·Basic Research·

An increased expression of CD40/CD40L costimulatory molecules in erythema nodosum of patients with Behçet's disease

Hong Wang[1,2], Pei-Zeng Yang[1], Xiao-Yan Peng[2], Meng Zhao[2], Hong-Yan Zhou[1], Xiang-Kun Huang[1]

Foundation items: National Natural Science Foundation of China (No.30400490); Beijing Natural Science Foundation of China (No. 7052023); Foundation of Bureau of Guangdong Traditional Chinese Medicine, China (No.1050171)

[1]Zhongshan Ophthalmic Center, Sun Yat-sen University; International Uveitis Study Laboratory; Uveitis Study Center, Sun Yat-sen University; State Key Laboratory of Ophthalmology; Guangzhou 510060, Guangdong Province, China

[2]Beijing Tongren Eye Center, Affiliate of Capital University of Medical Sciences, Beijing 100730, China

Correspondence to: Pei-Zeng Yang. Zhongshan Ophthalmic Center, Sun Yat-sen University, 54 S Xianlie Road, Guangzhou 510060, China. peizengy@126.com

Received: 2008-11-25 Accepted: 2009-01-10

Abstract

• AIM: To investigate the expression and the possible implication of CD40/CD40L costimulatory molecules in erythema nodosum of patients with Behçet's disease.

• METHODS: Sampling was done from erythema nodosum of 5 patients with Behçet's disease and normal skin of 2 healthy individuals. Immunohistochemical staining was performed to examine the expression of CD4, CD8, CD19, CD68, HLA-DR, CD40 and CD40L molecules in the obtained tissues.

• RESULTS: Approximately 90% of epidermic cells in erythema nodosum expressed CD40 molecule. In the dermis and subcutaneous tissue, a significantly increased number of $CD4^+$Tcells, $CD8^+$Tcells, $CD19^+$cells, $CD68^+$cells, HLA-DR^+cells, $CD40L^+$cells, and $CD40^+$cells were observed in the erythema nodosum as compared with that in normal skin. Double staining showed that CD40L molecules were expressed on 45% of $CD4^+$ T cells. CD40 molecules were expressed on 100% $CD68^+$ cells and 59.2% of HLA-DR^+cells respectively.

• CONCLUSION: A number of CD40/CD40L costimulatory molecules are upregulated in the erythema nodosum of patients with Behçet's disease.

• KEYWORDS: Behçet's disease; erythema nodosum; costimulatory molecules

Wang H, Yang PZ, Peng XY, Zhao M, Zhou HY, Huang XK. An increased expression of CD40/CD40L costimulatory molecules in erythema nodosum of patients with Behçet's disease. *Int J Ophthalmol* 2009;2(1):12-15

INTRODUCTION

Behçet's disease is common in areas of the world that line the ancient Silk Road, stretching from the Mediterranean basin to the Far East [1]. In China, Behçet's disease is one of common entities [2]. The major manifestations are ocular inflammation, oral aphtha, skin lesions such as erythema nodosum, and genital ulcers[1]. The ocular inflammation associated with Behçet's disease may have devastating effects on the patient's vision, and therefore has attracted a great deal of attention. The etiology and pathogenesis of this disease is still unknown, although intensive studies have been carried out during the last decades. Studies have revealed that autoimmune reaction[3-5], infection[6] and immunogenetic factors[7] are responsible for the development of Behçet's disease. Recent studies have shown that autoimmune reaction is particularly important and the activation of T cells plays a pivotal role in the pathogenesis of Behçet's disease[8].

Evidences have shown that the activation of T cells requires two distinct signals. The first one is antigen-specific and mediated through the T-cell receptor (TCR) recognizing an antigenic peptide bound to self MHC on the antigen presenting cell (APC). The other one is non-antigen specific signal delivered by costimulatory molecules expressed on APC. There are several costimulatory molecular pairs, such as CD40/CD40L, B7:CD28/CTLA-4, ICAM-1/LFA-1, CD2/LFA-3 and CD24/CD24L. Of these costimulatory molecules, the interaction of B7-CD28/CTLA-4 and CD40- CD40L plays a much more important role in the immune response[9-12].

A recent study by Bagenstose *et al* [13] revealed that the costimulatory molecules CD40 and CD40L play an important role in experimental autoimmune uveoretinitis in mice. However, their role in the pathogenesis of patients with Behçet's disease is not yet demonstrated. Behçet's disease is a multiple system disorder and erythema nodosum is a common lesion in this disease. Study on the expression of CD40/CD40L costimulatory molecules in erythema nodosum may provide insight into the mechanisms involved

in the pathogenesis of Behçet's disease. Our present study showed an increased expression of a number of CD40/ CD40L costimulatory molecules in the erythema nodosum of the patients with Behçet's disease.

MATERIALS AND METHODS

Patients and Biopsies Five patients aged 19-48 years with active Behçet's disease referred to Zhongshan ophthalmic center were enrolled in this study. The procedure of sampling on these five patients and two normal controls, and histological examination have been described in detail in our recent paper[14].

Immunohistochemistry Single and double immunohistochemistry was performed through using a panel of primary antibodies (Dako, Denmark and Santa Cruz, USA) to investigate the expression of CD40/CD40L costimulatory molecules. Working dilutions of all primary Abs used in this study were 1∶100. For single staining, the sections were incubated with primary Abs subsequently with alkaline phosphatase conjugated goat anti-mouse IgGs (Dako, Glostrup, Denmark) at 1∶200, and finally visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT). Immunohistochemistry was also performed by using biotinylated goat anti-rabbit IgGs (Southern Biotechnology, Birmingham, USA) at 1∶200, streptavidin: bionylated horseradish peroxidase (Strept ABComplexs) (Dako, Glostrup, Denmark) and 3-Amino-9-ethyl carbazole (AEC). Tissues were incubated with primary Abs for 1 hour, with secondary Abs and StreptABComplexes for 30 minutes at room temperature, followed by washing in phosphate-buffered saline (pH 7.4) for three times. All sections were blocked with H_2O_2 and normal goat serum. Stained sections were mounted by using aqueous mounting medium and coverslips.

Double staining was used to identify cells coexpressing molecules. The procedures were briefly described as follows. Specimens were incubated with mouse anti-human primary Abs, subsequently with rabbit anti-human primary Abs and StreptABComplexes. The labeled cells were visualized with AEC and BCIP/NBT. Double staining using CD4 and CD40L, CD8 and CD40L, CD19 and CD40, CD68 and CD40, HLA-DR and CD40, CD1a and CD40 was carried out in this study. Frozen sections of lymphoid tissue were used as the positive controls. Tissue sections incubated with PBS were used as negative controls.

Microscopy and Photography All microscopy was performed on a compound microscope (Olympus BX50, Olympus optical, Japan) and micrographs were obtained with a camera (Olympus PM-C35, Olympus optical, Japan). The positive cells were counted in three representative high power fields (×20) from each of the specimen within a 0.5mm×0.5mm grid under a microscope. A 0.1mm×1mm grid was used for cell counting.

Statistical Analysis Data analysis was performed using SPSS10.0 for windows software. Cell counts in normal subjects and in patients with erythema nodosum were compared using Wilcoxon rank sum W test for nonparametric data. The statistical significance risk rate was set at $P<0.05$.

Table 1 The expression of CD40 /CD40L costimulatory molecules in epidermis ($\bar{x}\pm s$, /mm^2)

Cell type	Normal skin	Erythema nodosum
CD4	0	0
CD8	0	0
CD19	0	0
CD68	0	0
HLA-DR	0	0
CD1a	200±280	270±210
CD40L	0	0
CD40	10±20	3020±440

[b]$P<0.01$ *vs* Normal skin

Table 2 The expression of CD40 /CD40L costimulatory molecules in dermis and subcutaneous tissue ($\bar{x}\pm s$, /mm^2)

Cell type	Normal skin	Erythema nodosum
CD4	72±41	1245±558[b]
CD8	67±25	102±65[a]
CD19	59±44	82±46[a]
CD68	94±95	328±83[b]
HLA-DR	51±48	635±497[b]
CD1a	51±47	95±49[b]
CD40L	0	633±309[b]
CD40	0	366±150[b]

[a]$P<0.05$, [b]$P<0.01$ *vs* Normal skin

RESULTS

Histological Findings of the Tissues The histological changes of the erythema nodosum have been reported previously, mainly showing massive infiltration of lymphocytes and macrophages[14].

Immunohistochemical Staining in Normal Skin In the epidermis of normal skin, CD1a was expressed on a few epidermic cells and $CD40^+$ cells were occasionally seen. No expression of $CD40L^+$ costimulatory cells was observed in the epidermis. In the dermis and subcutaneous tissue of normal skin, there was a small amount of perivascular cells expressing CD4, CD8, CD19, CD68, HLA-DR and CD1a, but no CD40L and CD40 molecules. Cell subpopulations and the expression of CD40/CD40L costimulatory molecules in the normal skin are shown in Table1,2.

Immunohistochemical Staining in Erythema Nodosum Nearly 90% of epidermic cells expressed CD40 molecules (Figure 1A), but no CD40L molecules (Table 2).

Focal accumulation of $CD4^+$T cells, scattered distribution of $CD8^+$T cells, $CD19^+$B cells and $CD1a^+$ cells, and diffuse distribution of $HLA\text{-}DR^+$ cells and $CD68^+$ cells (Figure 1B) were observed in the dermis and subcutaneous tissue. There was significantly increased expression of CD40L (Figure 1C) and CD40 in the dermis and subcutaneous tissue of erythema nodosum as compared with that in normal skin ($P<0.001$). $CD40L^+$ cells were more prominent in number than $CD40^+$ cells (Table 2). Positive cells were found around the blood vessels with a round morphology, or scattered in the tissue with a dendriform morphology and fusiform appearance.

As shown in Table 3, double staining of erythema nodosum

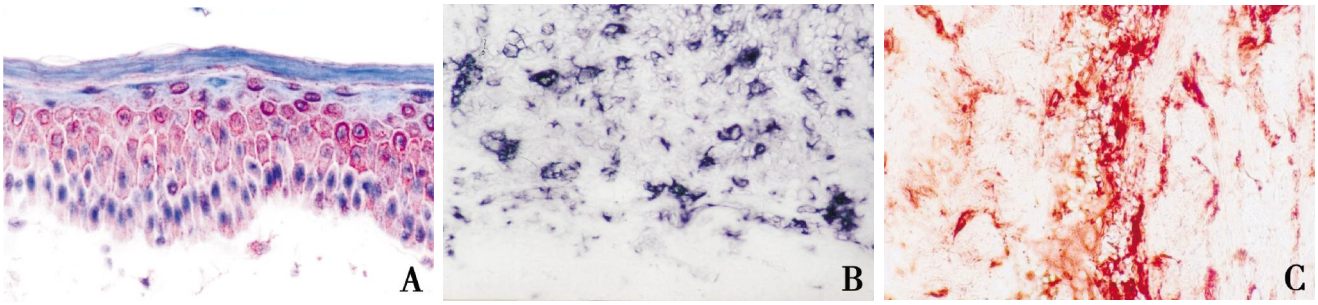

Figure 1 CD40 , CD68 & CD40L expression in the epidermis and subcutaneous tissue of erythema nodosum (×200) A: $CD40^+$(red) (AEC) in epidermis; B: $CD68^+$ (black) (BCIP/NBT) in subcutaneous tissue; C: CD40L (red) (AEC) in subcutaneous tissue

Figure 2 Double staining in the dermis and subcutaneous tissue of erythema nodosum (AEC and BCIP/NBT×200) A: CD4/CD40L (purple); B: CD68/CD40(purple); C: HLA-DR/CD40(purple)

showed that CD40L molecules were mainly expressed on $CD4^+$ T cells, whereas CD40 molecules predominantly expressed on macrophages and $HLA\text{-}DR^+$ cells . CD40L molecules were observed on 45% of $CD4^+$ T cells (Figure 2A). CD40 molecules were positive on all $CD68^+$ cells (Figure 2B) and 59.2% of $HLA\text{-}DR^+$cells (Figure 2C).

DISCUSSION

In the present study, we revealed an increased expression of CD40/CD40L costimulatory molecules in erythema nodosum. A variety of immuocompetent cells including T cells, macrophages, B cells, and antigen presenting cells, were observed in the erythema nodosum. T lymphocytes and macrophages were abundantly present in the erythema nodosum. In one patient we found a massive infiltration of neutrophils and ulceration. The most striking finding was that the number of CD40 and CD40L expressing cells was significantly increased in erythema nodosum, and that these molecules were highly expressed on immuocompetent cells. These results suggest that CD40/CD40L costimulatory molecules may play an important role in the development of this skin lesion.

CD40L is mainly expressed on activated $CD4^+$T cells, and occasionally on $CD8^+$T cells [11-13]. In this study, we found approximately 45% of $CD4^+$ T cell express CD40L in dermis and subcutaneous tissue of erythema nodosum, suggesting the presence of activated $CD4^+$T cells in the affected tissue. CD40, the receptor for CD40L, is mainly expressed on the surface of APC, including B cells, dendritic cells, macrophages, endothelial cells and fibroblasts[11-13].

Table 3 Double expression of CD40 /CD40L costimulatory molecules in dermis and subcutaneous tissue of erythema nodosum (%)

Cell identification*	Range	Mean ± SD
$CD4^+CD40L^+/CD4^+$	10->90	45.0 ± 29.2
$CD8^+CD40L^+/CD8^+$	0-10	3.64 ± 3.27
$CD19^+CD40^+/CD19^+$	100	100
$CD68^+CD40^+/CD68^+$	100	100
$HLA\text{-}DR^+CD40^+/HLA\text{-}DR^+$	40-90	59.2 ± 19.7
$CD1a^+CD40^+/CD1a^+$	40-70	53.3 ± 13.0

*The ratio of dual labeled cells to immunopositive cells

Our study revealed that there was no expression of CD40 in the normal skin, and that CD40 was mainly expressed on $CD68^+$ cells, $CD19^+$cells, $CD1a^+$cells and $HLA\text{-}DR^+$cells in dermis and subcutaneous tissue of erythema nodosum. We also found that approximately 90% of keratinocytes in the epidermis of erythema nodosum expressed CD40, suggesting a possible non-specific antigen-presenting role in the development of this lesion[15]. A number of $CD40L^+$ cells showed fusiform appearance and were distributed like fibroblasts or histocytes. This result suggests that fibroblasts or histocytes in the erythema nodosum may also express costimulatory molecules, which in turn activate the infiltrated cells. More studies are needed to confirm this hypothesis.

As CD40-CD40L interaction has been thought to play a role in T cell and macrophage activation and in initating inflammation response [16,17], the upregulated expression of CD40 and CD40L in erythema nodosum may therefore suggest a possible role of these molecules in the development of this skin lesion. In addition to a significantly increased number of CD40 and CD40L cells, our study also

showed that $CD4^+$ T cells were significantly increased in number in erythema nodosum as compared with those in the normal skin and the ratio of $CD4^+$ T cells to $CD8^+$ T cells was nearly 12∶1. This result suggests that $CD4^+$T cells were predominant in erythema nodosum. All these findings imply that ligation of CD40L by CD40 results in activation and proliferation of $CD4^+$T cell, which in turn contributes to the development of the inflammation in erythema nodosum.

In conclusion, our study illustrated a markedly increased expression of CD40L and CD40 in erythema nodosum. CD40L molecules were mainly expressed on $CD4^+$ T cells, whereas CD40 mainly on macrophages, antigen-presenting cells and keratinocytes. CD40/CD40L costimulatory pathway may be actively involved in the formation of erythema nodosum through activation of macrophages and T lymphocytes. As erythema nodosum is one of major lesions in patients with Behçet's disease, our study provides a clue for investigation of other lesions including uveitis in these patients.

REFERENCES

1 Suzuki Kurokawa M, Suzuki N. Behcet's disease. *Clin Exp Med* 2004:4:10-20

2 Yang P, Zhang Z, Zhou H, Li B, Huang X, Gao Y, Zhu L, Ren Y, Klooster J, Kijlstra A. Clinical patterns and characteristics of uveitis in a tertiary center for uveitis in China. *Curr Eye Res* 2005:30:943-948

3 Yates PA, Michelson JB. Behcet disease. *Int Ophthalmol Clin* 2006;46: 209-233

4 Ahn JK, Yu HG, Chung H, Park YG. Intraocular cytokine environment in active Behcet uveitis. *Am J Ophthalmol* 2006;142:429-434

5 Yamamoto JH, Minami M, Inaba G, Masuda K, Mochizuki M. Cellular autoimmunity to retinal specific antigens in patients with Behçet's disease. *Br J Ophthalmol* 1993;77:584-589

6 Eksioglu-Demiralp E, Direskeneli H, Kibaroglu A, Yavuz S, Ergun T, Akoglu T. Neutrophil activation in Behcet's disease. *Clin Exp Rheumatol* 2001;19: S19-24

7 Fietta P. Behcet's disease: familial clustering and immunogenetics. *Clin Exp Rheumatol* 2005;23:S96-105

8 Ben Ahmed M, Houman H, Miled M, Dellagi K, Louzir H. Involvement of chemokines and Th1 cytokines in the pathogenesis of mucocutaneous lesions of Behcet's disease. *Arthritis Rheum* 2004;50:2291-2295

9 Chang TT, Kuchroo VK, Sharpe AH. Role of the B7-CD28/CTLA-4 pathway in autoimmune disease. *Curr Dir Autoimmun* 2002;5:113-130

10 Magott-Procelewska M. Costimulatory pathways as a basic mechanisms of activating a tolerance signal in T cells. *Ann Transplant* 2004;9:13-18

11 Toubi E, Shoenfeld Y. The role of CD40-CD154 interactions in autoimmunity and the benefit of disrupting this pathway. *Autoimmunity* 2004;37:457-464

12 Howard LM, Miller SD. Immunotherapy targeting the CD40/CD154 costimulatory pathway for treatment of autoimmune disease. *Autoimmunity* 2004; 37:411-418

13 Bagenstose LM, Agarwal RK, Silver PB, Harlan DM, Hoffmann SC, Kampen RL, Chan CC, Caspi RR. Disruption of CD40/CD40-ligand interactions in a retinal autoimmunity model results in protection without tolerance. *Immunol* 2005;175:124-130

14 Wang H, Yang PZ, Peng XY, Zhou HY, Huang XK, Liu YX. The expression of costimulatory signals in the erythema nodule of Behcet patients. *J Capital Univ Medi Sci* 2005;26:258-262

15 Goodman RE, Nestle F, Naidu YM, Green JM, Thompson CB, Nickoloff BJ, Turka LA. Keratinocyte-derived T cell costimulation induces preferential production of IL-2 and IL-4 but not IFN-gamma. *J Immunol* 1994;152:5189-5198

16 Danese S, Sans M, Scaldaferri F, Sgambato A, Rutella S, Cittadini A, Pique JM, Panes J, Katz JA, Gasbarrini A, Fiocchi C. TNF-alpha blockade down-regulates the CD40/CD40L pathway in the mucosal microcirculation: a novel anti-inflammatory mechanism of infliximab in Crohn's disease. *J Immunol* 2006;176:2617-2624

17 Liu Z, Li N, Diaz LA. Inhibition of pemphigus vulgaris by targeting of the CD40-CD154 co-stimulatory pathway: a step toward antigen-specific therapy? *J Invest Dermatol* 2006;126:11-13