Article

The Effect of Anti-Human CD134 Monoclonal Antibody on Phytohemagglutinin-Induced mRNA Expression of Perforin in Peripheral Blood Mononuclear Cells

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CD134, a member of the tumor necrosis factor receptor superfamily, plays a crucial role in T cell survival. In this study, peripheral blood mononuclear cells (PBMCs) stimulated with phytohemagglutinin (PHA, 50 μg/ml) were treated by CD134 mAb (1 μg/ml, 5 μg/ml, 10 μg/ml) for 6 h, 12 h, 24 h and 48 h. The level of perforin mRNA was measured by reverse transcription-polymerase chain reaction (RT-PCR) technique. Our data showed that the expression of perforin mRNA in PBMCs was down-regulated by CD134 mAb in a dose-dependent manner in range of 1 μg/ml to 5 μg/ml and dropped down to its minimum on 24 h ($p < 0.05$). The level of perforin mRNA reached a plateau when the concentration of CD134 mAb exceeded 5 μg/ml. In conclusion, CD134 mAb can inhibit perforin expression, which may enhance the ability of T cells for survival. *Cellular & Molecular Immunology*. 2005;2(6): 467-471.

Key Words: CD134, perforin, phytohemagglutinin

Introduction

Mouse monoclonal antibodies anti-OX-40, now formally called as CD134, was developed in 1987 by immunizing BALB/C mice with phytohemagglutinin (PHA)-stimulated rat T lymphocytes (1). It is often quoted that CD134 is primarily expressed on CD4 T cells, however CD8 T cells can also bear CD134 molecule at least under certain conditions. Moreover, CD134 has now been detected on other cell types including B cells, dendritic cells and eosinophils (2). CD134 was not expressed on resting T cells; it was detectable predominantly *in vivo* on T cells after antigenic stimulation and immunization (3), such as in synovial fluid of patients with chronic synovitis, joints of mice undergoing rheumatoid arthritis (4, 5), lamina propria of mice undergoing colitis, gastrointestinal tract of patients with Celiac and Crohn's disease (3, 6), lymph nodes draining sites with growth of Leishmania major (7), and lungs of mice with allergic asthma (8).

CD134, a member of the tumor necrosis factor receptor (TNFR) superfamily, belongs to type I transmembrane glycoprotein and its relative molecular mass was about 48,000. The human CD134 gene of 1.4 kb nuclotides was cloned in 1994, which codes a single transmembrane protein with 277 amino acids. The gene is mapped to chromosome band lp36 and thus linked to the genes for CD30, 4-1BB, TNFR II and DR3 (9), which had similar functions. It was demonstrated that, for example, the CD30-initiated signals inhibited apoptosis through increasing the expression of anti-apoptotic molecules and down-regulating the expression of apoptosis molecules in a lymphoma cell line YT. Quantitative analysis of normalized expression of perforin mRNA after CD30 signaling revealed a 2-fold suppression of gene expression in microchip assays and RNase protection assay (10). It was observed recently that CD134 stimulation also promoted and maintained high level expression of anti-apoptosis genes and proteins, such as Bcl-xL and Bcl-2. They may control the survival of resting T cells and activated effector T cells, which suggested that CD134 signals can expand T cell survival ability (11). There are no reports so far that CD134 has similar function with CD30 to down-regulate the perforin expression to expand T cell survival ability. To explore this possible mechanism, the expression of perforin mRNA in PBMCs under the influence of CD134 mAb was determined in this study by RT-PCR technique.

Materials and Methods

Reagents

CD134 mAb, clone ACT35 and Ficoll-Hypague were

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Table 1. Effect of CD134 mAb on PHA-induced mRNA expression of perforin in human PBMC

Time (h)	Control	PHA (50 μg/ml)	PHA (50 μg/ml) + CD134 mAb					
			1 μg/ml	Inhibition ratio (%)	5 μg/ml	Inhibition ratio (%)	10 μg/ml	Inhibition ratio (%)
6	285.26 ± 25.73	326.69 ± 30.18*	298.72 ± 31.47	8.64 ± 1.45	273.89 ± 35.91	16.41 ± 3.55	276.02 ± 31.27	16.51 ± 5.33
12	272.03 ± 28.74	411.47 ± 35.25*	378.49 ± 34.73	8.05 ± 0.70	293.83 ± 26.31	28.61 ± 0.50	293.64 ± 30.33	28.51 ± 1.60
24	278.66 ± 26.93	504.66 ± 15.93*	176.15 ± 31.61	65.22 ± 5.25	130.01 ± 21.49	74.32 ± 3.49	129.75 ± 20.51	74.23 ± 4.93
48	271.62 ± 30.10	584.64 ± 30.36*	514.88 ± 41.51	12.03 ± 2.66	328.27 ± 24.00	43.90 ±1.37	328.31 ± 34.55	43.09 ± 2.73

PBMC were treated with 50 μg/ml of PHA without or with CD134 mAb (1 μg/ml, 5 μg/ml, 10 μg/ml) and then incubated for 6 h, 12 h, 24 h or 48 h. The expressions of perforin and β-actin were measured by the semi-quantitative RT-PCR, and the ratios of the number of perforin and β-actin copies were calculated. CD134 mAb was added to the culture medium at 48 h after PHA stimulation. The experiments were repeated for five times (n = 5). *, $q = 179.97$, $p < 0.01$ *vs* control group.

purchased from Pharmingen. PHA was purchased from Sigma. Onestep RT-PCR kits for human were purchased from Qiagen Company, and Trizol reagent was purchased from Life Technologies Company. Primers were synthetized by Sangon Company, China.

Isolation of PBMC

Venous blood (100 ml) from healthy adults were collected, antiagglutinated with 0.2% ethylenediaminetetraacetic acid-K_2, and sterilized with steam of high pressure, then diluted with an equal volume of cold Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution. The suspension was layered on Ficoll solution and PBMC were separated using Arnold V method (12), then counted by using 0.2% trypan blue, and live cells > 95%. PBMC (1×10^6/ml) were cultured in RPMI 1640, supplemented with 100 U/ml penicillin-streptomycin and 10% (vol/vol) heat-inactivated fetal calf serum (Gibco), and incubated in 24-well culture plates in 5% CO_2 at 37°C. All experiments were performed with approval of the Human Ethics Committee.

PBMC treatment

For dose-effect experiments, PBMC were randomly divided into three groups: 1) control group; 2) PHA group; 3) PHA + CD134 group. The study on the effect of CD134 mAb was performed as follows: 1) PBMCs of control group were cultured in RPMI 1640 medium only. 2) PBMC of PHA group were cultured in RPMI 1640 medium containing 50 μg/ml PHA. 3) PBMC of PHA + CD134 group were incubated RPMI 1640 medium firstly for 48 h. After that 50 μg/ml PHA was put into medium, then different end concentrations of CD134 mAb (1 μg/ml, 5 μg/ml and 10 μg/ml) were added and PBMC were incubated continuously. For time-course experiments, perforin mRNA was measured at 6 h, 12 h, 24 h, and 48 h in every group, and time of CD134 mAb addition was set up as zero.

Semi-quantitative RT-PCR

The cultured PBMC were washed twice with PBS at 1,500 rpm for 5 min and collected. Total ribonucleic acid (RNA) of PBMC was extracted with Trizol reagent and then used to synthesize cDNA and PCR amplificate with onestep RT-PCR kits. The primer sequences were as follows: perforin (13) (176 bp) forward: 5'-CAG TAC AGC TTC AGC ACT GAC (496-516)-3', and reverse: 5'-ATG AAG TGG GTG CCG TAG TTC (651-671)-3'; β-actin (14) (350 bp) forward: 5'-GGT CAC CCA CAC TGT GCC CAT (2,139-2,159)-3', and reverse: 5' -GGA TGC CAC AGG ACT CCA TGC (2,563-2,583)-3'. The 50 μl RT-PCR preparation contained: 27 μl RNase-free water, 10 μl 5 × QIAGEN OneStep RT-PCR Buffer, 400 μM of each dNTP, 0.6 μM Primer A, 0.6 μM Primer B, 2 μl QIAGEN OneStep RT-PCR Enzyme Mix, 5 μl template RNA. Template RNA (5 μl) was used with the above primers under the following conditions: 50°C for 30 min, 95°C for 15 min, 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min for 35 cycles, then 72°C for 10 min. The RNA was stored at -80°C until used. PCR products were analyzed by electrophoresis on a 2% agarose gel containing 0.5 μg/ml ethidium bromide. The resulting bands were observed and photographed under ultraviolet light and were measured as actual values by alphaimage 2,200. The relative values of perforin mRNA in PBMC were assessed as (perforin mRNA/β-actin mRNA) × 10^3. The result reported for each RNA sample is the mean of at least five RT-PCR assays.

Statistical analysis

All data were expressed as mean ± standard deviation. ANOVA and *q* test were used for comparison of more than two groups. All data were analyzed with SPSS13.0 for Windows.

Results

CD134 mAb inhibited perforin mRNA expression in PBMC

Expression of perforin mRNA was low and was not found to be statistically different for all times in the control group, while expression of perforin protein in the PHA group was increased by the time and higher than that in the control group ($p < 0.01$). *q* values of three CD134 mAb treated groups were 114.80, 200.36 and 199.93, respectively, when compared with the control group ($p < 0.001$). Table 1 showed

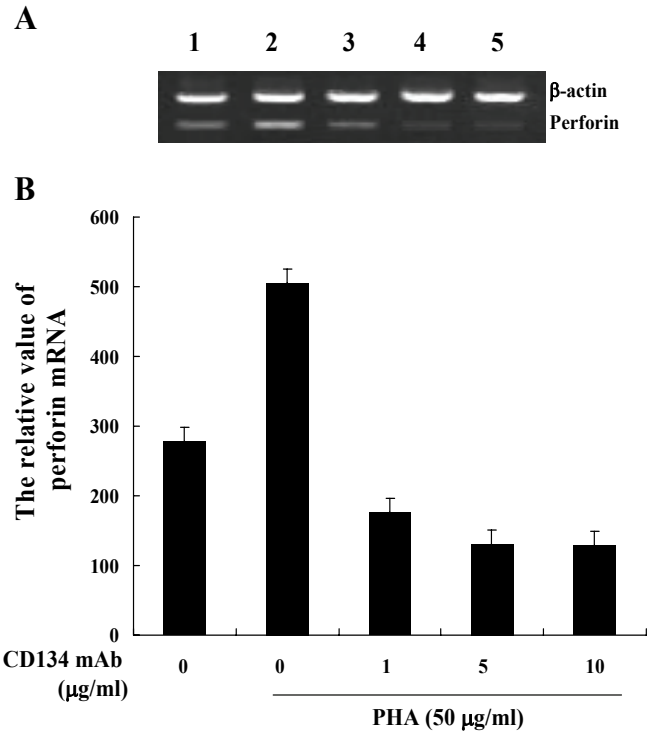

Figure 1. CD134 mAb inhibited perforin mRNA expression in PBMC in a dose-dependent manner. PBMC were incubated with medium only or medium containing 50 μg/ml PHA, 50 μg/ml PHA and 1, 5 10 μg/ml CD134 mAb for 24 h. Time of CD134 mAb addition was made as zero, CD134 mAb was added to the medium on 48 h after PHA stimulation. The expressions of perforin and β-actin were measured by semi-quantitative RT-PCR. (A) PCR products of perforin and β-actin were run on a 2% agarose gel. (B) The ratios of perforin and β-actin were calculated.

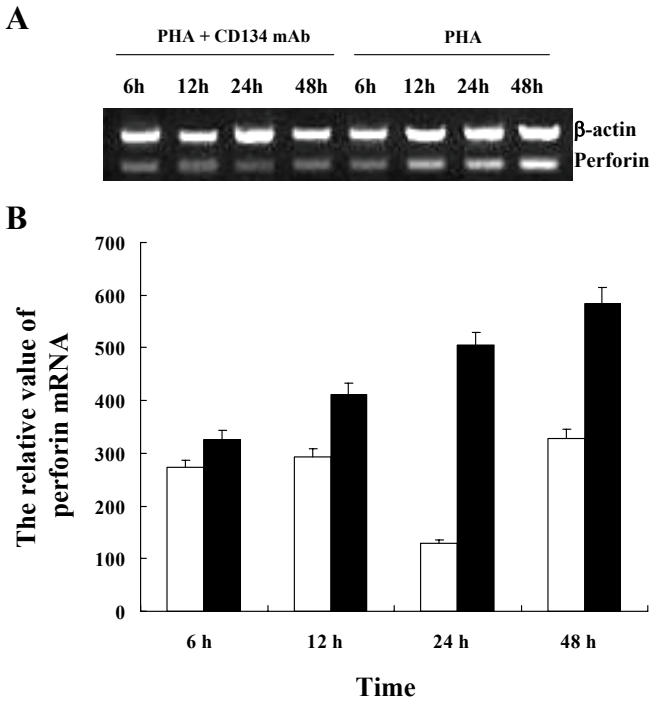

Figure 2. CD134 mAb inhibited perforin mRNA expression in PBMC in a time-dependent manner. PBMC (1×10^6 cells/ml) were treated with 50 μg/ml PHA without (■) or with 5 μg/ml antihuman CD134 mAb (□) and then incubated for 6 h, 12 h, 24 h or 48 h. Time of anti-human CD134 mAb addition was made as zero. Anti-human CD134 mAb was added to the medium at 48 h after PHA stimulation. (A) PCR products of perforin and β-actin were run on a 2% agarose gel. β-actin was used as a positive transcription control. (B) The ratios of perforin and β-actin were calculated.

that various concentrations of CD134 mAb had inhibitory effect on the expression of perforin mRNA in PBMC for different time, which reached its maximum at 24 h at the same concentration. Inhibition ratios of three CD134 mAb treated groups were 65.22 ± 5.25%, 74.32 ± 3.49% and 74.23 ± 4.93% at 24 h, compared with the PHA group.

Effect of various concentrations of CD134 mAb on expression of perforin mRNA in PBMC

For the same time, inhibitory effect of CD134 mAb on expression of perforin mRNA in PBMC of 5 μg/ml CD134 mAb treated group was obviously increased ($p < 0.001$) compared with that of 1 μg/ml CD134 mAb treated group. This tendency of elevation presented a dose-dependent manner ranging from 1 μg/ml to 5 μg/ml. However, there was no significant difference ($p > 0.05$) in perforin mRNA between 5 μg/ml CD134 mAb treated group and 10 μg/ml CD134 mAb treated group, and the inhibitory effect reached a plateau when the concentration of CD134 mAb exceeded 5 μg/ml. Figure 1 showed effect of various concentrations of CD134 mAb on the expression of perforin mRNA at 24 h.

Time-dependent effect of CD134 mAb on perforin mRNA expression in PBMC

In control and PHA groups, perforin mRNA in PBMC was increased in a time-dependent manner and it reached a peak at 48 h. In three CD134 mAb treated groups, perforin mRNA in PBMC was at the lowest level at 24 h. The levels of perforin mRNA were increased at 6 h, 12 h and 48 h compared with that at 24 h for the same group ($p < 0.05$). The inhibitory effect reached their maximum at 24 h at any concentration (Figure 3).

Discussion

CD134 can be induced by TCR/CD3 signals in isolation and initially appeared at 12-24 h after stimulation of naïve cells. Peak expression was seen after 2-3 days and then CD134 was downregulated, implying a delayed mode of action. A large number of *in vitro* studies that will not be detailed here, using either receptor specific antibodies or cells transfected with individual ligands, had shown that signals through CD134 can augment T cell responses, either in isolation or in combination with CD28 signals from B7. CD134 had activity

of costimulatory signal transduction and promoted proliferation, differentiation, cytokine secretion of T cells (15); it played a crucial role in inhibiting apoptosis and promoting survival of T cells. Potential mechanisms of promoting T cell survival (16), other than promoting cytokine transcription, could include up-regulation of members of the bcl family of proteins (11), inhibition of Fas, FasL and perforin expression or function, or overcoming down-regulatory signals through CTLA4, which may be used to treat the disease. The recent study indicated that the three main strategies by which CD134-mediated immunotherapies might be employed clinically. They included (17): inhibiting inflammation by blocking CD134-CD134L interaction using anti-CD134 or anti-CD134L mAb, enhancing vaccines by binding CD134 *in vivo* with anti-CD134 or anti-CD134L mAb, and deleting autoreactive T cells with a CD134-targeted immunotoxin (18, 19).

Perforin, stored mainly within the cytoplasmic granules of activated cytotoxic T lymphocytes and natural killer cells, was a kind of glycoprotein and perforin monomers polymerized into pore-forming aggregates in membrane of target cells (20). Pores of perforin allowed granzymes to enter the target cells and activate caspase to induce apoptosis (21, 22). In addition, some ions such as calcium that cannot enter cell under physiologic conditions may enter the target cells through the channels, which led to membrane deporlarization and elevated osmotic pressure of target cells, thus these cells would be lysed. Cell lysis initiated an inflammatory response, while increased apoptosis and lysis were observed. The large quantities of autoantigens could be made available in the individual predisposed to develop several autoimmune diseases during apoptosis and lysis (23), for instance, there was increased expression of perforin in peripheral blood lymphocytes from patients with active systemic lupus erythematosus (24); we can treat the disease if perforin expression was inhibited.

To study the concentration-response and time course of perforin mRNA expression in PBMC, perforin mRNA in PBMC under the effect of various concentrations of CD134 mAb was determined by RT-PCR. Our studies showed that various concentrations of CD134 mAb had inhibitory effects on expression of perforin mRNA in PBMC for different times, which reached its maximum at 24 h. For the same time, inhibitory effect of CD134 mAb on expression of perforin mRNA in PBMC was increased markedly in a dose-dependent fashion in the range of 1-5 μg/ml in CD134 mAb stimulated cultures, while the inhibitory effects reached a plateau when the concentration of CD134 mAb exceeded 5 μg/ml, which may be resulted from that CD134 in the membrane binding with CD134 mAb was saturated. In three CD134 mAb treated groups, perforin mRNA of PBMC were at the lowest level at 24 h. The level of perforin mRNA was increased at 6 h, 12 h and 48 h compared with that at 24 h at the same group ($p < 0.05$), which may be related with peak expression of CD134 induced after 72 h and then down-regulated.

In our study, we made time of CD134 mAb addition as zero, however at this time point PHA had been added to the medium for 48 h actually, peak expression of CD134 was induced at 24 h after CD134 mAb addition, so inhibitory effects of CD134 mAb was seen to reach its maximum at 24 h. It was addressed that CD134 mAb can inhibit perforin expression on transcription level and promote T cell survival, which may be used to treat several autoimmune diseases. The signal of CD134 was transmitted through tumor necrosis factor receptor associated factor (TRAF) and protein kinase B (PKB) signaling pathways, TRAF-1,2,3,5 bound intramembrane of CD134, thus influenced production of NF-κB and transmitting downstream signals, which had been substantiated by using immunoprecipitation and immunoblotting (2). Song found another molecule providing CD134 signals was PKB (25). PKB phosphorylation and kinase activity in wild-type T cells were strongly increased after stimulated with antigen plus agonist CD134 mAb, and then transduction CD134-deficient T cells with active (myristoylated) PKB, which maintained large amounts of anti-apoptosis molecules to promote survival of CD134-deficient T cells and restored the secretion of cytokine or inflammatory response, approximating functions of the wild-type phenotype, while retroviral transduction of a dominant-negative variant of PKB into activated wild-type T cells mimiced the CD134-deficient phenotype, expression of active PKB in responding CD134-deficient T cells reversed their defective long-term T cell survival and ability to mount *in vivo* inflammatory responses (25). It was possible that CD134 mAb inhibits perforin mRNA expression through the above pathways, which needs to be addressed further.

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