP), HSP27, HSP40, HSP70, HSP90β and HSC70, and HIF-1α were higher in the GFL than in the PNM region.[20—22] The levels of these transcripts were much higher in CD patients than those in the normal colon. These results indicate that GFL regions could be experiencing hypoxia.

DISCUSSION

The objectives of the present investigation were to identify transcript changes in mucosal samples from TNBS-treated mice, and to relate these changes to those of biopsy specimens from CD patients. The number of transcripts, 24, identified by both DNA microarray and cDNA subtraction studies appears to be small. This could have resulted from the small sample size (500 colonies) analyzed through cDNA subtraction. These experimental results were then compared to transcript changes in the intestinal mucosa of CD patients, which had been determined using cDNA mini-array analysis. Although numerous similarities such as transcripts related to inflammation and immune functions between the mouse model and CD patients have been documented,[5,9] it is possible that TNBS-treated mice may not fully represent an experimental model for studies of pathological events leading to human CD (Table 4). However, if the results from these models were compared to transcripts so far found, candidate transcript changes, yet unidentified or not previously considered, between early and advanced etiopathogenic events in CD could be identified.[23]

Within the same functional group, there were gene transcripts that increased with TNBS treatment, but others with the same or similar functions decreased. These results suggest expression redundancy, but more importantly, those genes whose functions were required for the maintenance of normal conditions were also required for pathological conditions induced by the TNBS treatment. Similarities found in transcript changes between TNBS-treated mice and human CD may be indicative of shared pathogenic events, whereas the dissimilarities may represent a different pathogenesis between acute TNBS model and chronic CD samples. In addition, whether the shared transcript changes between TNBS model and CD samples represent similar, if not the same, pathogenesis during early as well as extended periods may require further investigation.

Changes found both in DNA microarray and cDNA subtraction studies indicate that genes involved in cellular metabolism, transport, apoptosis, and transcription factors were decreased. Ephrin A1 is a proinflammatory cytokine induced by TNFα,[24] suggesting that inflammatory processes were initiated in a group of mice treated with TNBS. Rab 6, a member of the Ras superfamily, is a small GTP-binding protein involved in intra-Golgi transport,[25] suggesting that in the intestinal mucosa, membrane traffic from the Golgi apparatus to the endoplasmic reticulum (ER) could be disrupted by TNBS treatment. Reduction in neuronal apoptosis inhibiting protein (NAIP) suggests that TAK-dependent JNK1 activation and/or caspase-3-dependent apoptosis may be occurring.[26,27] This is supported by results from the DNA microarray analysis, in which transcripts of DNA repair and RNA processing genes decreased. However, the design of the present experiments did not allow us to assess the time courses of these changes, *i.e*., whether they occur as initial or as progressive events in the intestinal mucosa. Furthermore, morphological examination revealed that tissue fibrosis had not been initiated in mucosal tissues within 48 h after TNBS treatment, the time of tissue collection (data not shown), suggesting that integrins and other cell adhesion molecules were still effective against cell dissociation and/or fibrosis in mice treated with TNBS of the present regimen.

Changes in caspases 1 through 5 transcripts, which were higher in GFL samples than in PNM samples, may indicate that mucosal cell divisions had stopped or were about to stop.[28,29] Therefore, a thorough analysis of transcripts in healthy tissues *vs*. initial events (TNBS-treated mice) *vs*. advanced condition (CD patients), is required to single out transcript changes that reflect progressive events possibly leading to CD. Furthermore, changes in HLA and HSP transcripts suggest that this may have resulted from exaggeration of immunologic events such as self or non-self recognition processes involving HLA overexpression.

In summary, this study presents unique approaches for identifying genes previously not considered in samples from CD patients and/or TNBS-treated mice. DNA microarray analysis has been associated with a lack of reproducibility, but the present approach—a combination of DNA microarray

analysis, cDNA subtraction analysis, and subsequent RT-PCR validation—may overcome such problems. These combined analyses should be used to identify transcript changes possibly occurring during the initial and progressive stages of CD, and to dissect true pathological changes in gene expressions from unrelated or individual variations in gene expression often found in human samples. In addition, gene transcripts newly identified in this study require further validation within the context of early pathogenesis in CD patients, which may provide a clue for the identification of marker transcripts and/or targets for gene therapy.

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