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• BRIEF REPORTS •

mRNA expression profiling reveals a role of *Helicobacter pylori* vacuolating toxin in escaping host defense

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Abstract

AIM: To study the immune response of host to *Helicobacter pylori* VacA.

METHODS: The monocyte/macrophage-like U937 cells were infected with *Helicobacter pylori vacA*-positive strain NCTC 11638 or isogenic *vacA*-negative mutant. Differentially expressed genes were identified at 2, 6, 10, and 24 h post-infection by cDNA microarray. Differential expressions of some genes were confirmed by Northern blot.

RESULTS: More than 100 genes altered their mRNA expression at different time points respectively, many of which were identified to be related to immune evasion.

CONCLUSION: VacA is a crucial element for *H pylori* to escape from host immune defense by means of differentially regulating the expression of some related genes. These genes, previously known or unknown to be involved in the mechanism of immune evasion, deserve further investigation to unearth much more information complicated in the immune response.

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INTRODUCTION

Helicobacter pylori infects about half of the world' s population. Despite the induction of an immunological reaction, the infection of *H pylori* is commonly life-long, suggesting that this pathogen has evolved mechanisms to evade protective immune responses to achieve the state of host-microbial equilibrium[1]. Some products of *H pylori* have been determined to have immunosuppressive effects for prolonging the infection. A 100 ku *H pylori* protein inhibits proliferation of T-cell and macrophage[2], and VacA, a cytotoxin that has been found to cause massive vacuolation in several mammalian cell lines and in the gastric epithelia of patients with active chronic gastritis associated with *H pylori* infection[3], may perform targeted action to disable T cells. VacA interferes with proteolytic processing of tetanus toxin and toxoid and specifically inhibits the Ii-dependent pathway of antigen presentation mediated by newly synthesized major histocompatibility complex (MHC) class II, suggesting that VacA may contribute to the persistence of *H pylori* by interfering with protective immunity[4]. However, to the author' s knowledge, the exact mechanism of such an immunosuppression effect has not been fully studied. Hence, in this study, we performed a large scale measurement of gene expression alteration in host cells using gene microarray technology, which provided the crucial information for interpreting the mechanisms of immunosuppression.

MATERIALS AND METHODS

Bacterial strains and growth conditions

H pylori NCTC 11638 strain positive for vacA was given as a gift by Dr. Shi (Shanghai Institute of Gastroenterology). Isogenic vacA-negative mutant 11638-Δ vacA was constructed by substitution of a kanamycin resistant gene for a short fragment of *vacA* through homologous recombination, as described previously[5]. *H pylori* strains were cultured routinely on brain heart infusion (BHI) agar plates with 5% sheep blood in mixed air containing 100 ml/L CO_2, 50 mL/L O_2, and 850 mL/L N_2 at 37 ℃.

H pylori infection of monocyte/macrophage-like cell line U937

The U937 cells were maintained in RPMI 1640 medium (Gibco BRL, USA) with 2 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L HEPES, 1.0 mmol/L sodium pyruvate and 100 ml/L fetal bovine serum. The day before *H pylori* infection, fresh medium with 20 mL/L fetal calf serum was substituted. Eighteen hours later, the cells grown to 90% confluency were cocultured with *H pylori* isogenic strains at a multiplicity of infection of 10 in culture medium for 2, 6, 10, and 24 h.

Total RNA Isolation

U937 cells cocultured with NCTC 11638 and 11638-Δ vacA were collected at 2, 6, 10, and 24 h after the infection for mRNA extraction. Total RNA was isolated using TRIZOL reagent (Invitrogen, USA) according to the manufacturer' s instruction.

cDNA microarrays

The cDNA microarrays were designed by Shanghai BioStar Genechip Inc. In this study, microarrays with 8 464 human cDNAs were used, including full-length and partial complementary DNAs representing novel, known, and control genes.

Preparation and hybridization of fluorescent-labeled cDNA

Aliquots of 30 μg of total RNA were fluorescently labeled with Cy5- or Cy3-dCTP (Amersham Pharmacia, Sweden) by reverse transcription in the presence of 5 μg of oligo(dT) and 1 μL of SuperScriptII (Gibco-BRL, USA). The labeled cDNAs were purified using MicroSpin S-200 columns (Amersham

Pharmacia, Sweden) and lyophilized. The probes were resuspended in 20 μL hybridization solution containing 8 μg of poly(dA), 2 μg of yeast tRNA, 10 μg of human *Cot* I DNA (Gibco-BRL, USA). After heated to 95 ℃ for 2 min and then cooled to room temperature, the mixture was applied to the slides and covered by a coverslip. The slides were incubated in a humid cabinet of an incubator for 16-18 h at 42 ℃. Then the slides were washed at 60 ℃ for 10 min in solutions of 2×SSC with 2 g/L SDS, 0.1×SSC with 2 g/L SDS, and 0.1×SSC sequentially, and then dried at room temperature.

Array scanning and data processing

Each slide was scanned at 10 μm resolution on a GenePix 4000B scanner (Axon Instruments, Inc., Foster City, CA) at variable PMT voltage to obtain maximal signal intensities with no more than 1% probe saturation. The images were processed with GenePix Pro 3.0. Ratios were normalized by a linear regression between ln(Cy5) and ln(Cy3) of all the genes' background-corrected signal intensities on the microarray. Genes exhibited a 2-fold or greater change in expression level and exceeded 200 in signal intensity were considered true outliers.

Preparation of ^{32}P-labeled probes

The plasmids containing cDNA clone used for preparing probes were provided by Shanghai BioStar Genechip Inc. A total amount of 200 ng plasmids were used as templates for PCR amplification. PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, Germany). The probes were labeled using random primed Strip-EZ DNA Kit (Ambion, Austin, TX). A total amount of 25 ng purified DNA diluted in 9 μL TE (10 mmol/L Tris-HCl, pH8, 1 mmol/L EDTA) was denatured at 95 ℃ for 5 min and then immediately frozen in liquid nitrogen, thawed, microfuged, and placed on ice. Afterwards, the following reaction was assembled as follows and mixed gently: A 9.0 μL of denatured DNA, 2.5 μL of 10×Decamer solution, 5.0 μL of 5×buffer-dATP/-dCTP, 2.5 μL of 10×dCTP, 5.0 μL of [α-^{32}P]dATP (3 000 Ci/mmol, 10 mCi/mL), 1.0 μL of exonuclease-free Klenow, and nuclease-free water to 25 μL. After 20 min incubation at 37 ℃, 1 μL of 0.5 mol/L EDTA was added to stop the reaction.

Northern blot analysis

A 10 μg sample of total RNA per lane was subjected to electrophoresis on 12 g/L agarose gels containing 2.2 mol/L formaldehyde. RNAs were transferred onto Zeta-probe blotting membranes (Bio-Rad Laboratories, Hercules, CA) using Vacuum Blotter (model 785, Bio-Rad Laboratories) and baked under vacuum at 80 ℃ for 2 h. Membranes were hybridized for 16 h at 60 ℃ with ULTRArray hybridization solution (Ambion, Austin, TX) containing cDNA probes labeled with [α-32P] dCTP by random priming (Strip-EZ DNA labeling system, Ambion). The hybridized membranes were serially washed at 55 ℃ using 2×SSC with 1 g/L SDS solution, then exposed to a phosphoimager. Blots were scanned and quantified by a phosphoimager in combination with Optiquant software v. 2.50 (Cyclone Storage Phosphor System, Packard Instruments).

RESULTS

The mRNA expression in U937 cells was compared at 2, 6, 10, and 24 h after the infection with NCTC 11638 or 11638-Δ vacA. More than 100 genes altered their mRNA expression at different time points respectively, among which, the genes related to immune evasion were selected (Table 1). To confirm the differential expression profiling of the cDNA microarrays, two genes: *PSMD13* and *ILK* were chosen for northern blot analysis. As shown in Figure 1, the expression levels of these 2 genes were lower in the cells treated with *H pylori* NCTC11638 than those treated with 11638-Δ vacA. In addition, in both cases, the fold ratio changes detected by the microarray were confirmed by Northern blot (Table 2). The level of β-actin transcript was present at approximately the same level in both samples, providing an assessment of RNA content in each sample used for Northern blot analysis.

Table 1 Differentially expressed genes related to immune evasion

GeneBank_ID	Definition	Fold change			
		2 h	6 h	10 h	24 h
NM_004517	Integrin-linked kinase (ILK)	0.717	0.218	0.245	0.454
NM_001964	Early growth response 1 (EGR1)	3.184	1.149	–-	– -
NM_022555	Major histocompatibility complex, class II, DR beta 3 (HLA-DRB3)	1.000	0.448	0.518	0.621
NM_002116	Major histocompatibility complex, class I, A (HLA-A)	0.584	0.322	0.336	– -
NM_002124	Major histocompatibility complex, class II, DR beta 1 (HLA-DRB1)	–-	0.661	0.485	0.567
NM_002817	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 13 (PSMD13)	0.445	0.122	0.333	0.402
NM_004159	Proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional protease 7) (PSMB8)	0.573	0.150	0.304	0.254
NM_000616	CD4 antigen (p55) (CD4)	0.855	0.560	0.317	0.831
NM_015953	eNOS interacting protein (NOSIP),	0.857	0.517	0.450	0.463
NM_020998	Macrophage stimulating 1 (hepatocyte growth factor-like) (MST1)	0.690	0.562	0.353	– -
NM_005944	Antigen identified by monoclonal antibody MRC OX-2 (MOX2)	1.514	1.027	2.042	– -
NM_021138	TNF receptor-associated factor 2 (TRAF2)	0.817	0.773	0.713	0.465
NM_004001	Fc fragment of IgG, low affinity IIb, receptor for (CD32) (FCGR2B)	0.581	0.569	0.467	– -
NM_001100	Actin, alpha 1, skeletal muscle (ACTA1)	0.368	0.266	0.139	– -
NM_001615	Actin, gamma 2, smooth muscle, enteric (ACTG2)	0.419	0.229	0.191	- –
NM_005718	Actin related protein 2/3 complex, subunit 4 (20 ku) (ARPC4)	0.461	0.362	0.370	0.611
NM_020040	Tubulin, beta polypeptide 4, member Q (TUBB4Q)	0.462	0.345	0.335	0.573
NM_006009	Tubulin, alpha 3 (TUBA3)	1.062	0.710	0.484	0.885
NM_001665	*ras* homolog gene family, member G (*rho G*) (ARHG)	0.590	0.445	0.524	0.590
AB055890	*c-lbc* mRNA for guanine nucleotide exchange factor Lbc	1.065	0.315	0.701	0.433
NM_006400	Dynactin 2 (p50) (DCTN2)	0.865	0.361	0.334	– -
NM_005428	*vav* 1 oncogene (VAV1)	0.510	0.421	0.387	– -
NM_005993	Tubulin-specific chaperone d (TBCD)	0.770	0.485	0.524	– -
NM_001747	Capping protein (actin filament), gelsolin-like (CAPG)	0.512	0.213	0.191	0.323

Table 2 Ratio of expression values obtained in U937 cells cocultured with *H pylori* NCTC11638 versus those cocultured with 11638-Δ vacA as assessed by microarray analysis and Northern blot

GeneBank ID	Gene product	Microarray ratio				Northern blot ratio			
		2 h	6 h	10 h	24 h	2 h	6 h	10 h	24 h
NM_002817	PSMD13	0.445	0.122	0.333	0.402	0.07	0.71	0.25	0.33
NM_004517	ILK	0.717	0.218	0.245	0.454	0.57	0.19	0.43	0.15

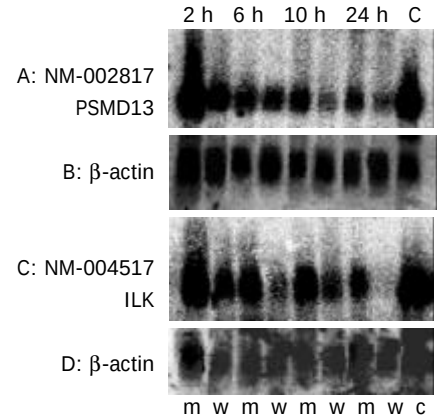

Figure 1 Northern blot analysis result. RNA from U937 cells cocultured with *H pylori* NCTC11638 (wild type, w) or 11638-Δ vacA (mutant type, m) or RNA from normal cells (c) were separated by agarose electrophoresis, transferred onto nylon membranes, and probed with the human cDNAs as follows: NM_002817 (PSMD13), and NM_004517 (ILK). Control blots with human β-actin were provided.

DISCUSSION

Helicobacter pylori has found its own way to thrive within host cells, despite the presence of a well-functioning immune system. In the present study, differential expression of macrophage upon stimulation of VacA gives clear evidence that this vacuolating cytotoxin has evolved with various tricks to escape from the innate and adaptive immunity of the host.

Recognition of pathogen-infected host cells by effector T lymphocytes requires intracellular processing of microbial antigens and their presentation on the surface of the antigen-presenting cells (APCs) in association with MHC molecules. To avoid immune recognition, many kinds of microorganisms have evolved mechanisms which interfere with antigen presentation pathways. These mechanisms may be pivotal, especially for those pathogens causing persistent infections. The present differential expressions provide clear evidence that VacA downregulates the expression of MHC class Iand class II molecules in macrophage. Thus, specific T lymphocytes may not be activated, resulting in the evasion of *H pylori* from the host' s immune response and colonization of the bacteria in gastric mucosa persistently, remaining "invisible" to both $CD8^+$ and $CD4^+$ T cells. Interference with the class II antigen presentation pathway by Listeria monocytogenes has been shown to result in a reduced proliferation of $CD4^+$ T lymphocytes in response to heterologous antigens[6]. Interactions between MHC class I and human immunodeficiency virus (HIV) resulted in down-regulation of MHC-Isurface expression, contributing to pathogenesis by suppressing the host' s immune response[7]. The role of MHC class I and class II-restricted functions in *H pylori* infection and immunity upon oral immunization has also been examined *in vivo*. It was found that experimental challenge with *H pylori* resulted in significantly greater colonization in MHC class I and class II mutant mice than in wild-type mice[8]. MHC class II-deficient mice were unable to respond to oral antigenic stimulation and remained persistently infected with *H pylori*[8].

Nitric oxide (NO) is an important mediator of biological processes including inflammatory response. It is synthesized from L-arginine by a family of nitric oxide synthases (NOS), in which, endothelial NOS (eNOS) is a constitutively expressed isoform present in vascular endothelial cells, cardiac myocytes, and blood platelets[9]. eNOS interacting protein (NOSIP) is a 34 ku protein specifically binds to the carboxylterminal region of the human eNOS oxygenase domain, overexpression of which in eNOS-expressing cells has been demonstrated to inhibit NO sythase activity[10]. Thus, down-regulation of this protein upon stimulation of VacA may relieve such inhibition, leading to more NO production. The original concept that the small quantities of NO generated in a pulsatile fashion by constitutive eNOS mainly fulfil regulatory functions required for normal homeostatic function of the vasculature, while the high amount of NO produced by inducible NOS (iNOS) exerts antimicrobial and cytotoxic effects in the immune system, has recently been modified[11]. The expression of eNOS is not restricted to endothelial cells, as it has been found to be present in monocytes/macrophages and in B and T lymphocytes[12]. eNOS can also assume typical immunological functions previously assigned to iNOS, such as the induction of apoptotic cell death and the control of viruses[11]. NO endows macrophages with cytostatic or cytotoxic activity against microbes and tumor cells[13]. Nevertheless, more and more evidences have demonstrated a role for NO in the induction of immunosuppression by inhibiting T-cell proliferation during G1/S transition[14-16]. In mouse models of T-cell-mediated autoimmunity, such as myelin antigen-induced EAE, the disease was exacerbated by genetic deletion of iNOS, indicating that NO suppressed T-cell-mediated immunity *in vivo*[17]. In addition, NO induces apoptosis, which, the ubiquitin/proteasome and NF-κB pathway have been determined to be involved in[18].

In eukaryotic cells, degradation of many proteins involves their initial modification by conjugation of ubiquitin (Ub). Ubiquinated proteins are rapidly degraded by the 26S proteasome[19]. NO can inhibit the activities of the 20S and 26S proteasomes, providing a likely mechanism for the accumulation of NO-induced pro-apoptotic proteins p53 and Bax, the substrates of the ubiquitin/proteasome system[18]. Apart from the indirect effect via NO functioning, the mRNA expression of proteasomes shows a significant downregulation as the result of being directly stimulated by VacA. Ub/proteasome pathway can catalyze the proteolytic processing of inactive 105 ku NF-κB precursor into 50 ku subunit. p50 is then maintained in the cytosol conjugated with the p65 subunit in an inactive complex bound to IκB. In addition, Ub/proteasome is involved in proteolytic digestion of IκB, which is required for NF-κB activation[19]. Therefore, decreased proteasome activity should inhibit the proteolysis of NF-κB precursor and inhibit IκB degradation, thus blocking NF-κB activation. Another involved protein, integrin-linked kinase (ILK), which has been determined to upregulate NF-κB activity[20], also shows decreased expression in this study. It might be the result of VacA induction, and moreover, ILK mRNA expression was found to be downregulated by NO[21], corresponding to the above speculation that more NO may be produced due to downregulation of NOSIP. NF-κB is known to be important

to cell survival. Fibroblasts and macrophages from Rel A (p65 subunit of NF-κB) (-/-) mice were sensitive to TNF-α-induced cell death, and reintroduction of Rel A enhanced cell survival[22]. Activation of NF-κB is required for inflammatory cytokine release by macrophages during infection[23]. Consequently, inhibition of NF-κB activation may be responsible for decreased cytokine release from macrophages and the resulting immunosuppression. Additionally, because ILK is an apoptosis suppressor[24], decreased production of this protein in macrophage may accelerate apoptosis of the cell that plays important roles in innate host defense and antigen presentation, leading to the evasion from host immunity against *H pylori*.

The capability of degrading proteins by the proteasome accounts for another important function to generate peptides presented on MHC-class I molecules to circulating lymphocytes. The presentation of these peptides enables the immune system to screen for and destroy cells expressing unusual polypeptides[19]. Selective preteasome inhibitors were determined to prevent MHC-class I presentation of the antigenic peptide[25]. Moreover, LMP2 and LMP7, two subunits of the proteasome, were found to be encoded in the major histocompatibility complex (MHC)[26-28]. The experiment using specific antibodies against LMP2 and LMP7 showed that they were co-expressed with MHC-class I molecules[29]. The levels of MHC-class I expression were shown to coincide perfectly with the LMP levels in different tissues, corresponding to the result in the present study, which shows simultaneous downregulation of MHC-class I and LMP7.

MSP, also known as HGF-like protein, is a serum protein belonging to the plasminogen-related growth factor family. It was originally discovered by Leonard and Skeel as a serum protein that stimulates shape change, movement, chemotaxis and phagocytosis of mouse peritoneal resident macrophages[30,31]. The other important effect of MSP on macrophages was to inhibit endotoxin- or cytokine-stimulated NO production[32]. Thereby, the decreased expression of MSP may contribute to deactivating macrophage and producing more NO, which, as described above, functions as an immunosuppressor.

The early growth response 1 (EGR1), a zinc-finger transcription factor that was shown to be significantly upregulated by 2 h postinfection in the present study, has been determined to induce downregulation of copper-zinc superoxide dismutase and manganese superoxide dismutase and stimulate the generation of reactive oxygen species (ROS) via the NADH/NADPH-oxidase system[33]. In addition to NO, ROS produced by NADPH oxidase also have the ability to inhibit the proliferation of lymphocytes by a mechanism that suppressor macrophages impair the proliferative response of T lymphocytes to antigens or mitogens[11]. Otsuji *et al*.[34] demonstrated that the oxidative stress from tumor-derived macrophages mediated the decrease of CD3ζ chain within T cells, which suppressed the antigen-specific T-cell responses. Pre-treatment of CTL or NK cells with nontoxic concentrations of H_2O_2 severely reduced their cytotoxic activity, leading to the speculation that macrophage-derived reactive oxygen metabolites contribute directly to alterations in signal transducing molecules of T cells and NK cells and to the mechanism of immunosuppression[35]. Furthermore, the defective expression of CD3ζ on lymphocytes has been related to some kind of carcinoma, including gastric adenocarcinoma[36,37].

From Table 1, we may find up-regulation of MRC OX-2 (the antigen identified by monoclonal antibody, MOX2), a broadly expressed membrane glycoprotein, which has been shown to be important for regulation of the macrophage lineage. In the OX-2-deficient spleens, the number of macrophages was nearly twice the number of those in normal spleens[38], implying a role of OX-2 in suppressing the activation of macrophage. The immunosuppression effects of OX-2 could be further determined by many other studies. For example, several studies reported that increased expression of OX-2 in mice receiving renal allografts was associated with immunosuppression leading to increased graft survival, along with the polarization of cytokine production to type 2 cytokines in lymphocytes harvested from the transplanted animals[39-41]. Furthermore, infusion of a mAb to OX-2 blocks both the increased graft survival and the altered cytokine production[42]. All these data make clear that upregulation of OX-2 does favor to the immune evasion of *H pylori*.

Table 1 also demonstrates significant downregulation of many cytoskeleton-related gene expression. For example, RhoG is a member of the Rho family of GTPases, which signals to actin assembly during phagocytosis[43]. Vav1 serves as a guanine nucleotide exchange factor (GEF) for Rho proteins, and establishes an essential and direct link between receptors with intrinsic or associated tyrosine kinase activity and the mitogenic and cytoskeletal pathways regulated by Rho proteins[44]. Cytoskeleton has been known to be an important structural basis for phagocytosis since cytochalasin B, a toxin that blocks actin polymerization, was shown to inhibit uptake of IgG-coated erythrocytes by mouse macrophages[45]. Phagocytosis is a process by which macrophages and leukocytes could ingest microbial pathogens to accomplish two essential immune functions, *i.e.*, to initiate the microbial death pathway, and to direct antigens to both MHC I and MHC II compartments. That is to say, phagocytosis serves not only as an innate immune effector but as a bridge between the innate and acquired immune responses[1]. Thus, to destruct cytoskeleton may be another trick of VacA to evade host immune response.

In conclusion, we have shown that *H pylori* VacA induces the alteration of a series of genes related to immune evasion in macrophage, which ultimately establishes a state of host-microbial equilibrium. Some of these genes are for the first time made an association with VacA stimulation. Further investigations of the previously uncharacterized genes should be made to help us see through the underlying mechanisms utilized by *H pylori* to escape host immunity.

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