Η συνενεργοποιητική οδός CD40-CDE40L, εμπλέκεται στην εξέλιξη πολλών αυτοανόσων νοσημάτων, ενώ τα αυξημένα επίπεδα διαλυτού CD40L στον ορό ασθενών με ΣΕΛ, έχουν σχετιστεί με την ενεργότητα της νόσου. Σκοπός της παρούσας μελέτης ήταν η διερεύνηση του ρόλου του διαλυτού CD40L στην ανάπτυξη της νεφρίτιδας του λύκου και τη ντροπή σχέση του με την κρυοσφαιριναιμία στο Σύνδρομο Sjögren (ΣS). Με τη μέθοδο ELISA προσδιορίστηκαν τα επίπεδα του CD40L σε ζεύγη ορού-υρών και ορού-σιέλου συνολικά εξήντα-τεσσάρων ασθενών με ΣΕΛ, πιθανή αρθρίτιδα και σS, καθώς και δεκαεπτά υγιών μαρτύρων, ενώ μελετήθηκαν και οι πιθανοί κλινικοί συσχετισμοί αυτών των επιπέδων με άλλες εργαστηριακές και κλινικές παραμέτρους του σS και του ΣΕΛ. Η μελέτη αυτή έδειξε στατιστικά σημαντικά αυξημένα επίπεδα του διαλυτού CD40L στον ορό ασθενών με ΣΕΛ και ΣS, ενώ δεν ανιχνεύθηκε δCD40L στα δείγματα των ούρων και των σιέλων. Τα ευρήματα αυτής της μελέτης απαιτούν μια πιο εκτεταμένη έρευνα προκειμένου να καθοριστεί ο πιθανός ρόλος αυτού του μορίου στη νεφρίτιδα του λύκου και στο Σύνδρομο Sjögren.
Elevated levels of soluble CD40 ligand (sCD40L) in serum of patients with systemic autoimmune diseases

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Abstract

The CD40–CD40L costimulatory pathway is involved in the evolution of many autoimmune diseases including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and Sjögren’s syndrome (SS). Increased levels of sCD40L in the serum have been associated with disease activity in SLE. The aim of this study was to investigate the role of sCD40L in the development of lupus nephritis and examine its possible association with cryoglobulinemia in Sjögren’s syndrome. We used a 2-site sandwich ELISA to measure the levels of sCD40L in sera, from 64 patients with SLE, RA and SS and 17 healthy blood donors. Biological specimens from the affected tissues such as urine from patients with lupus nephritis and saliwa from patients with SS were also tested. In this regard, paired sera and first morning urine samples from 6 SLE patients (3 with active lupus nephritis and 3 with inactive lupus nephritis) were tested with the sCD40L ELISA protocol as well as paired sera and salivary samples from 5 patients with SS and cryoglobulinemia, 5 patients with SS and anti-Ro or anti-La autoantibodies and 5 age-matched healthy control donors. We also examined possible correlations of sCD40L levels with several laboratory and clinical parameters in SS and SLE. We found that sera from SLE and SS patients had significantly higher levels of sCD40L compared to sera from healthy control donors. No sCD40L was detected, in urine samples of patients with either active or inactive nephritis and in salivary samples from SS patients or normal subjects. Soluble CD40L is elevated in sera of SS and SLE patients but further investigation is needed to determine its possible role in SLE nephritis and Sjögren’s syndrome.

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Keywords: Soluble CD40 ligand; sCD40L; Sjögren’s syndrome; Systemic lupus erythematosus; Autoimmunity

1. Introduction

T lymphocytes regulate the specificity of immune response to foreign antigens and control the function of other immune cells such as B lymphocytes, monocytes and dendritic cells. Activation of costimulatory pathways such as CD40–CD40L and CD28–B7.1/B7.2 is needed to achieve an efficient adaptive immune response that leads to the activation, proliferation and differentiation of the specific T-cell clone.

In the past years the CD40–CD40L pathway has been studied extensively. CD40 molecule, a member of the tumor necrosis factor (TNF)-receptor family, is a 39 kDa transmembranic protein expressed on the surface of antigen-presenting cells including B cells, activated macrophages, dendritic cells, monocytes and follicular dendritic cells [1,2] as well as a number of other cell types such as mesangial, endothelial and epithelial distal cells [3]. CD40L is a type II membrane protein of 33 kDa which belongs to the TNF gene family and is normally expressed on Th1, Th2 cells and activated platelets [4,5]. The CD40–CD40L ligation provides the signals required for B-cell activation and differentiation, immunoglobulin class switching and further activation of T cells. In addition,
CD40 ligation on DCs leads these cells to maturation in order to express an antigen presenting phenotype. CD40L has been also found to be expressed on a variety of other cell types such as platelets, B cells, mast cells, basophils and NK cells in many diseases [1]. CD40—CD40L interaction is involved in many autoimmune diseases such as systemic lupus erythematosus (SLE) [6], rheumatoid arthritis (RA) [7], experimental allergic encephalitis (EAE) [8], multiple sclerosis (MS) [9], inflammatory bowel diseases (IBD) [10], chronic cutaneous (CU) [11] and in allograft rejection [12]. CD40L circulates in a soluble form of 18–20 kDa in the serum of patients with SLE [13,14]. The soluble CD40L has been associated with disease activity and severity [14]. The major sources of sCD40L are activated T lymphocytes and activated platelets. Soluble CD40L circulates in monomer, dimer or trimer form and is considered functional and capable of inducing a CD40–CD40 ligation with CD40 bearing cells [13].

The involvement of CD40–CD40L pathway in the pathogenesis of lupus nephritis has been documented by several studies in humans [6,15–20] and murine models [21–25]. In addition, the blockade of the pathway by anti-CD40L antibodies has ameliorated the clinical and laboratory parameters of nephritis in both mice [26–29] and humans [30]. We assumed that the sCD40L is able to cross the glomerular barrier and potentially interact with CD40 bearing cells of renal tissue at the initial phase. Therefore, one of the aims of our study was to detect sCD40L in urine samples from SLE patients with active nephritis, considering that urine is originated directly from the injured tissue.

Similarly, few studies are referring to the role of the CD40–CD40L pathway in the pathogenesis of SS [31–34]. Recently, it has been shown that salivary gland epithelial cells from pSS patients express high levels of CD40 molecule [35]. We speculated that sCD40L might interact with the CD40 bearing epithelial cells of the injured glandular tissue and create a further induction of their antigen-presenting phenotype. In the present study, we measured the levels of sCD40L in sera and salivary samples from patients with pSS and evaluated possible correlations with clinical manifestations such as cryoglobulinemia, glomerulonephritis and peripheral neuropathy, as well as serologic findings such as anti-Ro/anti-La autoantibodies and RF.

2. Materials and methods

2.1. Patients and biological specimens

Blood was obtained from 23 consecutive SLE patients, 23 pSS patients, 16 RA patients and 17 healthy blood donors, and serum was separated immediately after phlebotomy by centrifugation at 2500 rpm and stored at −80 °C.

Paired serum and salivary samples were obtained from 10 patients including 5 with SS and cryoglobulinemia, 5 with SS and autoantibodies to Ro/SSA and/or La/SSB and 5 healthy individuals. Unstimulated whole salvia was collected from subjects over a period of 15–20 min. Saliva was centrifuged at 50,000 × g for 15 min to remove debris. The supernatants were stored at −80 °C until tested.

Paired serum and urine specimens were obtained from 3 SLE patients with active nephritis (defined as any of the following: proteinuria > 500 mg/day, macroscopic or microscopic hematuria, urinary casts and raised serum creatinine or reduced creatinine clearance) who underwent renal biopsy, and 3 SLE patients with inactive nephritis under treatment. The first SLE patient, with active lupus nephritis, had proteinuria of 590 mg/day and diffuse proliferative glomerulonephritis in renal biopsy (class IV according to WHO classification). The second patient had proteinuria of 2500 mg/day, microscopic hematuria of glomerular origin and focal segmental proliferative glomerulonephritis (class III according to WHO classification). The third patient had proteinuria of 1750 mg/day and membranous glomerulonephritis (class V according to WHO classification). Finally, from the 3 SLE patients who had inactive lupus nephritis, 2 disclosed histological features of mesangial lupus nephritis in renal biopsy (class II according to WHO classification) and 1 disclosed features of focal segmental proliferative glomerulonephritis (class III according to WHO classification). Serum was processed as described above. Morning urine specimens collected from patients were centrifuged at 2500 rpm for 10 min, and the supernatants were concentrated 10-fold and stored at −80 °C until tested.

SLE and RA patients satisfied the diagnostic criteria of the American College of Rheumatology (ACR) for SLE and RA, respectively [36,37] and SS patients met the validated European diagnostic criteria [38]. All patients were followed up at the outpatient Rheumatology department of pathophysiology (Medical School-University of Athens).

2.2. ELISA for sCD40L

The levels of soluble CD40L was measured by a 2-site sandwich ELISA, based on two anti-CD40L monoclonal antibodies, reacting with distinct epitopes on CD40L molecule. Polystyrene microtiter plates were coated with 100 μl in each well of the diluted anti-human CD40L monoclonal antibody (1 mg/ml, TRAP1 clone; Pharmingen, San Diego, CA) in carbonate-bicarbonate buffer 1:1000, or 1% bovine serum albumin (BSA) in PBS for control wells. After overnight incubation at 4 °C, the wells were washed 5 times with 200 μl PBS–TWEEN-20 (0.01%) in each well and blocked with 200 μl of nonfat milk 5% in PBS each well, for 3 h at room temperature. Serum samples were prepared in a dilution of 1:100 in PBS–1%BSA, salivary samples in a dilution of 1:10 in PBS–1%BSA and urine samples in a dilution of 1:1. Subsequently, the plates were washed 3 times with PBS–TWEEN 20 (0.01%) (200 μl/well) and then 100 μl of a diluted serum sample, salivary sample or urine sample, were added in duplicates and incubated overnight at 4 °C. After washing 5 times with PBS–TWEEN-20 (0.01%), 100 μl of biotin labeled-anti CD40L monoclonal antibody (1 mg/ml, 24–31 clone; Ancell, Bayport, MN, USA), diluted 1:1000 in PBS–1%BSA was added to each well and plates were incubated for 2 h at room temperature. After washing 3 times, streptavidin–alkaline phosphatase (1 mg/ml, Jackson,
Baltimore, MD, USA), diluted 1:5000 in PBS—1%BSA was added to each well and plates were incubated for 1 h at room temperature. The plates then were washed 3 times, 100 μl of phosphate substrate solution (pNPP, Sigma, St Louis, MO, USA) was added to each well and the optical density was measured at 410 nm.

3. Results

3.1. Evaluation and reliability of the assay used for the sCD40L detection in biological fluids

In order to assess the repeatability and reliability of the assay, preliminary studies were performed, using recombinant trimeric sCD40L in several concentrations in order to create a standard curve (data not shown). In addition we used as negative and positive controls, supernatants from a HEC-mock cell line and a HEC-mock cell line transfected with CD40L, respectively. Finally the influence of hemolysis was estimated using healthy control donors. We observed that binding of sCD40L in sera was correlated with the level of hemolysis (data not shown). Thus special care was taken during phlebotomy.

3.2. Determination of sCD40L levels in patients with autoimmune diseases

Serum samples from 23 SS patients, 23 SLE patients, 16 RA patients and 17 normal subjects were tested in the sCD40L ELISA in order to establish the role of sCD40L in the pathogenesis of Sjögren’s syndrome and SLE (Fig. 1). We expressed the levels of sCD40L in ELISA binding units. More specifically, we defined the value of 100 binding units as the cutoff value for the ELISA protocol, calculated as the mean normal sera binding units plus 3 standard deviations.

The median value of sera binding units of healthy controls was 38.0. The median value of sera binding units of SLE patients was 57.6 and 8 patients out of 23 (34.7%) had binding units values above the cut-off value (referred as “positive” SLE patients). The median value of sera binding units of SS patients was 61.4 and 8 patients out of 23 (34.7%) had binding units values above the cut-off value (referred as “positive” SS patients). The RA group had a median value of sera binding units of 52.6.

Sera of SLE patients had significantly higher levels of sCD40L than the control sera donors (p = 0.0128, by Mann–Whitney unpaired nonparametric test) and similarly sera of SS patients had significantly higher levels of sCD40L than the control sera donors (p = 0.0352, by Mann–Whitney unpaired nonparametric test).

3.3. Clinical and laboratory parameters of pSS and SLE patients

The SLE disease activity was determined by the ECLAM score. The 8 SLE “positive” patients (who had OD values above the cut-off value) had a median ECLAM score of 2.5 whereas the 15 SLE “negative” patients had a median ECLAM score of 2.0. From the 8 SLE “positive” patients, 5 patients (62.5%) had positive anti-dsDNA antibody, 6 patients (75%) had ENAs auto-antibodies and 6 patients (75%) had low C4 serum levels. From the 15 “negative” SLE patients, 5 patients (29%) had anti-dsDNA, 7 patients (46.6%) had ENAs auto-antibodies and 9 patients (60%) had low C4 serum levels. No SLE patient had low C3 serum value (Table 1).

In the SS “positive” group (8 patients), 1 patient (12.5%) had positive RF and 3 patients (37.5%) had Ro or La...

Table 1

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<td>Ro</td>
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autoantibodies. In the SS “negative” group, 5 patients (33.3%) had positive RF and 4 patients (26%) had Ro or La autoantibodies. All SS patients had normal C3 and C4 serum values. Clinical manifestations were also recorded at phlebotomy period. From the “positive” SS patients, 1 patient (12.5%) experienced Raynaud phenomenon, 2 patients (25%) had peripheral neuropathy and 5 patients (62.5%) had arthritis. From the 15 “negative” SS patients, 1 patient (6.6%) had peripheral neuropathy and 5 patients (62.5%) had arthritis. Similarly no SS patient had developed lymphoma (Table 2).

3.4. Correlation of sCD40L levels with rheumatoid factor (RF) activity in patients with pSS

We investigated a possible correlation between RF and sCD40L in pSS. RF is considered a common autoantibody in pSS. Furthermore, sCD40L showed a significant correlation with IgM-RF and IgG-RF titers in RA [39]. We performed a chi-square test, in pSS patients, which revealed no such correlation.

3.5. Detection of sCD40L in body fluids from the site of injury

Paired serum and first morning urine samples from 6 SLE patients, 3 with active nephritis and 3 with inactive nephritis were also tested with the sCD40L ELISA protocol. Preliminary studies with urine specimens from healthy control donors reveal no binding before and after dialysis for 48 h (data not shown). No binding was detected in the urine of the 3 SLE patients with recently diagnosed active nephritis who received no treatment. Similarly, no binding was detected in the 3 SLE patients with inactive nephritis under treatment. Urine samples form all 6 patients were concentrated 10-fold and tested with the ELISA protocol. No binding was detected either (Fig. 2).

Finally, paired serum and salivary samples from 5 patients with SS and cryoglobulinemia, 5 patients with SS and autoantibodies to Ro/SSA and/or La/SSB and 5 age-matched healthy control donors were detected for sCD40L. Salivary samples from all patients of each group had low values, independently from the respective OD values of the serum samples, including healthy control donors and pSS patients (Fig. 3).

4. Discussion

sCD40L is considered a proinflammatory and prothrombotic cytokine in cardiovascular diseases [40,41]. The major sources of the soluble form of sCD40L are activated CD4 lymphocytes [14] and activated platelets [5]. Increased levels of sCD40L have been found in SLE [13,14], RA [39], systemic sclerosis [42], MCTD [43], IBD [10] and ITP [44]. Increased levels of sCD40L have been correlated with disease activity in SLE [14] and many studies have documented the effectiveness of anti-CD40L therapy in lupus nephritis at many murine models [26–29]. It has also been found that lupus nephritis, at the initial phase, is auto-antibody dependent, but cell-to-cell interactions are further needed to maintain the tissue injury including the activation of the CD40–CD40L pathway [45]. Taking into consideration that sCD40L is a 18–20 kDa protein and circulates in monomer, dimer and trimer form [14], we assumed that the sCD40L might be involved in the pathogenesis of lupus nephritis.
We found that SLE patients have statistically significant higher binding units values than the healthy control donors, which indicates that serum level of sCD40L is increased in SLE patients. Consistent with this data, is the fact that a percentage of 34% of SLE patients had binding units values above the cut-off point. SLE-sCD40L positive patients had a tendency to develop more frequently anti-dsDNA (62.5% vs. 29%) and ENAs (75% vs. 46.6%) and had a median ECLAM score value higher than SLE negative patients (2.5 vs. 2.0). These findings confirm previous studies that correlate the presence of sCD40L with the disease severity.

We examined urine samples of 3 SLE patients with active nephritis, 3 SLE patients with inactive nephritis and healthy donors in order to investigate the role of sCD40L in the pathogenesis of SLE nephritis. Previous studies have reported that in normal kidney, CD40 is expressed on mesangial, endothelial cells and distal tubules but not in distal tubules. CD40 expression is up-regulated in lupus nephritis and is additionally expressed also in distal tubules [46]. Urine samples from healthy subjects as well as patients with active and inactive nephritis demonstrated no binding in the sCD40L assay, even after 10-fold concentration. The normal glomerular filtration barrier is characterized by size and charge selectivity. Low molecular substances such as β2 microglobulin, with a molecular weight of 25,000 Da, can easily cross the barrier. The glomerular basement membrane and endothelial cells possess a negative charge and therefore negatively charged substances such as albumin are repelled [47]. The monomer form of sCD40L is relatively small in size (18,000 Da) and possesses a positive charge (+5 at pH 7). These characteristics render the monomer form of sCD40L capable of crossing the renal protein barrier. In addition, the inflamed glomerulus in active lupus nephritis is likely to demonstrate increased permeability for such proteins. The fact that no sCD40L was detected in urine samples can be attributed to other parameters such as shape and flexibility. Alternately, sCD40L circulates also in dimmers or trimers with increased size and shape. Finally, sCD40L might be consumed within the tubules by CD40 bearing cells or might be reabsorbed by the renal tubular cells. Although the role of the CD40—CD40L pathway has been well documented in lupus nephritis, more intense research is needed to identify the possible involvement of sCD40L.

SS is a chronic autoimmune systemic disease, characterized by lymphocytic infiltration into the salivary and lacrimal glands [48]. About half of pSS patients will develop systemic disorders. The inflammatory infiltrate of the salivary glands in pSS consists mainly of activated CD4—CD40L bearing lymphocytes and B cells [49]. Recently, our laboratory has demonstrated that salivary gland epithelial cell lines derived from SS patients manifested increased constitutive surface expression of CD40 [35]. These findings indicate that CD40—CD40L interactions may contribute to disease progression. In this regard, sCD40L may act as an inflammatory cytokine which interacts with CD40 bearing epithelial cells, enhancing the antigen-presenting phenotype of the involved epithelium.

In the present study, we found that patients with pSS had significantly higher binding units values than normals and a 34% percentage of pSS patients had binding units values above the cut-off point, indicating that pSS patients have increased serum level of sCD40L. The increased levels of sCD40L were not correlated with any clinical or laboratory parameter. Previous studies reported a positive correlation between the levels of RF and sCD40L in RA [39]. Since RF is a common serologic finding in pSS, we examined the association between RF and sCD40L in pSS and found that such a correlation does not exist. It is noteworthy though that two SS “positive” patients had peripheral neuropathy (25%) versus one “negative” patient (6.6%). Taking into consideration that the CD40L molecule is expressed on activated T cells and not on resting T cells, we assume that most probably sCD40L is derived from the membrane-bound CD40L on activated T cells, after cleavage. This mechanism is not disease-specific and represents a means of down-regulation of the surface-exposed CD40L of biological importance, seen in many other autoimmune diseases such as SLE [14].

Furthermore, cryoglobulinemia in SS has been associated with palpable purpura, peripheral neuropathy, glomerulonephritis and lymphoproliferation as late sequelae of the disease.
suggests an antigenic T-cell dependent process \[52\] whereas B denotes an oligoclonal or monoclonal B-cell expansion, which in any of the three groups. The presence of cryoglobulinemia on biological material in pSS patients since it is directly collected from age-matched healthy controls. Saliva is considered a rare medium where T- and B-cells can interact via the CD40–CD40L pathway. The fact that sCD40L was not detected in salivary samples of pSS patients with cryoglobulinemia or in pSS patients with anti-Ro or anti-La antibodies, connotes that if the microenvironment of the infiltrated epithelium is a site of B–T cell interaction, sCD40L might be consumed by B cells and epithelial cells. Technical reasons such as entrapment of sCD40L into mucus and subsequent removal with debris might also contribute to the negative finding. We should also note that all pSS patients with cryoglobulinemia who participated in the study underwent immunoregulatory therapeutic intervention, which may alter the production of sCD40L. Although our data demonstrated increased levels of sCD40L in sera of pSS patients, the role of sCD40L in the pathogenesis of pSS and the development of cryoglobulinemia, lymphoproliferation and glomerulonephritis needs to be further investigated.

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