Gene expression levels of cytokines in peripheral blood mononuclear cells from patients with pulmonary embolism

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Abstract. The aim of this study was to investigate the differential gene expression of cytokines in peripheral blood mononuclear cells (PBMCs) from patients with pulmonary embolism (PE) and controls. Twenty patients with PE and twenty control patients matched for gender and age with the PE group were recruited into the study. Human cDNA microarray analysis was used to detect differences in the expression of cytokine-associated genes between the two groups. In PE patients, the expression levels of the genes encoding IFNα5, IFNα6, IFNα8, IFNα14, IFNκ, IFNω1, IFNε1 and IFNγ were significantly lower compared with controls (P<0.05). The expression levels of the genes encoding IL1α, IL2, IL3, IL9, IL13, IL17β, IL19, IL22, IL23α, IL24, IL25 and IL31 were significantly lower (P<0.05), while IL10 and IL28A mRNA expression levels were higher in PE patients compared with controls (P<0.05). In PE patients, Cxcl1, Cxcl2, Cxcl6, Cxcl13 and Cxcl14 mRNAs were significantly upregulated (P<0.05), however, Cxcl10 mRNA was significantly downregulated (P<0.01). In PE patients, the mRNA expression levels of TNF superfamily members 1, 9 and 13, and TNF receptor superfamily members 1A, 1B, 9, 10B, 10C, 10D and 19L, were significantly upregulated (P<0.05), whereas TNF receptor superfamily members 11B, 19 and 25 were significantly downregulated compared with controls (P<0.05). The mRNA expression levels of granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, erythropoietin, thrombopoietin and mast cell growth factor were significantly lower in PE patients compared with controls (P<0.05). In PE patients, the mRNA expression levels of a variety of cytokines were imbalanced and cellular immune function was downregulated compared with controls. Thus, PE patients may be more susceptible to infections caused by viruses, intracellular bacteria and parasites.

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Introduction

Deep vein thrombosis (DVT) and pulmonary embolism (PE) are collectively known as venous thromboembolism (VTE). PE includes acute pulmonary embolism (APE) and chronic thromboembolic pulmonary hypertension (CTEPH). PE is a major health problem, with a high incidence, mortality, misdiagnosis and low detection rate (1). There is much debate with regard to the diagnosis, treatment and prevention of PE, as the etiology and pathogenesis of this disease have not been well characterized. It has been reported that patients with APE and CTEPH possess lowered immune function (2,3). In this study, we used human cDNA microarray analysis to determine the mRNA expression levels of cytokines in PBMCs isolated from PE patients and controls. We designed this *in vitro* study to observe changes in the immune function of PE patients.

Patients and methods

Patients. Twenty patients with PE were recruited from the Tongji Hospital (Tongji University School of Medicine, Shanghai, China) between 2007 and 2008. A diagnosis of PE required any two of the following three criteria to be met: i) selective pulmonary angiography demonstrating pulmonary artery obstruction or filling defect; ii) lung ventilation/perfusion scan showing single or multiple blood perfusion defects, normal or; iii) clinical diagnosis: there are risk factors for PE and other cardiovascular diseases that are able to be excluded by clinical performance, electrocardiogram and chest film. Arterial blood gas analysis suggested that hypoxemia and hypocapnia, D-dimer detection, echocardiography and chest computed tomography support the diagnosis of PE. We selected 20 patients admitted to the Department of Cardiology, Tongji Hospital, at the same time as the controls used in the study. The patients were divided into two groups: i) the PE patient group: 20 patients (11 males and 9 females), with a mean age of 70±14 years (range, 44-89 years), including 3 cases of CTEPH; ii) the control group: 20 patients (11 males and 9 females) without PE, DVT, arterial thrombosis and congenital coagulation abnormality, with a mean age of 72±14 years (range, 44-91 years), who were matched for gender and age with the PE group. There was no significant statistical difference between the age of the two groups (P>0.05). The clinical trial was approved by the Ethics Committee of the Tongji University and written informed consent was obtained.

Gene expression profiling. Agilent G4112A Whole Human Genome Oligo Microarrays were purchased from Agilent Technologies (Palo Alto, CA, USA). The microarray was composed of 44,290 spots, including 41,675 genes or transcripts, 314 negative control spots, 1,924 positive control spots and 359 blank spots. The functions of >70% of the genes in the microarray were known. All patients were subjected to microarray analysis.

Total RNA isolation. A 5-ml sample of peripheral blood anticoagulated with EDTA was drawn from patients suspected with PE immediately following hospital admittance and from those patients without PE. Mononuclear cells were obtained through density gradient centrifugation (3,000 x g) with Ficoll solution and the remaining red blood cells were destroyed with erythrocyte lysis buffer (Qiagen, Hilden, Germany). Total mononuclear cell RNA was extracted with TRIzol (Invitrogen Life Technologies, Carlsbad, USA) and purified with RNeasy column (Qiagen) according to the manufacturer's instructions. Isolated total RNA was evident and quantified by means of Nanodrop ND-1000 spectrophotometer (Nanodrop Technology, Cambrige, UK).

Detection of gene expression. Approximately 1 μg of total RNA was reverse transcribed into double-strand cDNA. Following purification, *in vitro* amplification was performed using the Low RNA Input Linear Amplification kit (Agilent Technologies) and modified UTP [aaUTP, 5-(3-aminoallyl)-UTP] was used to replace UTP. The integrated aaUTP is capable of interacting with the Cy3 NHS ester-forming fluorescent products, which are then used for hybridization. The integration rate of fluorescence was determined using a Nanodrop ND-1000 spectrophotometer. Following this, the hybridization mixture was prepared with the oligonucleotide microarray *in situ* Hybridization Plus kit (Agilent Technologies). Approximately 750 ng of fluorescent products were fragmented at 60˚C and hybridization was conducted in Human Whole-Genome 60-mer oligo-chips (G4112F, Agilent Technologies) at 60˚C for 17 h at 10 rpm. Following hybridization, the chips were washed with Gene Expression Wash Buffer (Agilent Technologies) according to the manufacturer's instructions. Original signals were obtained using Scanner and Feature Extraction software (Agilent Technologies). The standardization of original signals was performed with RMA standardized methods and standardized signal values were used for the screening of differentially expressed genes.

RT-PCR. Three differentially expressed genes in the microarray analysis were selected and their expression was confirmed by RT-PCR. Among the genes with differential expression, three genes were randomly selected and subjected to RT-PCR along with the housekeeping gene (GAPDH). The relative expression was indicated as the expression of the target genes normalized by that of GAPDH ($2^{-\Delta\Delta Ct}$). Melting curve and $2^{-\Delta\Delta Ct}$ methods were used to compare differences in gene expression between the control and PE groups. Results from RT-PCR were consistent with those obtained from the microarray analysis.

Statistical analysis. The independent samples t-test was used to compare mRNA levels in samples from PE patients and controls. Statistical tests were performed using SPSS 17.0, and $P<0.05$ was considered to indicate a statistically significant result. Prior to the t-test, a test for equality of variances was performed; if variances were not equal, the t-test result was corrected.

Results

IFN mRNA expression levels. PBMCs obtained from PE patients demonstrated low IFN mRNA expression levels. Expression of the 12 genes encoding type I IFNs, as well as the gene encoding IFNγ, were examined (Fig. 1). In PBMCs from PE patients, the expression levels of genes encoding IFNα5, IFNα6, IFNα8, IFNα14, IFNκ, IFNω1 and IFNε1 were significantly lower than those detected in PBMCs from controls ($P<0.05$). IFNγ mRNA expression was significantly downregulated in PBMCs from PE patients compared with controls ($P<0.01$).

IL mRNA expression levels. The expression levels of 37 genes encoding ILs were examined (Fig. 2). The expression levels of genes encoding IL1α, IL2, IL3, IL9, IL13, IL17β, IL19, IL22, IL23α, IL24, IL25 and IL31 were significantly lower in PBMCs from PE patients compared with the controls ($P<0.05$). IL10 and IL28A gene expression levels were greater in PBMCs from PE patients than controls ($P<0.05$).

Th1/Th2 mRNA expression levels. Th1 cytokine mRNA expression levels including IFNγ and IL2 mRNAs were detected (Fig. 3), and in PE patients, both were significantly downregulated compared with the control group ($P<0.01$). Th2 cytokine mRNA expression levels, including IL4, IL6 and IL10 mRNAs were examined (Fig. 4). IL10 mRNA expression levels were significantly higher in PBMCs from PE patients than controls ($P<0.01$). No significant difference in IL4 and IL6 mRNA expression levels was observed between the two groups.

Chemokine mRNA expression levels. Twelve genes encoding CXC chemokines were detected (Fig. 5). In PE patients, mRNA expression levels of Cxcl1, Cxcl2, Cxcl6, Cxcl13 and Cxcl14 were significantly upregulated ($P<0.05$), and Cxcl10 mRNA expression levels were significantly downregulated compared with controls ($P<0.01$). Twenty-three genes encoding CC chemokines were examined (Figs. 6 and 7) and the mRNA expression levels of CC chemokines were significantly lower in PE patients than controls ($P<0.01$). C chemokine mRNA expression levels including Xcl1 and Xcl2 mRNAs were detected (Fig. 8), and Xcl1 mRNA expression was significantly lower in PE patients ($P<0.01$). No significant difference in Xcl2 mRNA expression levels was observed between the two groups. CX3C chemokine Cx3cl1 mRNA expression was detected, and there was no significant difference between the two groups.

TNF superfamily and TNF receptor superfamily mRNA expression levels. Thirty-eight genes encoding members of the TNF superfamily and TNF receptor superfamily were examined (Fig. 9). In PE patients, the mRNA expression levels of TNF superfamily members 1, 9 and 13, and TNF receptor superfamily members 1A, 1B, 9, 10B, 10C, 10D and 19L, were significantly upregulated ($P<0.05$), whereas TNF

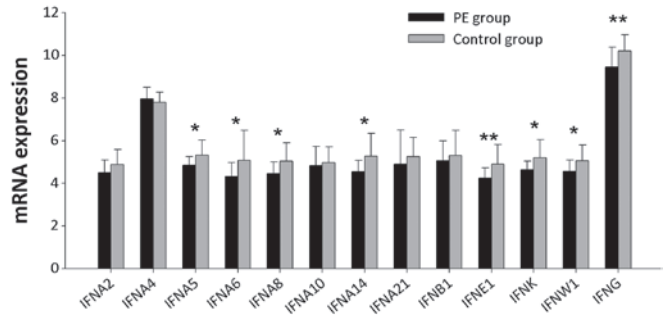

Figure 1. IFN mRNA expression levels in PBMCs from PE patients and controls. IFNA2, IFNA4, IFNA5, IFNA6, IFNA8, IFNA10, IFNA14, IFNA21, IFNB1, IFNE1, IFNK, IFNW1 and INFG represent the gene symbols of IFNα2, IFNα4, IFNα5, IFNα6, IFNα8, IFNα10, IFNα14, IFNα21, IFNβ1, IFNε1, IFNκ, IFNω1 and IFNγ, respectively. *P<0.05 and **P<0.01. PBMCs, peripheral blood mononuclear cells; PE, pulmonary embolism.

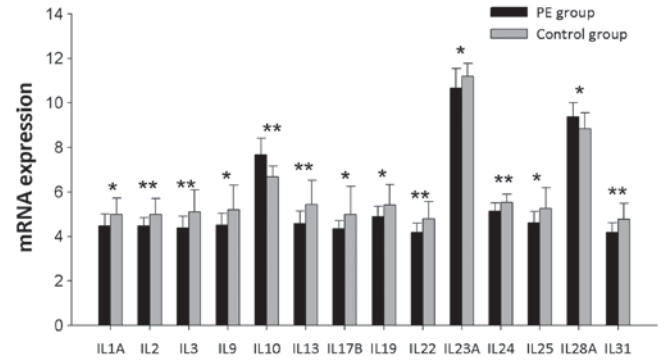

Figure 2. IL mRNA expression levels were significantly different in PBMCs from PE patients and controls. IL1A, IL2, IL3, IL9, IL10, IL13, IL17B, IL19, IL22, IL23A, IL24, IL25, IL28A, and IL31 represent the gene symbols of IL1α, IL2, IL3, IL9, IL10, IL13, IL17β, IL19, IL22, IL23α, IL24, IL25, IL28α and IL31, respectively. *P<0.05 and **P<0.01. PBMCs, peripheral blood mononuclear cells; PE, pulmonary embolism.

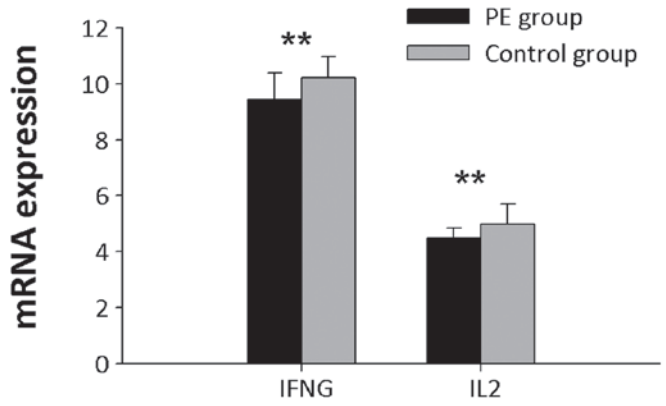

Figure 3. Th1 cytokine mRNA expression levels in PBMCs from PE patients and controls. IFNG and IL2 represent the gene symbols of IFNγ and IL2, respectively. *P<0.05 and **P<0.01. PBMCs, peripheral blood mononuclear cells; PE, pulmonary embolism.

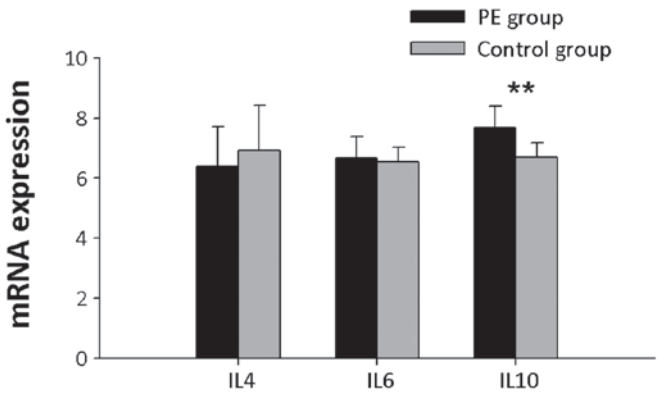

Figure 4. Th2 cytokine mRNA expression levels in PBMCs from PE patients and controls. IL4, IL6 and IL10 represent the gene symbols of IL4, IL6 and IL10, respectively. *P<0.05 and **P<0.01. PBMCs, peripheral blood mononuclear cells; PE, pulmonary embolism.

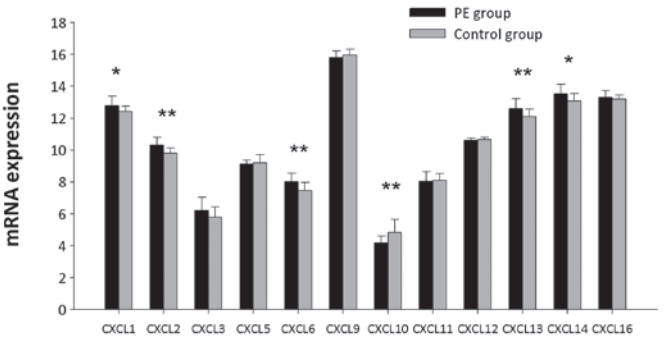

Figure 5. CXC chemokine mRNA expression levels in PBMCs from PE patients and controls. CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14 and CXCL16 represent the gene symbols of Cxcl1, Cxcl2, Cxcl3, Cxcl5, Cxcl6, Cxcl9, Cxcl10, Cxcl11, Cxcl12, Cxcl13, Cxcl14 and Cxcl16, respectively. *P<0.05 and **P<0.01. PBMCs, peripheral blood mononuclear cells; PE, pulmonary embolism.

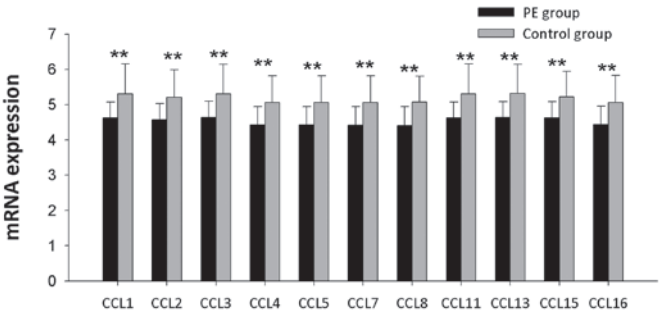

Figure 6. CC chemokine mRNA expression levels in PBMCs from PE patients and controls. CCL1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL15 and CCL16 represent the gene symbols of Ccl1, Ccl2, Ccl3, Ccl4, Ccl5, Ccl7, Ccl8, Ccl11, Ccl13, Ccl15 and Ccl16, respectively. *P<0.05 and **P<0.01. PBMCs, peripheral blood mononuclear cells; PE, pulmonary embolism.

receptor superfamily members 11B, 19 and 25, were significantly downregulated compared with controls (P<0.05).

Colony stimulating factor mRNA expression levels. Six genes encoding colony stimulating factors were detected (Fig. 10) and the mRNA expression levels of granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO), thrombopoietin (THPO) and mast cell growth factor (KITLG) were significantly lower in PBMCs from PE patients than controls (P<0.05).

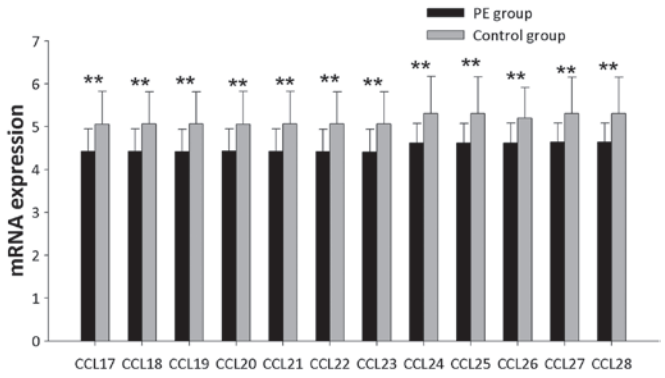

Figure 7. CC chemokine mRNA expression levels in PBMCs from PE patients and controls. CCL17, CCL18, CCL19, CCL20, CCL21, CCL22, CCL23, CCL24, CCL25, CCL26, CCL27 and CCL28 represent the gene symbols of Ccl17, Ccl18, Ccl19, Ccl20, Ccl21, Ccl22, Ccl23, Ccl24, Ccl25, Ccl26, Ccl27 and Ccl28, respectively. *P<0.05 and **P<0.01. PBMCs, peripheral blood mononuclear cells; PE, pulmonary embolism.

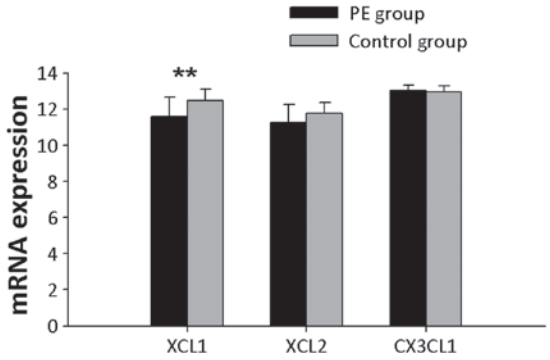

Figure 8. C and CX3C chemokine mRNA expression levels in PBMCs from PE patients and controls. XCL1, XCL2 and CX3CL1 represent the gene symbols of Xcl1, Xcl2 and Cx3cl1, respectively. *P<0.05 and **P<0.01. PBMCs, peripheral blood mononuclear cells; PE, pulmonary embolism.

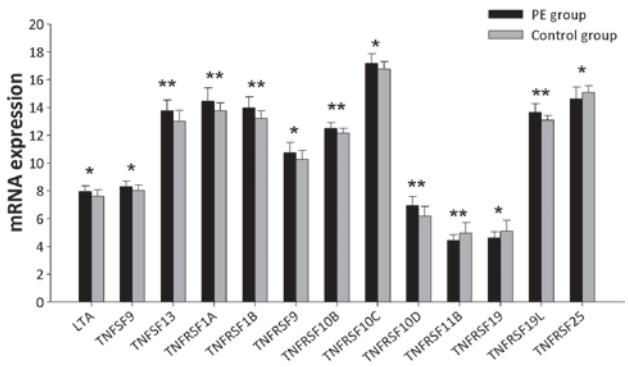

Figure 9. TNF superfamily and TNF receptor superfamily mRNA expression levels which were significantly different in PBMCs from PE patients and controls. LTA, TNFSF9, TNFSF13, TNFRSF1A, TNFRSF1B, TNFRSF9, TNFRSF10B, TNFRSF10C, TNFRSF10D, TNFRSF11B, TNFRSF19, TNFRSF19L, and TNFRSF25 represent the gene symbols of TNF superfamily members 1, 9 and 13, and TNF receptor superfamily members 1A, 1B, 9, 10B, 10C, 10D, 11B, 19, 19L and 25, respectively. *P<0.05 and **P<0.01. PBMCs, peripheral blood mononuclear cells; PE, pulmonary embolism.

Other cytokine mRNA expression levels. We examined eight genes associated with transforming growth factor (TGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) (Fig. 11). The mRNA expression levels of TGFβ1, TGFβ1-induced transcript 1, EGF and VEGF were significantly upregulated (P<0.01), whereas TGFβ3 mRNA was significantly downregulated (P<0.05) in PBMCs from PE patients compared with controls.

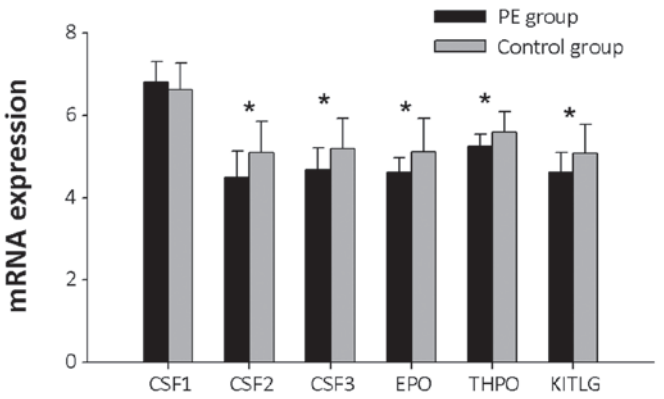

Figure 10. CSF mRNA expression levels in PBMCs from PE patients and controls. CSF1, CSF2, CSF3, EPO, THPO and KITLG represent the gene symbols of macrophage colony stimulating factor, granulocyte-macrophage colony stimulating factor, granulocyte colony stimulating factor, erythropoietin, thrombopoietin and mast cell growth factor, respectively. *P<0.05 and **P<0.01. PBMCs, peripheral blood mononuclear cells; PE, pulmonary embolism; CSF, colony stimulating factor; EPO, erythropoietin; THPO, thrombopoietin; KITLG, mast cell growth factor.

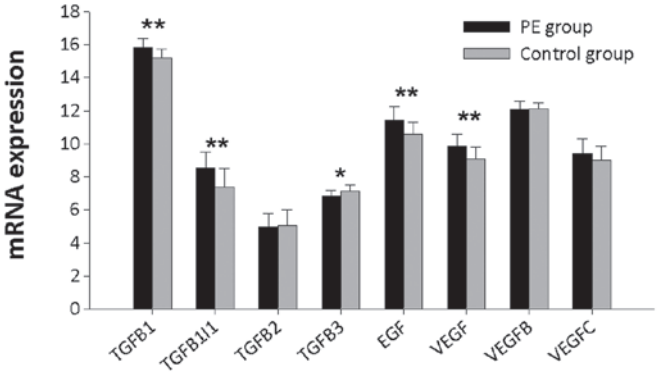

Figure 11. Other cytokine mRNA expression levels in PBMCs from PE patients and controls. TGFB1, TGFB1I1, TGFB2, TGFB3, EGF, VEGF, VEGFB and VEGFC represent the gene symbols of TGFβ1, TGFβ1-induced transcript 1, TGFβ2, TGFβ3, epidermal growth factor, VEGF, VEGFB and VEGFC, respectively. *P<0.05 and **P<0.01. PBMCs, peripheral blood mononuclear cells; PE, pulmonary embolism; VEGF, vascular endothelial growth factor; TGF, transforming growth factor; EGF, epidermal growth factor.

Discussion

IFNs are classified into two major groups: type I and type II. Type I IFNs include IFNα, IFNβ, IFNε, IFNω and IFNκ (4). We detected type I IFN mRNAs in the PE patients compared with controls. We demonstrated that IFNα5, IFNα6, IFNα8, IFNα14, IFNκ, IFNω1 and IFNε1 mRNA expression levels were significantly lower in PE patients compared with controls. The type I IFNs possess antiviral, antiproliferative and immunomodulatory effects (5). It has been reported that IFNα/β receptor knockout mice are susceptible to viral infection (6).

Mice treated with antibodies against IFNα/β may be lethally infected with filoviruses (7). Thus, our results suggest that PE patients are susceptible to viral infection. In this study, we also examined IFNγ mRNA expression levels which were observed to be significantly downregulated in PE patients compared with controls. IFNγ is important in the defence against intracellular bacteria (Mycobacterium, Listeria) and parasites (Leishmania) (8). A caudal vena cava thrombus in a dog with leishmaniasis has been reported (9). It has also been demonstrated that three patients with pulmonary tuberculosis suffered from PTE or DVT (10). Therefore, our results show that PE patients are susceptible to intracellular bacteria and parasites.

T-helper lymphocytes can be divided into Th1 and Th2 subsets based on their functional abilities and the profile of the cytokines they produce (11). Th1 and Th2 cells express different cytokines: Th1 cells produce IL2, IL12 and IFNγ, whereas Th2 cells produce IL4, IL5, IL6, IL10 and IL13 (12). Th1 cells promote cellular immunity, while Th2 cells induce humoral immunity and upregulate antibody production (13). The shift in Th1/Th2 balance leads to immune dysfunction. Our results demonstrated the presence of lower mRNA expression levels of Th1 cytokines (IFNγ and IL2) and higher mRNA expression levels of Th2 cytokine (IL10) in PE patients, suggesting a shift towards Th2 dominance in PE patients. Thus, our results suggest that an immune dysfunction in PE patients may exist.

Chemokines can be classified into four classes; CXC, CC, C and CX3C according to their structure. Previous studies have shown that a polymorphonuclear (PMN) activation in thrombosis is present, and that PMN activation may be important in the etiologies of thrombosis (14,15). CXC chemokines possess a potent chemotactic activity on neutrophils. Twelve genes encoding CXC chemokines and mRNA expression levels of Cxcl1, Cxcl2, Cxcl6, Cxcl13 and Cxcl14 were significantly upregulated in PE patients compared with controls, suggesting that a high expression of chemokines may induce neutrophil aggregation. The CC chemokines RANTES/CCL5, MIP-1α/CCL3 and MIP-1β/CCL4 produced by CD8+ T cells induce the inhibition of human immunodeficiency virus (HIV) (16). During chronic viral infection, virus-specific CD8 T cells generated low levels of cytokines and the cytotoxic ability of CD8 T cells was decreased in RANTES knockout mice (17). Deficiency of MIP-1α led to the recovery of higher levels of infectious viruses in comparison with fully competent mice in paramyxovirus infection (18). During mouse hepatitis virus infection, the cytokine production and cytolytic activity of CD8+ T cells were reduced in CCL3 knockout mice (19). Our results demonstrate the presence of lower mRNA expression levels of Ccl3, Ccl4 and Ccl5 in PE patients, which suggests that a reduced antiviral ability in PE patients may exist.

The interaction of costimulatory receptor CD137 (TNF receptor superfamily, member 9) and its ligand CD137L (TNF superfamily, member 9), which is involved in the TNF superfamily, is important in immune regulation (20). Blockade of the CD137-CD137L pathway significantly reduced the percentage of CD3+ CD8+ T cells in mice transplanted with donor bone marrow cells plus primary MLC spleen T cells (21). It has been reported that 4-1BB (also known as CD137)-deficient mice have enhanced susceptibility to Listeria monocytogenes (intracellular bacteria) infections (22). It has also been shown that the cytotoxic lymphocyte (CTL) response to the influenza virus was reduced in mice lacking 4-1BBL (23). In this study, mRNA expression levels of CD137 and CD137L were upregulated in PE patients, suggesting that PE patients may be more susceptible to infection. TNF may interfere with viral replication in several ways (24). TNF has two receptors, TNF receptor 1 (TNFR1) and 2 (TNFR2) (24). The TNF/TNFR pathway is important in the induction of apoptosis (25). It has been demonstrated that TNFR1 and TNFR2 induced evident apoptosis, including CD4 and CD8 T cells during HIV infection (26). In our study, mRNA expression levels of TNFR1 and TNFR2 were significantly greater in PE patients than controls. Thus, we hypothesize that peripheral T-cell apoptosis induced by TNFR1 and TNFR2 may exist, leading to a decreased immune function and enhanced susceptibility to virus expression in PE patients.

GM-CSF is important to the immune system. GM-CSF is capable of affecting the balance of Th1/Th2. It has been reported that mice lacking GM-CSF were not able to control an aerosol-delivered infection with *Mycobacterium tuberculosis* and died from severe necrosis in the lungs as it was not able to express a Th1 response (27). In this study, mRNA expression levels of GM-CSF were significantly downregulated in PE patients, suggesting that there may be decreased immune function and a reduced antipathogenic micro-organism ability in PE patients.

VEGF-A (also known as VEGF) is an important member of the VEGF family. PE-induced endothelium injury is important in the sequelae following PE (28). In this study, mRNA expression levels of VEGF in PE patients were significantly higher than controls, suggesting that the level of VEGF mRNA may increase compensatively to promote endothelial cell repair and vascular remodeling following PE-induced endothelium injury. Three types of isoform have been identified in mammals; TGFβ1, TGFβ2 and TGFβ3 (29). All three isoforms have similar properties *in vitro*, however, TGFβ1 is dominantly expressed in the immune system (30). It has been reported that the level of TGFβ1 increases during cytomegalovirus and hepatitis B virus infection (31,32). It has been shown that in HIV infection, TGFβ is capable of suppressing the cellular immune response and the humoral immune response (33). In this study, mRNA expression levels of TGF were upregulated significantly in PE patients, suggesting that a decreased immune function in PE patients may exist and this disease may be associated with viral infections.

From the characteristics of a variety of cytokine mRNA expression levels in PE patients, we conclude that the immune function and the ability of clearing viruses, intracellular bacteria and parasites are reduced in PE patients. Previously, we reported downregulated gene expression associated with NK and T cells in patients with PE (34), and the number of CD3+ and CD8+ T cells were significantly downregulated in patients with APE and CTEPH (2,3). In this study, we detected and analyzed the mRNA expression levels of cytokines in patients with PE and conclude that an immune dysfunction in PE patients exists, which is similar with the results of previously performed cytology tests. Our results may provide evidence for the etiology and pathogenesis of VTE.

Acknowledgements

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