Biochemical and Biophysical Research Communications **283,** 130–135 (2001)
doi:10.1006/bbrc.2001.4726, available online at http://www.idealibrary.com on IDEAL®

Identification of Genes Upregulated in the Inflamed Colonic Lesions of Crohn's Disease

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Received March 19, 2001

To identify the molecular mechanism which primarily plays a role in the pathogenesis of Crohn's disease (CD) without prior hypothesis, differential display method was employed to detect differentially expressed genes between the inflamed and uninflamed colonic samples from one patient with CD. The mRNA levels of these genes were subsequently semiquantitated in affected and unaffected tissues from six patients using reverse transcriptase polymerase chain reaction (RT-PCR). Six genes including long form FLICE inhibitory protein ($FLIP_L$) were found to be consistently overexpressed in the inflamed colonic CD tissues. Immunohistochemical studies revealed that $FLIP_L$ expressing cells were lamina propria lymphocytes (LPLs). The present study suggested that overexpression of $FLIP_L$ in the LPLs may be involved in the pathogenesis of CD through defective activation-induced cell death. In addition, this study provided evidence for a possible role of several previously unsuspected genes in the pathogenesis of CD. © 2001 Academic Press

Key Words: **differential display (DD); FLICE inhibitory protein (FLIP); activation induced cell death (AICD); Traf2 and Nck interacting kinase (TNIK); type 6 protein phosphatase regulated by interleukin-2 (PP6 regulated by IL-2); expression.**

Crohn's disease (CD) is an inflammatory bowel disease (IBD) of unknown etiology. Thus far, a number of studies demonstrated expression of various molecules in the affected tissues by focusing on a limited number of targets of interest (1–9). However, such previous observations have a limitation that only a few known genes strongly suspected for the involvement in the pathogenesis were examined.

We considered that comparison of genes that are differentially expressed between the involved and uninvolved tissues without prior hypothesis may provide new information on the pathogenesis of CD, which would potentially lead to the development of a novel therapeutic approach. For such a purpose, we applied differential display method (DD) (10, 11) on surgically resected tissue samples from patients with CD.

MATERIALS AND METHODS

Patients and samples. Macroscopically and microscopically inflamed/uninflamed intestinal tissues were obtained from surgically resected specimens from six patients with CD at Social Health Insurance Medical Center (Tokyo, Japan). Table 1 presents clinical characteristics of these patients. For immunohistochemistry, the inflamed/uninflamed colonic specimens were additionally obtained from three patients with CD. As a control, unaffected portion of the colonic tissue was obtained from a surgically removed specimen from a patient with colon cancer. This study was reviewed and approved by the Institutional Review Committees of boh institutes.

Differential display (DD). Genes differentially expressed between inflamed and uninflamed colonic tissues from patient No. 1 (Table 1) were analyzed by differential display (DD). Total RNA was extracted from the snap-frozen whole gut segments using Trizol reagent (Gibco BRL, Gaithersburg, MD), and first-strand cDNA was then synthesized from 3 μg of total RNA using MMLV reverse transcriptase (RT) (Clontech Laboratories Inc., Palo Alto, CA), according to the manufacturer's instructions. cDNA synthesized from 0.01 μg total RNA was amplified using Delta Differential Display Kit (Clontech). The first three cycles of polymerase chain reaction (PCR) were done under a low stringency condition (94°C, 5 min; 40°C, 5 min; 68°C, 5 min; 1 cycle, 94°C, 30 s; 40°C, 30 s; 68°C, 5 min; 2 cycles). The products were then amplified under a high stringency condition (94°C, 20 s; 60°C, 30 s; 68°C, 1 min; 30 cycles) in a Thermal Cycler MP (Takara, Kyoto, Japan). The amplified cDNAs were elec-

This study was supported by the Grant-in-Aid for Scientific Research on Priority Areas (C), the Grant-in-Aid for Scientific Research, and the Grant-in-Aid for Exploratory Research from the Ministry of Education, Culture, Sports, Science, and Technology.

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0006-291X/01 $35.00

TABLE 1

Clinical Characteristics of the Patients with Crohn's Disease at the Time of Surgery

Patient no.	Age	Sex	Perforation	Involvement*	Resected region	Treatment*
No. 1	27 y	Male	+	I, C	Stenotic region of descending C	ED
No. 2	43 y	Male	−	I	Bleeding region of I	ED, 5-ASA
No. 3	31 y	Male	+	I, C	Stenotic region of ascending C	ED, 5-ASA
No. 4	38 y	Male	−	I	Stenotic region of I	No treatment
No. 5	32 y	Male	+	I, C	Perforation of terminal I	SASP
No. 6	37 y	Female	+	I, C	Perforation of I and ascending C	No treatment

* I, the ileum; C, the colon; ED, elemental diet; 5-ASA, 5-aminosalicylic acid; SASP, sulfasalazine.

trophoresed on a 6% denaturing polyacrylamide gel and visualized by silver staining (Promega Corp., Madison, WI).

Comparisons were made between each of the two lanes representing RT-PCR products from 0.01 μg of total RNA either from uninflamed or inflamed tissues. The bands showing substantially different intensities between inflamed and uninflamed tissues were cut, eluted, and reamplified. The amplified fragments were purified using PCR-single strand conformation polymorphism method (12), reamplified again, cloned into the pCR-TOPO Vector (Invitrogen, Carlsbad, CA), and subjected to fluorescence-based automated cycle sequencing (ABI 310; PE Applied Biosystems, Foster City, CA) using dye-terminator method (ABI PRISM dRhodamine Terminator Cycle Sequencing-Ready Reaction Kit, PE Applied Biosystems). Homology search was performed using EMBL/GenBank database and the NCBI BLAST program (National Library of Medicine, MD).

Semi-quantitation of mRNA levels by RT-PCR. Differential expression of mRNAs was confirmed by semi-quantitative RT-PCR using the first strand cDNA as templates. cDNA concentration was adjusted to amplify equal amount of glyceraldehyde phosphate dehydrogenase (GAPDH). To find a linear range of amplification, preliminary PCR amplification was performed for 20 to 38 cycles. PCR primers used for semi-quantitative RT-PCR are shown in Table 2 (13–18). RT-PCR was carried out using GeneAmp reagents and AmpliTaq Gold DNA Polymerase (Perkin-Elmer, Norwalk, CT). The following PCR conditions were used for all reactions: 94°C for 10 min followed by 30 cycles of 94°C for 30 s; the annealing temperature shown in Table 2 for 30 s; 72°C for 30 s, with a final extension of 10 min at 72°C in a Thermal Cycler MP (Takara). The PCR products were electrophoresed on a 6% polyacrylamide gel and stained with SYBR Gold (Molecular Probes Inc., Eugene, OR). Densitometric values of PCR products obtained from scanning densitometry (Molecular Imager FX, Bio-Rad, Hercules, CA) were plotted against the dilution of the templates, and the dilutions which gave the equal densitometric values were compared between two samples.

Immunohistochemistry. Intestinal specimens were embedded in 50% OCT (Tissue-Tek, Sakura Finetech, Torrance, CA) in PBS, snap-frozen in liquid nitrogen, and then stored at −70°C until use. Serial cryostat sections (5 μm) were cut, air dried on microscope slides, and fixed in acetone for 20 min. Sections were preincubated with PBS containing 5% hydrogen peroxide, and then incubated for 45 min with the first antibodies. The antibodies were either rabbit polyclonal antibody against human $FLIP_L$ or human caspase-8 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted at 1:50 in PBS. After washing with PBS, sections were incubated with peroxidase conjugated goat anti-rabbit IgG (Nichirei Corp., Tokyo, Japan) for 30 min, followed by color development (Histofine Simplestain PO[R], Nichirei Corp.). Nuclei were counterstained with hematoxylin. The sections were observed under microscope, and the numbers of positively stained cells and total cells within × 200 power fields were counted.

RESULTS AND DISCUSSIONS

Identification of Genes Differentially Expressed between Inflamed and Uninflamed Colonic Tissues with CD

As a first step, we made an attempt to detect differentially expressed genes between inflamed and uninflamed colonic samples from one patient with CD (No. 1) using DD. Among the 90 differentially amplified fragments,

TABLE 2

PCR Primers and Conditions for Semi-Quantitative RT-PCR

Gene*	Chromosomal location	Size of product	Sense primer sequence	Antisense primer sequence	Annealing temperature
Cytochrome b	Mitochondria	195	CACATCAAGCCCGAATGATA	GTCTGCGGCTAGGAGTCAAT	50°C
Cytochrome oxidase subunit I	Mitochondria	201	ACGCACTCTCCCCTGAACT	GGGGAATGCTGGAGATTGTA	50°C
$FLIP_L$	2q33–34	204	CTCCAAGCAGCAATCCAAA	GATCCTAGGGGCTTGCTCT	50°C
GAPDH	12p13.31–p13.1	605	CGAGATCCCTCCAAAATCAA	ACCTGGTGCTCAGTGTAGCC	50°C
GRα	5q31	477	CCTAAGGACGGTCTCAAGAGC	GCCAAGTCTTGGCCCTCTAT	57°C
PP6 regulated by IL-2	Not identified	413	ACCCATTTTTCTGCCCTCTT	TCGTGCCCACTGAATAACAA	50°C
TNIK	Not identified	184	TGGTTCACACACTGGTTTCC	CCGGCCATAGGTGTTTACAT	50°C

* GAPDH, Glyceraldehyde phosphate dehydrogenase; GRα, Glucocorticoid receptor α; $FLIP_L$, long form FLICE inhibitory protein; TNIK, Traf2 and Nck interacting kinase; PP6 regulated by IL-2, type 6 protein phosphatase regulated by interleukin-2.

TABLE 3

Results of Semi-Quantitative RT-PCR Analysis

Patients:	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 6	No. 6
Sample	Inflamed C*/ uninflamed C	Inflamed I*/ uninflamed I	Inflamed C/ uninflamed C	Inflamed I/ uninflamed I	Inflamed TI*/ uninflamed TI	Inflamed C/ uninflamed C	Uninflamed C/ uninflamed I	Inflamed I/ uninflamed I
GRα	4/1	1.5/1	4/1	1/1	4/1	4/1	1/1	1/1
Cytochrome b	2/1	1/1	8/1	1/1	2/1	6/1	2/1	1/1.6
Cytochrome oxidase subunit 1	1.7/1	1/1	8/1	1/1.5	2/1	8/1	2.4/1	1/1.5
PP6 regulated by IL-2	3/1	2.7/1	8/1	2.7/1	1.7/1	3/1	4/1	1/2
FLIP long form	10/1	2/1	8/1	1/1	3/1	16/1	4/1	1/1
TNIK	4/1	1/1	5/1	1/1	2/1	13/1	2/1	1/2.5

Note. Semi-quantitation was performed by comparing the number of serial dilutions which gave the equal densitometric value of PCR products between two samples. *I, the ileum; C, the colon; TI, the terminal ileum.

eleven were confirmed to be differentially expressed genes using semi-quantitative RT-PCR. Five of them had no homology to sequences in the EMBL/GenBank database and were considered to be yet unidentified genes, while six of them showed perfect match with sequences of known genes, namely, glucocorticoid receptor α (GRα), cytochrome oxidase subunit 1, cytochrome b, type 6 protein phosphatase regulated by interleukin-2 (PP6 regulated by IL-2), Traf2 and Nck interacting kinase (TNIK), and long form FLICE inhibitory protein ($FLIP_L$). In this patient, these genes were preferentially expressed in the inflamed tissue by 1.7–10-fold compared with the uninflamed tissue (Table 3). All of these genes have not been previously shown to be upregulated in the inflamed tissue of CD.

Comparison of Expression Profiles in Tissues from Five Patients with CD

Next, we compared the mRNA level of these differentially expressed genes in the inflamed and uninflamed tissues from five other patients with CD, in order to evaluate the consistency of our findings among different patients. The results are summarized in Table 3. Expression of the six genes detected in the DD experiments of patient No. 1 was consistently upregulated in all three colonic samples and one terminal ileal sample. On the other hand, no significant difference was observed in three small bowel samples, suggesting a difference in the pathogenesis between colonic and small bowel lesions (19).

PP6 regulated by IL-2 is a recently identified phosphoprotein in lymphocyte extract and is upregulated by IL-2 in peripheral blood T cells (16). This protein is involved in the signaling pathway which leads to T cell proliferation in response to IL-2 (16). Therefore, overexpression of this molecule in the inflamed CD mucosa may reflect accumulation of proliferating T cells stimulated by IL-2 (4, 5).

Another interesting molecule is TNIK. TNIK is a recently characterized kinase which was shown to specifically activate JNK (18). Since JNK has been shown to upregulate the translation of TNF-α (20), upregulation of TNIK may serve as an accelerator of TNF-α-JNK positive feedback loop. The crucial role for TNF-α in the pathogenesis of CD has been established from a number of experimental and clinical studies (19–22).

Overexpression of mitochondrial genes in the inflamed CD mucosa is considered to reflect inflammation which produces reactive oxygen resulting in tissue damage (23, 24). Upregulation of GRα in the inflamed colonic CD mucosa may possibly imply compensation of the impaired function of GRα caused by the oxidative condition in the inflamed area (25–27).

Expression and Localization of $FLIP_L$ Proteins

Among these differentially expressed genes, $FLIP_L$ was considered likely to be directly involved in the pathogenesis, because the overexpression of $FLIP_L$ has been shown to inhibit activation-induced cell death (AICD) of the lymphocytes (19, 28) and thus to be associated with autoimmunity (29). In order to confirm the expression at the protein level and localization of $FLIP_L$, immunohistochemical staining was performed. As shown in Fig. 1, most of the $FLIP_L$ expressing cells were shown to be lamina propria lymphocytes (LPLs) (Fig. 1). The percentages of the $FLIP_L$ expressing cells per total cells were greatly increased in inflamed tissues from patients with CD compared with normal colonic mucosa from a patient with colon cancer, and moderately increased compared with uninflamed tissues from the same patients with CD (Table 4).

A number of studies have reported that $FLIP_L$ is downregulated following lymphocyte activation, which induces susceptibility to AICD (17, 28–30). It is therefore presumed that $FLIP_L$ expression should be downregulated in the inflamed tissues. However, this study revealed accumulation of $FLIP_L$ expressing lymphocytes in the actively inflamed CD colonic mucosa, suggesting that impaired downregulation of $FLIP_L$ after lymphocyte activation in a subset of

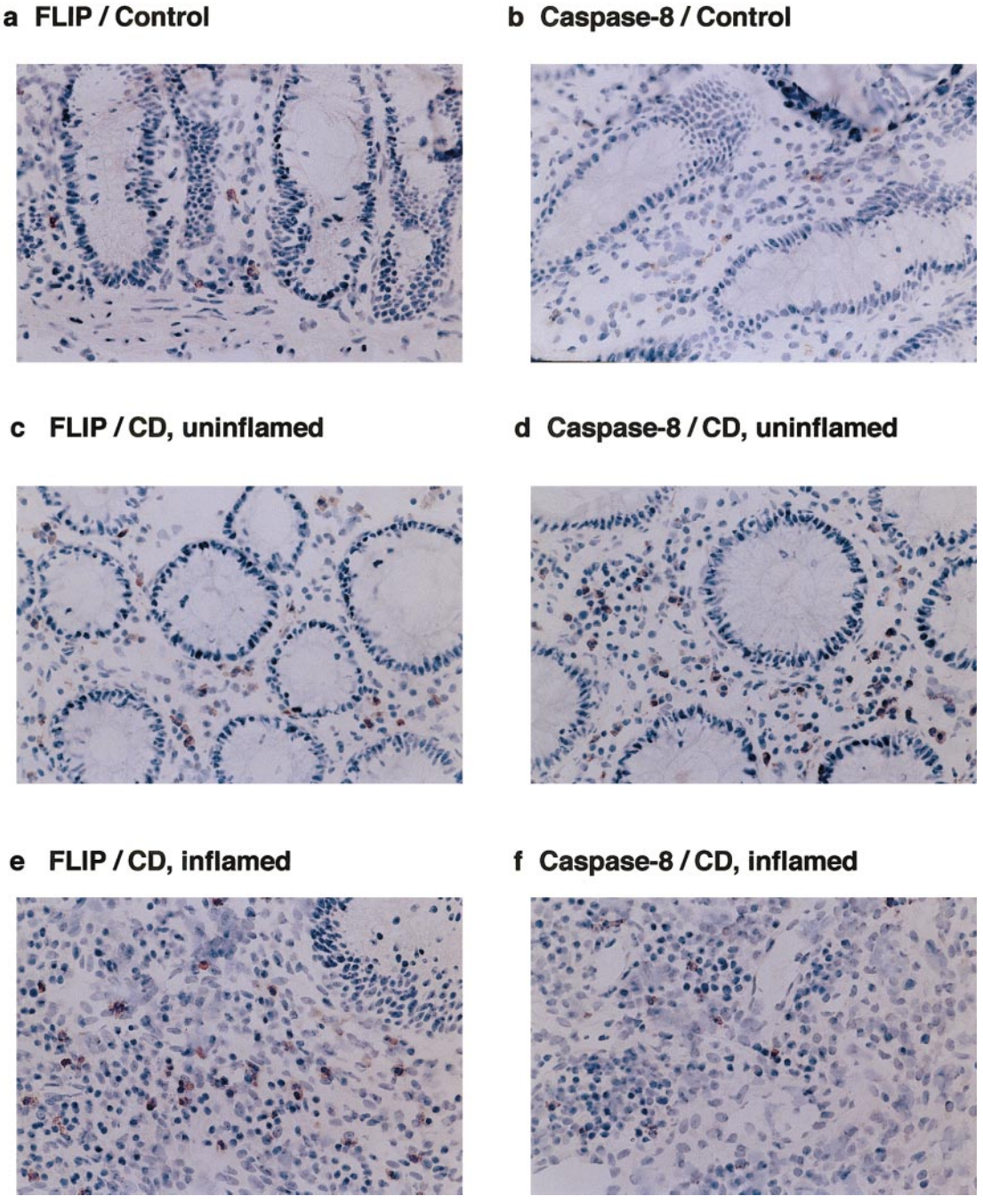

FIG. 1. Immunohistochemical detection of $FLIP_L$ and caspase-8 in the colonic mucosa. Sections of intestine were stained with rabbit polyconal antibody against human $FLIP_L$ and rabbit polyconal antibody against human caspase-8. Nuclei were counterstained with hematoxylin. (a) $FLIP_L$ expressing cells in the control colonic mucosa from the normal tissue from a patient with colon cancer. (b) Caspase-8 expressing cells in the control colonic mucosa. (c) Accumulation of $FLIP_L$ expressing lamina propria lymphocytes (LPLs) in the apparently uninflamed colonic mucosa from the patient with CD (CD1). (d) Accumulation of caspase-8 expressing LPLs in the apparently uninflamed colonic mucosa from the patient CD1. (e) Accumulation of $FLIP_L$ expressing LPLs is in the actively inflamed colonic mucosa from the patient CD1. (f) Caspase-8 expressing LPLs in the inflamed colonic mucosa from the patient CD1.

LPLs might be directly involved in the pathogenesis of CD. This possibility is also supported by the observation that mice lacking IL-2, which developed CD like colitis (31), showed impaired downregulation of $FLIP_L$ in T cells after activation (28) and defect in AICD (32).

TABLE 4

The Number of Lamina Propria Lymphocytes (LPLs) Expressing Long Form FLIP ($FLIP_L$) and Caspase-8 Per Total Cells

	$FLIP_L$ positive LPLs/total cells	Caspase-8 positive LPLs/total cells
Control mucosa	8/1234 (0.7%)	14/1380 (1.0%)
CD 1 Inflamed mucosa	112/1640 (6.8%)	13/1190 (1.1%)
Uninflamed mucosa	47/1130 (4.2%)	104/1010 (10.3%)
CD 2 Inflamed mucosa	58/1430 (4.1%)	23/1520 (1.5%)
Uninflamed mucosa	18/1240 (1.5%)	50/1390 (3.6%)
CD 3 Inflamed mucosa	64/1530 (4.2%)	42/1570 (2.7%)
Uninflamed mucosa	44/1520 (2.0%)	64/1400 (4.6%)

Note. The number of LPLs stained positive for $FLIP_L$, caspase-8 and the number of total cells in ×200 power fields were counted under the microscope. The specimens were derived from patients different from those examined by RT-PCR (Table 1).

Expression and Localization of Caspase-8 Proteins

To evaluate the significance of $FLIP_L$ expression, the expression of caspase-8 was also examined in the same tissues, because $FLIP_L$ is a direct inhibitor of caspase-8 (17). Most of the caspase-8 expressing cells were also found to be LPLs (Fig. 1). In contrast to $FLIP_L$, the percentage of caspase-8 positive LPLs were decreased in the inflamed compared with uninflamed tissues (Table 4). Surprisingly, caspase-8 positive LPLs were substantially increased in uninflamed tissues from CD compared with control normal tissue from a patient with colon cancer, suggesting the presence of abnormal signaling in the apparently uninflamed CD mucosa. Lower percentage of caspase-8 positive LPLs per total LPLs in the inflamed CD mucosa than those in the apparently uninflamed CD mucosa may indicate additional signaling in the inflamed CD mucosa which downregulates expression of caspase-8. Recently, epigenetic downregulation of caspase-8 expression has been demonstrated in neuroblastoma cells (33). Inadequate downregulation of caspase-8 expression in LPLs may also lead to impaired AICD in the inflamed area.

Interestingly, genes previously shown to be overexpressed in the inflamed tissue compared with the uninflamed tissue with CD (1–5) were not detected in this study. In our DD system, approximately 3000 fragments were detected on average, which is not supposed to cover all expressed genes. Nevertheless, DD proved useful in the detection of previously unsuspected genes of particular interest in this study.

In conclusion, the present study demonstrated several previously unsuspected molecular pathways which may be relevant to the pathogenesis of CD. Further genetic and functional studies concerning TNF-α-TNIK-JNK pathways and regulation of expression of apoptosis related molecules such as FLIP and caspase-8 may provide substantial information as to the pathogenesis of CD in the future.

ACKNOWLEDGMENTS

The authors are indebted to Kazuhiko Kobayashi (Department of Pathology, Social Health Insurance Medical Center), Yoshihito Ohtsuka (Department of Microbiology, Social Health Insurance Medical Center), Dr. Masatoshi Wakui (Department of Hematology, Keio University), and Dr. Shohei Chida and Daisuke Sakurai (Department of Human Genetics, University of Tokyo) for technical assistance and helpful suggestions.

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