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Alteration of gene expression in microvascular endothelial cells after exposure to foot-and-mouth disease virus 146S antigen

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Abstract: To investigate the global response of microvascular endothelial cells (MVECs) to foot-and-mouth (FMDV) disease vaccine (FMDV) antigen and to explore the role of MVECs in FMD vaccine immunity, the gene expression alterations of rat myocardium microvascular endothelial cells (RMMVECs) after stimulation with FMD virus (FMDV) 146S antigen were assayed using cDNA microarrays. We observed that 18 genes and 67 genes in RMMVECs were respectively altered by FMDV 146S antigen at 6 h and 24 h, respectively. The former were all up-regulated genes, and the latter included 60 up-regulated genes and 7 down-regulated genes. Among the differentially expressed genes, many were associated to immune response, such as the genes encoding chemokines, interleukins, complement components, and adhesion molecules. In particular, the chemokine genes had the greatest numbers and significant expression ratios. A part of the altered genes with immune function was validated by real-time reverse transcription-PCR. The influence of FMDV 146S antigen on the secretion of monocyte chemoattractant protein-1, interleukin-6 (IL-6), and IL-1 by RMMVECs was confirmed by enzyme-linked immunosorbent assay, and the expression of vascular cell adhesion molecule-1 was identified by immunocytochemistry. The results indicated that the FMDV 146S antigen induces the changes of immune-associated genes in RMMVECs, which suggests that MVECs play an important role in modulating how the innate immune system responds to FMD vaccine.

Key words: Microvascular endothelial cells, foot-and-mouth disease virus, 146S, microarray

Introduction

Foot-and-mouth disease (FMD) is a severe and highly contagious disease of cloven-hoofed animals or humans, which is widely believed to be the most economically devastating zoonosis in the world. Vaccination is a routine practice for FMD control in many countries, and the current FMD vaccines are inactivated whole-virus preparations, whose essential immunogenic component is FMD virus (FMDV) 146S particles (1). An important fact is that vaccination can protect animals, but the level of immune protection can wane such that animals need revaccinating after 6 months (2). The multiple serotypes of FMDV with no cross-protection between serotypes and the antigenic variability make the control of FMD by vaccination difficult. Although a great deal of knowledge about FMD has been obtained in the last 100 years, the goal

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of life-long immunity to FMDV antigens is still far from achievement. Most of the studies have focused on antigen sites of FMDV and immune responses to FMDV antigens of traditional immune cell, such as T or B cells. However, many new viewpoints and contents have already been added to the immunology field. For example, microvascular endothelial cells (MVECs) have been increasingly documented to be important immune cells (3).

The microvascular endothelium is a highly specialized cellular system lining capillary vessels everywhere in bodies and occupying strategic anatomical positions. MVECs play pivotal roles in physiological processes such as vascular homeostasis, nutrient delivery, and immune trafficking (4), and in pathological processes such as inflammation (5). The immune functions of MVECs particularly attract more and more attention for their critical intervention in leukocyte recruitment, lymphocyte homing, and leukocyte activation (6). Hemorrhage and inflammation in gut mucosal tissues and cardiac tissues, which are the typical pathological changes in FMDV-infected animals (7), and the expression of FMDV receptors in MVECs (8,9) suggest that there is a close relation between FMDV and MVECs. However, what changes of gene expression of MVECs the FMDV antigen could cause has not been reported.

This study aimed to investigate the global response of MVECs to FMD vaccine antigen and explore the role of MVECs in the immunity of FMD vaccines. Microarray technology is a high throughput method that provides a useful tool for identifying the repertoire change of mRNA levels in cells in responses to a given stimulus. Considering the availability of the rat microarray and the susceptivity of rat to FMDV (10), we used cDNA microarray to analyze gene expression changes in cultured rat myocardium microvascular endothelial cells (RMMVECs) after exposure to FMDV 146S antigen, and we confirmed part of the gene expression changes by real-time reverse transcription-PCR (RT-PCR), enzyme-linked immunosorbent assay (ELISA), and immunocytochemical staining.

Materials and methods

FMDV 146S antigen

BHK-21 cell cultures infected with the FMDV O/China99 strain were kindly gifted by the Lanzhou Veterinary Research Institute of the Chinese Academy of Agricultural Science (Lanzhou, China). The cell cultures with complete pathological change were collected and inactivated with binary ethylene imine (BEI). The FMDV 146S antigen was purified with PEG-6000 as described previously (11). The purified FMDV 146S particles were deeply stained with 1% uranyl acetate and observed by transmission electron microscope (TEM). The FMDV 146S particles were homogeneous, round, black-staining bodies by TEM, whose edges were smooth and whose diameters were about 25 nm (data not shown). After being quantitated by Bradford assay (12), the FMDV 146S antigen was stored at -20 °C.

Cell culture and stimulation with FMDV 146S antigen

The culture of RMMVECs was prepared as described by Nishida et al. (13) with modification. Briefly, Sprague-Dawley rats (15 days old) were anesthetized with pentobarbital sodium (30 mg/kg) by intraperitoneal injection and then heparinized with heparin sodium (1000 U/kg, i.m.). Hearts were rapidly excised and washed in ice-cold Hank's balanced salt solution (HBSS, Sigma, USA). After removal of connective tissue, the atria, right ventricle, and all valvular tissues, the left ventricular tissue was opened and immersed in 70% ethanol for 30 s. The epicardial and endocardial surfaces were removed, and the remaining left ventricle tissue was minced and digested with 0.2% collagenase II (Gibco, USA) and with 0.02% trypsin (Sigma, USA) respectively for 20 min at 37 °C. The digested solution was filtered through a 100-μm mesh filter, and the filtrate was centrifuged at 1000 rpm for 10 min. Cells were resuspended in DMEM (Gibco, USA) supplemented with 20% fetal bovine serum (FBS, PAA, Austria), 2 mmol/L of L-glutamine, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin at a concentration of 1×10^5 cells/mL, and plated in 12-well culture plates (Corning, USA) with 1 mL per well. After being cultured in a humidified atmosphere with 5% CO_2 at 37 °C for 2 h, attached cells were washed twice with DMEM to allow differential adhesion and cultured

in DMEM with 20% FBS, which was changed every 3 days. The confluent cells showed a cobblestone-like or polygonal appearance, and they were subcultured and identified by the positive immunofluorescent staining for CD31 (data not shown). All experiments were performed using second- to third-passage RMMVECs.

For microarray and quantitative RT-PCR analysis, cells were plated in 6-well plates at a density of 3×10^5 cells per well and cultured for 48 h, and the medium was replaced with maintenance medium (DMEM containing 2% FBS) overnight. Cells were then incubated in maintenance medium with or without 20 μg/mL of the FMDV 146S antigen. After 6 h and 24 h of stimulation, the medium was removed and washed twice with PBS, and the total RNA was isolated and processed as below. For ELISA, cells were seeded in 48-well plates for 48 h, and the subconfluent cells were incubated overnight in maintenance medium. Maintenance medium respectively containing 0.8, 4.0, and 20.0 μg/mL of the FMDV 146S antigen was used, and the control cells were incubated in maintenance medium without FMDV 146S antigen. The supernatants in triplicate were collected after 3, 6, 12, 24, 48, and 72 h incubation, respectively. For immunocytochemical staining, cells were subcultured on chamber slips in a 24-well plate. After incubation with or without 20.0 μg/mL of 146S for 24 h, the cells on slips were rinsed twice with PBS and fixed in 95% ethanol for 15 min at -20 °C.

DNA microarray and data analysis

According to the methods previously described (14), microarray analysis was carried out using 27K rat genome-wide oligonucleotide microarrays at CapitalBio Corporation (Beijing, China). Briefly, a *Rattus norvegicus* genome oligonucleotide set (version 3.0.5), consisting of 26,962 5' amino acid-modified 70-mer probes representing 22,012 genes and 27,044 gene transcripts, was purchased (Operon, USA) and printed on silanized glass slides using a SmartArray™ microarrayer (CapitalBio Corp.). Total cellular RNA was extracted with TRIzol (Invitrogen, USA) from FDMV 146S-treated and control RMMVECs. After being purified with NucleoSpin® RNA clean-up (Macherey-Nagel, Germany) and validated qualitatively with a denatured agarose gel containing formaldehyde, 5 μg of DNase-treated total RNA was prepared, and fluorescent dye (Cy5- and Cy3-dCTP)-labeled cDNA, produced through Eberwine's linear RNA amplification method (15) and subsequent enzymatic reaction, was then hybridized to an array at 42 °C overnight. Finally, arrays were scanned with a dual channel confocal LuxScan™ scanner (CapitalBio Corp.), and the obtained images were analyzed with LuxScan 3.0 (CapitalBio Corp.). A space- and intensity-dependent normalization based on a Lowess program was employed (16). Genes with the signal intensity (Cy3 or Cy5) > 800 were regarded as the expressed ones. For each test and control sample, 2 hybridizations were performed by using a reversal fluorescent strategy. Only genes whose alteration tendency kept consistent in both microarrays and whose mean expression ratios averaged above 2-fold were selected as differentially expressed genes.

Quantitative RT-PCR

To validate the microarray results, quantitative reverse transcription-PCR (qRT-PCR) was performed to analyze representative genes using the LightCycler FastStart DNA Master SYBR Green I PCR kit (Roche, Switzerland) in a Roche LightCycler 1.2 real-time PCR system according to the operator's manual. A total of 5 μg of RNAs extracted from each sample was digested with RNase-free DNase I (Promega, USA) and reverse transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen, USA). The reaction was taken in a 20 μL final volume following the manufacturer's instructions. The reaction mix contained 4 μL of first strand buffer (5×), 1 μL of dNTPs mix (2.5 mM each), 2 μL of oligo-dT15 primer (1 μg, Promega), 1 μL of RNase inhibitor (40 U/μL, Promega), 2 μL of DTT (0.1 M, Invitrogen), 1.5 μL of superscript II reverse transcriptase (200 U/μL, Invitrogen), 2.5 μg of template RNA, and RNase-free water. The real-time RT-PCR reactions in triplicate were carried out in a 20 μL final volume with the LightCycler FastStart DNA Master SYBR Green I Kit (Roche, Switzerland) using 1 μL of cDNA solution, and using sequence-specific oligonucleotide primers designed to yield approximately 200-bp sequences. All primers (Table 1) used in this study were designed with Primer Premier 5.0 software (Premier, Canada) and synthesized by Invitrogen. In detail, the master-mix for each PCR run was prepared as

Table 1. Primers and amplification size for RT-PCR analysis.

Gene name	Reference sequence	Primer type	Primer sequence (5'-3')	Amplicon size
Pla2g2a	NM_031598	Forward	TACCACCATCCCATCCAAGA	276
		Reverse	GTGCCACATCCACGTTTCTC	
Cxcl2	NM_053647	Forward	TCAATGCCTGACGACCCTAC	195
		Reverse	CAGTTAGCCTTGCCTTTGTTC	
Il6	NM_012589	Forward	GGATACCACCCACAACAGACC	254
		Reverse	AACGGAACTCCAGAAGACCAG	
Ccl2	NM_031530	Forward	GTCGGCTGGAGAACTACAAGA	203
		Reverse	TGCTGAAGTCCTTAGGGTTGA	
Il1a	NM_017019	Forward	AGGTGGTCAGTTAGATGCAGTG	260
		Reverse	TTCTTCCCGTCTTTAGATGGTT	
Vcam1	NM_012889	Forward	ACAGCTAAAGAACGGGGAACT	201
		Reverse	TCTTTGACGCTCTTAGATGGG	
Tgfb3	NM_013174	Forward	TCAGTTTACCAAGCCAAAGTCC	284
		Reverse	GAAGGTGTCTAGCCAAATGTCC	
GAPDH		Forward	TGCTGAGTATGTCGTGGAG	288
		Reverse	GTCTTCTGAGTGGCAGTGAT	

follows: 13.8 μL of water, 1.6 μL of $MgCl_2$ (3 mM), 0.8 μL of each primer (10 mM), 2 μL of FastStart DNA Master SYBR Green I, and 1 μL of reverse transcripts. The real-time RT-PCR cycles were as follows: initiation with a 10-min denaturation at 95 °C, followed by 40 cycles of amplification with 15 s of denaturation at 95 °C, 5 s of annealing according to the melting temperatures of each pair of primers, 15 s of extension at 72 °C, and reading of the plate for fluorescence data collection at 76 °C. To check the specificity of the amplified product, melting curve analyses were carried out at 75-95 °C, and 1.5% agarose gel electrophoresis was conducted. A reaction containing no reverse transcribed total RNA samples was processed to demonstrate the absence of genomic DNA contamination. The comparative threshold cycle (CT) method was used for the calculation of amplification fold. The expression level of each gene was normalized by dividing by the expression level of the "housekeeping" gene transcript.

Cytokine assay by ELISA

Chemokines and interleukins, which were differentially expressed in FMDV 146S antigen-stimulated RMMVECs, are important factors regulating the migration and activation of lymphocytes. To further characterize the translational level changes of interest genes, concentrations of monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), and IL-1β in cell culture supernatants were determined using commercially available ELISA kits (MCP-1 kit purchased from BioSource, USA; IL-6 kit and IL-1β kit purchased from Boster, China) according to manufacturer's instructions. Experiments for ELISA measurements were done in triplicate wells from each preparation. Results were expressed as mean ± standard errors of the means.

All statistical analyses of variance used Student's t-test. Differences in the data were considered significant or extremely significant at P values of less than 0.05 or 0.01.

Immunocytochemistry

Vascular cell adhesion molecule-1 (VCAM-1) was another important factor in immune response and inflammation whose gene expression was changed in FMDV 146S antigen-stimulated RMMVECs. Immunocytochemical staining with the streptavidin-peroxidase (SP) method was done to analyze the expression of VCAM-1 in RMMVECs after exposure to the FMDV 146S antigen. Briefly, fixed cells as described above were blocked respectively with 3% H_2O_2 for 10 min and 10% normal goat serum for

20 min at room temperature (RT), the slides were successively incubated with rabbit anti-rat VCAM-1 antibody (Zsbio, China) at a 1:100 dilution overnight at 4 °C, and goat anti-rabbit IgG was conjugated with biotin for 60 min at RT. For a negative control, the anti-rat VCAM-1 antibody was replaced with PBS. Following a 30-min incubation with streptavidin-peroxidase, the slides were stained with DAB solution for 5 min at RT. They were washed 3 times with 0.01 M PBS for 3 min each before each reagent, except the primary antibody, was added. Finally, the slips were mounted with gelatin and photomicrographs were taken by light microscopy.

Results

Expression profile of RMMVECs stimulated with FMDV 146S antigen

Rat 27K genome microarrays were used to investigate the responses of RMMVECs to FMDV 146S antigen. This cDNA-based microarray platform covers approximately 27,000 gene transcripts. The microarray analysis revealed that 18 genes were up-regulated in FMDV 146S antigen-stimulated RMMVECs at 6 h (Table 2), and 60 genes were up-regulated and 7 genes were down-regulated at 24 h (Table 3). The expression alteration of

Table 2. Differentially expressed genes in RMMVECs stimulated with FDMV 146S antigen for 6 h.[a]

Category and gene name	Gene ID number	Description	Relative mRNA expression ratio[b]
Colony-stimulating factors			
Csf3	ENSRNOG00000008525	Granulocyte colony-stimulating factor 3	3.76
Csf2	ENSRNOG00000026805	Granulocyte-macrophage colony-stimulating factor	2.77
Chemokines			
Cxcl1	ENSRNOG00000002802	Growth regulated protein precursor (CXCL1)	3.49
LOC60665	ENSRNOG00000002843	CXC chemokine LIX (CXCL5)	2.99
Ccl20	ENSRNOG00000015992	Macrophage inflammatory protein-3-alpha (CCL20)	2.84
Cxcl2	ENSRNOG00000002792	Macrophage inflammatory protein-2 (CXCL2)	2.71
Rn30026248	ENSRNOG00000028043	Macrophage inflammatory protein-2-beta (CXCL3)	2.56
Cx3cl1	ENSRNOG00000016326	Chemokine (C-X3-C motif) ligand 1	2.01
Interleukins			
Il19	ENSRNOG00000025571	Interleukin-19	2.30
Il6	ENSRNOG00000010278	Interleukin-6	2.25
Others			
Angptl4	ENSRNOG00000007545	Angiopoietin-like protein 4	2.44
Pla2g2a	ENSRNOG00000016945	Phospholipase A2	2.35
Ptx3	ENSRNOG00000012280	Pentaxin-related gene	2.31
RGD1306776	ENSRNOG00000009131	Similar to hypothetical protein mgc41320	2.30
Cyp7b1	ENSRNOG00000009730	Cytochrome p450 7b1	2.13
Rn30012520	ENSRNOG00000013621	Rho family GTPase 1	2.12
Ppap2b	ENSRNOG00000008116	ER transmembrane protein Dri 42	2.04
Lcn2	ENSRNOG00000013973	Lipocalin 2	2.04

[a]Total RNA was extracted from RMMVECs stimulated with 20 μg/mL of FMDV 146S antigen for 6 h and analyzed using microarray as described in the Materials and Methods section. Control RMMVECs were incubated in culture medium without FMDV 146S antigen. Genes whose expression in FMDV 146S antigen-stimulated RMMVECs was at least 2-fold higher than that of control RMMVECs were listed.

[b]Ratio of mRNA expression in FMDV 146S antigen-stimulated RMMVECs to that in control RMMVECs.

Table 3. Differentially expressed genes in RMMVECs stimulated with FDMV 146S antigen for 24 h.[a]

Category and gene name	Gene ID number	Description	Relative mRNA expression ratio[b]
Up-regulated genes			
Chemokines			
Rn30026248	ENSRNOG00000028043	Macrophage inflammatory protein-2-beta	4.65
LOC60665	ENSRNOG00000002843	CXC chemokine LIX	4.58
Ccl3	ENSRNOG00000011205	Macrophage inflammatory protein-1-alpha	3.09
Ccl20	ENSRNOG00000015992	Macrophage inflammatory protein-3-alpha	2.78
Cxcl1	ENSRNOG00000002802	Growth-regulated oncogene alpha	2.71
Ccl12_predicted	ENSRNOG00000000239	Monocyte chemoattractant protein-3	2.51
Cxcl2	ENSRNOG00000002792	Macrophage inflammatory protein-2	2.32
Ccl2	ENSRNOG00000007159	Monocyte chemoattractant protein-1	2.24
Rn30000167	ENSRNOG00000000239	Monocyte chemoattractant protein-3	2.16
Ccl4	ENSRNOG00000011406	Macrophage inflammatory protein-1-beta	2.08
Interleukins			
Il1b	ENSRNOG00000004649	Interleukin-1 beta	3.62
Il6	ENSRNOG00000010278	Interleukin-6	3.22
Il1a	ENSRNOG00000004575	Interleukin-1 alpha	2.54
Complement component			
C3	ENSRNOG00000019505	Complement component 3	2.61
C6	ENSRNOG00000024115	Complement component 6	2.45
C1s	ENSRNOG00000011971	Complement component 1	2.04
Adhesion molecule			
Vcam1	ENSRNOG00000014333	Vascular cell adhesion molecule 1	3.02
Metallothionein			
Mt1a	ENSRNOG00000027838	Metallothionein	4.53
Mt1a	ENSRNOG00000018756	Metallothionein-i	4.35
Rn30017416	ENSRNOG00000018926	Metallothionein-ii	3.68
Mt1a	ENSRNOG00000018756	Metallothionein-i	3.46
Mmp9	ENSRNOG00000017539	Matrix metalloproteinase-9	2.44
Enzymes			
Pla2g2a	ENSRNOG00000016945	Phospholipase A2	11.35
Enpp2	ENSRNOG00000004089	Phosphodiesterase 2	4.91
Npy	ENSRNOG00000009768	Neuropeptide y precursor	4.78
Sod2	ENSRNOG00000019048	Superoxide dismutase	3.93
Slpi	ENSRNOG00000013880	Secretory leukocyte protease inhibitor	3.19
Jak2	ENSRNOG00000015547	Tyrosine-protein kinase jak2	2.86
Jak2	ENSRNOG00000015547	Tyrosine-protein kinase jak2	2.86
Rn30014253	ENSRNOG00000015547	Tyrosine-protein kinase jak2	2.77
Irak3	ENSRNOG00000004226	Interleukin-1 receptor-associated kinase 3	2.66
Hpx	ENSRNOG00000018257	Hemopexin	2.52
Ptges	ENSRNOG00000006320	Prostaglandin E synthase	2.45
LOC171516	ENSRNOG00000017531	20 alpha-hydroxysteroid dehydrogenase	2.22
Xdh	ENSRNOG00000007081	Xanthine oxidoreductase	2.08
Adamts5	ENSRNOG00000001606	Aggrecanase-2	2.08
Hsd11b1	ENSRNOG00000005861	Hydroxysteroid 11-beta dehydrogenase 1	2.05
Serping1	ENSRNOG00000007457	Serine (or cysteine) proteinase inhibitor	2.01

Table 3. (Continued).

Category and gene name	Gene ID number	Description	Relative mRNA expression ratio[b]
Others			
Lcn2	ENSRNOG00000013973	Lipocalin 2	5.63
Rn30023225	ENSRNOG00000024905	Similar to hypothetical protein	5.14
Sfrp2	ENSRNOG00000009465	Putative secreted frizzled-related protein	4.84
Rn30020678	ENSRNOG00000022362	-[c]	4.52
Bf	ENSRNOG00000000419	B-factor, properdin	4.39
Hp	ENSRNOG00000014964	Haptoglobin precursor	4.25
Igfbp3	ENSRNOG00000008645	Insulin-like growth factor binding protein 3	3.15
Lnx1	ENSRNOG00000002272	Ligand of numb-protein X 1	2.84
Chi3l1	ENSRNOG00000003312	Glycoprotein-39	2.78
Ptx3	ENSRNOG00000012280	-	2.70
Tlr2	ENSRNOG00000009822	Toll-like receptor 2	2.58
Rn30009092	ENSRNOG00000009919	Similar to immune-responsive gene 1	2.54
Rn30018064	ENSRNOG00000019622	-	2.46
Rn30000470	ENSRNOG00000000569	-	2.36
Rn30018372	ENSRNOG00000019951	EGF-like module	2.24
Rn30024903	ENSRNOG00000026647	-	2.18
Rn30019109	ENSRNOG00000020733	Cathelicidin	2.17
RGD1309846	ENSRNOG00000026923	Similar to RIKEN cDNA 2010012F05	2.11
Bcl2a1	ENSRNOG00000013001	-	2.09
Map3k8	ENSRNOG00000016378	Tumor progression locus 2	2.08
RGD1309846	ENSRNOG00000017245	Similar to RIKEN cDNA 2010012F05	2.05
RSB-11-77	ENSRNOG00000016210	Rsb-11-77 protein	2.01
Down-regulated genes			
Ankrd1	ENSRNOG00000018598	Cardiac ankyrin repeat protein	0.47
Tpm1	ENSRNOG00000018184	Tropomyosin 1 alpha	0.45
Adarb1	ENSRNOG00000001227	Adenosine deaminase	0.45
Tgfb3	ENSRNOG00000009867	Transforming growth factor beta 3	0.44
Rn30022350	ENSRNOG00000024041	-	0.44
Fndc1	ENSRNOG00000018979	Fibronectin type III domain containing 1	0.42
Eln	ENSRNOG00000001469	Elastin	0.41

[a]Total RNA was extracted from RMMVECs stimulated with 20 μg/mL of FMDV 146S antigen for 24 h and analyzed using microarray as described in the Materials and Methods section. Control RMMVECs were incubated in culture medium without FMDV 146S antigen. Genes whose expression in FMDV 146S antigen-stimulated RMMVECs was at least 2-fold higher than that of control RMMVECs were listed.
[b]Ratio of mRNA expression in FMDV 146S antigen-stimulated RMMVECs to that in control RMMVECs.
[c]No description in the relative database.

genes encoding chemokines and interleukins was significantly induced by FMDV 146S for 6 h and 24 h. Colony-stimulating factor (CSF) gene expression was up-regulated only at 6 h, while the expression of genes encoding complement components, metallothioneins, and adhesion molecules (AM) was up-regulated at 24 h. Among the differentially expressed genes, many are involved in immune response, including the up-regulated genes of chemokines, interleukins, complement components, CSF, and AM, and the down-regulated transforming growth (TGF). Particularly, the chemokine genes had the greatest number and the most significantly up-regulated ratios. Interestingly, the subgroups of up-regulated chemokine genes were different in RMMVECs stimulated with FMDV 146S antigen for different times. At 6 h, 4 chemokine genes belonged to the CXC subgroup, including CXCL1, CXCL2, CXCL3, and CXCL5, and only the CCL20 gene belonged to the CC subgroup. The 4 genes of the CXC subgroup were still significantly altered at 24 h; however, more genes of the CC subgroup were up-regulated, which included CCL2, CCL3, CCL4, CCL7, CCL12, and CCL20.

Validation of cDNA microarray data using qRT-PCR

By real-time quantitative RT-PCR, the expression of 3 genes was determined at 6 h, and the expression of 7 genes was determined at 24 h. RNAs from samples and controls were extracted as described above. QRT-PCR analysis confirmed the alteration of these genes related with immune response or inflammatory reaction, whose expression ratios were in agreement with those by microarray analysis (Table 4). The specificity of quantitative RT-PCR products was identified with a melting curve showing a single peak and 1.5% native agarose gel electrophoresis showing a single specific band (data not shown).

Table 4. Quantitative RT-PCR confirmation of representative genes from microarray analysis.[a]

Gene name	Expression ratio			
	6 h		24 h	
	qRT-PCR[b]	Microarray[c]	qRT-PCR	Microarray
Pla2g2a	3.42	2.35	15.99	11.35
Cxcl2	3.04	2.71	5.33	4.65
Il6	2.21	2.25	1.66	3.22
Ccl2 (Mcp-1)	NT [d]	NT	1.48	2.24
Il1a	NT	NT	2.58	2.54
Mt1a	NT	NT	8.22	3.46
Vcam1	NT	NT	2.15	3.02
Tgfb3	NT	NT	0.35	0.44

[a]Total RNA was extracted from RMMVECs stimulated with 20 μg/mL of FMDV 146S antigen for 24 h and analyzed using qRT-PCR as described in the Materials and Methods section. Control RMMVECs were incubated in culture medium without FMDV 146S antigen.

[b]Expression ratios of qRT-PCR were calculated by the relative expression abundance compared with the internal control (GAPDH) in FMDV 146S antigen-stimulated RMMVECs to that in control RMMVECs.

[c]The ratios in microarray analysis.

[d]NT indicates that this gene was not tested at 6 h.

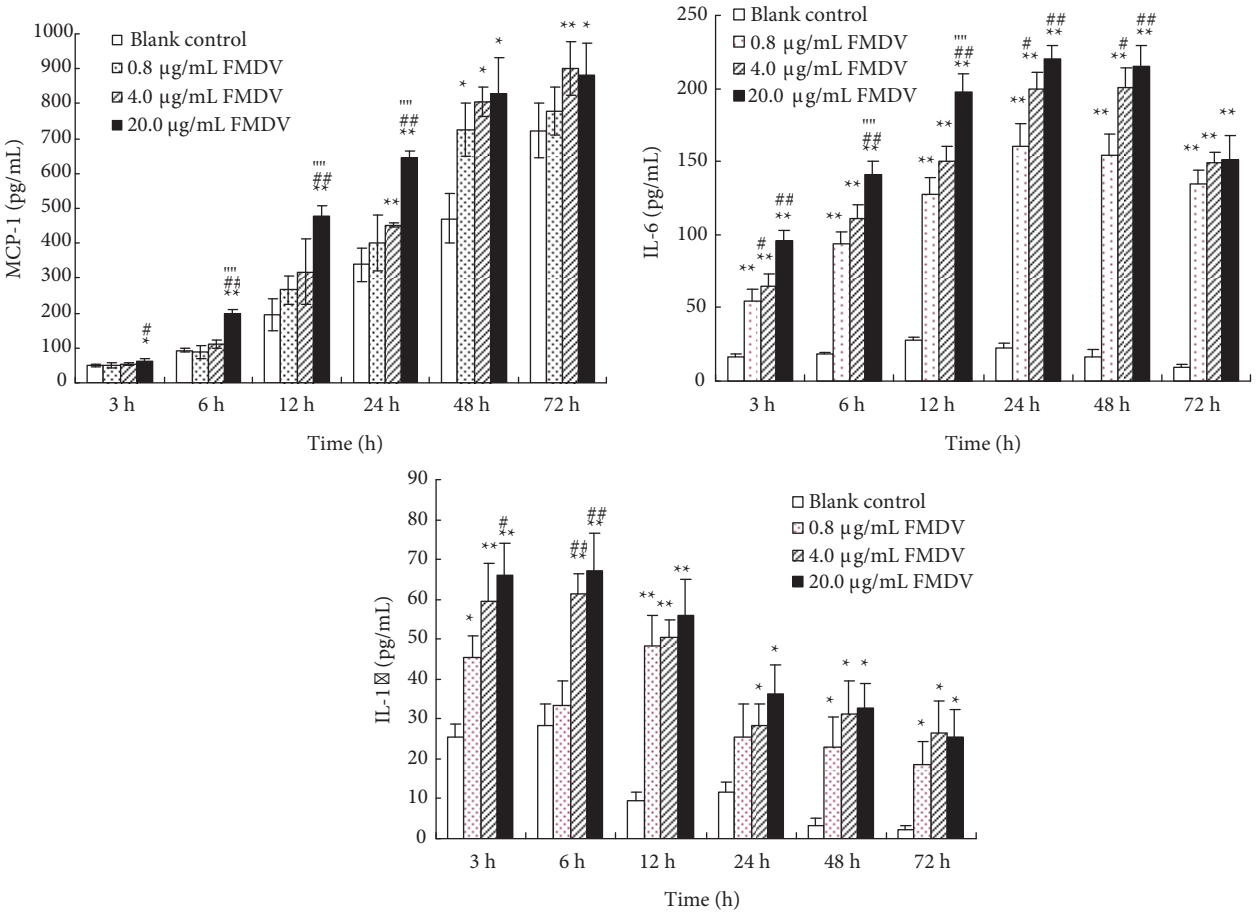

Figure 1. MCP-1 (CCL2), IL-6, and IL-1β production by RMMVECs in response to FMDV 146S antigen. Different doses of FMDV 146S antigen were respectively added to the RMMVEC culture medium and incubated respectively for 3, 6, 12, 24, 48, and 72 h. Supernatants were collected in triplicate for ELISA analysis. Blank control RMMVECs were incubated in culture media without FMDV 146S antigen. Representative findings are shown as mean and SDs from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ (compared with control), # $P < 0.05$, ## $P < 0.01$ (compared with 0.8 μg/mL FMDV 146S), ″ $P < 0.05$, ″″ $P < 0.01$ (compared with 4.0 μg/mL FMDV 146S).

Production of chemokines and interleukins in FMDV 146S antigen-stimulated RMMVECs

To further investigate the influence of the FMDV 146S antigen on the production of immune-associated factors by RMMVECs, we assayed the secretion of MCP-1 (CCL2), IL-6, and IL-1β by ELISA. As shown in Figure 1, RMMVECs constitutively produced MCP-1 (CCL2) at a low level; its production persistently increased with time from 3 h to 72 h, and the stimulation of FMDV 146S antigen significantly enhanced its production in a dose-dependent manner. The basal secretion of IL-6 and IL-1β was low and was also significantly elevated by FMDV 146S antigen, similar to its influence on MCP-1 (CCL2) production (Figure 1). However, the secreting dynamics of IL-6 and IL-1β were different from that of MCP-1 (CCL2), and the concentrations of IL-6 and IL-1β, respectively, reached their peaks at 24 h and 6 h.

Expression of VCAM-1 in RMMVECs after exposure to FMDV 146S antigen

VCAM-1 was the only classical adhesion molecule whose mRNA expression was altered in microarray analysis. By immunocytochemical staining, we assayed VCAM-1 protein expression in RMMVECs stimulated with the FMDV 146S antigen. The result showed that the expression of VCAM-1 was weakly positive in normal RMMVECs, and the light brown

staining was presented within the cytoplasm and cell membrane, but not within cell nuclei (Figure 2A). FMDV 146S-stimulated RMMVECs were more strongly positive for VCAM-1 (Figure 2B), with brownish-yellow marks within the cytoplasm and cell membrane, and the negative control slip had no positive staining (Figure 2C). In a word, the FMDV 146S antigen could up-regulate VCAM-1 expression both at the transcription level and at the translation level.

Discussion

MVECs are a highly heterogeneous population of cells with the ability to interact with and modulate the function of immune cells (3). To comprehensively understand the immunoreactive mechanism of FMD vaccine antigens, it was essential to investigate the response of MVECs to them and to explore the potential role of MVECs. The intact 146S particles are the major immunogenic component of conventional FMD vaccines, whose degradation greatly reduces the potency of the vaccines (1), and whose concentration measurement is the single most useful parameter for the formulation of effective FMD vaccines (2). Therefore, the FMDV 146S antigen was used in our experiments, and its concentration and its exposure period in microarray analysis were based an earlier study demonstrating that they provided optimal transcription.

By microarray analysis, we found that many immune-associated genes were changed by the stimulation of the FMDV 146S antigen. Although heterogeneity exists in MVECs from various species or different organs, their general response to the same stimulus should be similar. Consequently, the results obtained from RMMVECs can reflect the reaction of MVECs of susceptible animals to the FMDV 146S antigen, which indicates that the functional changes of MVECs are related to immune response. Interestingly, only 18 genes and 67 genes, among a total of 15,000 genes checked in the RMMVECs, were significantly altered by FMDV 146S antigen stimulation for 6 h and 24 h. The numbers of differentially expressed genes were relatively few, which suggests that the gene expression profile response to FMDV 146S antigen stimulation is specific and relatively well defined.

Among the differentially up-regulated genes at 6 h and 24 h, chemokine genes are particularly worth noticing, as their members were the most numerous and their gene changes were significant. Chemokines are a large cytokine family regulating leukocyte trafficking and activation and include 4 subgroups, whose functions are different. In our microarray analysis, the up-regulated chemokine genes belonged to the CC subgroup and CXC subgroup. The CXC chemokines are primarily in attraction of neutrophils, while the CC chemokines have powerful chemoattractant and activator properties for monocytes and T cells (17,18), as well as probably for dendritic cells (DCs) and NK cells (19). At 6 h, the up-regulated chemokine genes included 4 CXC subgroup members and 1 CC subgroup member, while at 24 h, there were 4 CXC subgroup members and 6 CC subgroup members up-regulated. Alternatively, as the stimulation time was prolonged,

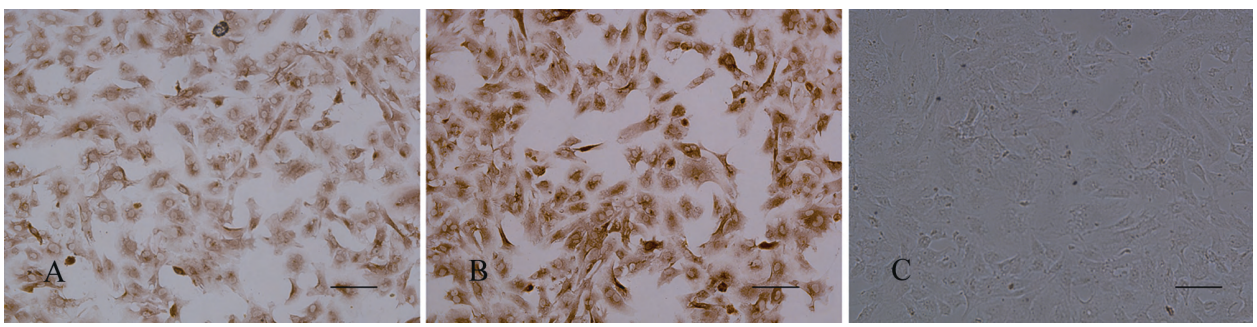

Figure 2. Up-regulated expression of VCAM-1 in RMMVECs by FDMV 146S antigen stimulation. RMMVECs were incubated with 20 μg/mL FMDV 146S antigen for 24 h and fixed with 95% ethanol for streptavidin-peroxidase immunocytochemical staining. A) VCAM-1 expressed constitutively at a low level in RMMVECs, B) expression of VCAM-1 in FMDV 146S antigen-stimulated RMMVECs was strongly positive, C) negative control was not stained. Scale bar = 50 μm.

the predominant chemokine subgroups were shifted from the CXC subgroup to the CC subgroup, which may indicate that the roles of MVECs shift from regulating nonspecific immunity to regulating specific immunity. In addition, among the CC chemokines, monocyte inflammatory protein-3α (MIP-3α, CCL20) was up-regulated at 6 h and 24 h, while MIP-3β (CCL19) expression was not changed. It was reported that MIP-3α plays an important role in the recruitment of immature DCs, and that MIP-3β is important to the accumulation of antigen-loaded mature DCs (20).

IL-6 and IL-1 were up-regulated interleukin genes in our microarray analysis, whose protein secretion was proved to be enhanced by the FMDV 146S antigen in a dose-dependent manner. IL-6 is traditionally considered a proinflammatory cytokine; however, its immune activities, such as resolving innate immunity and promoting acquired immune responses (21), are increasingly drawing attention. In vivo studies have documented that the FMD vaccine could induce the elevation of IL-6 production in vaccinated animals (22,23), which is supported at the cellular level by our data. It was reported that ectogenic IL-1 could increase the immune response to the FMDV antigen (24). Our data show that the FMDV 146S antigen increases the secretion of IL-1 by RMMVECs, which is helpful to the FMD immunity. On the other hand, as a potent proinflammatory cytokine, the up-regulation of IL-1 will have the potential of provoking inflammatory injury.

Compared to the up-regulated genes, the down-regulated genes in RMMVECs stimulated with the FMDV 146S antigen were few. The altered genes included no down-regulated genes at 6 h, and 7 down-regulated genes at 24 h. Transforming growth factor beta-3 (TGF-β3) was one potent immunosuppressive factor (25) whose gene expression was down-regulated by FMDV 146S antigen stimulation. However, little is known regarding the functions of other down-regulated genes, and the biological significance of their down-regulation requires further investigation.

In summary, we examined the general gene expression changes in RMMVECs after exposure to the FMDV 146S antigen and brought forth a new idea to understand the immunity of the FMD vaccine antigen at the fundamental level. Our findings show that many altered genes in FMDV 146S antigen-induced RMMVECs are associated with immune responses, which suggests that MVECs play an important immunomodulatory role in how the immune system, particularly the innate immune system, responds to the FMD vaccine.

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